FOOD SAFETY AND SECURITY OF SAGO STARCH IN RURAL PAPUA NEW GUINEA

A Thesis submitted by
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I declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university of other institution of tertiary education. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references given.

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STATEMENT ON THE CONTRIBUTION OF OTHERS

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DECLARATION OF ETHICS

Relevant research reported in this thesis received approval of the Papua New Guinea Medical Research Advisory Council (MRAC number 05/25) and the James Cook University Ethics Review Committee (Human ethics number H2167).

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ABSTRACT

Sago starch is an important source of dietary carbohydrate in lowland and coastal areas of Papua New Guinea (PNG). There have been sporadic reports of severe haemolytic illness resulting from sago starch consumption, termed sago haemolytic disease (SHD), with most reports coming from the Western Province. Despite the occurrence of SHD, and a high likelihood of less severe foodborne illness resulting from consumption of indigenous foods in general in PNG, there have been no detailed studies of the microbiology of sago starch. The aim of this study was to establish a broad basal knowledge of the microbiology of traditionally prepared sago starch in PNG.

Sago starch samples and sociological information were collected in two of the main sago eating regions of PNG, the East Sepik Province and the Western Province. Sago starch samples were collected predominantly from the houses of sago starch producers in rural areas of the two provinces, and to a lesser degree from markets in some villages in the East Sepik Province. In addition to these samples considered 'fit for consumption', two samples of sago starch that had been associated with outbreaks of SHD were also analysed.

Analysis of the sago starch for common bacterial pathogens was done using accepted methods, and where possible was based on the relevant Australian Standards. The findings suggest that faecal contamination of sago starch is widespread, with over three-quarters of all samples tested for faecal coliforms at the upper limit of detection. The human pathogen *Salmonella* spp. was isolated from approximately 7% of samples tested. The presence of emerging human pathogens such as *Citrobacter freundii* and *Enterobacter sakazakii* was tested, with the former being present in a low percentage of samples tested. Other important bacterial food pathogens such as *Staphylococcus aureus* and *Bacillus cereus* were also detected in sago starch, but none of the 57 samples tested for *Listeria monocytogenes* was positive.

Mycological analysis of sago starch revealed a variety of fungal contaminants. Commonly occurring genera of filamentous fungi included *Penicillium, Scytalidium, Aspergillus,* and *Acremonium*. Mycotoxin analysis of sago starch revealed that the

common mycotoxins such as aflatoxins, ochratoxin A, cyclopiazonic acid, sterigmatocystin, zearalenone and citrinin were not present. Selected fungal isolates were tested for the presence of mycotoxin production in pure culture, with two-thirds found to be capable of citrinin production and one isolate capable of sterigmatocystin synthesis.

In an attempt to determine the aetiological agent of SHD, bacterial and fungal isolates were screened for haemolytic activity on blood agar. A suitable semi-quantitative assay was developed, and extracts from bacterial and fungal cultures were tested. Particular attention was paid to the haemolytic activity of fungal extracts, given the long speculated role of mycotoxins in the aetiology of SHD. The haemolytic activity of numerous fungal species has been demonstrated for the first time, and steps in the optimised extraction and purification of the haemolytic component of some isolates has been completed. Further work was conducted on extracts from *Penicillium steckii*, a common contaminant of sago starch. The chemical properties of the extract suggest that a novel fungal metabolite is responsible for haemolytic activity.

Detailed studies on the microbial ecology of stored sago starch have been conducted, primarily to garner a greater understanding of factors that contribute to the microbial safety of the food. The study has established that sago starch is a naturally fermented product, and this fermentation process contributes greatly to the food safety of the product. Bacterial pathogens such as *B. cereus, L. monocytogenes, S. aureus* and *Salmonella* sp. do not survive well in actively fermenting sago starch, primarily due to the production of weak acids. Furthermore, numbers of filamentous fungi are low in actively fermenting sago starch, presumably due to the reduced oxygen availability.

On the basis of the sociological data and microbial studies, a hazard analysis and critical control point (HACCP) plan was devised that was considered appropriate for application in village based production of sago starch in rural PNG. Through a greater understanding of the microbiology of sago starch, together with the development of an appropriate HACCP plan, this research will lead to increased food safety and food security for sago consumers in rural and remote lowland areas of

PNG. Moreover, studies of the haemolytic metabolites of fungi isolated from sago starch pave the way for further research to determine the aetiology of SHD.

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COMMONLY USED ABBREVIATIONS

ANOVA	Analysis of variance
ATCC	American type culture collection
a_w	Water activity
BHC	Balimo Health Centre
BHIB	Brain heart infusion broth
BPW	Buffered peptone water
BSA	Bismuth sulphite agar
CAST	Council for Agricultural Science and Technology
CCP	Critical control point
cfu	Colony forming unit
CPA	Cyclopiazonic acid
Da	Dalton
DRBC	Dichloran rose Bengal chloramphenicol agar
EHEC	Enterohaemorrhagic *Escherichia coli*
g	gram
g	gravity
HACCP	Hazard Analysis and Critical Control Point
Hb	Haemoglobin
HBA	Human blood agar
HBA-chlor	Human blood agar with chloramphenicol
HPLC	High performance liquid chromatography
HUS	Haemolytic uraemic syndrome
JCU	James Cook University
kDa	kilo Dalton
kg	kilogram
l	litre
LD50	50% lethal dose
M	molar
ml	millilitre
min	minute
MPN	Most probable number
NACMCF	National Advisory Committee on Microbiological Criteria for Foods
nm	nanometre
PBS	Phosphate buffered saline
PLC	Preparatory layer chromatography
PMGH	Port Moresby General Hospital
PNG	Papua New Guinea
ppm	parts per million
ppb	parts per billion (10^9)
Rh	Rhesus factor
rpm	revolutions per minute
RR	Reference range
RTX	Repeat in toxin
SBA	Sheep blood agar
SBA-chlor	Sheep blood agar with chloramphenicol
SBA-gluc	Sheep blood agar with glucose

SHD	Sago haemolytic disease
SMA	Synthetic *Mucor* agar
STEC	Shiga-toxigenic *Escherichia coli*
stx	Shiga toxin
TDS	Toxin diluent solution
TEF	Toluene: ethyl acetate: formic acid
TLC	Thin layer chromatography
UV	Ultraviolet
VFA	Volatile fatty acid
WBC	White blood cell
WHO	World Health Organisation
×	Multiplication
ºC	degrees Celsius
µl	microlitre
µg	microgram

CHAPTER 1: GENERAL INTRODUCTION

1.1 Background

The health and nutritional status of the people of Papua New Guinea (PNG) remains one of the biggest challenges faced by that country. Statistics from relevant organizations point to one of the lowest life expectancies, highest infant and child mortality rates and lowest health care expenditures in the Pacific region (PNG Ministry of Health, 2000; WHO, 2004). Basic needs such as access to safe water and food security are yet to be adequately met. Diarrhoea is one of the leading causes of morbidity and mortality in the country, and typhoid and malnutrition also feature in the leading 15 causes of death (PNG Ministry of Health, 2000).

Undoubtedly, improved access to safe water, which currently stands at about 40% (PNG Ministry of Health, 2000) would help reduce the incidence of diarrhoea. However, the importance of foodborne illness should not be underestimated. Based on the findings of two World Health Organisation (WHO) reports, Ehiri and Prowse (1999) suggested that worldwide over 70% of cases of diarrhoea in children under five years old are attributable to the consumption of contaminated food. Other factors that may be less tangible than the incidence of diarrhoea also contribute to food safety, and should not be overlooked. Although it is difficult to relate to national health statistics, exposure to mycotoxins, for example, is likely to have numerous detrimental health effects, particularly in developing countries (FAO, 2001), such as PNG.

One of the most important food crops in PNG is the endemic sago palm, *Metroxylon sagu* Rottboell. The palm covers an estimated one million hectares of the country (Power, 1999; McClatchey *et al.*, 2004), and is an important source of carbohydrates for approximately 10% of the population (Rhoads, 1980; Sopade, 2001). Dependence on sago starch is greatest in lowland areas prone to intertidal flooding, where very few other sources of carbohydrate will grow. In such areas, life

is based on subsistence agriculture, household income is very low, infrastructure is poor and access to health services is limited.

Sago starch is extracted from the macerated pith of *M. sagu* using traditional methods, and is typically stored for up to two months. In the 1970s, Taufa (1974) described an illness that was linked to the consumption of old stored sago. This illness, known as sago haemolytic disease (SHD), was initially thought to be confined to the Maprik region of the East Sepik Province, but two years later, two outbreaks were reported in the Western Province (Donovan *et al.*, 1976). Sporadic outbreaks continue to occur, with high mortality rates, but to date little is known about the aetiology, epidemiology or pathophysiology of the disease.

1.2 The Purpose of the Study

On the basis of the current knowledge of SHD (Taufa, 1974; Donovan *et al.*, 1976; Donovan *et al.*, 1977), it seems likely that the as yet unidentified aetiological agent of the disease is microbial. The purpose of this study is to establish detailed basal knowledge of the microbiology of sago starch, which will form a foundation for further investigation. Specifically, the presence of pathogenic microbes and associated toxins in sago starch will be determined, and the conditions under which their presence can be limited will be established. Particular attention will be given to haemolytic organisms. It is envisaged that by conducting a thorough study of the microbial biota of sago starch, a greater understanding of the aetiology of SHD will be gained. Furthermore, the results of the study will contribute broadly to improve food safety, and ultimately food security, for the rural, subsistence people of lowland PNG who depend heavily upon sago starch as a staple energy source.

In brief, the study seeks to address the following hypotheses:

1. Sago starch is a reservoir of haemolytic organisms which may contribute to SHD
2. Sago starch is a reservoir of other foodborne pathogens
3. Factors of processing and storage influence the survival and persistence of these organisms

4. Establishing this knowledge base will aid in reducing SHD and other foodborne illnesses.

1.3 The Study

Two study regions were established in lowland areas of PNG. In the north of the country in the East Sepik Province, samples of sago starch and sociological data were collected predominantly from Ambunti and Angoram villages, and surrounds. Some samples were also collected in the Wewak region. In the south of the country samples were collected from throughout the southern and central regions of the Western Province. Many of the samples were collected from villages along the Aramia River in the Balimo district. Further samples were collected from the Morehead, Suki, Mabawe and Lake Murray districts. Figure 1.01 shows the regions of sample collection in each province. Sample collection was based around the East Sepik and Western Provinces primarily because both provinces have regions of high sago starch dependence, and they are the only two provinces from which cases of SHD have been documented in the scientific literature (Taufa, 1974; Donovan *et al.*, 1976; Donovan *et al.*, 1977). There have been no known reports of SHD occurring in recent times in the East Sepik Province, however outbreaks continue to sporadically occur in the Western Province (Chapter 3).

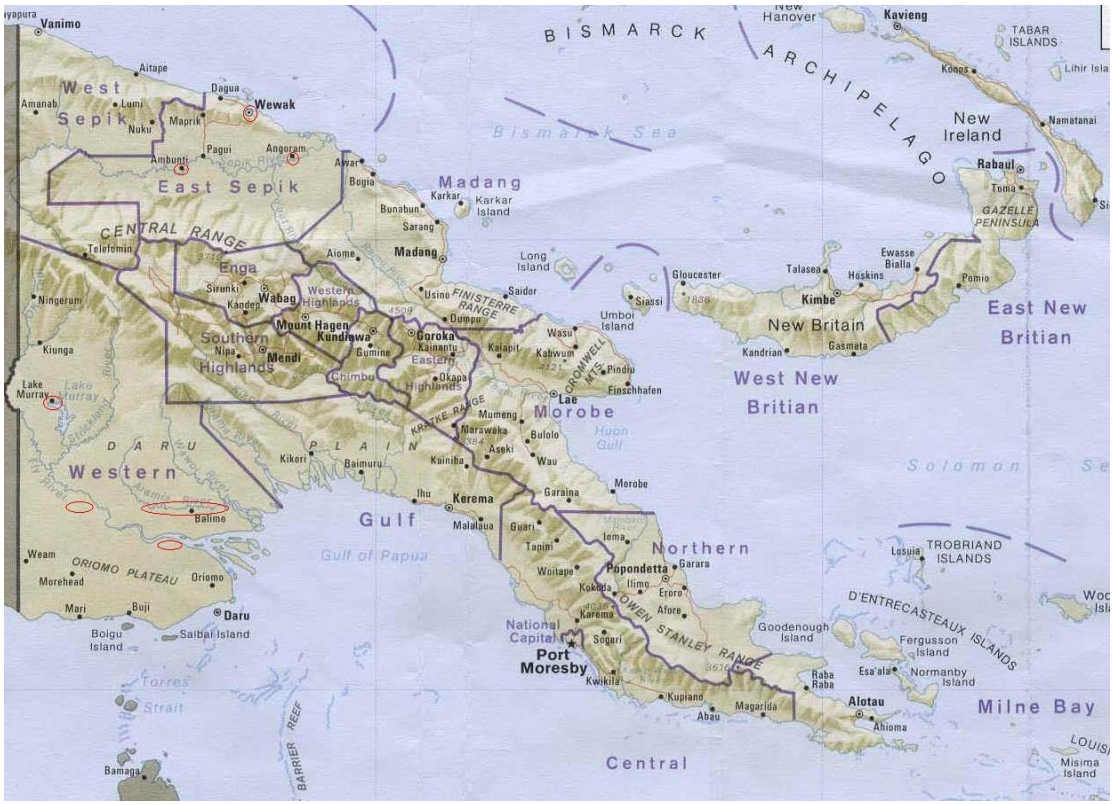


Figure 1.01: Map of PNG showing provincial borders. Areas of sample collection are marked with red. The site east of Balimo is Suki, and south of Balimo is Mabawe district.

The thesis commences with a review of the literature, followed by a chapter pertaining to sociological aspects of sago consumption, then research chapters concentrating primarily on the microbiology of sago starch. The broad aims of this work are:

- To garner knowledge pertaining to sago starch production and storage which might influence the microbial ecology of sago starch, to introduce the reader to important sociological aspects of sago production and storage, and to provide evidence of ongoing outbreaks of SHD in the Western Province
- To conduct a thorough survey of common foodborne bacterial pathogens and indicator organisms in sago starch
- To conduct a thorough mycological survey of sago starch, paying particular attention to the identification of filamentous fungi
- To test for the presence of common mycotoxins in sago starch
- To test for the presence of microorganisms that demonstrate haemolytic activity *in vitro*, and develop a semi-quantitative haemolytic assay that can be

used for activity driven fractionation of haemolytic components from microbial cultures

- To gain an understanding of the microbial ecology of sago starch, enabling the identification of optimal storage and preservation conditions for sago starch
- To apply a hazard analysis and critical control point (HACCP) plan to sago starch production and storage at the village level, based on the findings of preceding studies.

In doing so, it is expected that recommendations can be made that will help reduce the incidence of SHD and other foodborne illnesses resulting from sago starch consumption. Furthermore, it is hoped that this study will pave the way for future research into the problem of SHD, enabling the aetiological agent to be determined, and the pathophysiology of the disease to be elucidated.

CHAPTER 2: LITERATURE REVIEW

2.1 Sago

2.1.1 The sago palm

The term ‘sago palm’ has been applied to numerous genera of palms worldwide, but is usually reserved for palms with an edible pith. *Metroxylon sagu* is the most common source of palm derived sago, and is considered the true sago palm (Vaughan and Geissler, 1997; McClatchey *et al.*, 2004). For the purpose of this study the term sago palm will be used to describe *M. sagu* only, and sago starch will refer to starch derived from that palm.

Metroxylon sagu is thought to have originated in New Guinea and the Moluccas (now part of Indonesia), with PNG considered the centre of sago palm diversity (Flach and Schuiling, 1989; Vaughan and Geissler, 1997). The palm has subsequently spread through parts of South East Asia and the Pacific, with much of its current distribution outside Melanesia attributed to ancient anthropogenic introduction (McClatchey *et al.*, 2004). It is estimated that PNG is home to over one million hectares of sago stands, equating to approximately 40% of the world’s sago palms (Power, 1999; Sopade, 1999). The palm occurs at an altitude of up to 1,000 metres, although this is probably beyond its natural altitude limit (Paijmans, 1980). Rasyad and Wasito (1985) suggested an upper altitudinal limit of 700 metres in Indonesia, with higher starch yields in palms grown in areas of up to 400 metres above sea level. Figure 2.01 shows the broad area within the Asia–Pacific region in which *M. sagu* occurs naturally.

Figure 2.01: Distribution of *Metroxylon* spp. in the Asia–Pacific region. The distribution range was taken from Ruddle et al. (1978). The map was adapted from www.gcir.org.

As a food plant, *M. sagu* requires very little management, and can grow on marginal agricultural land. The palm occurs naturally in tropical peat lands, which are mainly found in coastal and sub-coastal lowland areas. Peat land soil is characterised by being poorly drained, typically waterlogged for at least part of the year, and is often acidic. Few plants are able to tolerate such conditions, but the sago palm can do so (Ulijaszek, 1983; Purwanto *et al.*, 2002). The palm also grows on silt, clay and sand, and can grow in brackish environments (Paijmans, 1980). Given that huge tracts of land in PNG are swampy intertidal land, the sago palm provides food where the cultivation of other food plants is very difficult (Shimoda and Power, 1985). Figure 2.02 illustrates the swampy lowland and coastal areas where sago palms are most likely to thrive. Figure 2.03 illustrates the waterlogged conditions often encountered by *M. sagu*. Such areas are predominantly in the West Sepik, East Sepik, Western, and Gulf Provinces. Sago palms are said to grow in every province in PNG (Sopade, 1999), although much of the current sago palm distribution is anthropogenic (Rhoads, 1980).

Figure 2.02: A map of PNG showing areas suitable for growth of the sago palm, *M. sagu*, in green. Small patches of palms also grow on many of the coastal fringes of islands off the coast of mainland PNG. Based on information from Paijmans (1980) and Ulijaszek (1983). The cartographer is unknown.

Figure 2.03: Sago palms growing in waterlogged conditions in the Western Province, PNG.

The taxonomy of the *Metroxylon* genus remains open to debate, with little coherence between researchers on the issue. Beccari (1918: cited in Schuiling, 1995) divided the genus into three species, with 23 subspecies. Fifty years later Backer and Backhuizen van den Brink (1968: cited in Sastrapradja, 1985) suggested a single species, *M. sagu*. More recently Rasyad and Wasito (1985) listed five distinct species of the genus, namely *M. longispinum* Martius, *M. microconthum* Martius, *M. rumphii* Martius, *M. sagus* Rottbal, and *M. silvester* Martius. However, at around the same time, Rauwerdink (1986) proposed one species, namely *M. sagu* Rottboell, with four subspecies. Regardless of the taxonomy, it is evident that the genus *Metroxylon*, the true sago palm, covers a group of closely related, but variable palms.

2.1.2 Uses of the sago palm

2.1.2.1 Sago starch

Sago starch has been a highly utilised source of carbohydrates for many thousands of years (Gwaiseuk, 2001). It is claimed to be one of the oldest known food plants (Takamura, 1991), although anthropological evidence is difficult to obtain. Both Crosby (1976) and Rhoads (1980) argued that the widespread use of the sago palms (*M. sagu* and others) and the similarities between starch extraction techniques throughout the Asia–Pacific region are indicative that palm starch as a food source has “great antiquity”.

Sago starch consumption occurs widely throughout PNG. Dependency is greatest in lowland swampy areas (see Figure 2.02) where other food plants cannot survive. Indeed, if not for the availability of sago starch, it is likely these areas would be largely uninhabited. It is also used as an important supplementary source of carbohydrates for some people living in the Western Highlands and Southern Highlands Provinces, and most of the coastal and island provinces (Ruddle *et al.*, 1978), although its use in many such areas may have declined in recent years.

Sago starch is an excellent source of carbohydrates. The carbohydrate content exceeds most other commonly consumed carbohydrates such as wheat, corn, sweet potato or rice, with dried sago reported to be 94% starch (Vaughan and Geissler,

1997; Sopade, 2001). Ohtsuka (1977) estimated that the Oriomo people obtain 70% of their total calorie intake from sago starch. More recently, Ulijaszek (1991) estimated that the people of Koravake village in the Purari Delta region depend on sago for 43% of their total dietary energy intake. Similar levels of dependence on sago starch are expected in sago eating areas of the Gulf, West Sepik, East Sepik and Western Provinces. Many people from West Papua also depend heavily on sago starch (Glazebrook, 2001).

While sago starch is an excellent source of carbohydrates it is nutritionally poor, being low in protein, vitamins and minerals (Vaughan and Geissler, 1997; Sopade, 2001). Throughout PNG protein can be difficult to obtain. Many people from sago eating areas of PNG catch fish, prawns and wild game, and occasionally consume pork in feasts and ceremonies. Some sago eating communities have sufficient elevated ground to grow vegetables, adding valuable vitamins and nutrients to their diet. However, many people in sago eating areas are likely to be lacking essential nutrients from their diet, and may also suffer protein energy malnutrition. The nutritional status of Papua New Guinean people is discussed in detail in Section 2.2.

2.1.2.2 Secondary food products

The sago palm has been used for the production of foods other than starch. Perhaps the most important secondary food derived from the palm are sago grubs (Ruddle *et al.*, 1978). The grubs are the larval stage of the palm weevil, *Rhynchophorus ferrugineus papuanus* Kirsch, which are a pest of living palms (Mercer, 1994). Grubs are collected from growing palms, and are also cultivated on unprocessed portions of felled palms. Some inferior quality palms are felled specifically for the cultivation of sago grubs (Ruddle *et al.*, 1978; Rhoads, 1982; Mercer, 1994). The grubs are an important source of protein, and their cultivation is considered by some to be one of the earliest forms of agriculture (McClatchey *et al.*, 2004).

The spent pith resulting from starch extraction from the sago palm is commonly used as animal feed, predominantly for pigs and domestic fowls (Ruddle *et al.*, 1978; Quartermain, 1999). This has typically occurred in a haphazard manner to date, with

the by-products often left where they were produced (at the sago extraction site) and animals allowed access to them. Felled palms may be used to maintain a bonding between humans and semi-wild pigs, and hollowed logs can also be used to trap feral pigs (Quartermain, 1999).

The production of a salty ash from sago palms has been documented in areas of PNG that do not have access to natural salt (Townsend *et al.*, 1973). The ash is typically prepared from the base and midribs of leaves of sago palm, which are essentially by-products of a palm felled for starch extraction. It is unlikely that this process is commonly practised today, due to the widespread use of commercially available table salt in the country.

2.1.2.3 Building products and tools

In addition to being an invaluable food source, *M. sagu* is also used as a building material by indigenous people throughout the Asia–Pacific region where the palm grows (McClatchey *et al.*, 2004). The outer cortex of the palm is used for walls, flooring and planking. Leaves are commonly used for thatch for roofs and walls (Figure 2.04). Following starch extraction, bark and fronds are left to dry and subsequently used as fuel wood. Parts of the palm are also commonly used during the sago extraction process (Section 2.1.3).

2.1.2.4 Sago as a trade commodity

It is likely that sago starch has been used in trade for thousands of years. Lowland sago eaters bartered sago starch for goods that they did not have, such as pottery, other foods and shell ornaments (Kari, 1978; Ella, 2002). Harding (1994) describes in detail facets of pre-colonial trading in PNG, of which sago starch featured prominently. Today sago is a commonly traded item, and can be seen at markets all around the country. By-products of the sago palm, such as roof thatching (see Figure 2.04), are also sold at local markets on occasions.

Figure 2.04: A young boy stands in front of thatch made from the leaves of the sago palm, *M. sagu*. This thatch was being prepared for sale at the local market. The roof of the hut in the background consists of non-thatched sago palm leaves. A stand of sago palms can be seen in the background.

International trade in sago starch has been active for hundreds of years. Ruddle *et al.* (1978) discuss the interesting commodity transactions whereby Chinese storekeepers in Sarawak (Malaysia) sold their wares in return for sago. This was then on-sold to a dealer who would pool the starch and sell it to businessmen. Collectively, the sago was exported to Singapore for processing in Chinese owned factories, and exported to worldwide markets. There was a decrease in sago trade through much of the 1900s, but in recent years considerable effort has been put into re-establishing a market. Sago starch can be used in the production of a variety of goods, including numerous foods, ethanol, glue, fructose and maltose syrups, chemicals and pharmaceuticals (Sudwikatmono, 1991).

Reflecting this renewed interest in sago starch, both the government and the private sector in PNG have expressed interest in the establishment of large-scale sago extraction factories for the production and exportation of high quality food grade starch (Haiveta, 1999; Power, 1999). Similar operations to those proposed already operate in Malaysia and Indonesia. These factories are highly mechanised, resulting

in a considerably more efficient extraction of sago than traditional methods. The main problem faced in Malaysia and Indonesia is sustainability, with the factory operators unable to obtain sufficient sago palms (Power, 1999). Given the expansive areas of sago stands in PNG, with appropriate planning and community consultation a sustainable sago industry might be feasible.

A well-planned, sustainable and viable sago industry would have considerable flow on effects to the community. It is recognised that food security is not simply a case of self-sufficiency, and that growing cash crops can increase food security (Foster, 1992). Increased incomes that individuals and landowners would derive from employment and royalties could contribute to a better diet and greater food availability. Temu and Saweri (2001) state that in the PNG context, if money is spent wisely the nutritional status of the population would greatly improve, particularly for children. However, the foreseeable irony of increased income from sago starch exports being largely used on the purchase of imported foods must be avoided (Gwaiseuk *et al.*, 1999).

2.1.2.5 Overview of the cultural significance of sago

The long history of sago starch consumption has ensured that the sago palm is culturally important, and has had an influence on society well beyond simply being a source of dietary carbohydrate. Given the great cultural diversity in Papua New Guinea, it is impossible to broadly generalise about the role sago plays in society. Nonetheless, some traditional beliefs are worthy of mention to help illustrate the cultural significance of sago starch. The Gogodala people (from the middle Fly region of the Western Province) have a belief that there is a close relationship between humans and sago, with the first sago palm said to have arisen from a man who defecated a seed that grew into a sago palm (Dundon, 2002). The Gebusi people (from the Upper Strickland-Bosavi region of the Western Province) use sago starch to determine the guilt of sorcerers who are accused of committing murder. The accused cooks sago, which, if hard all the way through, proves the accused is innocent, and if soft in the centre, proves the accused is guilty (Strathern, 1994). There is evidence in the literature of sago starch being consumed in feasts (Connell and Hamnett, 1978) and rituals marking death (Telban, 1997), practises that are still

thought to be widespread in PNG. Sago helps define gender roles (Busse, 1990; Dundon, 2005), with extraction often considered women's work in many regions, but in some cases women are actively excluded when menstruating (Williamson, 1979). Ruddle *et al*. (1978) discuss at length the significance of sago in myth and ritual in PNG and other traditional sago consuming cultures.

2.1.3 Production of sago starch

2.1.3.1 Extraction of starch from the palm

In PNG sago starch is extracted using traditional methods that are likely to have been used for thousands of years (Crosby, 1976). The task is very labour intensive and time consuming, usually involving the cooperation of a small group of people or family (Dwyer, 1985). The palm is felled using an axe, and then the bark is stripped back to expose the pith of the plant. The pith is macerated using an adze or similar tool, as illustrated in Figure 2.05. Close human contact with the macerated pith is common during this stage of the process. In rare cases a crude rasp is used to macerate the pith, saving time and energy. In PNG rasps are found in only a small number of villages, and have only been used since the 1960s (Nakau, 1999).

The extraction of the starch from the macerated pith requires a large volume of water. The pith is repeatedly washed and the starch squeezed from it. The apparatus used to enable this process to occur is often constructed from the outer layers and leaves of the sago palm (Figure 2.06). Hands may be used to squeeze the leached starch from the pith, as shown in Figure 2.06, or the pith can be placed in a woven bag and force applied with bare feet. The starch washes from the pith and is collected in a large vessel along with the water. Often a dugout canoe is used as the receptacle, but modern plastic containers are sometimes used also. The starch is left to settle to the bottom of the receptacle and the excess water can be tipped off, leaving the moist starch.

Figure 2.05: Macerating the pith of a sago palm using traditional tools. The women are sitting on the spine of a sago palm frond as they work. Note also the close proximity of their feet to the macerated pith, a potential source of contamination.

Figure 2.06: Extraction of sago starch. The large funnel-like structure is an outer layer of bark from the sago palm.

Microbiologically, it is likely that the quality of water used will have considerable bearing on the quality of starch extracted. Often the palms grow in swampy areas a long distance from fresh running water, so a well is dug in the swamp to collect water for processing. When available, river water is used to process sago palms grown near the villages. The rivers are also used for bathing and faecal waste disposal, thus compromising the water quality and consequently the safety of the sago starch. A minority of villages in PNG have access to safe drinking water (PNG Ministry of Health, 2000), thus it is likely that the water used in sago production is rarely fit for consumption or food production.

The relative similarity of traditional extraction techniques employed throughout the Asia–Pacific region where sago is consumed is considered indicative of a long history spanning many thousands of years (Crosby, 1976; Rhoads, 1977; Rhoads, 1980). For further information on starch extraction, the reader is referred to Ruddle *et al*. (1978).

2.1.3.2 Storage of sago starch

After extraction, moist sago starch is stored until consumed. Common storage techniques include wrapping the starch in sago leaves, banana leaves or occasionally pandanus leaves, storing in woven bags made of natural fibre, storing in plastic bags and storing in ceramic pots covered with water. In some villages the sago is lightly smoked after being wrapped in leaves, and some people store their sago above the cooking fire, which smoulders most of the day. The smoking of sago is also likely to contribute to preservation (Davidson, 1997). Two of the commonly used traditional methods are illustrated in Figure 2.07.

(a): Densely packed moist sago starch wrapped in sago leaves. Sago stored in this method is commonly referred to as a sago bundle.

(b): A bag made of woven natural fibre used for sago storage.

Figure 2.07: Two commonly used methods of sago storage: (a) wrapped in leaves; (b) placed in a woven bag.

2.2 Health and Nutrition in Papua New Guinea

2.2.1 Overview

The health and nutritional status of the people of PNG has been well documented over the past 30 years. Studies invariably point to poor nutritional status, and general health indicators below international standards. According to the PNG National Health Plan 2001–2010 (PNG Ministry of Health, 2000), the country has the highest infant mortality rate (73/1,000 births) and highest mortality rate for children aged one to five years old (102/1,000 births) in the Pacific region. The maternal mortality rate (370/100,000) is the second highest in the region. Furthermore, 70% of rural communities do not have access to safe drinking water, and very few have access to safe disposal of human and animal waste. Consequently, the incidence of diarrhoea is high, and accounts for around 3% of all deaths in PNG (see Section 2.2.3).

When the impact of sub-optimal nutrition (Section 2.2.2) and the high incidence of infectious disease (Section 2.2.3) are taken into account, it is evident that further

research in health and nutrition in PNG is required, followed by the implementation of appropriate policy.

2.2.2 Nutrition

2.2.2.1 Occurrence of malnutrition

Many of PNG's health concerns are at least partly due to poor nutrition, and in particular protein-energy malnutrition. Studies have repeatedly demonstrated nutritional deficiencies (Korte, 1975; Heywood and Heywood, 1988; McNair and Learoyd, 1989; Gibson *et al.*, 1991; Gibson, 2001; Mueller, 2001; Mutambek and Tumana, 2001). The nutritional status of infants and children is of particular concern in developing countries, with PNG being no exception. According to the PNG National Health Plan 2001–2010 (PNG Ministry of Health, 2000), 40% of children under five years old are not receiving adequate nutrition.

Various researchers have observed the low protein nature of the PNG diet and have suggested that it may be a significant factor contributing to malnutrition (Gibson *et al.*, 1991; Mueller, 2001; Temu and Saweri, 2001). However, low protein is not the only contributing factor. Despite Papua New Guineans having access to high starch content foods such as sweet potato, taro, banana and sago starch, researchers have repeatedly observed that energy malnutrition is also an important factor. Korte (1975) found that in the Sepik, energy deficiency was a greater problem than protein deficiency. Many people in the Sepik region have good access to seafood, which probably ensures sufficient protein intake. More recently, Gibson (2001) found that 42% of the population did not reach the target energy requirement of 2000 calories per day; a target that is below the recommended daily allowance suggested by the PNG Department of Health. Given the reasonably good supply of high carbohydrate foods in most areas of PNG, Gibson (2001) was probably right to suggest that education, particularly maternal education, will play an important role in improving the nutrition of children.

Malnutrition in adults tends not to be studied to the same extent as it is in children, but it is not exclusively a childhood problem. Particular concern is expressed about

malnutrition of women at childbearing age. Malnourished pregnant women are likely to give birth to small babies, and are unlikely to produce enough milk for their babies (Mutambek and Tumana, 2001). This obviously leads to malnourished children who are then more susceptible to disease (Section 2.2.3.). Furthermore, malnourished adults are likely to be less productive than adequately nourished adults, having an adverse effect on family income and food availability.

2.2.2.2 Variation of nutritional status within PNG

Typically, anthropometric assessment has been employed to determine height-for-age, weight-for-age and weight-for-height values in children. Some such surveys have been used to make inter-provincial comparisons. However, Heywood *et al.* (1988) found the length and weight of children in PNG to differ according to altitude. Highland children tend to be shorter and heavier than lowland children, with children from the highland fringe being the shortest. Thus, a straight comparison of data between the highland and lowland provinces may not give an accurate picture of comparative health and nutrition. The authors did not clearly identify the cause of the height difference, but it seems likely that genetics play an important role.

Non-comparative studies have demonstrated that poor nutrition is a problem in both highland rural communities, where the main staple foods are root crops such as sweet potato and yam (King and Mascie-Taylor, 2002), and lowland communities that have a higher dependency on sago starch (McNair and Learoyd, 1989; Gibson *et al.*, 1991; Mutambek and Tumana, 2001). Despite evidence of malnutrition throughout PNG, Mueller (2001) claimed that high consumption of sago was correlated to lighter children and poorer growth than children in areas where the consumption of sweet potato, cassava or purchased food (e.g. rice and tinned meats) dominated. However, given the finding of Heywood *et al.* (1988), it is unlikely that Mueller's finding can be attributed to diet alone. Mueller (2001) also proposed a correlation between socio-economic status and good growth in children, a finding supported by Temu and Saweri (2001). In fact, Temu and Saweri (2001) found that people in the poorest two quartiles have a greater dependency on sago starch that those in the richest two. On this basis, one could theorise that sago starch is not necessarily an inferior food source compared to other forms of carbohydrate, but rather those with the greatest

dependency on sago do not have sufficient income to supplement their diet with high protein foods.

2.2.3 The impact of infectious disease on health

The percentage of deaths that are a result of infectious disease in PNG is staggering. Cumulatively, pneumonia, malaria, tuberculosis, meningitis, diarrhoea, septicaemia and typhoid are the cause of around 50% of all deaths in the country. The National Health Plan 2001–2010 (PNG Ministry of Health, 2000) predicted that infection rate of HIV in high risk sectors of the community will reach 20% in the near future, presumably leading to a further increase in the death rate due to infectious disease.

The main infectious agents responsible for mortality and morbidity in PNG fall outside the scope of this study, however the incidence of diarrhoea and typhoid are of some interest. According to Naraqi *et al.* (2003) dysentery and typhoid fever account for 12% and 8% respectively of adult admissions to Port Moresby General Hospital (PMGH). In children less than five years old, diarrhoea is implicated in 15% of admissions, with a fatality rate of 3.9% (Han *et al.*, 1995). A more accurate indication of the significance of diarrhoea in PNG could be gained if outpatient data from rural health centres were included (Vince, 1995), especially given the lack of safe drinking water and sanitary disposal, and higher dependency on traditional foods in these areas.

Only limited work has been conducted on the main causes of diarrhoea in PNG, and this tends to concentrate mainly on infantile diarrhoea. In a small study conducted by Miwatani *et al.* (1990) samples were taken from 52 children, of which 45 were positive for bacterial pathogens. Almost 40% of samples when cultured demonstrated *Shigella* spp., with enterotoxigenic *Escherichia coli* present in nearly 20% of samples. Of the nine cases of enterotoxigenic *E. coli*, heat stable toxin was present in all. Other bacterial pathogens isolated were *Bacillus cereus*, *Staphylococcus aureus*, *Campylobacter jejuni* and *Aeromonas hydrophila*.

Rotavirus is known to be a common cause of diarrhoea worldwide, particularly in children. In one study in PNG, it was detected in 9% of moderate and 18% of severe

cases of infantile diarrhoea (Bukenya *et al.*, 1990). Han *et al.* (1995) observed two seasonal peaks in admissions for infantile diarrhoea in PMGH and proposed that rotavirus may be responsible for the cool season peak (May to July), and enterotoxigenic *E. coli* responsible for the hot season peak (December and January).

2.2.4 The relationship between nutrition and immunity

Malnutrition is estimated to be directly responsible for 1% of deaths in PNG (PNG Ministry of Health, 2000). Perhaps more importantly, malnutrition is likely to play a role in compromising the health and immunity of the population, thus predisposing many people to other illnesses. Through the epidemiological analysis of data from 53 developing countries, Pelletier *et al.* (1995) determined that 56% of deaths in children (6 to 59 months) were due to the potentiating effects of malnutrition. In the vast majority of cases it was mild to moderate malnutrition, rather than severe malnutrition, that contributed to the deaths.

Research suggests there is a link between malnutrition and diarrhoea. Early work by Scrimshaw *et al.* (1968) in Central America suggested that malnutrition predisposes children to diarrhoea, and that diarrhoea results in growth retardation and malnutrition. In PNG, Vince (1995) states that malnourished children are more susceptible to diarrhoea, are more likely to develop persistent diarrhoea (lasting 14 days or more), and are likely to suffer higher mortality than well nourished children. Han *et al.* (1995) demonstrated malnutrition to be a significant risk factor in the development of persistent diarrhoea in children in PNG. Although Lehmann *et al.* (1988) were unable to demonstrate a link between malnutrition and predisposition to diarrhoea, they suggested that cases were more severe in undernourished children.

The incidence of respiratory disease and malaria is also a very important issue in PNG. Lehmann *et al.* (1988) suggested a link between increased risk and severity of acute lower respiratory tract infection and malnutrition. The authors found in a previous unpublished study that children in Tari, Southern Highlands Province, weighing less than 2.5 kg at birth are at four times the risk of dying of acute lower tract respiratory infections in the first year of life than are those born heavier. This is

alarming given that according to the PNG National health Plan 2001–2010 (PNG Ministry of Health, 2000) only 10% of children in PNG weigh over 2.5 kg at birth.

There have been contradictory reports on the relationship between malaria and nutritional status. Lehmann and co-workers (1988) found no link between malnutrition and incidence of malaria in young children in PNG. Subsequently, Genton and co-workers (1998) suggested that moderate malnutrition in PNG children up to 10 years old confers a protective effect against falciparum malaria. Much of the recent work, conducted predominantly in Africa, has shown a relationship between protein-energy malnutrition and malaria. Intervention studies have shown that malaria has a detrimental effect on child nutrition (Bradley-Moore *et al.*, 1985; ter Kuile *et al.*, 2003), with at least one statistical analysis suggesting the effect to be greatest in the first two years (Nyakeriga *et al.*, 2004). The effects may continue well beyond early childhood, with Friedman *et al.* (2003) concluding that *Plasmodium falciparum* parasitaemia has a detrimental effect on the nutritional status of early adolescents also.

Nonetheless, the effect of nutrition on parasitaemia remains open to debate. Friedman and co-workers (2005) established a link between protein energy malnutrition and indicators of malarial infection. They found that stunted children were more likely to succumb to malaria infection and illness than non-stunted children, although the study design did not enable them to determine causality. Upon the analysis of previously published studies, Caulfield *et al*. (2004) concluded that well-nourished individuals are better able to mount an immune response and have a greater ability to withstand and clear infection. This conclusion was supported by studies conducted in PNG that showed vitamin A and zinc supplements may help reduce morbidity due to *P. falciparum* infection (Shankar *et al.*, 1999; Shankar *et al.*, 2000).

Although it is difficult to explicate precisely, a relationship does exist between adequate nutrition and the immune and overall health status of an individual. This is particularly the case for infants and young children. As such, food security is a very important issue in developing countries, and can directly influence the health of a given population.

2.3 Sago Haemolytic Disease

2.3.1 Symptoms

Sago haemolytic disease presents as an acute haemolytic crisis, with patients suffering fever, vomiting, jaundice and haematuria. In some cases splenomegaly or hepatosplenomegaly and high bilirubin levels have been observed (Taufa, 1974; Donovan *et al.*, 1977). These signs and symptoms are typical of those associated with intravascular haemolytic anaemia (McKenzie, 1996). The worst affected are severely anaemic, may become irrational or uncontrollable, and can lose consciousness (Donovan *et al.*, 1977). The onset of symptoms is rapid, occurring within 12–24 hours of consumption. The rate of recovery is likely to be dependent on the severity of illness and level of treatment received. In one of the case studies described by Donovan *et al.*(1977), recovery of the patients was spontaneous but variable, taking between a few days to some weeks. In the other case study described, recovery, following treatment, occurred in three days. Treatment with blood transfusion is the most effective measure (Taufa, 1974; Donovan *et al.*, 1977).

2.3.2 Epidemiology

The occurrence of SHD has been linked to the consumption of ‘stale’ sago starch in the literature (Taufa, 1974; Donovan *et al.*, 1976; Donovan *et al.*, 1977) and anecdotally (Melrose, pers. com.). However, the significance of this perceived risk factor needs further elucidation, as currently unexplored factors may be wholly or partially attributable to the aetiology of the disease. There is a need for a controlled longitudinal study to determine risk factors associated with the illness.

The disease is an acute and often fatal illness associated with consumption of sago starch that has been stored for relatively long periods. Despite local knowledge of the disease dating back to 1961, and the first documentation of the disease in the scientific literature occurring over 30 years ago (Taufa, 1974), little is known of its aetiology. Following Taufa’s initial description of the SHD in the East Sepik Province, Donovan *et al.* (1976) documented further outbreaks of the disease in the Western Province. Sporadic outbreaks of the disease have since been observed in the

Western Province (Melrose, pers. com.; Warner, pers. com.; Wissink, pers. com.) and Madang Province (Stace *et al.*, unpublished).

Although not a common disease, the scarcity of documented cases of SHD in recent years may be indicative of the paucity of health professionals in rural areas of PNG, rather than a true reflection of the incidence of the disease. Interestingly, there are no known reports of sago haemolytic disease in other countries in the South East Asia–Pacific region. This is probably in part due to the lesser (and ever decreasing) dependence on sago starch throughout most of South East Asia, where rice is now more commonly consumed.

2.3.3 Aetiological studies

2.3.3.1 The microflora of sago starch

Donovan and co-workers (1977) were the first to investigate the aetiology of SHD, although their study was superficial, being limited by equipment and resources. The researchers obtained a very small sample of sago implicated in an outbreak of the disease and tested it for the presence of bacteria, fungi and mycotoxins. Three species of bacteria were isolated, all belonging to the genus *Bacillus*. One of the species isolated, *B. cereus*, is well known as a food pathogen causing vomiting and diarrhoea. It is not known to cause acute haemolytic disease, although its presence is of some interest given the haemolytic nature of the organism *in vitro*.

Four fungal isolates were identified, three yeasts and one mould (*Paecilomyces*). None of the yeasts was considered pathogenic. In recent years, the mould *Paecilomyces* has had pathogenic isolates associated with it. For example, *Paecilomyces variotii* is known to produce the mycotoxin patulin in silage (Frisvad and Thrane, 2000), and has been isolated from the cerebrospinal fluid of cancer patients (Kantarcioglu *et al.*, 2003). *Paecilomyces lilacinus* has been reported as causing disseminated infection in advanced HIV positive patients (Lovell *et al.*, 2002). Human illness associated with this genus is rare, and is typically the result of invasive sepsis, not toxicosis (although toxicosis is possible in animals, given the occurrence of patulin in silage). Currently, there are no reported cases of

Paecilomyces spp. causing acute intravascular haemolysis in humans or animals. In the study of Donovan and co-workers (1977), only one colony of this *Paecilomyces* sp. was isolated, leading the authors to question the significance of the finding and to acknowledge that it was unlikely to be associated with SHD. Moreover, there is no suggestion that the species isolated by Donovan *et al*. (1977) is the same as those discussed by Frisvad and Thrane (2000) or Lovell *et al*. (2002).

In addition to studying the microflora of sago starch, Donovan *et al.* (1977) screened for the presence of the mycotoxins aflatoxin, ochratoxin, zearalenone and byssochlamic acid. Although none was detected, the authors did not rule out mycotoxins as a possible aetiological agent.

A microbial study of a sample of sago starch associated with an outbreak of SHD in Madang was conducted in the 1980s (Stace *et al.*, unpublished). The sago was tested for the presence of the common food poisoning bacteria *Salmonella, Staphylococcus* and *Clostridium*, but none was detected. Mycological analysis demonstrated "significant" numbers of *Trichoderma harzianum* and *Gliocladium virens*. Toxicology studies were conducted using mice as an animal model. Mice were fed the implicated sago, and in a separate experiment were fed mixed cultures of *T. hazianum* and *G. virens*. Gross organ anatomy was inconsistent in mice fed sago starch implicated in SHD, and no mice died within five days of commencement of feeding trials. When fed the mixed culture of fungi, mice died within three days. However, only two mice were subjected to the latter trial, and no controls were used.

Despite further sporadic outbreaks of SHD, little work has been done to determine the aetiological agent over the past thirty years. In recent years there as been a small renewal of interest in the field, with both microbial flora and the incidence of mycotoxins in sago starch being investigated (Amoa *et al.*, 1999; Omoloso, 1999).

Omoloso (1999) found sago starch harboured the bacterial pathogens *Staphylococcus aureus* and *Salmonella* spp. These results should be interpreted with caution, as there is no documentation of confirmatory tests being conducted. Coliforms were also detected, although they were not confirmed as faecal coliforms, so the significance of their presence is unknown. Yeasts and moulds were present in

most sago starch samples tested, but identifications were not made. Omoloso concluded that the water used to extract the starch, the equipment used, and those handling the starch were likely to be the main sources of contamination. This conclusion seemed to be based more on a sound understanding of food handling and production than from results obtained in the study.

Amoa and co-workers (1999) conducted a preliminary study on the fungal flora of sago starch and screened for the presence of aflatoxins. *Aspergillus* spp. were isolated from most samples tested, but no attempt was made to speciate these isolates. Yeasts were also commonly present in sago starch, but were not identified.

Aflatoxin was detected in many of the samples tested, including relatively fresh sago samples. Levels were between 5 and 15 parts per billion (ppb), which the authors reported to be within the national guidelines for food in PNG.

2.3.3.2 The possibility of host genetic predisposition

To date no studies have been published addressing the possibility of a host genetic predisposition being a contributing factor to SHD. However, both Taufa (1974) and Donovan *et al.* (1977) suggested that genetic predisposition in the patients may be a contributing factor. Donovan *et al.* (1977) cited the occurrence of acute haemolytic anaemia caused by fava bean ingestion in glucose-6-phosphate dehydrogenase deficient individuals as an example (see Section 2.6.5.1).

2.3.3.3 The need for further research

There have been many limitations of the studies conducted on the microflora of sago starch to date. A paucity of bacterial and fungal species has been isolated from sago starch. Furthermore, the screening of only a limited number of mycotoxins has been conducted. A more thorough investigation is required to identify the main fungal species, to test for the presence of a greater number of bacterial pathogens, and to screen for the presence of common and novel haemolysins that might be the aetiological agent of SHD. If evidence of a direct toxicosis is not forthcoming, other factors such as the role of genetic host predisposition and immune haemolytic

anaemia warrant investigation. Indeed, the possibility that consumption of stale sago starch prior to onset of the disease is no more than a coincidence should also be addressed in future studies.

Due to the unknown aetiology of the disease, no data are currently available on the possible effects of continuous or prolonged exposure to low levels of the toxic agent causing SHD. Chronic exposure to some microbial toxins are now considered to have detrimental long term health effects (Malloy and Marr, 1997; Williams *et al.*, 2004). Such issues may need to be addressed in the future, following the elucidation of the aetiological agent.

2.4 Bacterial Food Pathogens

2.4.1 Introduction

Bacterial pathogens are currently the best described, and probably the major, contributor to foodborne illness. While it is currently unknown what role, if any, common bacterial food pathogens play in the aetiology of SHD, it is likely that their presence in sago starch leads to gastrointestinal illness, thereby contributing to morbidity and mortality in PNG. Those foodborne bacterial pathogens considered most important in the context of sago starch consumption are briefly described below. The reader is referred to specific food microbiology texts for more detailed reviews of these and other important foodborne pathogens (Doyle *et al.*, 1997; Lund *et al.*, 2000a; Hocking, 2003; Jay *et al.*, 2005). Review of the following selected pathogens does not suggest that they are the only bacterial pathogens which might be significant as foodborne contaminants in PNG.

2.4.2 *Bacillus cereus*

The association of episodes of *B. cereus* food poisoning with farinaceous products, in addition to the ubiquitous nature and spore-forming ability of the organism, make it of particular interest in relation to sago starch. *Bacillus cereus* is widespread in both raw and processed foods (Jenson and Moir, 2003), and it once has been detected in

sago starch, although no effort was made to enumerate it (Donovan *et al.*, 1977). Outbreaks of diarrhoeal syndrome have been linked to various products, including meat, potatoes, vegetable stew, cooked noodles, soups, puddings and dairy products (Granum, 1997; Granum and Baird-Parker, 2000), whereas production of cereulide, the emetic toxin, is commonly, but not exclusively, associated with rice and other starchy foods (Melling *et al.*, 1976; Agata *et al.*, 2002). Cereulide is heat stable (Melling and Capel, 1978), thus, cooking food does not necessarily prevent *B. cereus*-associated emetic illness.

The incidence *B. cereus* food poisoning is rare relative to the ubiquitous nature of organism. There may be numerous factors that contribute to this phenomenon, but recent studies have shown that foodborne isolates are often positive for the various genes that encode diarrhoeal toxin production, but production of high levels of toxin is not common (Guinebretiere *et al.*, 2002). Additionally, genotyping by Ehling-Schulz *et al.* (2005) suggests that the emetic strains are closely related, and the ability to produce emetic toxin may be recently acquired relative to the evolutionary lineage of *B. cereus*. Thus, production of emetic toxin is probably not widespread among *B. cereus* strains.

2.4.3 *Clostridium* spp. food poisoning

The two species of clostridia of most significance in food microbiology are *Clostridium botulinum* and *Clostridium perfringens*. *Clostridium botulinum* produces the most potent group of neurotoxins known to humans, while *C. perfringens* type A infection can result in mild gastroenteritis. Of greatest interest in PNG is the disease manifestation caused by *C. perfringens* type C, namely severe enteritis necroticans, commonly known as pig bel. The aetiology of pig bel is interesting, and highlights the role nutritional status and dietary intake can play in disease aetiology. Classically, pig bel affects individuals who normally have a protein deficient diet, and depend heavily on sweet potato (*Ipomaea batatas* Lam.) as a carbohydrate source. Outbreaks occur when a meal of meat (usually a feast of pork) contaminated with *C. perfringens* type C is consumed. The low protein diet leads to a reduction in pancreatic tryptic activity. Sweet potato also has antitrypsin activity. These two factors combine to decrease the detoxification of *C. perfringens* toxin, thus pig bel

ensues (Lawrence and Walker, 1976; Murrell and Walker, 1991). Following consumption of the contaminated meal there may be a latent period of hours to days. The symptoms are abdominal pain, vomiting, nausea and severe diarrhoea, often with blood present. The disease is most common in children, suggesting natural immunity in adults. Indeed, immunisation of highland children has dramatically decreased the incidence of pig bel (Murrell and Walker, 1991).

2.4.4 *Escherichia coli*

In recent years, numerous strains of *E. coli* have been associated with foodborne illness. Arguably, the most important pathogenic strains of *E. coli* are the enteroinvasive (EIEC), the enteropathogenic (EPEC), the enterotoxigenic (ETEC), and the enterohaemorrhagic (EHEC). The presence of EIEC, EPEC and ETEC in food and water is indicative of human faecal contamination, and can come directly from the food handler, or from contaminated water. The intestinal tract of ruminant animals, in particular sheep and cattle, is the primary reservoir of EHEC strains (Desmarchelier and Grau, 1997). Other important and emerging strains of pathogenic *E. coli* include enteroaggregative (EAEC) and diffusely adherent (DAEC).

Due to the countless reviews published in recent years on all of the aforementioned pathogenic strains of *E. coli*, they will not be discussed in any further detail here (Clarke *et al.*, 2003; Huang and Dupont, 2004; Chen and Frankel, 2005; Parsot, 2005; Qadri *et al.*, 2005; Servin, 2005; Welinder-Olsson and Kaijser, 2005; Le Bouguenec and Servin, 2006; Spears *et al.*, 2006).

2.4.5 *Listeria monocytogenes*

Although not often associated with gastrointestinal illness, *L. monocytogenes* is now recognised as an important food pathogen. On the basis of current evidence, the vast majority of strains are thought to be capable of pathogenesis (McLauchlin *et al.*, 2004). Listeriosis is an important cause of meningitis in neonates, with fatality rates somewhere in the order of 15 to 30% in developed countries (Braden, 2003; McLauchlin *et al.*, 2004). Data are not available for developing countries, but mortality is expected to be higher. Moreover, microbial infection is a major cause of

stillbirth, particularly in developing countries. Although *L. monocytogenes* is a minor contributor to the problem relative to non-foodborne infections such as syphilis and malaria (Goldenberg and Thompson, 2003), its presence in food is an important public health concern that warrants investigation.

2.4.6 *Salmonella* species

There are numerous reports of salmonellosis in PNG (Morahan, 1968; Gratten *et al.*, 1983; Bukenya *et al.*, 1986; Passey, 1995), with typhoid fever being one of the major contributors to morbidity and mortality (PNG Ministry of Health, 2000). The primary reservoir of *Salmonella* spp. is the intestinal tract of vertebrate animals (Jay *et al.*, 2003), thus infection is often the result of insufficient separation of food and drinking water from faecal waste. Close association with animals, which is common in rural PNG, is also likely to be an important route of infection (Gratten *et al.*, 1983).

Transmission of typhoid fever in PNG is thought to be predominantly person-to-person, rather than waterborne (Passey, 1995). Food might play an important role as a vector in person-to-person transmission, with one study at a mine site in PNG showing that over 3% of individuals tested were carriers for *Salmonella* spp. prior to intervention strategies being implemented. In the broader community of PNG, where sanitation levels are lower, the carrier rate is higher (Schuurkamp *et al.*, 1990). An important factor in the transmission of *Salmonella* food poisoning is the finding that only very small numbers of the organism need to be ingested to cause illness (D'Aoust *et al.*, 1985; Kapperud *et al.*, 1990).

2.4.7 *Staphylococcus aureus*

Numerous studies have been published on the incidence of *S. aureus* in foods in developing countries (Sokari, 1991; Ombui *et al.*, 1992; Desmarchelier *et al.*, 1994; Erku and Ashenafi, 1998; Muleta and Ashenafi, 2001; Mensah *et al.*, 2002; Witthuhn *et al.*, 2005), but there is a paucity of information pertaining to the significance of *S. aureus* foodborne illness in such countries. This is probably in part due to the relatively mild symptoms: as such, the illness goes unreported. The organism is recognised as an important food pathogen in the developed world, although the

incidence has decreased in recent years (Baird-Parker, 2000b). Given that poor handling and storage, and human hygiene are major factors contributing to the incidence of the disease, it seems likely that *S. aureus* is an important contributor to gastrointestinal illness in developing countries such as PNG.

2.5 Mycotoxins and Mycotoxicosis

2.5.1 Introduction

2.5.1.1 Brief overview and definition of mycotoxins

The term mycotoxin first came into use in the early 1960s, following the outbreak of turkey X disease, a mycotoxicosis that resulted in the death of approximately 100,000 turkey poults. Mycotoxins are secondary metabolites produced by filamentous fungi that are toxic to humans and higher animals. They are generally low molecular weight compounds, with most known mycotoxins ranging from 98 to 710 atomic mass units (Cole and Cox, 1981). They are diverse in structure, existing as single heterocyclic rings, six to eight member rings, cyclic peptides and lipid analogues (Hocking and Pitt, 2003).

It is difficult to determine the number of known mycotoxins. Cole and Cox (1981) describe approximately 275 secondary fungal metabolites, of which the majority are toxic to vertebrate animals. In contrast, the Council for Agricultural Science and Technology (CAST) task force report (2003) suggests that there is the potential for between 20,000 and 300,000 unique mycotoxins to exist. This varied estimate is due, in part, to how one defines a mycotoxin. Gravesen *et al.* (1994) described mycotoxins as secondary metabolites of filamentous fungi that can cause chronic or acute disease in vertebrates in small concentrations. There is an obvious need to exclude non-vertebrate animals when defining a mycotoxin, otherwise the term would describe the majority of fungal secondary metabolites. Thus, the number of mycotoxins currently known is probably close to 400 to 500 compounds, the majority of which have only been associated with toxicity in the laboratory environment. Considerably fewer have been linked to human or animal disease

(Kurata, 1990; Bennett and Klich, 2003). Nonetheless, it is likely that there are still many genuine mycotoxins yet to be discovered.

2.5.1.2 Mode of exposure to mycotoxins

Mycotoxins can enter the body through skin contact with mouldy substrates and by inhalation of toxin producing fungal spores, but by far the most common route is through the consumption of contaminated foods (Bennett and Klich, 2003). Episodes of mycotoxicosis are most common in domestic animals, probably due to the less stringent food standards applied to animal feeds compared with food for human consumption. However, humans are still at risk of mycotoxicosis, especially given that many mycotoxins are heat stable compounds, enabling them to maintain toxicity throughout most cooking processes. There are about 20 known mycotoxins that occur in human food and/or animal feed (Kurata, 1990; Trucksess, 2001).

2.5.1.3 Conditions that influence mycotoxin production

Numerous factors interact to dictate mycotoxin production. It is well recognised that not all isolates of a mycotoxigenic species are capable of mycotoxin synthesis (Moss, 1991; Ominski *et al.*, 1994; Pitt *et al.*, 2000). Recent studies have determined at least some of the genes involved in the biosynthetic pathway of various polyketide mycotoxins (Yu *et al.*, 1995; Yu *et al.*, 2002; Ehrlich *et al.*, 2004; Ehrlich *et al.*, 2005; Karolewiez and Geisen, 2005; Kim *et al.*, 2005; Shimizu *et al.*, 2005; Gaffoor and Trail, 2006; Lysoe *et al.*, 2006; Proctor *et al.*, 2006, and others), and molecular techniques have been used to try to determine relatedness between mycotoxigenic and non-mycotoxigenic strains (Geiser *et al.*, 2000; Baird *et al.*, 2006). Yu and Keller (2005) review in detail the current understanding of genetic regulation of secondary metabolite production.

In addition to the mycotoxigenic ability of fungal isolates, various physiochemical parameters influence mycotoxin production. Lillehoj *et al.* (1974) noted increased aflatoxin production when corn was enriched with various trace metals. In a poorly described study, Cuero (2001) concluded that the effect of metal ions on mycotoxin production was dependent on substrate. Further examples of the influence of

substrate and addition of trace metals are provided by Ominski *et al.* (1994). Oxygen levels have also been shown to influence mycotoxin production, with decreased oxygen availability and increased carbon dioxide concentrations leading to lower levels of mycotoxins (Larsen *et al.*, 1998; Filtenborg *et al.*, 2000; Cairns-Fuller *et al.*, 2005).

Rarely does any one factor alone dictate mycotoxin production. Consequently, recent studies have concentrated on the effect of two or more physiochemical parameters on mycotoxin production. Studies have investigated the influence of temperature and water activity (a_w) (Hajjaji *et al.*, 2006; Ramirez *et al.*, 2006; Ribeiro *et al.*, 2006; Valero *et al.*, 2006), a_w and fungal growth duration (Belli *et al.*, 2004), a_w and pH (Patterson and Damoglou, 1986), and pH and fungal growth duration (Esteban *et al.*, 2005), to list just a few. Recently Hope *et al.* (2005) used predictive modelling to estimate deoxynivalenol (DON) concentrations over a time and a_w gradient at different temperatures. The use of such multi-factorial models is widespread in bacteriology to predict growth of spoilage organisms, and is likely to be applied to mycotoxin production more commonly in the future.

Biological factors are also known to influence mycotoxin production. One such factor is insect infestation. Insect damage to crops during growth, and insect infestation of grains during storage, have regularly been associated with high mycotoxin levels (Pier, 1992; Sinha, 1994; Miller, 2001). Another biological factor shown to influence mycotoxin production is growth of competing microbes, but it is difficult to predict what effect they will have. Various yeasts were shown to inhibit ochratoxin A production on artificial media (Masoud and Kaltoft, 2006). However, Lee and Magan (1999) demonstrated increased ochratoxin A production by *Aspergillus ochraceus* when grown in competition with some other fungal isolates on artificial media. Valero (2006) found the effect of competing microbes on ochratoxin production varied with other conditions, such as temperature and water activity.

Due to the broad spectrum of fungal species, mycotoxins, and interactions among physiochemical conditions that have been studied to date, it is difficult, and perhaps misleading, to generalise about the effect of any given parameter on mycotoxin

production. However, it can be concluded that factors such as nutrient availability, a_w, pH, temperature and gas exchange influence mycotoxin production. Furthermore, biological factors such as growth of competitive organisms could result in either an increase or decrease in mycotoxin production.

2.5.1.4 Global distribution of mycotoxin contaminated foods and mycotoxicosis

Mycotoxin contaminated foods are found worldwide, however for numerous reasons the greatest problems are encountered in the developing world. First, many of the world's developing countries are in the tropics, where climatic conditions are well suited for the rapid growth of filamentous fungi (Nicolaides *et al.*, 1998), which can result in mycotoxin production. Secondly, the most commonly regulated mycotoxins (the aflatoxins), are regulated in approximately 50 countries (Moss, 2000), the majority of which are relatively affluent. Thus, most developing countries do not regulate mycotoxin levels, and in those less affluent countries where regulation does occur, such as India and China, maximum allowable limits are considerably higher than in developed countries. As such, people from developing countries are much more likely to be exposed to mycotoxins.

Thirdly, many of the known mycotoxins are produced by species of the genera *Aspergillus* and *Penicillium* (see Section 2.5.2.1), which are associated with commodity storage (Sweeney and Dobson, 1998; Hocking and Pitt, 2003). Traditional storage methods, as used in developing countries, are generally not as well regulated as the modern storage techniques utilised in the developed world. Post harvest losses are a problem worldwide, but more so in the tropics (Aidoo, 1991), where people depend mostly on tradition storage methods. Studies have been conducted on a variety of stored commodities in tropical countries, including limited studies pertaining to stored products in PNG (Shaw *et al.*, 1972; Greve *et al.*, 1994).

Finally, there may be a link between mycotoxicosis and malnutrition. Numerous studies have demonstrated a correlation between dietary aflatoxin exposure and Kwashiorkor, a form of protein-energy malnutrition common in Africa (Hendrickse, 1984; Coulter *et al.*, 1986; Ramjee *et al.*, 1992; Hatem *et al.*, 2005) (see Section 2.5.2.2). As with other disease-nutrition interactions, it is difficult to determine

causality (see Section 2.2.4) but, cumulatively, mycotoxin exposure and malnutrition may have serious health implications.

2.5.2 The main mycotoxins and their producers

2.5.2.1 Mycotoxigenic fungi

The three most important fungal genera in terms of food invasion are *Aspergillus*, *Penicillium* and *Fusarium*, and it is these three genera that also produce the most important mycotoxins (Hocking and Pitt, 2003). In addition, *Claviceps* spp. produces an important group of mycotoxins called the ergot alkaloids. Table 2.01 lists the most important known mycotoxins and the organisms responsible for their production.

The genus *Aspergillus* consists of over 100 species, typically growing in decaying vegetation and also on a variety of human and animal foods (Smith and Ross, 1991; Hocking and Pitt, 2003). It is worldwide in distribution, but particularly abundant in sub-tropical and warm temperate regions. Many species are xerophilic, making them well-adapted to growth in a variety of foods and storage grain. Cereal grains, groundnuts and certain animal feeds are almost exclusively colonised by species of *Aspergillus* and/or *Penicillium* (Smith and Ross, 1991)

The genus *Penicillium* is comprised of approximately 150 species (Pitt, 1979), many of which have been associated with mycotoxin production. The discovery of penicillin in 1929 led to the search for other *Penicillium* metabolites with antibiotic properties. Many of the metabolites identified, such as citrinin and patulin, were found to be toxic to higher animals as well as bacteria, and were subsequently termed toxic antibiotics. They are now recognised as mycotoxins.

Various species of the genus *Fusarium* have been reported to be mycotoxin producers. However, there exist many anomalies in the literature as to which mycotoxins are produced by which species. This problem is due in part to the difficulty of identifying fusaria to species level. Marasas (1991) considered *F. sporotrichades*, *F. poae*, *F. equiseti*, *F. graminearum* and *F. moniliforme* to be the most important toxigenic species of fusaria occurring in animal foodstuffs.

The common mycotoxins that have been associated with human toxicosis through ingestion of contaminated food are discussed below. It is by no means a complete list of mycotoxins, and many that have not been included for discussion may be important aetiological agents in human disease.

Table 2.01: Common mycotoxins and associated human diseases.

Mycotoxin	Main fungal species	Disease/symptoms in humans
Aflatoxins	*Aspergillus parasiticus*, *A. flavus* & *A. nomius*	Hepatitis, hepatic cancer, gastrointestinal haemorrhage, immunosupression
Sterigmatocystin	*A. versicolor*, *A. flavus*, *A. parasiticus*	Possible human carcinogen
Ochratoxin A	*A. ochraceus*, *A. carbonarius* *Penicillium verucosum*	Kidney disease
Cyclopiazonic acid	Numerous penicillia & aspergilli	Kodo poisoning, potentiating agent?
Penitrem	*P. crustosum*	Possible human neurotoxin
Patulin	*P. expansum*	Possible liver & kidney damage
Citrinin	Predominantly *Penicillium* spp.	Kidney disease, potentiating agent?
Tricothecenes	*Fusarium* spp.	Alimentary Toxic Aleukia
Fumonisins	*F. verticillioides*	Oesophageal cancer
Zearalenone	*F. graminearum*	mycoestrogen, dubious link to early onset menarche
Ergot	*Claviceps* spp.	Ergotism: gangrene, ataxia, effects central nervous system

Information from Bennett and Klich (2003) and Hocking and Pitt (2003).

2.5.2.2 Aflatoxins

The term aflatoxin usually refers to a group of four compounds: aflatoxin B_1, B_2, G_1 and G_2. There are other aflatoxins that are described in the literature, but they are the products of microbial or animal metabolism, e.g., aflatoxin M_1 and M_2, which are excreted in cows' milk. Aflatoxins are typically produced by just three species of aspergilli; *Aspergillus parasiticus*, and the closely related *Aspergillus flavus* and *Aspergillus nomius* (Smith and Ross, 1991). Another closely related species, *Aspergillus tamarii*, has also been reported to produce aflatoxin (Goto *et al.*, 1996), although there is some debate over the validity of the finding. Most strains of *A. parasiticus* are potential mycotoxin producers and are able to produce all four

main aflatoxins. In contrast, *A. flavus* typically produces only aflatoxins B_1 and B_2, and only some strains have that ability.

Aflatoxin contamination is primarily a post harvest problem, a result of poor storage practises. However, aflatoxin contamination of crops such as peanuts, corn and cottonseed does occur during growth of the plant. These commodities are also at risk of post-harvest contamination, as too are various nuts, copra, sultanas, spices, pulses, cereal grains, cassava, dried fish and fermented beverages (Denning, 1987; Pittet, 2001).

Aflatoxins are difuranocoumarin derivatives produced by the polyketide pathway. Sweeney and Dobson (1998) discuss in greater detail the synthesis of aflatoxins, as well as other important mycotoxins. Aflatoxin has been ranked as the most potent animal carcinogen (Squire, 1981), although susceptibility to aflatoxins varies greatly with species, age and nutritional status. Experimentally, aflatoxicosis has been shown to occur in trout, pigs, cattle and poultry, as well as other species (CAST, 2003).

In humans, aflatoxin ingestion has been linked to hepatitis and acute encephalopathy in western India, Malaysia and Thailand (Shank *et al.*, 1971; Krishnamachari *et al.*, 1975; Lye *et al.*, 1995). Invariably, it is difficult to obtain conclusive evidence linking such diseases to an aetiological agent. Nevertheless, sufficient evidence exists to suggest that outbreaks of aflatoxicosis occur at least sporadically, particularly in developing countries. Furthermore, individual cases probably occur more frequently, but due to the difficulty of diagnosis remain unrecognised (Denning, 1987; Peraica *et al.*, 1999).

Studies have also linked aflatoxin consumption to two diseases of unknown aetiology, namely Reye's syndrome (Ryan *et al.*, 1979) and Kwashiorkor (Hatem *et al.*, 2005). No clear link between Reye's syndrome and aflatoxin ingestion exists, and indeed there are studies that have claimed aflatoxin plays no role in the aetiology of the disease (Rogan *et al.*, 1985). It was once thought aflatoxin ingestion in combination with viral infection might be the cause of the syndrome (Olso *et al.*, 1971; Becroft and Webster, 1972), but now it is more commonly linked with the treatment of viral infection with aspirin. The cause remains unknown (van Bever *et*

al., 2004), but Vashishtha (2004) suggests that Reye's syndrome is the name given to a heterogeneous group of disorders that have various aetiologies.

Various researchers have suggested a link between aflatoxin exposure and Kwashiorkor (Hendrickse *et al.*, 1982; Apeagyei *et al.*, 1986; Coulter *et al.*, 1986; Hatem *et al.*, 2005). As with other examples of chronic mycotoxicosis, it is very difficult to prove an association between long-term exposure and illness. Nonetheless, aflatoxin exposure is thought to play a role in the disease, but is unlikely to be the sole aetiological agent (Ramjee *et al.*, 1992; Oyelami *et al.*, 1995).

2.5.2.3 Sterigmatocystin

Sterigmatocystin is closely related to the aflatoxins, although it is considerably less toxic. It is predominantly produced by *Aspergillus versicolor, A. flavus* and *A. parasiticus*. In the latter two species it is produced as an intermediate in aflatoxin production (Smith and Ross, 1991; Hocking and Pitt, 2003). While numerous studies have shown sterigmatocystin to be carcinogenic in laboratory animals (Ohtsubo *et al.*, 1978; Mabuchi, 1979; Maekawa *et al.*, 1979; Hendricks *et al.*, 1980), it has not been implicated in human disease to date.

2.5.2.4 Ochratoxins

Ochratoxin A is produced by several species of aspergilli and penicillia, predominantly *Aspergillus ochraeceus* and *Penicillium verrucosum*. In warm and tropical environments, occurrence is typically due to *Aspergillus* spp. (namely *A. carbonaris* and *A. niger*), whereas *P. verrucosum* production dominates in cooler and temperate climates. Contamination can occur pre-harvest, but post-harvest contamination predominates (Pittet, 2001). Ochratoxin A is the most potent of the ochratoxins. It was first described in laboratory studies, but was later found occurring naturally on corn. It has since been found to occur in a vast array of cereals, nuts and feed products (Smith and Ross, 1991).

In humans, ochratoxin A has been associated with outbreaks of Balkan endemic nephropathy, a kidney disease first described by local specialists in the 1950s

(Castegnaro *et al.*, 2006). Studies in the former Yugoslavia demonstrated high levels of ochratoxin A in grains, and the presence of ochratoxin A in some serum samples of people suffering Balkan endemic nephropathy (Krogh *et al.*, 1977; Plestina *et al.*, 1990; Petkova-Bocharova and Castegnaro, 1991). There still remains some doubt about the aetiology of the disease, and factors other than, or in addition to, ochratoxin A contamination of food have been speculated, as too has genetic predisposition (Castegnaro *et al.*, 1991; Abouzied *et al.*, 2002; Castegnaro *et al.*, 2006). It seems probable that ochratoxin A plays some role in the disease, as ochratoxin A has long been known to be nephrotoxic in experimental animals (Theron *et al.*, 1966; Prior *et al.*, 1980; Tapia and Seawright, 1984). Moreover, studies have demonstrated nephrotoxicity of ochratoxin A using human cell lines (Schwerdt *et al.*, 1999; Sauvant *et al.*, 2005). A disease similar to Balkan endemic nephropathy also occurs in northern Africa, where levels of ochratoxin contamination of food is commonplace (Maaroufi *et al.*, 1995a; Maaroufi *et al.*, 1995b; Filali *et al.*, 2002).

2.5.2.5 Various mycotoxins produced by *Penicillium* species and other genera

There are numerous mycotoxins commonly produced by the genus *Penicillium*, and in some instances by other genera also, that have been shown to be detrimental to animals, but their effect on humans is largely unknown.

Cyclopiazonic acid (CPA) is produced by numerous aspergilli and penicillia. Due to the variety of species associated with CPA production it is potentially ubiquitous, but fortunately is less toxic than many mycotoxins. Much of the research to date has been conducted on broiler chickens (Cullen *et al.*, 1988; Smith *et al.*, 1992; Balachandran and Parthasarathy, 1995; Gentles *et al.*, 1999; Kamalavenkatesh *et al.*, 2005), which are susceptible to loss of condition, immunosuppression and liver and kidney damage. Smith *et al.* (1992) found that when administered together in chickens, aflatoxin and CPA could have an additive effect. Retrospectively, it has been suggested that CPA, in addition to aflatoxin, was a contributing aetiological factor to turkey X disease (Cole, 1986).

Cyclopiazonic acid has rarely been associated with human illness, although it has been linked to kodo poisoning in northern India. Symptoms of kodo poisoning

include nausea, vomiting, delirium, intoxication and unconsciousness (Antony *et al.*, 2003). Kodo millet is the staple food for some people in the region, which can become heavily contaminated with *Aspergillus* spp. Lalitha Rao and Husain (1985) found high levels of CPA in millet implicated in human illness.

Penitrem is a potent neurotoxin produced by *Penicillium* spp. It has been associated with disease in various domestic animals (Cysewski *et al.*, 1975; Penny *et al.*, 1979; Peterson *et al.*, 1982; Jortner *et al.*, 1986; Cavanagh *et al.*, 1998), but most naturally occurring outbreaks have been reported in dogs (Arp and Richard, 1979; Hocking *et al.*, 1988; Boysen *et al.*, 2002; Naude *et al.*, 2002; Young *et al.*, 2003). One suspected outbreak in humans has been reported, resulting in tremor, headache, nausea, vomiting, double vision, weakness and bloody diarrhoea (CAST, 2003). In addition to penitrem, there are numerous other tremorgens, produced mainly by *Penicillium* spp., *Aspergillus* spp. and *Claviceps* spp. (Bennett and Klich, 2003).

Patulin is produced by numerous species of *Penicillium*, with perhaps the most important one being *Penicillium expansum.* Much of the initial work concentrated on the antimicrobial properties of patulin, but when it was found to be toxic to animals it was reclassified as a mycotoxin. *Penicillium expansum* is an important post-harvest pathogen of apples and pears, and patulin can often be detected in products such as juices and ciders. Patulin has been shown to be toxic at high doses in laboratory animals, but the occurrence of human poisoning is not well documented (Bennett and Klich, 2003; Hocking and Pitt, 2003).

Citrinin is produced by numerous species of *Penicillium*, and fewer species of *Aspergillus*. It is known to be nephrotoxic in various experimental animals (Carlton *et al.*, 1974; Mehdi *et al.*, 1981; Mehdi *et al.*, 1984; Lura *et al.*, 2004), and has been linked to disease in cattle (Griffiths and Done, 1991). The role of citrinin in human illness is unknown, although it has been suggested that it may have a synergistic effect with other mycotoxins. It has been hypothesised that citrinin plays a potentiating role in the aetiology of Balkan endemic nephropathy (Pfohl-Leszkowicz *et al.*, 2002). Furthermore, citrinin has been associated with yellow rice syndrome. After the Second World War, Japan was heavily dependent on imported rice. Much of this rice (along with locally produced rice) was heavily contaminated with storage

fungi, predominantly *Aspergillus* spp. and *Penicillium* spp. Citrinin was one of many toxins produced by fungal isolates, and was shown to cause renal damage in laboratory animals. Recommendations were made to the Japanese government to regulate the quality of imported rice on the findings of toxicology studies using laboratory animals, perhaps preventing human disease (Saito *et al.*, 1971; Udagawa and Tatsuno, 2004: abstract only, article in Japanese).

2.5.2.6 Trichothecenes

The trichothecenes are the largest family of mycotoxins, some of which are highly toxic (Cole and Cox, 1981). They are produced predominantly by *Fusarium* spp., but other genera, including *Trichothecium*, *Trichoderma*, *Myrothecium* and *Stachybotrys*, are also known to produce trichothecenes (CAST, 2003). The trichothecenes inhibit protein synthesis and have been shown to be toxic to a variety of laboratory animals. It has been suggested that they also have an immunosuppressive effect on laboratory animals (Niyo *et al.*, 1988; Rotter *et al.*, 1996). General symptoms of trichothecene toxicosis in animals include weight loss, feed refusal, haemorrhage, vomiting and bloody diarrhoea, among others (CAST, 2003).

In humans trichothecenes are thought to be responsible for mouldy grain intoxications in Japan (Bennett and Klich, 2003), and have been associated with Alimentary Toxic Aleukia. The disease is endemic to the former USSR, and has been observed sporadically throughout the 20th century. The disease occurs after the consumption of over-wintered grains that have become infected with *Fusarium*. Within hours, mild inflammation of the mouth and stomach develop into gastroenteritis. Several weeks later there is evidence of damage to haematopoietic tissues and bleeding from various orifices (Joffe, 1978; Shank, 1978; Bennett and Klich, 2003). Alimentary Toxic Aleukia is a classic example of synergistic effects of food security and mycotoxicosis. Outbreaks of the disease were most widespread and severe in the early 1940s, during the Second World War. During this time of hardship people were reduced to consuming grain that had been covered with snow during winter and spring. The disease was more common in rural areas and populations fed a balanced diet were less susceptible to the illness (Joffe, 1978).

2.5.2.7 Fumonisins

Fumonisins are produced by various species of *Fusarium*, most notably *Fusarium verticillioides*. This species is found on virtually all corn samples, but only a few strains produce the toxin. Fumonisins are responsible for animal diseases such as Equine Leukoencephalomalacia and Porcine Pulmonary Oedema. They have also been demonstrated to be hepatotoxic and carcinogenic to rats (CAST, 2003). In humans fumonisins have been linked to oesophagus cancer in the Transkei region of South Africa and in parts of China (Marasas *et al.*, 1988; Chu and Li, 1994). The reader is referred to Marasas (2001) for a review of the discovery of fumonisins and their carcinogenic activity.

2.5.2.8 Zearalenone

Zearalenone, produced by *Fusarium graminearum*, is not a toxin in the true sense of the word, but is biologically active because it resembles oestrogen. Thus it is better described as a non-steroidal oestrogen. Zearalenone has been reported to be the cause of various reproductive problems in domestic animals, particularly pigs (Dacasto *et al.*, 1995) . In humans little is known about the effect of zearalenone, however it was speculated that it might be responsible for the precocious sexual development in children in Puerto Rico (Schoental, 1983).

2.5.2.9 Ergot alkaloids

Ergotism is the oldest known mycotoxicosis. The common structure of the ergot alkaloids was first determined in 1934 (Bennett and Klich, 2003), thirty years prior to the term mycotoxin even being coined. But the history of ergotism in humans probably predates the discovery of its structure by thousands of years. Anecdotal evidence exists for cases of ergotism in biblical times, and more substantial evidence suggests it was epidemic in the Middle Ages (Shank, 1978; Bennett and Klich, 2003).

Ergot alkaloids are produced by toxigenic strains of *Claviceps purpurea* and *Claviceps paspali*, which are common pathogens of various grasses. The fungi

invade the carpel of the host plant and replace it, producing a sclerotium. Ergotism can affect either the central nervous system, resulting in convulsions and hallucinations, or the blood supply, resulting in gangrene (CAST, 2003). With increased understanding of the cause of ergotism, its occurrence in humans has decreased greatly, however outbreaks still occur in animals (Blaney *et al.*, 2000; Botha *et al.*, 2004; Handeland and Vikoren, 2005).

2.6 Biological Toxins with a Demonstrated Haemolytic Activity

2.6.1 Introduction

Compounds of diverse origin exhibit haemolytic activity *in vitro*. These compounds are typically also active against cell types other than erythrocytes, thus could be more accurately termed cytolysins. The term haemolysin is commonly used because erythrocytes are used in laboratory studies to test for the presence of cytolytic activity (Bernheimer, 1988; Ludwig, 1996). For consistency, the term haemolysin will be used throughout this review for compounds with demonstrated haemolytic activity.

To date, haemolysins of bacterial origin have attracted the most attention, but haemolysins of fungal, protist, protozoan, plant and animal origin have also been isolated (see ensuing discussion for details). The role haemolysins play in the various organisms differs. Haemolysins of microbial origin, particularly the bacterial haemolysins, often confer virulence, whereas cytolysins produced by invertebrate animals are associated with toxicity (Eno *et al.*, 1998; Lagos *et al.*, 2001; Santamaria *et al.*, 2002), and are thought to play a role in immunity (Rowe and Welch, 1994).

Despite the role microbial haemolysins play in virulence, very few cause *in vivo* haemolysis in humans or other animals. Similarly, haemolysins of plant and animal origin are seldom associated with *in vivo* haemolysis in humans. A review of the major haemolysins follows, with an emphasis on organisms and their haemolysins that are known to produce *in vivo* clinical haemolytic crisis.

2.6.2 Bacterial haemolysins

2.6.2.1 Overview of bacterial haemolysins

Haemolysins are produced by a wide variety of both Gram positive and Gram negative bacteria. Indeed, the list of bacterial genera that demonstrate haemolysis *in vitro* is exhaustive, and include common pathogens such as *Aeromonas, Bacillus, Clostridium, Escherichia, Listeria, Pasteurella, Staphylococcus, Streptococcus* and *Vibrio* (Braun and Focareta, 1991), to name but a few.

Bacterial haemolysins can be categorised as enzymatic, pore-forming or surfactant, according to the mechanism of lysis of target cells. Enzymatic haemolysins include the phospholipases and sphingomyelinase found in Gram positive bacteria. They are highly substrate specific, and are active on more than one target cell, i.e., they can be re-used. The pore-forming haemolysins form transmembrane pores. The toxin binds to and penetrates the target membrane and increases membrane permeability. Surfactants are highly hydrophobic and have a detergent like action on cell membranes. They are typically thermostable, but are considerably less haemolytic than enzyme haemolysins and pore-forming haemolysins (Rowe and Welch, 1994). Examples of each category are provided in the ensuing discussion.

2.6.2.2 Clostridial haemolysins

Numerous toxins have been isolated and characterised from *Clostridium* spp., many of which are haemolytic *in vitro*. For pathogenic species of clostridia, toxins have been assigned a letter of the Greek alphabet in order of discovery. Consequently, no relation necessarily exists between clostridial toxins across species that share the same name. For this reason it is difficult to discuss the clostridial toxins collectively, other than to say that they are a group of biologically active proteins, which are often lethal to a variety of animals including humans, and have molecular masses of between 22 and 600 kDa. Many clostridial species, including *C. tetani, C. perfringens, C. sordellii, C. haemolyticum, C. histolyticum* and *C. septicum* produce haemolytic toxins (Hatheway, 1990).

In terms of *in vivo* haemolysis, the most important species of the genera, and possibly the most important of all bacterial species, is *C. perfringens*. This organism has been associated with sepsis and subsequent acute intravascular haemolysis for over 50 years, with reports of such incidences still commonplace, particularly in those with underlying malignancies or other illness (Felix and Davey, 1987; Borrego *et al.*, 1991; Pun and Wehner, 1996; Perseghin *et al.*, 1997; Alvarez *et al.*, 1999; Barrett *et al.*, 2002; Fukuhara *et al.*, 2002; Hamoda and Chamberlain, 2002; Jimenez *et al.*, 2002; Kreidl *et al.*, 2002; Vaiopoulos *et al.*, 2004; Au and Lau, 2005; Pirrotta *et al.*, 2005; McArthur *et al.*, 2006). Interestingly, one report documents acute intravascular haemolysis resulting from foodborne infection (Lantelme *et al.*, 1995).

Despite multiple reports of acute intravascular haemolysis as a sequela of *C. perfringens* bacteraemia, little is known about the pathophysiology of this progression, which is commonly fatal. The most potent *C. perfringens* toxin is α-toxin, an extracellular enzyme. In a series of complex studies using erythrocytes from various mammals, Ochi and co-workers have investigated the mechanisms of haemolysis in α-toxin (Ochi *et al.*, 1996; Nagahama *et al.*, 2002; Ochi *et al.*, 2003; Ochi *et al.*, 2004). The toxin is able to hydrolyse phosphatidylcholine and sphingomyelin, both of which are important erythrocyte membrane components. On the basis of its high toxicity and intense haemolytic nature, it is thought that α-toxin, a phospholipase C, is responsible for episodes of acute intravascular haemolysis associated with *C. perfringens* sepsis (Batge *et al.*, 1992; Lee, 1993; McKenzie, 1996). Currently, it is the only bacterial toxin known to directly cause haemolysis in humans (Berkowitz, 1991).

Other examples of acute intravascular haemolysis resulting from clostridial infection exist. There is one example of *C. hathewayi* septicaemia resulting in haemolytic crisis and subsequent death in a human (Linscott *et al.*, 2005). In domestic animals, pathology results suggest that an autoimmune haemolytic anaemia results as a sequela of *C. perfringens* infection in horses. The mechanisms by which *C. perfringens* induce immune mediated haemolysis are unknown, but clostridial toxins are thought to play a role (Reef, 1983; Weiss and Moritz, 2003). In cattle *C. haemolyticum* has been linked to red water disease, an acute haemolytic crisis with a high fatality rate (Hall, 1929; Hatheway, 1990).

2.6.2.3 Haemolysins from other Gram positive bacteria

Many of the toxins produced by *B. cereus* are haemolytic, including haemolysin BL, cytotoxin K, haemolysin I (cereolysin), haemolysin II, haemolysin III, and phospholipase C (Bernheimer and Grushoff, 1967; Beecher and MacMillan, 1991; Baida and Kuzmin, 1996; Lund *et al.*, 2000b; Miles *et al.*, 2002), some of which have been shown to act synergistically (Beecher and Wong, 2000). The role of cytotoxin K and haemolysin BL in foodborne illness are well described. However, Jensen and Moir (2003) indicate that, in terms of *B. cereus* toxins, the greatest focus to date has concentrated on identifying the toxic moieties rather than determining pathogenicity. On rare occasions *B. cereus* has been associated with clinical cases of *in vivo* haemolysis (Rodgers *et al.*, 1980; Arnaout *et al.*, 1999), but what role, if any, the known haemolysins play is yet to be determined. Rodgers *et al.* (1980) found that cell free supernatant of a *B. cereus* strain isolated from a fatal case of septicaemia was unable to lyse erythrocytes, whereas the culture material was, which suggests that secreted toxins were not responsible.

Staphylococcus aureus produces numerous haemolytic compounds, including the α, β, γ and δ haemolysins. Of these toxins, α haemolysin is the most thoroughly studied, and is responsible for the complete haemolysis most *S. aureus* strains exhibit when grown on blood agar (Dinges *et al.*, 2000). It is thought to be involved in various disease states caused by *S. aureus*, but the role of the toxin has not been conclusively established. The formation of pores in membranes of various cell types is likely the most important mechanism of pathogenesis (Dinges *et al.*, 2000; Menestrina *et al.*, 2001). The toxin is not associated with human haemolysis, and in fact is poorly haemolytic towards human erythrocytes (Bhakdi and Tranum-Jensen, 1991). The main function of the proteinaceous staphylococcal toxins is probably to convert infected tissue into nutrients required for bacterial growth (Dinges *et al.*, 2000).

Four other haemolysins from Gram positive bacteria that are worthy of brief mention are perfringolysin (θ-toxin) from *C. perfringens*, listeriolysin O, from *L. monocytogenes*, streptolysin O from *Streptococcus pyogenes*, and pneumolysin from *Streptococcus pneumoniae*. These haemolysins are a closely related group

known as cholesterol-dependent cytolysins, or thiol-activated haemolysins. They become inactive at low concentrations of cholesterol, or when exposed to oxygen (Braun and Focareta, 1991). These toxins are known to be lethal in animal models, and it has recently been shown that they lose lethality if their haemolytic activity is inhibited (Watanabe *et al.,* 2006).

The bacterial species that produce the cholesterol-dependent cytotoxins, and also *Staphylococcus* spp., are well known human pathogens, but are not generally associated with *in vivo* haemolysis in humans. Lee (1993) cites examples of haemolysis of children infected with streptococcal, staphylococcal or pneumococcal infection, however the most recent reference is 45 years old. This suggests that either such sequelae are extremely rare, or that the aetiology of the haemolysis was falsely attributed to the bacterial infection in times when conclusive aetiological evidence was harder to attain.

2.6.2.4 Haemolysins from *Escherichia coli*

Most haemolysins found in the Gram negative bacteria (often more accurately referred to as cytolysins) are pore-forming protein haemolysins. The Gram negative haemolysins differ from Gram positive haemolysins in that they are usually synthesised as inactive precursor proteins which are activated through proteolytic processing in the cytoplasm or membrane, or post-secretion. Many of the Gram negative haemolysins are structurally and functionally related pore-forming proteins containing a repeated nine-residue amino acid sequence. Consequently they are commonly referred to as repeat in toxin (RTX) toxins (Braun and Focareta, 1991; Ludwig, 1996).

The most extensively studied RTX toxin is α-haemolysin from *E. coli*. As well as being haemolytic, α-haemolysin is active against various nucleated cells, including leukocytes (Bhakdi *et al.,* 1990). The toxin is associated with strains of *E. coli* causing extra-intestinal infections, and its role in virulence has been demonstrated in animal models (Welch *et al.,* 1981; Hacker *et al.,* 1983).

Gastrointestinal infection by some strains of *E. coli*, namely the EHEC, can result in haemolytic uraemic syndrome (HUS) (Karmali *et al.*, 1983). This syndrome eventuates in about 10% of EHEC infections, with children under 10 years old and the elderly being the most susceptible (Nataro and Kaper, 1998). The nomenclature of this group of *E. coli* is rather confusing, with EHEC being the pathogenic subset of a group of organisms sometimes called shigatoxigenic *E. coli* (STEC), and sometimes referred to as verotoxigenic *E. coli* (VTEC). The major virulence factor of EHEC is shiga toxin. There are two main groups of shiga toxin, stx 1 and stx 2, and an EHEC may have one or both of these toxins present. The stx toxins cause occlusion of the small blood vessels in the kidney glomeruli, leading to decreased kidney function, and resulting in kidney failure (Nataro and Kaper, 1998). Numerous recent review articles are available for a more detailed discussion of the occurrence and pathogenesis of EHEC and resulting HUS (Karmali, 2004; Karch *et al.*, 2005; Tarr *et al.*, 2005).

Although α-haemolysin has been shown to be a virulence factor for extra-intestinal *E. coli* infections, it seems to play no role in HUS, being present in only a small percentage of pathogenic isolates. However, a different RTX haemolysin, enterohaemolysin, is commonly associated with pathogenic strains. Beutin *et al.* (1989) found the vast majority of STEC strains tested were positive for enterohaemolysin (89%), but only 5% had α-haemolysin. A more recent study with a larger sample size detected enterohaemolysin in 85% of STEC strains (Beutin *et al.*, 2004). Similarly, Taneike and co-workers found over 98% of EHEC associated with illness in Japan in 1996 were positive for enterohaemolysin (Taneike *et al*., 1998, cited in Taneike *et al.*, 2002).

The association of enterohaemolysin with strains of EHEC has led researchers to conclude that enterohaemolysin is a virulence factor. The precise role of enterohaemolysin in pathogenesis is yet to be elucidated, but various findings suggest that it plays an important role. It is commonly associated with strains that cause HUS, but less commonly associated with non-HUS strains (Schmidt and Karch, 1996); EHEC strains that did not produce stx but did produce enterohaemolysin have been isolated from HUS patients (Schmidt *et al.*, 1999); and enterohaemolysin lyses erythrocytes, with the liberated haeme and haemoglobin

stimulating growth of the bacteria *in vitro* (Law and Kelly, 1995). Despite these findings, enterohaemolysin is not likely to be the primary cause of haemolysis in HUS. Erythrocytes are thought to become damaged during traversal of the occulated blood vessels in the kidney (Nataro and Kaper, 1998). These resulting schistocytes are prone to extravascular haemolysis.

Escherichia coli is not the only organism that has been aetiologically linked to HUS, with numerous outbreaks having been associated with *Shigella dysenteriae* (Kovitangkoon *et al.*, 1990; Nathoo *et al.*, 1995; Bhimma *et al.*, 1997; Azim *et al.*, 1999; Oneko *et al.*, 2001; Houdouin *et al.*, 2004), and rare cases associated with *Salmonella* sp. (Albaqali *et al.*, 2003) and *Campylobacter* sp. (Chamovitz *et al.*, 1983).

2.6.2.5 Haemolysins from other Gram negative bacteria

Other Gram negative bacteria are also haemolytic, including (but not confined to) genera of *Enterobacteriaceae*. Koronakis *et al.* (1987) showed haemolysins in two species of *Proteus* and one species of *Morganella* to be related to *E. coli* α-haemolysin, and Prada and Beutin (1991) detected genes for α-haemolysin in *Enterobacter cloacae*. Other RTX toxins have been identified in *Pasteurella haemolytica* (Thumbikat *et al.*, 2003), *Pasteurella aerogenes* (Kuhnert *et al.*, 2000) and *Actinobacillus pleuropneumoniae* (Maier *et al.*, 1996).

Gram negative genera worthy of brief mention are *Serratia, Vibrio, Aeromonas* and *Pseudomonas*. The two partner secretion system in *Serratia* pore-forming haemolysins is notably different to other well described modes of haemolysis such as that found in *E. coli*, *S. aureus* or β-haemolytic streptococci. Similar toxins to those found in *Serratia* are though to exist in various other Gram negative bacteria (Hertle, 2005).

The role of haemolysins in the pathogenesis of *Vibrio* spp. is unclear. The classical biotype of *V. cholerae* is non-haemolytic, but the El Tor biotype is capable of extracellular haemolysin production (Ludwig, 1996). Clinical isolates of *Vibrio parahaemolyticus* are typically capable of haemolysis under specific

conditions, known as the Kanagawa phenomenon. This species also produces other haemolysins that are not linked to pathogenesis (Desmarchelier, 2003).

Aerolysin is a pore-forming haemolysin isolated from *A. hydrophila* and *A. sobria*. Chakrabotry *et al*. (1987) demonstrated an association between virulence and aerolysin using an animal model, and in separate work suggested aerolysin shared some similarities to *S. aureus* α-toxin (Chakraborty *et al.*, 1990).

Finally, *Pseudomonas aeruginosa* produces two non-pore-forming haemolysins; a heat-labile phospholipase C, and a heat-stable haemolysin consisting of two acidic glycolipids. The heat stable haemolysin is active through a detergent-like mechanism (Fujita *et al.*, 1988). Haemolysis observed on blood agar plates is attributed to the phospholipase C, as the heat stable haemolysin is inhibited by serum (Johnson and Boese-Marrazzo, 1980).

2.6.3 Fungal haemolysins

2.6.3.1 Overview of fungal haemolysins

Studies of the haemolytic nature of fungi commenced over half a century ago, but there are considerably fewer fungal haemolysins described in the literature than there are bacterial haemolysins. This is indicative of the pathogenic nature of the two groups of organisms. Bacteria are commonly associated with infection and sepsis of humans and other higher animals. By comparison, fungi only rarely cause infection and sepsis in humans and higher animals.

2.6.3.2 Haemolysins from yeasts

The haemolytic activity of yeasts was first described in the early 1950s, when Salvin (1951) observed haemolytic activity in *Candida albicans, Cryptococcus neoformans, Histoplasma capsulatum* and *Blastomyces dermatitidis*. Very little work was published on the haemolytic activity of yeasts over the next four decades, but in recent years studies have concentrated on the haemolytic activity of *Candida* spp. A study on the haemolytic activity of *C. albicans* by Manns *et al.* (1994) revealed three

main points of interest. First, haemolytic activity of *C. albicans* was much more pronounced on blood agar supplemented with 3% glucose than on normal blood agar. This is an important finding for studies of haemolytic activity in yeasts, and suggests that glucose plays a role in haemolytic activity of yeasts. Secondly, *C. albicans* was shown to more readily lyse complement-opsonised red blood cells than non-opsonised red blood cells, suggesting that complement mediated haemolysis might occur *in vivo*. Finally, the hyphal form demonstrated greater haemolytic activity than the yeast form. Following on from the later finding of Manns and co-workers, Watanabe *et al.* (1997) found there to be more haemoglobin receptors on hyphal cells than on yeast cells in *C. albicans*, and found hyphal cells had the ability to use haemoglobin as an iron source, but yeasts cells did not.

In subsequent work the same research team suggested that the haemolytic factor in *C. albicans* is a secreted, heat stable mannoprotein, of which the sugar moiety is particularly important in the haemolytic process (Watanabe *et al.*, 1999). The researchers concluded that mannoprotein induced haemolysis around the site of infection might enhance *C. albicans* growth, but the compound was unlikely to cause systemic haemolysis.

It is unclear as to whether the mannoprotein is the sole contributor to haemolytic activity in *Candida* spp. Despite its presence in some *C. albicans* strains, it seems unlikely that phospholipase B contributes to haemolysis. Samaranayake *et al.* (2005) found no statistical correlation between haemolytic activity and expression of the phospholipase gene. In a different species, namely *C. glabrata*, Lachke *et al.* (2000) detected a gene for a haemolysin-like protein. The deduced amino acid sequence was similar to that of haemolysins from various bacteria and eukaryotic organisms. However, haemolytic activity was not tested for in this study. Also using *C. glabrata*, Luo *et al.* (2001) were able to discount phospholipase A on account of its low rate of presence in haemolytic isolates. In a later study Luo *et al.* (2004) found a correlation between haemolytic activity on blood agar (using arbitrary units) and expression of the haemolysin-like protein gene. Despite the rudimentary method of determining haemolytic activity, it suggests further study into the structure and nature of haemolytic compounds from yeasts is warranted.

2.6.3.3 Haemolysins from filamentous fungi

As with investigations into the haemolytic activity of yeasts, the study of haemolytic activity of filamentous fungi commenced some time ago (Henrici, 1939), but little progress was made over the next four decades. Early work was conducted on *Aspergillus* spp., investigating a number of virulence factors. One species, *Aspergillus fumigatus*, was found to be haemolytic (Budzko and Negroni, 1975).

Since the mid 1970s considerable work has been conducted on haemolytic compounds from *A. fumigatus*, much of it by the one group of Japanese researchers. This group lay claim to having purified the first haemolysin from a fungi: asp-haemolysin from *A. fumigatus*. The haemolysin was described as a sugar which contains protein (as opposed to being a protein, like many of the prokaryotic haemolysins), with a molecular weight of approximately 30 kDa (Yokota *et al.*, 1977). This is significantly larger than any of the mycotoxins listed in Cole and Cox (1981) and is more analogous to bacterial haemolysins. Considerable work has been conducted on the nature of asp-haemolysin since, but much of this work centres on its potential use as a chemotherapeutic agent rather than any role it might play in virulence (Kudo *et al.*, 1999; Kudo *et al.*, 2001; Kudo *et al.*, 2002) . The haemolysin has been found to be inactivated by serum (Fukuchi *et al.*, 1996; Fukuchi, 2001), so is unlikely to cause *in vivo* haemolysis.

In some of the more recent work to be conducted on fungal haemolysins, Vesper and co-workers (Vesper *et al.*, 1999; Vesper *et al.*, 2000; Vesper *et al.*, 2001) looked at the haemolytic activity of *Stachybotrys chartarum* strains. This fungus has been linked to various human and veterinary diseases, including pulmonary haemorrhage and haemosiderosis (Vesper *et al.*, 2000). As with most other microbial toxins described to date, *S. chartarum* haemolysin (stachylysin) does not cause acute intravascular haemolysis, but might play a role in the pathophysiology of pulmonary haemorrhage. Vesper and Vesper (2002) suggested that stachylysin was involved in haemorrhaging in experimental earth worms, however the extrapolation of these findings to humans should be interpreted with some caution. The authors have suggested a link between haemolysis production in *S. chartarum* and virulence,

(Vesper *et al.*, 1999; Vesper *et al.*, 2000), although they have thus far been unable to prove it.

A small number of other filamentous fungi or their metabolites demonstrate haemolytic activity. The polypeptide paracelsin, isolated from *Trichoderma reesei*, was shown to be haemolytic by Bruckner and co-workers (1984). The pathogenic dermatophytic *Trichophyton* spp. were shown to be haemolytic on blood agar (Schaufuss and Steller, 2003; Schaufuss *et al.*, 2005). Recently, the haemolysins chrysolysin and nigerlysin have been isolated from *P. chrysogenum* and *A. niger*, respectively (Donohue *et al.*, 2005; Donohue *et al.*, 2006).

2.6.3.4 Haemolytic activity of common mycotoxins

In addition to high molecular weight haemolysins, some of the common mycotoxins are also haemolytic. The effect of the trichothecene T-2 on erythrocytes has been shown to vary with species. Khachatourians (1990) suggested that T-2 has a haemolytic affect on erythrocytes from non-ruminant animals, including humans, but not on ruminant erythrocytes. This trend was linked to the presence of phosphatidylcholine in the erythrocyte membrane of non-ruminants, and its absence in ruminants.

The effect of T-2 toxin on erythrocytes has also been shown to vary according to osmolarity of the solution. The toxin provided a protective effect to guinea pig erythrocytes in hypotonic solutions, but was detrimental in isotonic solutions (Gyongyossy-Issa *et al.*, 1986). Although the authors claimed that this finding helped explain the interaction between erythrocyte membranes and T-2, it is unlikely to be of any clinical significance, given that erythrocytes are not subjected to hypotonic conditions *in vivo*.

Using rat erythrocytes, Rizzo and co-workers determined that T-2 toxin was only haemolytic at concentrations above 130 mg/l *in vitro*, and speculated the mode of action of the toxin on erythrocytes. However, this finding is of little practical use, as the lethal dose that results in 50% mortality (LD50) in various animals in considerably lower than the concentration required for haemolytic activity. Grizzle *et*

al. (2004) found the lethal dose required to kill half of the animals (LD50) of T-2 toxin in quail to be 14.7 mg/kg body weight, and in rats the LD50 was 3.71 mg/kg body weight (McKean *et al.*, 2006). Thus, one would not expect T-2 toxin to be haemolytic *in vivo*, and indeed testing for haemolytic activity at such high concentrations is of questionable scientific merit.

The haemolytic activity of aflatoxin, generally considered the most potent of the mycotoxins, on rabbit erythrocytes has been studied (Verma and Raval, 1991). The authors found aflatoxin to be haemolytic at a concentration of 1.4 mg/l and above. This is approximately half the LD50 of rats, found to be 2.71 mg/kg body weight (McKean *et al.*, 2006). Given that data for haemolysis and LD50 were obtained using different species, it is difficult conclusively to determine the role of aflatoxin in haemolysis *in vivo*. Nonetheless, on the basis of information presented, it is feasible that aflatoxin could have a haemolytic effect *in vivo*.

2.6.3.5 Haemolysins from higher fungi

Higher fungi, specifically mushrooms, are well known for their ability to produce potent toxins. The general toxicity of higher fungi is discussed in detail elsewhere (Karlson-Stiber and Persson, 2003; Berger and Guss, 2005a; Berger and Guss, 2005b). Some of the toxins isolated from higher fungi have demonstrated haemolytic activity under laboratory conditions. Seeger (1975a; 1975b) described phallolysin, a protein extracted from *Amanita phalloides*, and found it to be haemolytic (Seeger *et al.*, 1976) *in vitro*. Seitz (1981) suggested the mode of haemolysis of phallolysin was through cation leakage, resulting in osmotic damage to erythrocytes. Although phallolysin is highly toxic to various laboratory animals when administered intravenously, when given orally phallolysin was not toxic to rats (Odenthal *et al.*, 1975).

Numerous other higher fungi are also known to produce haemolysins. Seeger *et al.* (1981) demonstrated rubescenslysin, a protein isolated from *Amanita rubescens*, to be toxic to mice and rats, causing intravascular haemolysis. Odenthal *et al*. (1982) demonstrated that rubescenslysin was haemolytic, and generally toxic to multiple organs and cell types. Pelger and Watling (1982) listed two species of *Amanita*,

namely *Amanita rubescens* and *Amanita vaginata*, that produce an unknown haemolysin. Thorough cooking prior to consumption was recommended to destroy the heat labile toxin.

Bernheimer and Oppenheim (1987) found flammutoxin from the edible mushroom *Flammulina velutipes* to be haemolytic, and suggested its mode of action was similar to that of phallolysin. Recent studies have shown flammutoxin to be present as a protoxin which is activated by truncation, a phenomena commonly observed in a variety of prokaryotic and eukaryotic cytolysins (Tomita *et al.*, 2004a). Studies of haemolysins of higher fungi continue, but as stated by Tomita *et al.* (2004b), the physiological function of mushroom cytolysins remains enigmatic.

Cases of severe clinical haemolysis have been reported in humans in Western Europe following consumption of the edible mushroom *Paxillus involutus* (Winkelmann *et al.*, 1982: abstract only, article in German; Winkelmann *et al.*, 1986). It seems that after repeated ingestion some individuals produce an immune response to mushroom antigen. Immune complexes bind to erythrocytes, which are considered 'innocent bystanders', and activation of the complement system results in haemolysis. The illness can be fatal. For further details of 'innocent bystander' haemolysis, refer to McKenzie (1996).

2.6.4 Haemolytic anaemia associated with protozoan infections

Parasitaemia has been linked to *in vivo* haemolysis in humans, particularly malaria infection, but also toxoplasmosis and trypanosomiasis. No specific haemolysins have been associated with haemolytic parasitaemias. In *Plasmodium* spp. infection, the mechanism of invasion has numerous detrimental effects on erythrocytes including the increased destruction of erythrocytes in the spleen, decreased life span, increased osmotic fragility and changes to membrane lipids. Moreover, even non-parasitised erythrocytes are destroyed at increased rates (Lee, 1993).

Malaria infection usually results in only mild anaemia. However, a rare complication of falciparum malaria known as Blackwater fever, results in an acute haemolytic crisis. The aetiology of Blackwater fever remains unresolved, although many of the

early studies linked the disease with quinone treatment. The disease was common in Europeans living in tropical areas who had little or no natural immunity against malaria (Rogier *et al.*, 2003). It became uncommon in the latter half of the 1900s, when chloroquine was the most widely used antimalarial drug, but with increased resistance to chloroquine, quinine has been used increasingly in recent years, resulting in a re-emergence of Blackwater fever (Gobbi *et al.*, 2005).

For further details of haemolytic parasitaemias, including malaria and associated complications, the reader is referred to Lee (1993).

2.6.5 Haemolysins of plant and animal origin

2.6.5.1 Haemolysins of plant and algal origin and plant associated haemolytic anaemia

Saponins have been isolated from a wide variety of trees and shrubs (Gestetner *et al.*, 1971a; Gestetner *et al.*, 1971b; Hahn *et al.*, 1989; Nose *et al.*, 1989; Rickling and Glombitza, 1993; Guinea *et al.*, 1994; Santos *et al.*, 1997; Oda *et al.*, 2000; Apers *et al.*, 2001; Woldemichael and Wink, 2001). They are amphipathic glycosides consisting of a hydrophobic aglycone and hydrophilic sugar chains (Oda *et al.*, 2000). Saponins are able to bind free or membrane associated cholesterol, with the capability to form pores in biological membranes (Campbell and Peerbaye, 1992). Saponins have received attention because they demonstrate adjuvant activity, however their haemolytic activity has ensured that to date they are not widely used in vaccines (Campbell and Peerbaye, 1992; Santos *et al.*, 1997; Oda *et al.*, 2000). The exact mechanism of haemolytic activity remains unknown, as too does the genuine potential for *in vivo* haemolysis.

Haemolytic compounds not considered to be saponins have also been isolated from algae and bracken ferns (Igarashi *et al.*, 1998; Tjatur Rasa *et al.*, 1999a; Tjatur Rasa *et al.*, 1999b). Bracken ferns are known to cause intoxication in a variety of animals, and it has been suggested that a haemolytic compound contributes to their toxicity (Tjatur Rasa *et al.*, 1999a).

The occurrence of *in vivo* haemolysis as a result of consumption of plants and plant products is known in humans. In particular, the association between fava bean (*Vicia fava*) ingestion and haemolysis in people with glucose-6-phosphate dehydrogenase deficiency is well documented. The illness, termed favism, most commonly occurs in children, but is capricious in occurrence. The onset of symptoms occurs 5–24 hours after ingestion of fava beans, and are typical of those of haemolytic crisis (Lukens, 1993). Glucose-6-phosphate dehydrogenase deficiency, and other enzyme deficiencies, have also been linked to haemolytic crisis in individuals on exposure to certain drugs and infections (Lukens, 1993).

Haemolytic anaemia following the consumption of certain foods has been reported in domestic animals. Onions can induce haemolytic anaemia in dogs (Stallbaumer, 1981; Harvey and Rackear, 1985; Yamoto and Maede, 1992), brassicas have been linked to haemolysis in ruminant animals (Taljaard, 1993; Prache, 1994), and experimentally, bracken fern extract has caused haemolysis in guinea pigs (Tjatur Rasa *et al.*, 1999a).

2.6.5.2 Haemolysins of animal origin

Some marine invertebrates are known to produce haemolytic compounds. Sea cucumbers and starfish produce saponins and saponin-like compounds, some of which are haemolytic (Kaul and Daftari, 1986; Kalinin *et al.*, 1996; Kouriki-Nagatomo *et al.*, 1999). A variety of toxins and biologically active substances have been isolated from sea anemones, including some which are haemolytic *in vitro* (Shiomi *et al.*, 1985; Eno *et al.*, 1998; Huerta *et al.*, 2001; Lagos *et al.*, 2001; Santamaria *et al.*, 2002). Haemolysins from sea anemones are generally basic proteins with molecular weights of 15 to 20 kDa (Shiomi *et al.*, 1985; Santamaria *et al.*, 2002) The exact mechanism of haemolysis remains unknown, although it is likely that more than one mode of haemolytic activity is involved. Santamaria *et al.* (2002) found that oxidative stress at least partially contributed to haemolytic effect, while Alvarez *et al.* (2003) demonstrated that the sea anemone toxins sticholysin I and sticholysin II form pores in membranes, resulting in the leakage of potassium from erythrocytes.

In addition to the production of haemolysins for prey acquisition and repulsion of predators, it has been suggested that haemolysins are active in the humoral immune response of some invertebrates (Rowe and Welch, 1994; Hatakeyama *et al.*, 1995).

Clinically there are no reports of marine invertebrates causing haemolysis in humans. However, on occasions envenomation by other animals including spiders, snakes and bees has resulted in haemolysis in humans (Lee, 1993; Williams *et al.*, 1995; Malaque *et al.*, 2002; van Meeteren *et al.*, 2004).

2.6.6 Justification and summary

The preceding discussion highlights the fact that haemolysins that have been isolated, purified and characterised in laboratory studies are often associated with virulence or toxicity, but are only rarely associated with the aetiology of acute intravascular haemolytic crisis. Indeed, aside from the role of phospholipase C (α toxin) in *C. perfringens* (Berkowitz, 1991), known haemolysins play no role (or an as yet undetermined role) in acute intravascular haemolysis. A review of the literature suggests that episodes of acute intravascular haemolysis typically have complex aetiologies, often involving an immune reaction to chemicals, and/or a genetic host predisposition (Berkowitz, 1991; Lee, 1993; Lukens, 1993; McKenzie, 1996).

Nonetheless, the screening of microorganisms for *in vitro* haemolytic activity when trying to determine the cause of an acute intravascular haemolysis such as SHD has merit for numerous reasons. Basic scientific principles suggest that if SHD is a toxicosis, the toxin that produces haemolysis *in vivo* would be likely to produce haemolysis *in vitro*. There are circumstances under which this assumption would not hold true, such as host genetic predisposition.

Very little is known about SHD, and it is difficult to access patients during the illness due to its sporadic nature and the logistical difficulties faced in rural PNG. With these limitations in mind, an investigation into the aetiology of the disease should commence with the screening for the presence of haemolytic compounds, and microorganisms capable of producing them. In the absence of such microbes or

microbial metabolites, more complex aetiological agents should be addressed. Furthermore, mycotoxins have long been speculated as a potential cause of SHD (Taufa, 1974; Donovan *et al.*, 1977), and as such need to be thoroughly investigated.

In a rare insight into the pathophysiology of SHD, Melrose (pers. com.), suggested that the haemolysis might be due to exposure to a powerful toxin. Having viewed a blood smear of a patient suffering SHD, Melrose reported a lack of schistocytes or other evidence of damaged erythrocytes, despite the patient being severely anaemic. This is indicative of acute intravascular haemolysis, possibly the result of a powerful haemolytic toxin.

CHAPTER 3: SOCIOLOGICAL ASPECTS OF SAGO USE AND THE EPIDEMIOLOGY OF SAGO HAEMOLYTIC DISEASE

3.1 Introduction

Sago starch has long been recognised as an important source of carbohydrates for people living in lowland and coastal PNG (see Chapter 2). However, dietary trends are changing in PNG, partially due to need and partially due to desire. In recent years the population has increased rapidly, from approximately 3,500,000 in the 1990 national census to over 5,000,000 in the 2000 census, with over 85% of the population living in rural areas (NSO, 2006). This is likely to put enormous pressure on traditional farming practices to meet the demand for food. There is a perception that pollution from industrial activities in some areas of the country has decimated sago swamps and once arable land, making subsistence farming all but impossible for some communities (Hyndman, 1988; Wissink *et al.*, 2001). Furthermore, "Western" style foods are deemed by many to be more appealing than traditional foods (Ulijaszek and Pumuye, 1985; Ogle, 2001; Saweri, 2001).

Food safety issues compound the problems of food supply. Poorly regulated traditional methods of food production and storage may result in a higher risk of foodborne illness than processed foods in which physiochemical parameters are regulated (Nout, 1994; Montville, 1997; Jay *et al.*, 2005). One such example of foodborne illness associated with traditional foods in PNG is SHD. Occurrences of SHD have been speculated to more commonly occur in times of food shortage (J. Warner, pers. com.). Such problems in food adequacy are often the result of drought or other agricultural disaster.

Since the early reports of SHD (Taufa, 1974; Donovan *et al.*, 1977) there have been no case studies of the disease documented in the scientific literature. However, non-documented evidence is indicative of ongoing disease outbreaks (J. Warner, pers.

com.). This study aims to meet two main objectives. First, a sociological survey will provide an insight into the processes of sago production and storage. In understanding these processes, their effect as variables that predispose microbial contamination and persistence can be tested. Also, in communicating findings of the project, it is important to determine who the primary users are in these communities. Secondly, records at the Balimo Health Centre will be reviewed to determine the frequency of hospitalisation resulting from SHD, and to gain valuable knowledge on signs, symptoms and mortality rates. In revealing and publishing this information this work will aid in describing a case definition that may provide further insights into the epidemiology and diagnosis of this condition in the future.

3.2 Materials and Methods

3.2.1 Sociological survey of sago consumers in the Western and East Sepik Provinces

A sociological survey was developed in collaboration with Dr Elizabeth Kopel, a sociologist from The University of Papua New Guinea. The survey instrument was designed to gain insight into the management and cultivation of sago palms (particularly in terms of gender roles), harvesting of sago palms and subsequent extraction of the starch, storage of sago starch, preparation and consumption, and social and cultural significance. An example of the instrument can be seen in Appendix 1.1.

The survey was conducted in lowland areas of the East Sepik Province and Western Province, two areas of high dependence on sago starch. Individuals who provided sago samples used for microbial and toxin analysis were invited to participate in the survey. Participation was voluntary. In some cases where multiple samples were collected from one village, the community would participate in the one survey, providing general answers for what was typical for that village. All surveys were conducted in conjunction with a Papua New Guinean national to aid facilitation of the survey. Appropriate ethics approval was sought, and granted, from James Cook

University (ethics approval number H2167) and the PNG Medical Research Advisory Council (research and ethical clearance number MRAC 05/25).

3.2.2. Review of hospital records at the Balimo Health Centre

Hospital records at the Balimo Health Centre (BHC) were reviewed with permission from the health secretary, Mr Segedaba Wiyawa. Records of hospital admissions from 1994 to 2002 were reviewed for occurrences of SHD and other food poisoning/intoxication conditions.

3.2.3 Outbreaks of sago haemolytic disease during course of this study

Since June 2005, reports of two outbreaks of SHD have been made to the team of researchers at JCU involved in this study. Samples of sago starch implicated in these outbreaks have been sent to JCU for microbial analysis. Organoleptic qualities were reported for the two sago samples that were sent to JCU, as well as two previously collected samples of sago starch that were 'fit for consumption' for comparison.

3.3 Results

3.3.1 Sociological survey of sago consumers in the Western and East Sepik Provinces

3.3.1.1 General findings

A total of 49 surveys were conducted, 27 in the East Sepik Province and 22 in the Western Province. Trends were observed in the data and presented as percentage of respondents, with binomial 95% confidence intervals in parenthesis. Surveys were conducted mainly in areas already known to be highly reliant on sago starch as a food source.

The analysis of survey results demonstrated that there is a dependence on both cultivated sago palms and naturally growing palms, with no sago consumers

dependant on naturally occurring sago palms alone. In the East Sepik Province 15% (5–35%) of respondents use only cultivated palms for starch extraction, with the remaining 85% (65–95%) using a combination of cultivated and naturally occurring palms. In the Western Province the figures were 36% (17–59%) and 64% (41–83%) respectively.

In the East Sepik Province, the majority of respondents (85%; 66–96%) indicated that they typically harvest the palm during flowering, with the remaining 15% (4–34%) harvesting the palm either just before, or during, flowering. In the Western Province, responses were varied, with no trends evident. Approximately 27% (11–50%) of respondents fell the palm before it flowers, and a further 14% (3–35%) harvest before, or during, flowering. Many of the respondents (32%; 14–55%) harvest at around the time of flowering, regardless whether it is slightly before, during, or slightly after flowering.

3.3.1.2 Division of labour

Analyses of survey results suggest that most tasks associated with sago cultivation and starch production are shared among both men and women (Table 3.01). In the East Sepik Province the sago palms are felled by men predominantly, but both men and women do all other tasks. Starch extraction, as a whole, is done by both men and women, but roles within the process are gender specific. In the majority of responses, males were said to extract and macerate the pith, while women wash the pith to extract the starch. In the Western Province, most jobs are shared between the men and women, as is the case for the East Sepik Province. However, men do not extract starch as commonly as women.

Table 3.01: Division of labour between men (M) and women (W) for main tasks involved in sago palm cultivation and starch production. Results are presented as percentage of respondents, with 95% confidence limits in parenthesis.

	East Sepik Province (n = 27)			Western Province (n = 22)		
	M	**W**	**M & W**	**M**	**W**	**M & W**
Clearing area for planting	15% (4–34%)	0% (0–11%)	85% (66–96%)	27% (11–50%)	0% (0–13%)	73% (50–89%)
Planting sago palms	11% (2–29%)	0% (0–11%)	89% (71–98%)	9% (2–31%)	0% (0–13%)	91% (69–98%)
Removing excess growth	19% (6–38%)	0% (0–11%)	81% (62–94%)	45% (25–67%)	0% (0–13%)	55% (33–75%)
Felling palm	81% (62–94%)	0% (0–11%)	19% (6–38%)	9% (1–29%)	5% (0–23%)	86% (65–97%)
Starch extraction	Gender specific roles*			0% (0–13%)	59% (36–79%)	41% (21–64%)

* Men typically macerate the pith, women wash the pith to extract the starch

The transfer of knowledge from one generation to the next is influenced by gender roles (Table 3.02). In the East Sepik Province, transfer of knowledge for planting and growing sago palms, and for the sago extraction process, is from parent to child through observation and participation. None of the respondents stipulates gender in the transfer of knowledge. In the Western Province transfer of knowledge for both tasks is most commonly non-gender-specific, from parent to child. However, just under half the respondents identified mother to daughter as the most important route of knowledge transfer for starch extraction in the Western Province, whereas no respondents reported father to son transfer of knowledge for that task.

Table 3.02: Transfer of sago cultivation and extraction knowledge from one generation to the next in the East Sepik Province and the Western Province. Results are presented as percentage of respondents, with 95% confidence limits in parenthesis.

	Sepik Province (n = 27)			Western Province (n = 22)		
	F–S	**M–D**	**P–C**	**F–S**	**M–D**	**P–C**
Planting and growth	0% (0–11%)	0% (0–11%)	100% (90–100%)	28% (10–50%)	5% (0–22%)	67% (45–86%)
Extraction process	0% (0–11%)	0% (0–11%)	100% (90–100%)	0% (0–13%)	41% (21–64%)	59% (36–79%)

F–S: Father to son; M–D: Mother to daughter; P–C: Parent to child (not gender specific).

3.3.1.3 Extraction of sago starch and factors that can contribute to microbial contamination of the starch

The equipment and practises used to extract sago starch are similar in the two regions surveyed (See Section 2.1.3 for description of method). Some of the practises could potentially contribute to microbial contamination of the sago starch. Following the felling of the sago palm, it is common to store the trunks prior to maceration of the pith and extraction of sago starch. In the East Sepik Province 93% (76–99%) of respondents store trunks prior to extraction, mostly dry (on the ground), but occasionally underwater. A similar trend was observed in the Western Province, with 73% (50–89%) of respondents claiming to store sago trunks at least some of the time. Trunks might be stored in water in the wet season, but are more commonly stored on dry land. The remaining 27% (11–50%) of respondents from the Western Province generally do not store trunks between felling the palm and processing the sago starch.

Direct comparison of cleaning regimens of extraction equipment between the East Sepik Province and the Western Province is difficult, given the different level of detail obtained in the two provinces. In both provinces equipment is cleaned more often than not, although the difference is not statistically significant in the Western Province (Table 3.03). In the Sepik Province, equipment is usually cleaned to some degree.

Table 3.03: Percentage of respondents who wash extraction equipment following the maceration of sago palms. Results are presented as percentage of respondents, with 95% confidence limits in parenthesis.

	Sepik Province (n = 27)	**Western Province (n = 22)**
Washing of extraction equipment	78%: Yes, to some degree (58–91%)	33%: Yes, with water (16–56%) 13%: Sometimes (3–35%) 9%: Excess pith material removed, water not used (2–31%)
	22%: No (13–49%)	45%: No (25–67%)

The source of water used in the sago extraction process varies greatly, and is presumably largely dependent on seasonal availability and proximity to the sago

palm being processed. The vast majority of respondents in both provinces listed at least two common sources of water. In the Sepik Province bore water and river water were commonly identified as the primary water source, with creek water and rainwater also used regularly. In the Western Province swamp water and river water are the most commonly used water sources, followed by lagoon water. When using swamp water a small well is dug to access the water just below ground level (Figure 3.01).

Figure 3.01: Example of a well dug in a sago swamp to access water for starch extraction. Note that the water is very close to the ground surface (within 50 cm).

In most village situations pit toilets are employed as the primary mode of human faecal waste disposal (Table 3.04), although some villages that are situated in close proximity to water have toilets that empty straight into the water (Figure 3.02). When at ‘camping places’ processing sago starch, the people of the Western Province rely heavily on bush disposal of human waste. Data was not collected on how people in the East Sepik Province disposed of faecal waste when out in the bush making sago. Nonetheless, disposal was often in close proximity to areas of starch extraction.

Table 3.04: Methods of human faecal waste disposal, and the distance of disposal from site of sago extraction. Results are presented as percentage of respondents, with 95% confidence limits in parenthesis.

	Sepik Province (n = 27)	Western Province (n = 22)
In the village		
Pit toilet	93% (76–99%)	86% (65–97%)
Over water	7% (1–24%)	9% (1–29%)
Bush disposal	0% (0–11%)	5% (0–23%)
When sago making away from village		
Bush disposal	NA	95% (77–100%)
Distance from where sago is made		
< 100 m	44% (25–65%)	70% (45–86%)
100–500 m	48% (29–68%)	18% (5–40%)
> 500 m	8% (1–24%)	12% (3–35%)

NA: Data not collected.

Figure 3.02: Picture of two toilets (circled in red) that drain directly into the Sepik River in close proximity to where sago starch was being extracted. An additional toilet was located just outside the field of view to the right

3.3.1.4 Storage of sago starch

The sociological survey demonstrated differences in sago storage preferences between the East Sepik Province and the Western Province (Table 3.05). In the East Sepik Province the preferred storage method is in watertight containers. Traditionally clay pots have been used, but more recently saucepans and buckets have come into use. A total of 19% (6–38%) of all respondents from the East Sepik Province stated a preference for clay pots, and 19% (6–38%) for saucepans, buckets or similar. A further 47% (29–68%) of respondents did not disclose an individual preference for clay pots, saucepans or buckets. In total, 85% (66–96%) of respondents from the Sepik region stated a preference for the aforementioned storage vessels. The remaining 15% (4–34%) of people from the East Sepik Province preferentially store sago starch wrapped in leaves.

In contrast, the vast majority (85%: 65–97%) of respondents in the Western Province preferentially store sago wrapped in leaves, with the remaining 15% (3–35%) opting for bags or baskets woven from natural fibres such as tree bark. The majority of people in the Western Province use woven bags, woven baskets and plastic bags as their second storage preference. In the East Sepik Province sago that is produced for sale at the market is usually stored in baskets, leaves or plastic bags.

Table 3.05: Preferred methods of sago starch storage in the East Sepik Province and the Western Province. Results are presented as percentage of respondents, with 95% confidence limits in parenthesis.

Preferred Storage Method	**Sepik Province (n = 27)**	**Western Province (n = 22)**
Clay pots	19% (6–38%)	0% (0–13%)
Saucepan or buckets	19% (6–38%)	0% (0–13%)
Clay pots, saucepans or buckets	47% (29–68%)	0% (0–13%)
Wrapped in leaves	15% (4–34%)	82% (60–95%)
Woven fibre bags and baskets	0% (0–11%)	18% (5–40%)

Examples of some storage methods are illustrated in Section 2.1.3.2.

In the East Sepik Province, lemon is used as an additive to sago starch during storage by 33% (17–54%) of respondents. It is said to keep the sago white. No data were collected on how commonly lemon is used, or how much is added.

Sago starch is typically made when required, and as such consumption of sago starch commences very soon after extraction, within a day in most cases. In both provinces surveyed, sago starch is typically kept for 2–4 weeks, but sometimes up to 2–3 months.

Sago starch that is no longer considered suitable for human consumption is commonly thrown out in both the East Sepik Province and the Western Province (Table 3.06). Starch that is thrown out in the East Sepik Province is often fed to animals. Interestingly, almost one quarter of respondents from the Western Province stated that they only discarded stale sago if they had sufficient food supply. In times of food shortage old sago is likely to be consumed.

The most commonly used characteristic to determine suitability of sago starch for consumption is change of colour (Table 3.06). However, the colour of sago starch varies considerably, so there is no definitive colour that signifies sago is unsuitable for consumption. A vast array of colours was used to describe stale sago in both provinces, including various shades of grey, brown, black, red and yellow. In the Western Province, 72% (50–89%) of respondents described stale sago as having some degree of yellow colouration, often in conjunction with other colours. One respondent from the same province suggested black and brown spots were indicative of stale sago. Anecdotal evidence suggests that visible mould growth is more commonly used as an indicator of poor quality sago starch in the East Sepik Province than in the Western Province.

Table 3.06: Use of stale sago starch in the East Sepik and Western Provinces, and characteristics used to determine suitability for consumption. Results are presented as percentage of respondents, with 95% confidence limits in parenthesis.

	Sepik Province (n = 27)	**Western Province (n = 22)**
Thrown out	81% (62–94%)	50% (28–72%)
All eaten	19% (6–38%)	27% (11–50%)
Dependent on food supply	0% (0–11%)	23% (8–45%)
Characteristics Used to Determine Suitability For Consumption	Changes colour Does not mix well during food preparation Visible mould growth Goes hard	Changes colour Smell Taste: hot, sour Texture: dry, loose

3.3.1.5 Sago starch preparation and consumption

In both provinces surveyed, a variety of methods are used to cook sago starch, but nonetheless, a clear trend is evident. In the East Sepik Province, the preferred method of cooking sago is by boiling, followed by cooking 'flat bread' in a frying pan. In the Western Province the frying pan method is clearly favoured, followed by sago wrapped in leaves and cooked over the fire. Only rarely is sago prepared by boiling in the Western Province. Other methods of preparation used include: placed inside bamboo or tree bark and cooked, rolled into balls and cooked in hot ashes, in a soup, or mixed with banana and served sweet as a cake.

Table 3.07: Primary and secondary methods of sago preparation in the East Sepik Province and the Western Province. Results are presented as percentage of respondents, with 95% confidence limits in parenthesis.

	Sepik Province (n = 27)	**Western Province (n = 22)**
Primary cooking method		
Boiled	89% (71–98%)	0% (0–13%)
Frying pan	11% (2–29%)	91% (71–99%)
Wrapped in leaves	0 (0–11%)	9% (1–29%)
Secondary cooking method		
Frying pan	78% (58–91%)	4% (0–23%)
Wrapped in leaves	7% (1–24%)	91% (71–99%)
Other	15% (5–35%)	5% (0–23%)

3.3.1.6 Importance of sago starch as a food source

Sago starch remains the staple carbohydrate for the majority of respondents throughout lowland areas of the East Sepik Province and Western Province (Table 3.08). In most villages there has been no change in dependency upon sago starch as the dietary staple, but some villages described a decrease in dependency. In the East Sepik Province, this decrease was solely attributed to an increase in consumption of garden foods and purchased foods, namely rice and flour. While the same reason was given for a decrease in sago consumption by some respondents in the Western Province, equally as important was the decreased availability of sago palms.

Table 3.08: The significance of sago starch as a food source and changes in dependency in recent years. Results are presented as percentage of respondents, with 95% confidence limits in parenthesis.

	Sepik Province (n = 27)	**Western Province (n = 22)**
Significance as food source		
Staple carbohydrate	81% (62–94%)	95% (77–100%)
Highly used carbohydrate source	19% (6–38%)	0% (0–13%)
Minor carbohydrate source	0% (0–11%)	5% (0–23%)
Change in dependency		
No change	70% (50–86%)	68% (45–86%)
Less consumed now than 10 years ago	22% (9–42%)	27% (11–50%)
More consumed now than 10 years ago	8% (1–24%)	5% (0–23%)

People of all ages eat sago starch, from the very young to the very elderly. In the East Sepik Province all respondents stated that infants from six months of age eat sago starch, whereas many respondents in the Western Province stated that infants are two years or older when they start eating sago starch. Differences in answers may be in part attributable to different personnel assisting with the survey in the different provinces, and differences in weaning ages of infants. Regardless, it is evident that young children commonly eat sago starch.

3.3.1.7 Cultural importance of sago starch

For some sago consumers, sago is an important food source, but is not highly regarded in a cultural sense. In the East Sepik Province the majority of respondents

stated that sago was not culturally significant, but when asked about the role sago plays in ceremonies, usually stated that it was important, particularly at weddings and funerals. Many respondents from the Western Province acknowledged the cultural importance of sago starch, where it also plays an important role in ceremonies. Selected responses to cultural aspects of sago usage are given in Table 3.09. These responses are not necessarily representative of all opinions and cultural beliefs in the respective province, but provide an insight into the beliefs of some sago users. In the Western Province multiple respondents stated that the traditional prayers asking for “more than enough sago” are not as common as they once were, or are restricted to the older generation, as Christianity has taken over from traditional beliefs.

Table 3.09: Selected responses from survey participants pertaining to cultural aspects of sago use

	Sepik Province	**Western Province**
Importance	"Rice bilong ol Sepik" (sago is the rice of the Sepik)	"Sago is life" Must have sago every meal
Ceremonial use	Large quantities eaten at ceremonies particularly weddings and funerals	Always eaten at ceremonies Cooked a special way for special occasions, especially funerals
Culture and traditions	Patches of sago stands given as wedding gift to newlyweds by both bride and groom's family Sago can be exchanged for pigs	Sago swamps given for bride price Highly revered as source of food and building materials Sago planting, management and processing is part of the culture
Myths and customs	Only women can pack sago into storage pots and change the water Women are not allowed to process sago while menstruating or men will get sick with asthma-like illness Women are not allowed to jump over sago The person who planted the sago must not eat from his/her own harvest or they will die	In weddings sago is sprinkled on ground for newlyweds to walk on People paint themselves with sago starch at Christmas time Pray for "more than enough" sago when planting and extracting Place hot sago from the fire on wounds to facilitate healing Red sago tastes good. To make red sago, sit white sago in the sun

3.3.1.8 Problems associated with sago starch

The growth of sago palms and subsequent extraction of the starch is laborious, with almost all respondents from both regions describing it as hard work. Table 3.10 shows some of the common responses from the East Sepik Province and the Western Province when asked about problems associated with sago starch production.

Table 3.10: Problems associated with sago palm cultivation and starch extraction in the East Sepik Province and the Western Province.

Sepik Province	Western Province
For many it is a long distance to sago stands.	For many it is a long distance to sago stands (up to 2 days travel).
Hard work, particularly starch extraction.	Hard work, particularly starch extraction.
Population increases: more people to feed.	Population increases: more people to feed (1 respondent only).
Land ownership (compounded by increasing population).	Injuries (cuts, thorns, backache, snake bites) and illness (exposure to malaria in swamps).
Sago starch does not keep well (relative to flour and rice).	Lower starch yields now than in years gone by, possibly due to pollution.
Fire has destroyed sago stands.	Fire has destroyed sago stands.
Long time for sago palms to grow.	Wild animals and insects destroy sago palms.
Have to change water every day to keep sago fresh during storage.	

3.3.1.9 Illness associated with sago starch consumption

Respondents in the Western Province are more familiar with illness associated with sago consumption than those in the East Sepik Province. Reports of mild to moderate illness associated with sago consumption are not uncommon in the Western Province, but in general the people of the East Sepik Province are not familiar with sago-borne food poisoning. A number of respondents from the Western Province also described a number of SHD-like illnesses that had occurred in recent years. Table 3.11 gives brief accounts of these illnesses.

Table 3.11: Previously unreported illnesses with similar symptoms and epidemiologies to SHD in the Western Province.

Village	Details of illness
Kaniya	One child died following the consumption of "yellow coloured sago" in 1998. Parents were also ill, but survived.
Garaita	Pregnant mother and seven year old son died after eating sago in 1998. No confirmation of associated haemolytic crisis. Five girls fell ill at same time after sago consumption in 2002. All recovered.
Mipan	Grandparents and small grandchild very ill after consumption of sago starch, passed blood in urine. Male adolescent/young man also ate the sago, but did not suffer severe illness (he was mildly ill). The child died. Exact date unknown (2002/2003).
Manda	Boy died of illness with similar symptoms to SHD in 2002.

3.3.2 Review of hospital records at the Balimo Health Centre

Two outbreaks of SHD have resulted in the hospitalisation of multiple casualties at BHC in recent years. An outbreak in Musula, in the south-western reaches of the Southern Highlands Province, in March 1998 resulted in the admission of five people. In April 2000 four people from Ali, upstream from Balimo on the Aramia River in the Western Province, were admitted. Selected incidences of food poisoning that resulted in hospital admission in recent years were also noted.

3.3.2.1 Outbreak of sago haemolytic disease in Musula

Three adults and two children were admitted to BHC in mid March 1998 following the deaths of two children suffering similar symptoms the previous day. Onset of illness occurred following a meal of old sago, and was concurrent for all affected. Details and symptoms of each individual admitted are listed below.

Patient A1: A 39 year old female, the mother of the two children that died prior to admission, was admitted to BHC on 13.3.1998. She developed the illness after the consumption of sago and bananas. Symptoms included headache, dizziness and malaise. A general examination revealed pus in eyes, discharge from the nose, pallor of mucus membranes, and shortness of breath. She was suffering neck stiffness and pain, and generalised abdominal pain with an enlarged spleen and liver. Urination

was painful, and blood was observed in her urine. One day after admission her temperature was 39.3 ºC, pulse rate 116/min and respiration rate 40/min.

Urine analysis, routine haematology and some biochemistry tests were conducted on the day of admission. Urine pH was 6.0, and protein was detected in the urine (0.3 g/l). No urine microscopy results were recorded. Haemoglobin (Hb) was 68 g/l, considerably lower than the reference range (RR) of 100–130 g/l, and the haematocrit reading 18% (RR 35–50%). White blood cell (WBC) count was 13.0×10^9/l (RR 4.0–11.0 $\times 10^9$/l). No malaria parasites were observed under microscopic examination.

A blood transfusion was given on the day of admission. The patient was blood type O Rh (D) positive (Rh: Rhesus factor). Various chemotherapies were administered, including quinine, sulfadoxine-pyrimethamine (Fansidar®), albendazole, chloramphenicol, codeine and an iron and folate supplement. The patient recovered and was discharged on 23.3.98, at which time her Hb was 82 g/l and WBC count was 4.5×10^9/l.

Patient A2: A 47 year old male, the father of the two children that died prior to admission, was admitted to BHC on the same day as patient A1. Following the consumption of sago he vomited, and was febrile and weak. A general examination upon admission revealed pallor, a coated tongue, shortness of breath, abdominal tenderness and pain radiating to the back. The patient also had slight pain in the neck and ears. One day after admission his temperature was 38.2 ºC, pulse rate 82/min and respiration rate 20/min.

Urine analysis and routine haematology was conducted on the day of admission. Urine pH was 5.0, and protein was detected in the urine (0.3 g/l). Polymorphonuclear leucocytes (20/µl) and erythrocytes (>100/µl) were observed in the urine. There was no bacterial growth upon urine culture. Haemoglobin was 82 g/l (RR 100–130 g/l) and the haematocrit reading 20% (RR 35–50%). The WBC count was 16×10^9/l (RR 4.0 –11.0 $\times 10^9$/l). No malaria parasites were observed under microscopic examination.

Two days after admission the patient's Hb had dropped to 37 g/l, and he was consequently given a blood transfusion. His blood type was O Rh (D) positive. Various chemotherapies were administered, including quinine, sulfadoxine–pyrimethamine, maxalon, lasinx, chloramphenicol, and an iron and folate supplement. The patient recovered.

Patient A3: A 30 year old female developed symptoms at the same time as the previous two patients. Her symptoms included high fever, headache, neck stiffness, abdominal pain, diarrhoea and vomiting. There was no documentation of sago consumption in the patient records, which were less detailed than for the previously described patients.

On the day of admission the patient's urine was pH 5.0 and protein level was 0.3 g/l. Polymorphonuclearcytes (70/µl) and erythrocytes (70/µl) were observed in the urine. Haemoglobin was 112 g/l (RR 100–130 g/l) and the haematocrit reading 27% (RR 35–50%). White blood cell count was 4.3×10^9/l (RR $4.0 – 11.0 \times 10^9$/l). No malaria parasites were observed under microscopic examination.

One day after admission her temperature was 37.5 ºC, and vomiting and abdominal pain continued. Treatments included quinine, chloramphenicol and intravenous fluids. Ten days after admission her Hb was 116 g/l and WBC count was 6.9×10^9/l. The patient recovered and was discharged.

Patient A4: The 8 year old daughter of patients A1 and A2 (and sister of the two children who died) was admitted to BHC at the same time her parents were, having become ill after the consumption of sago starch. She was repeatedly vomiting, passing "yellowish' urine, febrile, had diarrhoea and dysentery, and was generally weak. Vomitus material was described as "green stuff".

General examination revealed pallid eyes with pus present, nasal discharge, pallor of the mouth mucous membranes, and an enlarged abdomen. The lower abdomen was hard and painful to touch. She was also suffering head pain, neck stiffness and pain, and dysuria. Blood was observed in the urine. Regular episodes of vomiting

continued for the following two days. Her temperature was documented as 38.3 ºC, pulse rate 128/min, and respiration rate 32/min.

Urine analysis revealed a pH of 5.0, glucose level of 60 mM, and high protein levels (20.0 g/l). Granular casts were observed. Haematology showed an Hb of 48 g/l (RR 100–130 g/l) and a haematocrit reading 12% (RR 35–50%). The WBC count was 18.0×10^9/l (RR 4.0–11.0×10^9/l).

The patient's blood was typed (O Rh (D) positive) and a blood transfusion given on the day of admission. She received numerous chemotherapeutics, including quinine, frasimide, sulfadoxine–pyrimethamine, penicillin, chloramphenicol, folic acid supplement and multivitamins. After 10 days in hospital the patient was discharged. Her Hb was 82 g/l and WBC count 4.5×10^9/l.

Patient A5: The younger brother of patient A4, whose precise age was not known (documented as 4–6 years old) was also admitted to BHC on 13.3.1998, following the consumption of sago starch, bananas and kau kau (sweet potato). His symptoms were similar to those of his sister (patient A4), with repeated vomiting of "green stuff", fever, haematuria and weakness. Vomiting continued for several days after admission.

General examination revealed pallid eyes with pus present, nasal discharge, pallor of mouth mucous membranes, and an enlarged abdomen. He was also suffering mild headaches, neck stiffness and pain, and dysuria. Blood was observed in the urine. One day after admission the boy's temperature was 38.6 ºC, his pulse rate was 140/min and his respiration rate 44/min.

On the day of admission haematology tests were conducted. He had a Hb of 56 g/l (RR 100–130 g/l), haematocrit reading of 11% (RR 35–50%) and WBC count of 29×10^9/l (RR 4.0–11.0×10^9/l). His blood was typed (O Rh (D) positive) and he was given a blood transfusion on the same day. Chemotherapeutics used were essentially the same as for patient A4, namely quinine, primaquine, frasimide, sulfadoxine–pyrimethamine, albendazole, penicillin, chloramphenicol, folic acid

supplement and multivitamins. After 10 days in hospital the patient was discharged. His Hb was 86 g/l and WBC count 9.3×10^9/l.

3.3.2.2 Outbreak of sago haemolytic disease in Ali

One adult, two adolescents and one young child were admitted to BHC in late April, 2000 following the deaths of one adult male and one infant male who had suffered a similar illness. The onset of illness occurred following a meal of old sago, and was concurrent for all affected. None of the individuals admitted had any previous medical history. Details and symptoms of each individual admitted are listed below.

Patient B1: A middle aged female patient of disputable age (conflicting ages were recorded, but probably 45 years old), was admitted to BHC on 29.4.2000. She was the wife of the adult male and grandmother of infant male that died before admission. She shared a meal of old sago with family members, including the recently deceased, about one week prior to admission, and had been ill for the six days preceding admission. Symptoms included vomiting, diarrhoea with blood and haematuria. Upon admission to BHC she was conscious but appeared very ill, with fever, pallor of skin and general weakness. Routine laboratory tests revealed polymorphonuclear leucocytes (60/µl) and Gram negative rods in the urine. Her Hb was 68 g/l (RR 100–130 g/l), the haematocrit reading 21% (RR 35–50%), reticulocytes 2.4% (RR 0.2–2.0%) and WBC count 6.3×10^9/l (RR $4.0–11.0 \times 10^9$/l).

The patient's blood was typed (B Rh (D) positive) and she was given one unit of whole blood. Chemotherapeutics included chloroquine, albendazole, intravenous fluids and folic acid and iron supplement. The patient recovered and was discharged on 8.5.2000.

Patient B2: The 17 year old daughter of patient B1 was admitted to BHC on the same day as her mother and other family members. Symptoms developed six days prior to admission, after eating "very old, dry sago", and included repeated vomiting, fever, weakness and haematuria. Upon admission she was pale, weak, febrile (38.2 ºC), anaemic, and restless, with partial consciousness. She was also diarrhoeal,

short of breath, having occasional fits, and her abdomen soft to touch with enlarged organs. Haematuria was observed.

Treatment included intravenous fluids, quinine and penicillin. The patient went into cardiac arrest and was pronounced dead at 6 pm on the day of arrival.

Patient B3: The 16 year old son of patient B1 was admitted to BHC on the same day as his mother and other family members. Symptoms developed six days prior to admission after eating old sago with other family members. The patient had been febrile and had haematuria prior to admission. Upon presentation he was looking pale and weak. A general examination reported that he had a rapid heartbeat, his abdomen was soft with enlarged organs, and he was vomiting and diarrhoeal. Dysuria and haematuria were present. He was conscious, but had had fits. Respiration was wheezy, his chest indrawing, and he was cyanotic. On the day of admission his temperature was 37.8 ºC. A separate entry documented a temperature of 37.4 ºC, pulse rate 88/min, and respiration rate 24/min, although it is not clear if these readings were taken on the day of admission or some days later.

Routine laboratory tests revealed polymorphonuclear leucocytes (60/µl) and Gram negative rods in the urine. His Hb was 68 g/l (RR 100–130 g/l), the haematocrit reading 21% (RR 35–50%), reticulocytes 2.2% (RR 0.2–2.0%) and WBC count 6.8×10^9/l (RR 4.0–11.0 $\times 10^9$/l). Treatments included chloroquine, primaquine, albendazole, and folic acid and iron supplement. There was no reference in the hospital records of a blood transfusion. He was discharged on 8.5.2000.

Patient B4: The granddaughter of patient B1, aged 2 years and 9 months, was admitted on BHC on 29.4.2000. She was the sister of the infant boy who died of a similar illness prior to admission. Symptoms started six days prior to admission, following sago starch consumption. The girl was febrile and had haematuria prior to admission, and upon admission looked very ill, with skin pallor, and was vomiting. Her temperature was 39.2 ºC, pulse rate 130/min and respiration rate 36/min. Her blood was typed (O Rh (D) positive) and she was given two units of whole blood on the day of admission. Two days after the transfusion her Hb was 68 g/l (RR 100–130 g/l), the haematocrit reading 25% (RR 35–50%), reticulocytes 2.8% (RR 0.2–2.0%)

and WBC count 6.1 × 10^9/l (RR 4.0–11.0 × 10^9/l). The numerous chemotherapeutics administered were quinine, primaquine, camoquine, imferon, amoxil, penicillin, intravenous fluids, folic acid and iron supplement and multivitamins. The girl survived, and was discharged on 8.5.2000.

3.3.2.3 Perusal of hospital records for admissions due to food poisoning

Time did not permit the thorough examination of hospital admission and discharge books. However, while looking for cases of SHD, frequent hospital admissions attributed to food poisoning were noted. Most admissions were singular, as opposed to familial, as is typically the case in SHD. There seemed to be no seasonal variation in incidence of foodborne illness, although no analysis was conducted.

3.3.3 Outbreaks of sago haemolytic disease during the course of this study

3.3.3.1 Suki, May 2005

An outbreak of severe food poisoning diagnosed as SHD occurred at Aewe village, near Suki, Western Province, on May 28, 2005. Onset of illness followed the consumption of sago that had been stored in a woven bag for approximately four weeks. Eight family members were affected, namely the parents and their six children. One child, an 11 year old boy, died within two days of consuming the sago. He was the only member of the family that mixed the sago starch with coconut and wrapped it around a catfish, although it was not documented as to how the other family members prepared their sago. The mother and her oldest daughter were both antenatal at the time of the illness, and subsequently suffered stillbirths.

A sample of sago starch was sent to JCU for microbial analysis (W0605-01). It arrived approximately three weeks after the onset of illness, and had been stored refrigerated since it was collected shortly after the outbreak. The sample was tested for the presence of bacterial pathogens, filamentous fungi were isolated and identified, haemolytic screening of common microbes conducted, and the presence of common mycotoxins tested. Results can be seen in the relevant chapters to follow.

Organoleptic characteristics of sample W0605-01 were described as having no distinctive flavour, not even a fermented flavour typically present in sago starch, and a grainy texture. The starch had an earthy, musty aroma. The colour of the starch was off-white with a yellow tinge. Microscopic examination revealed clumping of the starch, yellow-orange pigmentation and the presence of hyphae. These properties were compared to two previously collected samples of sago starch considered 'fit for consumption'. Sample W0405-13 was slightly orange in colour, with no distinctive smell or taste, and a smooth texture. Under microscopic examination there was no clumping, no orange pigmentation and no visible hyphae. Sample W0405-09 was white in colour, with a fermented smell, a slightly fermented taste, and a smooth texture. Under microscopic examination there was no clumping, no orange pigmentation and no visible hyphae.

Metal analysis was conducted on the implicated sample of sago starch (W0605-01), and two other samples from the Western Province that had been previously collected (W0405-13 and W0405-09, as above). Results are documented in Appendix 1.2.

3.3.3.2 Erecta, November 2005

A sample of sago starch (W1105-01) that had been associated with an outbreak of food poisoning in a family of three at Erecta, near Kiunga, in the north Fly region of the Western Province was sent to JCU. The family was sent to Kiunga for medical attention. The two parents were described as being mildly ill, and their 8 year old very ill. Intravascular haemolysis was observed in all patients. All three recovered.

Over six weeks passed between the outbreak of SHD and the arrival of the sago sample at JCU. Consequently, very little bacterial analysis was conducted on the sample, but thorough mycological studies were conducted (see Chapters 5 and 6). Organoleptic characteristics were described as having an earthy, musty aroma and no noticeable flavour, not even a fermented flavour typically present in sago starch. The starch was very dry and clumped, and varied in colour with black clumps and areas with an orange tinge.

3.3.4 Case definition and incidence of sago haemolytic disease

On the basis of information currently available, SHD should be used to describe illness following consumption of sago starch characterised by haematuria, anaemia, and gastrointestinal symptoms. Presentation of signs and symptoms is rapid, and familial outbreaks are typical.

The application of the case definition enables the incidence of the disease to be determined. Using the cases described in the hospital records (Section 3.3.2), and the recent outbreaks (Section 3.3.3), there was an annual incidence of 2.0 cases/100,000 people in the Western Province between 1998 and 2005. Additional cases as described in Table 3.11 were not used to determine the incidence, as there were insufficient details available to confirm the illnesses as SHD. Thus, the estimated incidence of the disease is a minimum.

3.4 Discussion

The survey employed in this study was used primarily to determine the influence of sociological aspects of sago starch production on human health. Although some of the findings may have little direct impact on human health, they expand our knowledge of sago dependent subsistence living and the many challenges that need to be met to improve health in these communities. Moreover, the survey garnered baseline data that will enable the fulfilment of other aspects of this study such as trialing commonly used storage techniques for microbial survival, effective fermentation and the presence of haemolytic microorganisms. Gaining information on who is primarily responsible for sago starch extraction and processing will enable better communication of the findings of this research project, by ensuring the appropriate people in the community are targeted.

Notable differences in certain aspects of sago production in the East Sepik Province and the Western Province are evident, as well as many similarities. On the basis of this study, it appears that men play a greater role in sago production in the East Sepik Province than in the Western Province. This is evident by comparing the division of

labour (Table 3.01), and also the transfer of knowledge (Table 3.02). That almost 60% of respondents from the Western Province identified starch extraction as women's work, and all other respondents said that it was shared between men and women, suggests that in reality women do the vast majority of starch extraction. The transfer of knowledge of starch extraction from mother to daughter, as is commonly the case in the Western Province, lends further weight to this conclusion.

Clearly, the gender roles in sago production vary from one area to another, even within provinces. Nonetheless, other researchers have noted that sago extraction is women's work in the Western Province (Ohtsuka, 1977; Busse, 1990; Dundon, 2005). Moreover, Busse (1990) suggests that sago is an important feminine symbol, and Dundon (2005) describes at length the role sago production and consumption plays in establishing the 'sense' of being a Gogodala woman. She likens the relationship between women and the sago they produce to that of a woman and her husband.

Some researchers have suggested an inverse relationship between the input of men into sago extraction and processing, and the level of dependency that cultural group has on sago starch (Ohtsuka, 1977; Townsend, 1977; Connell and Hamnett, 1978). Overall, the people of the Sepik are still highly dependent on sago starch, with most respondents describing it as their staple food. It seems other factors may also contribute to the level of input by men into sago processing. In isolated cases at least, cultural beliefs may be a factor (Williamson, 1979).

The process whereby the pith is removed from the trunk and the starch extracted varies little between the two regions surveyed, and indeed across the country (see Section 2.1.3). However, preferred storage methods differ in the two provinces. Traditional clay pots, and modern replacements such as large saucepans and buckets, are the preferred method of sago storage in the East Sepik Province. Throughout the course of the study this method was never encountered in the Western Province. It is likely that the clay pots are of some cultural significance in the Sepik region, but this issue was not explored in the survey.

While the preferred method of storage in the Sepik appears to be in pots or ostensibly similar modern equivalents, starch that is produced for sale in the markets is stored using methods similar to those of the Western Province. In the major towns in the East Sepik, such as Wewak, Angoram and Ambunti, many people obtain their sago from the market. No sago purchasers were questioned in the survey, only sago producers. It is not known how these people store their sago after purchase, but in most cases it is likely to remain in the packaging in which it was purchased. Thus, while 85% of respondents from this area prefer to use pots, it is unlikely that such a high percentage of sago starch is actually stored that way.

The method of storage may have some influence on preferred cooking methods. Sago stored submersed in water in pots obviously has a very high moisture content. Such sago is probably well suited to boiling, whereby more water is added and a thick paste is produced. The moisture content is probably too high for frying, and it is hypothesised that much of the sago that is fried in the East Sepik Province is not stored in pots. In the Western Province where stored sago has lower moisture content, it is better suited to frying.

Table 3.08 highlights the importance of sago as a food source in parts of the East Sepik and Western Provinces. In the vast majority of villages visited sago remains the staple food. This dependence on sago is not indicative of the entire East Sepik and Western Provinces, as the sample collection was biased towards areas with high sago consumption. However, it does suggest that there are areas that are still highly dependent on sago starch.

In some villages in the East Sepik Province, namely Makendo, Kainde, Parom and Wariman, sago starch is still highly utilised, but its consumption is in decline (data not shown). Rice, flour and garden foods are taking the place of sago starch as a carbohydrate source. This might be in part due to an increased disposable income in some areas of the East Sepik Province, where vanilla farming has been very profitable in recent years. In contrast, in the Western Province, the main reason given for those villages that are eating less sago now is a lack of trees to harvest. Villagers from Alagi, Gawi and Kaenewa along the Aramia River stated that fires have destroyed sago stands in recent years. Given the high reliance upon sago starch in the

Western Province, but the decrease in availability of harvestable trees, sago is likely to be stored for longer. Consequently, illness associated with microbial contamination is more likely.

It is evident that as well as being a dietary staple, cultural links to sago starch and the process by which it is made remain strong. It is commonly used in ceremonies, there is an obvious reverence towards it (with comments such as “Rice bilong ol Sepik” and “Sago is life”), and it still plays a role in culture, traditions, myths and customs. Many other researchers have acknowledged the close relationship lowlanders in PNG have with the sago palm (Johnson, 1976; Townsend, 1977). The findings of this study suggest that even today, with the introduction of foods such as flour and rice into the more accessible areas of lowland PNG, the cultural importance of sago starch should not be overlooked. As such, the use of sago starch should be encouraged when considering future food supply and food security issues in PNG.

During the survey various problems pertaining to sago palm cultivation and starch extraction were raised. Indeed, many of these problems have implications for food security. Issues such as land ownership disputes, increasing population, lower starch yields and the greater distance to sago stands could have a detrimental effect on food security. To help overcome the burden of increasing population and distance to sago stands, more advanced agronomic principles should be employed. Studies have been conducted on plant spacing, fertiliser application, and starch yield in various countries in the Asia–Pacific region (Haska, 1995; Jong *et al.*, 1995; Shimoda, 2000; Yamamoto *et al.*, 2003; Kakuda *et al.*, 2005), but more work is required. With further research some of these principles, such as optimal plant spacing, could be implemented in PNG with minimal financial, social or environmental cost.

While work has progressed on the agronomy of the sago palm, to date little has been conducted on the microbiology of sago starch, and its implications for human health. This survey has identified numerous steps in the sago making process where contamination could occur, such as use of uncleaned implements for sago production, contamination of the sago log if stored prior to starch extraction, the extraction process, and more specifically the source of water. Further contamination might eventuate during storage. The role these steps play in microbial contamination

of sago starch, and how contamination can be minimised, will be addressed in detail in Chapter 10 through a hazard analysis and critical control point (HACCP) plan.

Analysis of survey results suggests there is a greater association between foodborne illness and the consumption of sago starch in the Western Province than in the East Sepik Province. This finding could be for one of many reasons. For example, it simply might be that people more commonly become ill following sago consumption in the Western Province. Or alternatively, it might be that other constituents of a meal that includes sago starch are actually responsible for foodborne illness, but people incorrectly assume sago to be the aetiological agent of the illness. Regardless, evidence gained in the sociological survey, along with information gained from reviewing the records at BHC (Section 3.3.2), and the recent outbreaks of SHD in the Western Province (Section 3.3.3), suggest that concern over the food safety of sago starch is not without merit.

Despite the limited detail contained in many of the hospital records, and the discrepancies occasionally observed, the exercise of reviewing them for cases of SHD was a valuable one. Symptoms reported in the two outbreaks documented above are similar to those observed in early communications about the disease (Taufa, 1974; Donovan *et al.*, 1976; Donovan *et al.*, 1977). Those symptoms that were commonly documented in the Musula and Ali outbreaks included haematuria, fever, headache, neck stiffness, pallor of mucous membranes, abdominal pain with enlarged organs, and diarrhoea. Many of these signs and symptoms are typical manifestations of haemolytic anaemia (Hoffbrand and Pettit, 1993). These observations, in conjunction with the previously published symptoms, enabled the development of a case definition (Section 3.3.4). Such a definition can be used in the future to ensure more accurate diagnosis and documentation of this condition.

Notwithstanding the limited capacity at BHC, results of tests completed provide insight into the aetiology of the illness. All patients except patient A3 had Hb levels well below the standard range for PNG, and haematocrit readings were invariably low, confirming patients were anaemic. Observation of blood films for malaria parasites was documented for only three patients, but all were negative. This suggests that malaria is not involved in the aetiology of the disease, although cases of

Blackwater fever (Section 2.6.4) are often characterised by low or absent parasitaemia (Rogier *et al.*, 2003).

Reticulocyte counts for patients in the Ali outbreak were within the normal range for healthy adults (McKenzie, 1996), discounting a proliferative anaemia. White blood cell counts were slightly elevated in some cases, which is consistent with inflammation, infection or intoxication. Protein was detected in urine in four of the five patients from Musula, suggesting presence of haematuria and/or kidney malfunction: in some of the patients erythrocytes were observed in urine. This is probably the result of decreased kidney function rather than evidence of haemorrhaging.

The recent outbreaks of SHD give some credence to the notion that the disease occurs more commonly than has been reported. If one were dependent on hospital admissions to determine the incidence of the disease, one of the two recent cases would have gone undetected. If not for the close contacts with appropriate people in the Western Province, researchers at JCU would not have become aware of the incidents. Only the patients in the outbreak in Erecta in late 2005 were sent to a hospital. The three patients were sent to Kiunga, in the northern region of the Western Province. The current research team has not reviewed records from this hospital. Although sago is not as heavily consumed in the northern regions of the province, it is possible that people suffering from SHD have been admitted to this hospital in the past also. Furthermore, while conducting the sociological survey, respondents from four villages in the Western Province described an illness with some similarities to SHD, which was typically associated with the consumption of old sago starch.

While it would be interesting to review the records of health centres and other hospitals in all rural sago-eating areas of PNG, again it is unlikely they would reveal the true incidence of the disease. When outpatients attend rural health centres in PNG, they take with them their own medical logbook. This book remains in the possession of the patient at all times, and is the only record of patient treatment that is kept. Although it is unlikely an individual suffering SHD would remain an outpatient, it might occur on rare occasions. In such cases it is unlikely that details of

SHD outbreaks would be available from rural health centres. In other regions of PNG health staff might not be familiar with SHD, especially if outbreaks are only sporadic. Thus, it is conceivable that occasional outbreaks of disease are undiagnosed or misdiagnosed in other sago eating areas of PNG. With these factors in mind, it is conceivable that the incidence of SHD is higher than that calculated in this study (2/100,000).

On the basis of the sociological survey and review of hospital records, evidence exists to suggest that people in the Western Province of PNG are sporadically suffering from foodborne illness following the consumption of sago starch. It seems likely that there are two distinct presentations of illness resulting from sago starch consumption. First, a mild, self-limiting foodborne illness resulting in diarrhoea and/or vomiting is likely to occur as a result of predominantly environmental and faecal contamination. Secondly, SHD may occur, the aetiology of which little is currently known. Notwithstanding the other possible confounding factors that may be responsible for SHD, given past reports and evidence collected so far, it follows that a thorough microbiological study of sago starch should be conducted and aetiological agents/compounds capable of acute destruction of erythrocytes should be sought. The findings of the sociological study will enable appropriate experimental design of studies described in forthcoming chapters, and will greatly assist in the final communication of findings to sago consumers.

CHAPTER 4: THE PREVALENCE OF FOODBORNE BACTERIAL PATHOGENS IN SAGO STARCH

4.1 Introduction

Food and waterborne microorganisms are a major contributor to the worldwide incidence of diarrhoea, particularly in the developing world (WHO, 2004; WHO, 2005). In PNG, gastrointestinal illness is one of the major causes of hospital admission (Han *et al.*, 1995; Vince, 1995; Naraqi *et al.*, 2003). Dysentery and typhoid fever account for 20% of adult admissions to PMGH (Naraqi *et al.*, 2003), and diarrhoea accounts for 15% of infant admissions (Han *et al.*, 1995). The burden is particularly heavy on infants where the fatality rate of diarrhoeal hospital admissions is 3.9% (Han *et al.*, 1995) compared to a national average of approximately 1.6% (PNG Ministry of Health, 2000). One of the major factors contributing to the incidence of gastrointestinal illness is inadequate supply of safe drinking water. The National Health Plan 2001–2010 states that only 30% of drinking water in PNG is safe (PNG Ministry of Health, 2000).

The dependence on unsafe drinking water and consumption of unsafe foods in many parts of PNG, together with a low standard of hygiene, are likely to be major contributors to the incidence of gastrointestinal disease. To date, only one limited study of the presence of bacterial pathogens in sago starch has been conducted on sago starch from the Morobe Province (Omoloso, 1999). This study will build on current knowledge by extending the analysis of sago for foodborne bacteria of significance and indicators of faecal contamination from varying regions of PNG where sago is consumed as a staple carbohydrate. In doing so, the prevalence of bacterial foodborne pathogens in sago starch and their impact on food safety and food security in lowland rural PNG will be determined.

4.2 Materials and Methods

4.2.1 Sample collection and general procedures

4.2.1.1 Sample collection and storage

A total of 70 samples of sago starch were analysed from among those collected from the East Sepik, Western and Milne Bay Provinces of PNG in five sampling trips (see Section 1.3 and Figure 1.01 for introduction to study regions). The first trip was to the East Sepik Province in November 2002, where 10 samples were collected for microbial analysis (S1102-01 to S1102-10). During this expedition one sample was also obtained from Misima Island, Milne Bay Province (M1102-01) (subsequent expeditions did not allow for the return to Misima Island, thus only one sample was collected from this site during the study). Sixteen samples collected in the Western Province in March 2003 were analysed for bacterial pathogens (W0303-01 to W0303-20, not all samples analysed), and 15 samples collected from the East Sepik Province in June 2003 (S0603-01 to S0603-20, not all samples analysed). In April 2004 a further 15 samples collected from the Western Province were analysed (W0404-01 to W0404-22, not all samples analysed), and in April 2005 the final sample collection expedition resulted in 12 samples for bacterial analysis (W0405-01 to W0405-12). One additional sample of interest arrived at James Cook University following an outbreak of SHD in Suki, Western Province, in June 2005 (W0605-01). The other sample associated with an outbreak of SHD, W1105-01, was not analysed for bacterial pathogens (see Section 3.3.3.2).

All samples were placed in a cool insulated container (‘Esky’) immediately following collection, where they remained for a maximum of two days. Samples were transferred to a Quirk’s autofridge (Quirk’s Victory Light Co., Pty Ltd, Botany, New South Wales) portable refrigerator as soon as possible after collection, where they were stored at 8 ºC until they were transported to Australia for analysis. Upon arrival in Australia samples were stored at 4 ºC until microbial analysis was completed, at which time they were stored at -20 ºC for later toxin analysis.

At the time of collection the storage technique used by the sago consumers and the storage duration (time since sago starch was extracted from the palm) was documented. This information was subsequently used in statistical analyses (see Section 4.2.8).

4.2.1.2 Determination of pH

The pH value of 59 sago samples was determined using a TPS pH Cube (Crown Scientific, Acacia Ridge, Queensland). Five grams of sago starch was placed in a stoppered bottle with 10 ml of distilled water. The suspension was shaken at 5 min intervals for 30 min, and then the pH value of the liquid was measured.

4.2.1.3 Determination of water activity

The water activity (a_w) of 46 sago samples was determined using a dew point microvoltmeter, model HR-33T (Wescor Inc, Utah). The dew point for each sample was recorded then plotted on a graph against a standard curve consisting of standards of known molality. The range of standards was from 0.1 molal to 1.2 molal.

4.2.1.4 General microbiological procedures

In isolating and enumerating microbes from food, analysis should be conducted as soon as possible following sampling. In this study, there was insufficient time available to analyse all sago samples for all foodborne pathogens. As such, sago samples were more commonly analysed for those pathogens and indicator organisms considered the most relevant/important.

Where applicable, all isolation and enumeration techniques were based on the relevant Australian Standard. The general methods for food microbiology pertaining to preparation of dilutions (AS 1766.1.2 – 1991), pour plates (AS 1766.1.3 – 1991), colony counts (AS 1766.1.4 – 1991), most probable number (MPN) (AS 1766.1.6 – 1991) and standard plate counts (AS 1766.2.1 – 1991) were adhered to (Standards Australia, 1991c; Standards Australia, 1991d; Standards Australia, 1991e; Standards Australia, 1991a; Standards Australia, 1991b).

4.2.2 Enumeration of *Bacillus cereus*

A total of 43 sago samples was tested for *B. cereus*. Organisms isolated from samples from the first collection expedition to the East Sepik (10 samples) were enumerated using surface spread plates, all subsequent samples were enumerated using the most probable number (MPN) technique. For both methods, 10 g ± 0.2 g sago starch was aseptically weighed into a stomacher bag (Sarstedt Australia Pty Ltd, Ingle Farm, South Australia) and diluted in 90 ml of diluent (Appendix 2.1.1.1). The sample was mixed for 2 min using a Seward Stomacher 400 (John Morris Pty Ltd, Bowen Hills, Queensland). For plate counts, appropriate dilutions were made and plated onto duplicate, dried polymyxin egg yolk emulsion mannitol bromothymol blue agar (PEMBA) plates (Appendix 2.1.2.1). Plates were incubated at 37 ºC for 24 hours, and at room temperature for a further 24 hours. Presumptive positive colonies were confirmed using a Holbrook and Anderson lipid globule spore stain (Appendix 2.1.2.3).

For the MPN method, appropriate dilutions were added to triplicate tubes of tryptone soy polymyxin broth (Appendix 2.1.2.2). Tubes were incubated at 30 ºC for 48 hours and examined for turbidity. Tubes demonstrating turbidity were plated onto PEMBA agar, incubated at 37 ºC for 24 hours, and examined for typical colonies. Presumptive positive colonies were confirmed using a Holbrook and Anderson lipid globule spore stain.

All methods used to enumerate *B. cereus* were based on AS 1766.2.6 – 1991 (Standards Australia, 1991g). *B. cereus* ATCC 10876 and *B. subtilis* ATCC 6633 were used throughout as control organisms.

4.2.3 Enumeration of *Clostridium perfringens* and detection of saccharolytic clostridia

Clostridium perfringens enumeration was conducted on 16 sago samples. Ten grams (± 0.2 g) of sago starch was diluted in 90 ml diluent and mixed for 2 min using a Seward stomacher, as above. Serial dilutions were prepared, and triplicate tubes of cooked meat medium with neomycin (Appendix 2.1.3.1) were inoculated with 1 ml

of 10^{-1} dilution, 10^{-2} dilution and 10^{-3} dilution, resulting in a total of nine tubes. Tubes were incubated at 37 ºC for 48 hours. Tubes showing turbidity were plated onto tryptose sulphite cycloserine (TSC) agar (Appendix 2.1.3.2).

After the MPN had been set up, the 10^{-1} dilution tubes were heated to 80 ºC for 10 min to kill the vegetative cells (Barrow and Feltham, 1993). One hundred microlitres was plated on to saccharolytic clostridia agar (Gottschalk *et al.*, 1981) (Appendix 2.1.3.3). After incubation (as outlined below), colonies were counted and all colony morphology types were Gram stained (Appendix 2.1.1.2). Gram positive rods were then confirmed as *Clostridium* spp. using a Holbrook and Anderson spore stain. Those colony morphologies identified as *Clostridium* spp. were subsequently enumerated.

All plates (for both *C. perfringens* enumeration and saccharolytic clostridia) were incubated anaerobically in an anaerobic jar with an anaerobic atmosphere generation system (Oxoid Ltd, Thebarton, S.A.) at 37 ºC for 24 hours. Typical *C. perfringens* colonies were confirmed using Gram stain (Appendix 2.1.1.2), lactose gelatine medium (Appendix 2.1.3.4) and motility nitrate medium (Appendix 2.1.3.5).

The methods used were based on AS 1766.2.8 – 1991 (Standards Australia, 1991h), with some amendments to enable detection of saccharolytic clostridia. *Clostridium perfringens* ATCC 13124 and *Clostridium sordellii* ATCC 9714 were used as control organisms.

4.2.4 Isolation of various species of potential pathogens from family *Enterobacteriaceae*

4.2.4.1 Enumeration of total coliforms

Total coliforms from 57 samples were enumerated using the MPN technique based on AS 1766.2.3 – 1992 (Standards Australia, 1992). A 10 g (± 0.2 g) sample of sago starch was weighed out and diluted in 90 ml of diluent. This 10^{-1} dilution was mixed in a Seward stomacher, as previously. A dilution series was produced using 9 ml aliquots of diluent. The three appropriate dilutions were then added to triplicate tubes

of lauryl tryptose (LT) broth (Appendix 2.1.4.1). The tubes were incubated at 30 ºC for 24–48 hours for total coliform analysis. Tubes were examined for turbidity and gas production. Presumptive positives were plated onto eosin methylene blue (EMB) agar (Appendix 2.1.4.2) and incubated at 30 ºC for 24 hours. Plates showing a green metallic sheen, dark red/purple or mucoid pink colonies were recorded as positive. *E. coli* ATCC 25922 and *E. aerogenes* ATCC 13048 were used as control organisms.

4.2.4.2 Enumeration of faecal coliforms and *Escherichia coli*

Faecal coliforms and *E. coli* were enumerated from 69 samples using the MPN technique based on AS 1766.2.3 – 1992 (Standards Australia, 1992). A 10 g (± 0.2 g) sample of sago starch was weighed out and diluted in 90 ml of diluent. This 10^{-1} dilution was mixed in a Seward stomacher, as previously. A dilution series was produced in 9 ml aliquots of diluent. The three appropriate dilutions were then added to triplicate tubes of LT broth. Tubes were incubated at 37 ºC for 24–48 hours. Tubes were examined for turbidity and gas production, and presumptive positives plated onto EMB agar, incubated at 37 ºC for 24 hours. Plates were examined for metallic green, dark red/purple or mucoid pink colonies. Furthermore, a loopful of broth from each presumptive positive tube was transferred to EC broth (Appendix 2.1.4.3) and tryptone water (Appendix 2.1.4.4). The EC broth was incubated at 44 ºC for 24–48 hours, and observed for turbidity and gas production. The tryptone water was incubated at 44 ºC for 24 hours, and then 0.5 ml Kovac's reagent (Appendix 2.1.4.5) was added to test for indole production. *Escherichia coli* ATCC 25922 and *E. aerogenes* ATCC 13048 were used as control organisms throughout the protocol.

4.2.4.3 Isolation of *Salmonella* species

Salmonella spp. isolation was conducted on 69 samples. Twenty five grams (± 0.2 g) of sago starch was weighed into a stomacher bag and diluted in 225 ml of buffered peptone water (BPW) (Appendix 2.1.4.8). The bag was mixed for 2 min using a Seward stomacher. The pH of the contents of the bag was adjusted to pH 7.0 (± 0.2) using 1 M HCl or 1 M NaOH, then sealed and incubated at 37 ºC for 18–20 hours. Tubes of mannitol selenite cystine (MSC) enrichment broth (Appendix 2.1.4.9) and Rappaport-Vassiliadis (RV) enrichment broth (Appendix 2.1.4.10) were inoculated

with the pre-enrichment BPW. The MSC broth was incubated at 37 ºC for 18–24 hours, and the RV broth at 42 ºC for 18–20 hours. A loopful of each enrichment broth was plated onto both xylose lysine desoxycholate (XLD) agar (Appendix 2.1.4.11) and bismuth sulphite agar (BSA) (Appendix 2.1.4.12). The XLD plates were examined after 18–24 hours at 37 ºC for red/pink colonies, typically (but not always) with black centres. The BSA plates were examined after 18–24 hours at 37 ºC, and re-incubated for a further 24 hours if required. Typical colonies appear dark green/black, with a metallic sheen in the surrounding medium.

Typical colonies were confirmed using lysine decarboxylase broth (Appendix 2.1.4.13), ONPG broth (Appendix 2.1.4.14), API 20 E (bioMérieux Australia Pty Ltd, Baulkham Hills, New South Wales) and polyvalent 'O' and polyvalent 'H' antiserum (Oxoid Ltd, Thebarton, South Australia). The procedure used was based on AS 1766.2.5 – 1991 (Standards Australia, 1991f). This method detects only the presence/absence of *Salmonella* spp. *Salmonella salford* ACM 3762 was used as a control organism throughout the isolations.

4.2.4.4 Isolation of *Enterobacter sakazakii*

Thirteen samples were analysed for the presence *E. sakazakii*, using two different methods. Method 1 was based on that of Kandhai *et al.* (2004). A sample of 25 g (± 0.2 g) was weighed into a stomacher bag, and 225 ml of BPW was added. The sample was left for 30 min, and then mixed using a Seward stomacher for 2 min. It was then incubated for 18–20 hours. A loopful of BPW was then streaked onto violet red bile lactose (VRBL) agar (Appendix 2.1.4.15). Typical coliform colonies were plated onto tryptone soy agar (TSA) (Appendix 2.1.1.8) and incubated at 37 ºC for 24 hours. Yellow pigmented, oxidase negative colonies were tested for α-glucosidase activity (Appendix 2.1.4.16) and sorbitol fermentation (Appendix 2.1.4.17). Positive isolates were stored at –80 ºC for later confirmation using Vitek® II (bioMérieux Australia Pty Ltd, Baulkham Hills, New South Wales).

In the second method used to isolate *E. sakazakii*, a dilution series was prepared according to AS 1766 1.2 – 1991 (Standards Australia, 1991a) and plated onto nitrogen free agar (Appendix 2.1.1.3). Agar plates were incubated at 30 ºC for

72 hours. Large colonies were picked off the nitrogen free agar and subcultured onto VRBL agar and incubated at 37 ºC for 24 hours. Selected colonies were inoculated on citrate and urease slopes to isolate citrate positive, urease negative organisms. Confirmation using α-glucosidase activity was conducted according to the method of Kandhai *et al.* (2004). Positive isolates were stored at –80 ºC for later confirmation using Vitek® II (bioMérieux Australia Pty Ltd, Baulkham Hills, New South Wales).

4.2.4.5 Isolation of *Citrobacter freundii*

Thirteen samples were analysed for the presence of *C. freundii*, using two different methods. In the first method, selected colonies isolated during the enumeration of faecal coliforms and *E. coli* (Section 4.2.5.2) were picked off EMB agar and grown on citrate and urease slopes (Appendices 1.4.6 and 1.4.7) at 37 ºC for 24 hours. Isolates that were citrate positive were stored at –80 ºC for later confirmation using Vitek® II as above.

In the second method, a dilution series was prepared according to AS 1766 1.2 – 1991 (Standards Australia, 1991a) and plated onto nitrogen free agar. Agar plates were incubated at 30 ºC for 72 hours. Large colonies were picked off the nitrogen free agar and subcultured onto VRBL agar and incubated at 37 ºC for 24 hours. Selected colonies were inoculated on citrate and urease slopes. Citrate positive, urease variable isolates were stored at –80 ºC for later confirmation using Vitek® II, as above.

4.2.5 Isolation of *Listeria monocytogenes*

Detection of *L. monocytogenes* was conducted on 57 sago samples. Twenty-five grams (± 0.2 g) of sago was placed in a stomacher bag, and 225 ml of half strength Fraser broth (Appendix 2.1.5.1) was added. The contents of the bag were mixed using a Seward stomacher for 2 min, and then incubated at 30 ºC for 24 hours. A secondary enrichment in full strength Fraser broth (Appendix 2.1.5.2) was inoculated then incubated for 48 hours at 37 ºC. A loopful of the secondary enrichment broth was plated onto both Oxford agar (Appendix 2.1.5.3) and PALCAM agar (Appendix 2.1.5.4), and incubated at 37 ºC for 24–48 hours. Plates were examined for colonies

typical of *Listeria* spp. Presumptive positive colonies were plated onto blood agar (Appendix 2.1.1.4) confirmed using Gram stain, catalase, nitrate motility media (Appendix 2.1.3.5) and CAMP test (Sutherland *et al.*, 2003).

Control strains of *L. monocytogenes* ACM 4986, *L. ivanovii* ACM 4985 and *L innocula* ACM 4984 were used throughout the isolation process. The method used was based on AS/NZS 1766.2.16.1:1998 (Standards Australia, 1998). This method detects only the presence/absence of *L. monocytogenes*.

4.2.6 Enumeration of coagulase positive staphylococci

Coagulase positive staphylococci (typically *S. aureus*) were enumerated from 57 sago samples using a method based on AS 1766.2.4 – 1994 (Standards Australia, 1994b). A 10 g (± 0.2 g) sample of sago starch was weighed out and diluted in 90 ml of diluent. This 10^{-1} dilution was mixed in a Seward stomacher for 2 min. A dilution series was conducted in 9 ml aliquots of diluent and the appropriate dilutions were plated on Baird-Parker agar (Appendix 2.1.6.1). Plates were incubated at 37 ºC for 48 hours and examined for typical colonies. Presumptive colonies were subcultured in brain heart infusion broth (Appendix 2.1.1.7) then confirmed using a rabbit plasma coagulation test (Appendix 2.1.6.2). *Staphylococcus aureus* ATCC 25923 and *Staphylococcus epidermidis* ATCC 12228 were used throughout the test as control organisms.

4.2.7 Enumeration of total culturable aerobic bacteria

Standard plate counts were conducted on 69 sago samples to determine total culturable bacterial counts according to AS 1766.2.1 – 1991 (Standards Australia, 1991e). A 10 g (± 0.2 g) test potion was weighed into a stomacher bag, and 90 ml diluent added. The resulting 10^{-1} dilution was mixed using a Seward stomacher, then further diluted using 9 ml diluents. Appropriate dilutions were dispensed in empty Petri plates and molten standard plate count agar (Appendix 2.1.1.9) was added. Plates were incubated at 30 ºC for 72 hours. All colonies were then counted.

4.2.8 Statistical analysis

Statistical analyses were employed to determine whether relationships existed between bacterial pathogens and storage conditions of the sago starch. The SPSS 12.0.1 for Windows package (SPSS Australasia Pty Ltd, North Sydney, NSW) was used for all statistical analysis. Data that were normally distributed were analysed using the least significant difference one-way analysis of variance (ANOVA). A significance level of $P \leq 0.05$ was used. Data that were not normally distributed were analysed using Fisher's exact test, also using a significance level of $P \leq 0.05$. Analysis was conducted to determine whether parameters such as those listed in Table 4.01 (namely storage duration, storage technique, pH and a_w) have an influence on numbers of bacterial pathogens in sago starch. The influence of province of origin was also assessed.

To enable statistical analysis, parameters were categorized. In designating categories, two goals had to be met. First, categories that split the data into similarly sized groups where few, if any, categories did not have any data points were required for statistical analysis. Secondly, the categories required biological relevance. In keeping with these requirements, each parameter was split into four or five groups for normally distributed data, and two or three groups for data not normally distributed. The categories used for ANOVA and Fisher's exact test are listed in Table 4.01.

Table 4.01: Parameters that might affect microbial growth in sago starch and their categorisation for statistical analysis.

ANOVA	**Storage duration (weeks)**	**Storage technique**	**Starch pH**	**Starch a_w**
1	< 1.00	Fresh	< 4.00	≥ 0.990
2	1.00–2.99	Leaves	4.00–4.99	0.989–0.970
3	3.00–4.99	Plastic bag	5.00–5.99	0.969–0.940
4	≥ 5	Woven fibre	6.00–6.99	< 0.940
5		Other, unknown	7.00–7.99	
Fisher's exact test	**Storage duration (weeks)**	**Storage technique**	**Starch pH**	**Starch a_w**
1	< 3	Fresh	< 6	≥ 0.970
2	≥ 3	Leaves, woven fibre	≥ 6	< 0.970
3		Plastic, other, unknown		

4.3 Results

4.3.1 Sample information

Information about each sample analysed, including the approximate age, the method of storage, the area from which it originated, the sample pH and sample a_w is listed in Appendix 3.1. The majority of samples were stored wrapped in leaves, in woven bags or in plastic bags. They ranged in storage duration from freshly made to approximately one year old (one sample only). The majority of samples were less than six weeks old. The pH of the sago starch ranged from 2.73 to 7.34 and the a_w was invariably sufficiently high to allow bacterial growth.

4.3.2 *Bacillus cereus* enumeration

Bacillus cereus was present in 32 of the 43 sago samples tested. However, numbers were typically low, with less than 1.0×10^2 colony forming units per gram (cfu/g) being detected in 58% (25/43) of samples. A further 23% (10/43) of samples had between 1.0×10^2 cfu/g and 1.0×10^3 cfu/g. Thus, over 80% of all samples tested had less than 1.0×10^3 cfu/g of *B. cereus*. The number of *B. cereus* isolated from each sago sample tested is shown in Appendix 3.2.

Statistical analysis was conducted to determine whether any relationships existed between the $\log_{10}$ of *B. cereus* numbers and various parameters that might influence microbial growth in sago starch. Storage duration, storage technique and starch pH were considered. The data set did not lend itself to analysis for a_w. Table 4.02 shows the level of significance using least significant difference, post hoc analysis of variance. Sago starch less than one week old had significantly lower numbers of *B. cereus* than that of older sago. Fresh sago has significantly less growth of *B. cereus* than sago stored in traditional woven containers or wrapped in leaves. There are significantly higher numbers of *B. cereus* in sago with a pH below 4.00 than in sago with a pH greater than 5.00. No correlation between province of origin and numbers of *B. cereus* was observed.

Bacillus cereus was not detected in the implicated sago (W0605-01, see Section 3.3.3.1) using AS1766.2.6 – 1991.

Table 4.02: Statistical analysis (using analysis of variance) of the influence of storage duration, storage technique and pH on numbers of *B. cereus* isolated from sago starch.

Parameter	Group 1	Group 2	Mean difference	Significance
Storage duration	< 1.0 week	1.0–2.9 weeks	-1.034	0.037
Storage duration	< 1.0 week	3.0–4.9 weeks	-0.920	0.044
Storage duration	< 1.0 week	≥ 5.0 weeks	-1.711	0.002
Storage method	Fresh	Leaves	-1.296	0.023
Storage method	Fresh	Woven	-2.187	0.008
pH	< 4.00	5.00 - 5.99	1.589	0.005
pH	< 4.00	6.00 - 6.99	1.336	0.037
pH	< 4.00	≥ 7.00	1.274	0.065
pH	4.00 - 4.99	5.00 - 5.99	1.063	0.018

4.3.3 Enumeration of *Clostridium perfringens* and detection of saccharolytic clostridia

Of the 16 samples tested for *C. perfringens* and saccharolytic clostridia, only three tested positive for *C. perfringens* and one positive for saccharolytic clostridia (Appendix 3.3). The remaining 13 samples of *C. perfringens* had < 3 organisms/g, the lower limit of detection for the MPN method used. Saccharolytic clostridia were detected in only one sample, W0605-01 (the sample of sago implicated in the Suki outbreak of SHD). All other samples had <100 cfu/g, the lower limit for detection using the spread plate technique. All samples in which clostridia were detected had only low numbers. The data set was not suitable for the application of statistical analysis.

4.3.4 Isolation and enumeration of various species of potential pathogens from family *Enterobacteriaceae*

Members of the family *Enterobacteriaceae* were commonly isolated from sago starch. Total coliforms, which comprise members of the family *Enterobacteriaceae*, along with bacteria of some other families, were commonly isolated from sago starch at the upper limits of detection. Faecal coliforms were also commonly isolated, and

often at the upper limits for detection. In samples with high faecal coliform numbers, *E. coli* was common. Results of the enumeration of total coliforms, faecal coliforms and *E. coli*, and the presence of *Salmonella* spp., are shown in Appendix 3.4. Of the 69 samples tested for the presence of *Salmonella* spp, the analysis of five demonstrated the presence of the organism. None of the 13 samples tested was positive for *E. sakazakii*, and only one of the 13 samples, W0405-11, was positive for *C. freundii*.

Test results from the analysis of the samples collected on the first four sample collection expeditions (samples S1102-01 to S1102-10, M1102-01, W0303-01 to W0303-20 and S0603-01 to S0603-20) demonstrate that total and faecal coliform numbers were often at the upper limit of detection (1.1×10^3 cfu/g). In surveying the samples collected on the subsequent collection expeditions, further dilutions were conducted, resulting in the upper limit of detection being 1.1×10^4 cfu/g for samples W0404-01 to W0404-22 and 1.1×10^5 cfu/g for samples W0405-01 to W0405-12.

Statistical analysis was done to determine whether an association existed between the presence or absence of total coliforms, faecal coliforms, *E. coli* and *Salmonella* spp. with parameters such as the following: the province the sago was collected from (Western Province compared to East Sepik Province), the age of the sago sample at time of collection (< 3 weeks compared to ≥ 3 weeks), the pH of the sample (< 6 compared to ≥ 6), the a_w of the sample (< 0.997 compared to ≥ 0.997) and storage of the samples (stored in leaves or woven bags compared to stored in plastic bags and other less common methods). All analysis was done using Fisher's exact test. Table 4.03 shows the results of the analysis that was conducted. It can be seen that an association exists between the age of the sample and total coliforms and *E. coli*, and between the pH of the sago and faecal coliforms/*E. coli*. There were no other statistically significant correlations ($P < 0.05$) between organisms and the parameters tested.

Table 4.03: Significance levels using Fisher's exact test of the relationship between presence/absence of total coliforms, faecal coliforms, *E. coli* and *Salmonella* spp. with province of origin for sago starch, the age of the sample at time of collection, the pH of the sample, and the water activity of the sample.

	Total coliforms	**Faecal coliforms**	**E. coli**	***Salmonella***
Province	P = 0.606	P = 0.583	P = 0.276	P = 0.375
Age of sample	**P = 0.032**	P = 0.080	**P = 0.007**	P = 0.605
Sago pH	P = 0.053	**P = 0.037**	**P = 0.007**	P = 0.197
Water activity	P = 0.366	P = 0.275	P = 0.486	P = 0.792
Storage	P = 0.557	P = 0.447	P = 0.509	P = 0.087

4.3.5 Isolation of *Listeria monocytogenes* from sago starch

Listeria monocytogenes was not detected in any of the 57 samples tested.

4.3.6 Enumeration of coagulase positive staphylococci

Coagulase positive *Staphylococcus* spp. were isolated from 12 of 57 (21%) sago samples. The remaining 45 samples had $< 1.0 \times 10^2$ cfu/g, the lower detection limit of the plate count method used. Appendix 3.5 demonstrates the number of colony forming units of coagulase positive staphylococci per gram of sago starch.

Statistical analysis was done to determine whether an association existed between the presence or absence of coagulase positive staphylococci with parameters such as the province the sago was collected from, the age of the sago sample at time of collection, the pH of the sample and the a_w of the sample. Using Fisher's exact test ($P < 0.05$), no association was detected between the presence/absence of *S. aureus* and age (P = 0.543), water activity (P = 0.596), province (Western Province compared to East Sepik Province, P = 0.099) or storage (stored in leaves or woven bag compared to plastic bags P = 0.425). An association was found for the presence of *S. aureus* with 'neutral' pH (for pH > 6.00, P = 0.043).

4.3.7 Enumeration of total culturable aerobic bacteria

Standard plate counts were conducted to enumerate total culturable aerobic counts in sago starch. Bacteria were isolated from the vast majority of sago samples analysed. Results can be seen in Appendix 3.6.

Statistical analysis was conducted to determine whether any relationships existed between the $\log_{10}$ of total viable bacterial numbers and various parameters that might influence microbial growth in sago starch. Storage duration, storage pH and starch pH were considered. The data set did not lend itself to analysis for a_w using ANOVA as there was at least one group with less than two cases. Table 4.04 shows the level of significance using ANOVA least significant difference post hoc analysis. The analysis shows an association between older samples and total viable bacteria, and also between low pH and total viable bacteria. There was no association between total culturable bacteria and province (analysis not shown).

Table 4.04: Statistical analysis of the influence of pH and storage duration on numbers of total culturable bacteria isolated from sago starch.

Parameter	Group 1	Group 2	Mean difference	Significance
Storage duration	< 1.0 week	≥ 5.0 weeks	-1.296	**0.003**
Storage duration	1.0–2.9 weeks	≥ 5.0 weeks	-2.187	**0.011**
Storage duration	3–4.9 weeks	≥ 5.0 weeks	-1.034	0.072
pH	< 4	4.00–4.99	1.589	**0.005**
pH	< 4	5.00–5.99	1.336	**0.037**
pH	< 4	6.00–6.99	1.274	0.065
pH	< 4	≥ 7.00	1.063	0.018

4.4 Discussion

Given the conditions under which sago starch is extracted and stored (as described in Chapters 2 and 3), high levels of bacterial contamination are to be expected. In general, total bacterial numbers were high, as demonstrated in Section 4.3.7. There was a high prevalence of total coliforms and faecal coliforms, with the majority of sago samples being positive for these indicator organisms. Common foodborne bacterial pathogens were also isolated from sago starch.

One microbe of public health significance that frequently was isolated from sago starch was *B. cereus*. *Bacillus* species are environmental organisms often found in soil and on materials of plant and animal origin (Turnbull and Kramer, 1995). Consequently, raw foods of plant origin are a common source of *B. cereus*. The organism has been isolated from a wide variety of food products (Jenson and Moir, 2003), but have a particular association with farinaceous products. Moreover, outbreaks of *B. cereus* emetic food poisoning are usually linked to starchy products (Mortimer and McCann, 1974; Agata *et al.*, 2002).

This is the first study to isolate and enumerate *B. cereus* from sago starch, although it has commonly been isolated from other indigenous fermented foods (Ashenafi and Busse, 1991; Cook *et al.*, 1991; BeiZhong *et al.*, 2001). Studies summarised by Jenson and Moir (2003) have shown that while there is often a high prevalence of *B. cereus* in a variety of foods, numbers are usually low. The findings of this study support such a trend, with *B. cereus* being isolated from 32 of the 43 samples tested (74%), but generally in low numbers. The high prevalence is probably at least partly attributable to the ubiquitous nature of the organism, and its ability to produce spores. Furthermore, the extraction and processing techniques employed in sago production contribute to the high level of incidence of *B. cereus* contamination in sago starch.

With such factors in mind, it might have been expected that numbers of *B. cereus* would have been consistently high. This was not the case, with 6 of the 43 samples tested having greater than 1.0×10^3 cfu/g, of which only 1 sample had greater than 1.0×10^4 cfu/g. Sago starch is cooked prior to consumption, presumably resulting in a considerable decrease in vegetative cell numbers. Thus, under typical circumstances, only low numbers of *B. cereus* vegetative cells would be consumed in sago starch. Consequently, diarrhoeal food poisoning due to *B. cereus* is unlikely to be a major contributor to gastroenteritis in PNG.

Most food poisoning episodes due to *B. cereus* emetic toxin are the result of spore survival in the cooking process, then subsequent outgrowth in stored foods. As with other foods, it is unlikely that the sago cooking process is sufficient to kill all

B. cereus spores. Sago starch is typically consumed soon after production, but in cases where cooked sago starch is stored for later consumption there is an increased risk of emetic food poisoning.

The statistical analysis of *B. cereus* numbers in relation to pH, storage method and storage duration suggested an association between high bacterial numbers and low pH. Higher numbers of *B. cereus* were found in sago samples with a pH < 4.00 compared to sago with pH 5.00 to 5.99, and pH 6.00 to 6.99. The difference between *B. cereus* numbers at pH < 4.00 and pH ≥ 7.00 was not statistically significant to the 0.05 level (P = 0.065), however samples with pH 4.00 to 4.99 had significantly higher numbers than samples with pH 5.00 to 5.99. Thus, an overall trend of higher numbers of *B. cereus* at low pH was observed. Such a trend seems to defy reports within the literature, with the lower limit for growth of *B. cereus* documented to be in the range of 4.3 to 5.0 (Thomas *et al.*, 1993; Lund and Eklund, 2000). The results observed in this study are indicative of spore survival in sago starch. Spores are known to be more resistant to harsh environmental conditions than vegetative cells (Nicholson *et al.*, 2000; Nicholson *et al.*, 2002), and may be able to survive in highly acidic sago. Clavel *et al.* (2004) demonstrated survival of *B. cereus* spores over six hours in highly acidic simulated gastric conditions, although it is unknown what effect much longer exposure would have. Spores present in sago are likely to germinate under suitable conditions, as was the case on the relatively nutrient rich agar used for enumeration.

Samples that were stored in leaves or woven bags were a more favourable environment for *B. cereus* than fresh sago, and older sago samples consistently had higher numbers than samples under one week old. It is likely that over time sago is contaminated with spores. *Bacillus cereus* is a well known environmental contaminant, and has recently been proposed as a symbiont in the gut of invertebrate animals (Jensen *et al.*, 2003). Insects and other arthropods are commonly observed in old stored sago. Thus, with increased exposure to invertebrate animals and the environment over time, there may be an increase in spore numbers, resulting in higher numbers being cultured from old sago. In this case, the increase in culturable *B. cereus* might not be associated with an increased public health risk.

The genus *Clostridium* is important in public health due to the numerous clostridial toxins that have been associated with human foodborne illness. The incidence of *Clostridium* spp. in sago starch was low, with only 3 of the 16 samples testing positive. All positive samples had low numbers of clostridia. Interestingly, the sample of sago implicated in an outbreak of SHD (W0605-01) was positive for both saccharolytic clostridia and *C. perfringens*. As with the other positive samples, numbers were well below those associated with clostridial foodborne illness. According to Bates and Bodnaruk (2003) large numbers of cells have to be ingested to cause perfringens food poisoning, usually 10^6/g of food. Despite the anaerobic environment of tightly packed sago starch, this research suggests that such high numbers of clostridia are unlikely to be reached in appropriately stored sago. This might be attributable to a number of factors:

- *C. perfringens* and *C. botulinum* are more commonly associated with, although not restricted to, meat, fish and other high protein products
- Outbreaks are often associated with foods that have undergone heat treatment (particularly *C. botulinum*), providing a competition free environment for clostridia spores to germinate and multiply
- The minimum pH for growth of *C. perfringens* and *C. botulinum* is around 4.6 to 5.0 (Lund and Eklund, 2000). Nevertheless, botulism has been associated with traditional fermented foods in the northern hemisphere, but these are typically high protein foods in which levels of fermentable carbohydrates are too low to enable a rapid decrease in pH (Dodds and Austin, 1997).

The methodology used to enumerate saccharolytic clostridia may have contributed to the low numbers isolated, as Lund and Peck (2000) state that heating samples to 80 °C for 10 min is likely to inactivate spores of non-proteolytic strains of *C. botulinum*. This may hold true for other species of clostridia also. Nevertheless, the low incidence of saccharolytic clostridia is consistent with that of *C. perfringens* in sago starch.

Indicator organisms were used to gauge the level of faecal contamination in sago starch. The perceived validity of the indicator organisms used in this study, namely coliforms, faecal coliforms and *E. coli*, has been discussed in detail elsewhere (Cox

et al., 1988; Craven *et al.*, 2003). Based on the enumeration of indicator organisms, it is clear that the incidence and level of faecal contamination in sago starch is high. Using coliforms as indicator organisms, less than 20% (11/57) of samples were free of contamination, while 77% of samples were at the upper limit of detection. Faecal coliforms and *E. coli* are often considered a better indicator of faecal contamination than coliforms. The incidence of both faecal coliforms and *E. coli* was high, with the former being detected in over 75% (52/69) of samples, and *E. coli* in approximately 70% of samples.

Coliforms have been isolated from a number of traditional fermented foods, occasionally reaching over 10^3 cfu/g (Mugula *et al.*, 2003b; Muyanja *et al.*, 2003; Tsav-Wua *et al.*, 2004). However, it seems that in many cases coliform numbers decrease as fermentation progresses (Nout, 1991; Masha *et al.*, 1998; Mugula *et al.*, 2003b). The statistical analysis suggests that a similar trend occurs in sago starch. Sago over three weeks old was less likely to have total coliforms or *E. coli* present, which might be due to the progression of fermentation. The effect of fermentation on coliform numbers in sago starch will be further investigated in Chapter 9.

Salmonellae were isolated from approximately 7% (5/69) sago samples tested. This corresponds well with a survey of sorghum based flour by Kunene (1999), where *Salmonella* spp. were isolated from 7% of non-fermented samples. However, no *Salmonella* spp. were isolated from the fermented cooked product. The survival of salmonellae in other traditional fermented foods has been well studied, and their survival in sago starch was investigated in this study (see Chapter 9).

The incidence of *Salmonella* spp. in sago is perhaps not surprising when one considers the high levels of faecal contamination in sago starch. Although *Salmonella* spp. is more commonly associated with foods of animal origin, it can survive for months outside the animal host, particularly in association with soil, but also on some foods of non-animal origin (Natvig *et al.*, 2002; Islam *et al.*, 2004; Holley *et al.*, 2006; Uesugi *et al.*, 2006; You *et al.*, 2006). In the late 1960s the sporadic occurrence of salmonellosis was reported in the East Sepik Province of PNG (Morahan, 1968). The study found 33 of approximately 1100 stool specimens positive for *Salmonella* spp.

In recent years *E. sakazakii* has been recognized as a potential human pathogen, mainly in neonates (Lai, 2001). Contaminated food has been associated with illness, particularly infant formula (Seo and Brackett, 2005). It has also been isolated from indigenous fermented foods, but numbers tend to decrease as fermentative activity increases (Nout, 1991; Coulin *et al.*, 2006). The low pH of sago starch and other factors associated with fermentation (see Chapter 9) are expected to inhibit the growth of *E. sakazakii*.

No *L. monocytogenes* was isolated from sago starch. The absence of *L. monocytogenes* in the 57 samples tested is important, given the serious public health implications of the organism. There are numerous reports of outbreaks of listeriosis in a variety of food products, with many outbreaks having mortality rates in excess of 20% (Sutherland *et al.*, 2003). The majority of the cases listed by Sutherland *et al.* (2003) occurred in developed countries, which are better equipped to conduct the necessary epidemiological studies and laboratory confirmation. It is likely that listeriosis occurs in developing countries also, but goes undetected. Factors that influence the growth and survival of *L. monocytogenes* in sago starch will be addressed in Chapter 9.

Coagulase positive staphylococci were isolated from approximately 21% of sago samples tested. There are at least three species of *Staphylococcus* capable of coagulating rabbit plasma (Barrow and Feltham, 1993), but *S. aureus* is the most important food contaminant, and is associated with the vast majority of outbreaks of staphylococcal food poisoning (Stewart, 2003). Consequently, in food microbiology the term coagulase positive staphylococci usually refer to *S. aureus*.

Numbers of coagulase positive staphylococci were often low, in the range of 10^2-10^3 cfu/g. Despite there being many variables that contribute to production of staphylococcal enterotoxin, it is generally considered that at least 10^5 cfu/g are required to produce sufficient toxin to cause foodborne illness (Jablonski and Bohach, 1997; Stewart, 2003). Thus, the presence of coagulase positive staphylococci in sago starch at low levels should not be considered a public health risk. Four sago samples had coagulase positive staphylococci present at 4.50×10^4

cfu/g or higher, suggesting that in some cases numbers do get high enough to become a risk to human health.

Statistical analysis suggested an association between sago above pH 6.0 and the presence of coagulase positive staphylococci. A cursory examination of the raw data reveals that all samples that were positive were well within the pH growth range of *S. aureus*, which is approximately pH 4 to 10 (Lund and Eklund, 2000). *S. aureus* is said to be a poor competitor in complex microbial communities and in fermented foods (Stewart, 2003). The association with coagulase positive staphylococci and relatively high pH suggests that the organism may survive better in sago that does not undergo rapid and thorough fermentation.

Contamination of foods with *S. aureus* is indicative of human or other animal contamination, with humans being the main reservoir (Jablonski and Bohach, 1997). Repeated handling of sago starch is likely to be the main source of contamination.

Total bacterial counts in sago starch were generally very high, typically in the range of 10^7 to 10^8 cfu/g, but up to 5.9×10^9 cfu/g. The vast majority of samples tested exceed the Indonesian standard for sago starch, as stated by Sopade (1999), of 10^6 cfu/g. The high numbers of total aerobic bacteria is indicative of the high level of contamination of sago starch from the environment, and from the water used to extract sago. Statistical analysis suggests that the high counts might also be attributable to fermentation of sago starch. The analysis shows an association between low pH and high bacterial numbers. There is also an association between storage duration and bacterial numbers, with sago five weeks or older having higher total bacterial counts than samples that had been stored for a lesser duration.

It is difficult to assess the suitability of sago starch based on total bacterial counts alone, as such a count might represent potential pathogens, indicator organisms, harmless organisms and potentially beneficial organisms. However, tests for specific bacteria of public health significance suggest that pathogenic bacteria are present in sago starch. Statistical analysis suggests that in many cases there is an association between pH of the sample and growth of certain bacterial organisms. Although there was no association between storage method and pathogen numbers, storage method

might influence other parameters. The influence of selected storage methods and pH (more specifically fermentation) on the growth and survival of pathogenic bacteria was investigated (Chapter 9).

CHAPTER 5: THE PREVALENCE AND DETERMINANTS OF FUNGI AND ACTINOMYCETES IN SAGO STARCH

5.1 Introduction

Following the discovery of mycotoxins and their implications for human and animal health in the 1960s, the importance of foodborne fungi has been realised. Fungi can no longer be considered as merely undesirable, for many genera are capable of producing mycotoxins. Furthermore, it is likely that novel mycotoxins will continue to be discovered in the future, possibly produced by fungi not currently thought to be toxigenic. The presence of known mycotoxins in sago starch will be addressed in Chapter 6.

Actinomycetes have been associated with toxin production and adverse human health effects. Recent studies have suggested that actinomycetes are capable of producing tetrodotoxin, which is associated with puffer fish intoxication (Imada, 2005; Wu *et al.*, 2005). Actinomycetes are also known to produce a variety of secondary metabolites, including potentially immunosuppressive compounds (Komatsu *et al.*, 2004), and compounds associated with allergenic responses (Dutkiewicz *et al.*, 2002; Huttunen *et al.*, 2004; Hirvonen *et al.*, 2005).

Toxin production is just one problem associated with microbial contamination. The growth of unwanted fungi, and to a lesser extent actinomycetes, in food results in several types of spoilage, including: the changes in nutrient status and aesthetic value; the development of off flavours and smells; discolouration; rotting; and the formation of allergenic propagules (Blaney and Williams, 1991; Pitt and Hocking, 1991; Fleet, 1997; Filtenborg *et al.*, 2000). Moreover, the social and economic costs of fungal spoilage are enormous. Although monetary values are difficult to apply, Pitt and Hocking (1997) estimate losses due to fungal spoilage in Australia alone to be in excess of AUD$10,000,000 per annum. Aidoo (1991) estimates that globally

10% of durable crops are lost post-harvest. In the tropics, 5–20% losses have been estimated, rising to 25% for perishable foods (Aidoo, 1991; Pitt and Hocking, 1997). In countries with variable food security, like PNG, such losses can have a large effect on human diet.

Since the first reports of SHD, it has been speculated that fungi and associated mycotoxins could be involved in the disease (Taufa, 1974; Donovan *et al.*, 1977). Furthermore, sociological surveys revealed that sago considered unfit for human consumption often has changed in colour, taste or smell (see Chapter 3). Such changes might be indicative of fungal contamination.

Numerous mycological surveys have been conducted on tropical agricultural commodities, including studies pertaining specifically to foods produced and consumed in PNG (Shaw *et al.*, 1972; Greve *et al.*, 1994; Amoa *et al.*, 1999). Studies involving implicated sago (Donovan *et al.*, 1977; Stace *et al.*, unpublished) and sago from markets (Amoa *et al.*, 1999) have included fungal isolation. However, the studies of Stace *et al.* (unpublished) and Donovan *et al.* (1977) were inconclusive, and the work of Amoa and co-workers (1999) was limited to just six sago samples. This study addresses the need for a comprehensive survey of fungi and actinomycetes to determine their prevalence in sago starch, and the factors that contribute to their presence. The inclusion of actinomycetes with the fungi is on account of their relatively slow growth and filamentous habit.

5.2 Materials and Methods

5.2.1 Enumeration and identification of yeasts and moulds

Sago samples, as described in Section 4.2.1.1, were analysed to determine the extent of fungal contamination. In addition to the 70 samples tested for pathogenic bacteria, mycological analysis was also conducted on the second sago sample associated with an outbreak of SHD (W1105-01). Despite the extended storage duration of sample W1105-01, it was deemed worthwhile conducting mycological analysis given the speculation that fungal metabolites play a role in SHD.

As with the detection and enumeration of bacterial pathogens in sago starch (Section 4.2.1.4), time did not permit the analysis of all sago samples for all groups of filamentous fungi and actinomycetes.

Seventy-one sago samples were enumerated for yeasts and moulds (filamentous fungi) using methods based on AS 1766.2.2 – 1994 (Standards Australia, 1994a). A 10 g ± 0.2 g representative sample of sago starch was aseptically weighed into a stomacher bag and diluted in 90 ml of diluent (Appendix 2.1.1.1). The sample was mixed for 2 min using a Seward Stomacher 400 (John Morris Pty Ltd, Bowen Hills, Queensland). Appropriate dilutions were made and plated onto duplicate, dried dichloran rose-bengal chloramphenicol (DRBC) agar plates (Appendix 2.2.1.1). Plates were incubated at 25 ºC for 72 hours, after which time yeast colonies were enumerated. Plates were then incubated for a further 48–96 hours, and mould colonies subsequently enumerated.

Following mould enumeration, representative mould isolates were taken from DRBC agar and subcultured onto potato carrot agar (Appendix 2.2.1.2). Plates were incubated for 5–7 days at 25 ºC. Following incubation, representative spore bearing structures were removed and stained with trypan blue and examined microscopically using a Carl Zeiss compound microscope. Moulds were identified to genus level where possible using appropriate keys (Barron, 1968; Zycha and Siepmann, 1969; von Arx, 1970; Pitt, 1979).

Further identification was conducted as required. For *Penicillium* spp., isolates were grown on Czapek yeast autolysate agar (CYA agar) (Appendix 2.2.1.3), malt extract agar (MEA) (Appendix 2.2.1.4), and glycerol nitrate (GN25) agar (Appendix 2.2.1.5). The plating regimen as outlined in Pitt (1979) was followed. Each *Penicillium* isolate was plated onto CYA agar in triplicate, with individual plates being incubated at a different temperature (5 ºC, 25 ºC and 37 ºC). Isolates plated onto MEA and G25N agar were incubated at 25 ºC. All plates were incubated for 7 days, and colony diameters subsequently measured.

Isolates of *Trichoderma* spp. were grown on MEA in the dark and in the light at ambient laboratory conditions (~ 21ºC), as described in Gams and Bissett (1998). Appropriate keys, illustrations and diagrams were used to enable identification (Bissett, 1991; Gams and Bissett, 1998).

Isolates of interest were sent to Centraalbureau voor Schimmelcultures at the Institute of the Royal Netherlands Academy of Arts and Sciences for definitive identification.

5.2.2 Enumeration of mucoraceous moulds

A total of 27 samples was analysed for the presence of mucoraceous moulds. A 10 g ± 0.2 g representative sample of sago starch was aseptically weighed into a stomacher bag and diluted in 90 ml of diluent. The sample was mixed for 2 min using a Seward Stomacher, as above. Appropriate dilutions were made and plated onto duplicate, dried synthetic *Mucor* agar (SMA) plates (Appendix 2.2.1.6). The SMA used was based on that of Hesseltine (1954), with chloramphenicol used in place of penicillin and streptomycin. Plates were incubated at 25 ºC for 72 hours and examined for growth of *Mucor* and related species. Plates that did not demonstrate growth after 72 hours were incubated for a further 48–96 hours, and plates were again examined for *Mucor* and related species.

5.2.3 Enumeration of *Geotrichum* species

A total of 42 samples was analysed for the presence of *Geotrichum* spp. A 10 g ± 0.2 g representative sample of sago starch was aseptically weighed into a stomacher bag and diluted in 90 ml of diluent. The sample was mixed for 2 min using a Seward Stomacher, as above. Appropriate dilutions were made and plated onto duplicate, dried *Geotrichum* agar plates (Appendix 2.1.7) based on the medium of Tsai and Hsieh (1999). Plates were incubated at 25 ºC for 72 hours and examined for growth of *Geotrichum* spp.

5.2.4 Isolation of actinomycetes from sago starch

Thirty-one samples were analysed for the presence of actinomycetes. A 10 g ± 0.2 g representative sample of sago starch was aseptically weighed into a stomacher bag and diluted in 90 ml of diluent. The sample was mixed for 2 min using a Seward Stomacher, as above. Appropriate dilutions were made and plated onto duplicate, actinomycete agar (Appendix 2.2.1.8) (Waksman, 1967). Plates were incubated at 30 ºC for 5 days and examined for growth of typical actinomycetes.

5.2.5 Additional techniques used for sago samples implicated in SHD

5.2.5.1 Additional fungal isolation techniques applied to the implicated sago

The sago samples implicated in SHD that arrived late in the project (after the general survey sago starch had been completed) were tested for the presence of fungi and actinomycetes using the same methods as outlined above (Section 5.2.1, 5.2.2, 5.2.3 and 5.2.4). Additional methods were also used to overcome the problem of plates being overrun by rapidly growing isolates, so enabling a more thorough investigation of fungal species present. Due to the low pH of most sago samples, fungal enumeration was conducted on DRBC agar with increased acidity (pH 4.5). Plates were incubated and enumerated under the same conditions as regular DRBC agar (Section 5.2.1). Serial dilutions were also plated onto one-quarter strength Czapek Dox agar (Appendix 2.2.1.9) and incubated at 25 ºC for 5–7 days. Resulting isolates were subcultured for later identification and haemolysis screening.

Individual pigmented clumps of sago starch were plated directly onto DRBC agar and one-quarter strength Czapek Dox agar to determine whether fungal growth was responsible for the clump formation. Colonies were subcultured for later identification and haemolysis screening.

5.2.5.2 Direct culture of fungi on sago starch

Clumps from the implicated sago were placed in sterile Petri plates with moist cotton wool to provide a humid atmosphere conducive to fungal growth. The sago starch

was examined regularly for the presence of fungal growth. Where possible resulting growth was subcultured onto one-quarter strength Czapek Dox medium for later identification and haemolysis screening. Alternatively, morphological features were used to identify fungi to genus level using direct microscopy.

5.2.6 Ergosterol analysis of sago starch

Forty-six samples were analysed for ergosterol content as an indicator of fungal biomass. A 10 g ± 0.2 g representative sample of sago starch was weighed into a flat bottom QuickFit® flask. Sixty millilitres of saponifying reagent (Appendix 2.2.2.1) was added and refluxed at boiling point on a hot plate for 30 min. After cooling, the suspension was filtered into a 250 ml separating funnel using Whatman No. 4 filter paper (Crown Scientific, Acacia Ridge, Queensland). The flask was washed with 15 ml of methanol, which was added to the separating funnel. Fifteen millilitres of water and 50 ml of hexane subsequently was added to the separating funnel, and shaken for 1 min. The lower layer was collected into a conical flask. The upper layer was run through a 4 cm column of anhydrous granular sodium sulphate (Ajax Chemicals, Sydney, New South Wales) in a filter funnel to remove water. The resulting fraction was collected into a 250 ml round bottom QuickFit® flask. The previously reserved lower layer was returned to the separating funnel and the conical flask rinsed with 5 ml of methanol and added to the separating funnel. The conical flask was further rinsed with 50 ml of hexane, which was then placed in the separating funnel. The funnel was shaken for 1 min, and then the lower layer discarded. The upper layer was run through the anhydrous granular sodium sulphate column (as above) and collected in the same round bottom QuickFit® flask. The column was washed twice with 5 ml of hexane.

The hexane extract was evaporated to near dryness using a rotary evaporator (Buchi, Switzerland) at 42 ºC, and then taken to complete dryness under a stream of nitrogen. The residue was dissolved in 2 ml of hexane and filtered using a 0.22 µm PVDF syringe filter (Biolab, Mulgrave, Victoria) into an autosampler vial for analysis by high performance liquid chromatography (HPLC).

High performance liquid chromatography was conducted on a Shimadzu LC-10AT VP liquid chromatograph fitted with a Shimadzu SIL-10 AD VP auto injector, a Shimadzu CTO-10A VP column oven (set at 30 ºC) and Shimadzu SCL-10A VP system controller (Shimadzu Scientific Instruments, Eagle Farm, Queensland). A 10 µl sample was injected into a Waters Novapak silica 60 Å normal phase column (150 × 3.9 mm, 4 µm particle size) (Waters Australia, Rydalmere, New South Wales), using a mobile phase of 1% isopropanol in hexane at a flow rate of 1 ml/min. Ergosterol was detected using a Shimadzu SIL-10AD VP diode array detector (Shimadzu Scientific Instruments, Eagle Farm, Queensland) set at 282 nm.

5.2.7 Statistical analysis

Statistical analyses were used to determine whether relationships existed between the number of fungi, ergosterol content and storage conditions of the sago starch. The SPSS 12.0.1 for Windows package (SPSS Australasia Pty Ltd, North Sydney, New South Wales) was used for all statistical analysis. Data that were normally distributed were analysed using the least significant difference one-way ANOVA. A significance level of $P \leq 0.05$ was used. Data that were not normally distributed were analysed using Fisher's exact test, also using a significance level of $P \leq 0.05$. Data were grouped into categories to enable statistical analysis to be conducted. The categories used for each test are listed in Table 4.01. The influence of province of origin was also assessed.

A T-test and Pearson correlation test were also conducted to compare yeast and mould numbers, and to determine whether a relationship existed between ergosterol levels and fungal numbers.

5.3 Results

5.3.1 Enumeration of yeasts and moulds and prevalence of fungal genera

5.3.1.1 Enumeration of yeasts and moulds and statistical analysis

Of the 69 sago samples analysed (excluding the implicated sago), yeasts were isolated from all but 2 samples, while moulds were present in all but 5 samples. Appendix 4.1 lists the numbers of yeasts and moulds isolated from each sample. In general yeast numbers were higher than mould numbers ($t = 3.925$; $df = 70$; $P = 0.000$).

Analyses were conducted to determine whether correlations existed between fungal numbers and factors such as storage duration, storage technique, pH, and a_w. Analysis of numbers ($\log_{10}$) suggested that yeast numbers increased over the first few weeks of storage, then decreased in sago stored for over five weeks. Yeast numbers were significantly lower in samples greater than five weeks compared to samples aged one to three weeks ($F = 2.32$, $df = 3$, $P = 0.014$). However, there was no significant difference between yeast numbers in samples one to three weeks old compared to samples three to five weeks old ($F = 2.32$, $df = 3$, $P = 0.170$), or in sago samples one to three weeks old than in samples less than one week old ($F = 2.32$, $df = 3$, $P = 0.067$).

Sago samples stored in natural woven containers had significantly more yeasts than fresh sago ($F = 4.44$, $df = 4$, $P = 0.020$) and sago categorised as ‘other and unknown’ (including in earthenware pots and saucepans covered with water and smoked samples) ($F = 4.44$, $df = 4$, $P = 0.000$). Sago samples in the ‘other and unknown’ category also had lower yeast numbers than sago starch stored in leaves ($F = 4.44$, $df = 4$, $P = 0.004$) and plastic bags ($F = 4.44$, $df = 4$, $P = 0.023$).

Yeast numbers were lower at pH < 4.0 compared to all other pH categories, although the only value that reached statistical significance (to $P = 0.05$) was the comparison between the pH < 4.0 and pH 4–4.99 ($F = 2.68$, $df = 4$, $P = 0.002$). Yeast numbers

were greatest within the pH range 4–4.99, although there was no significant difference between numbers in this category compared to higher pH categories (5.00–5.99; 6.00–6.99; > 7.00).

Mould numbers increased in old sago. Samples greater than five weeks old had significantly higher mould numbers than samples less than one week old ($F = 3.76$, $df = 3$, $P = 0.004$) and samples one to three weeks old ($F = 3.76$, $df = 3$, $P = 0.016$). Sago samples aged between three and five weeks also had significantly higher mould numbers than sago less than one week old ($F = 3.76$, $df = 3$, $P = 0.046$).

Analysis of mould numbers subjected to different storage techniques shows significantly higher mould numbers in sago stored in natural fibre woven containers compared to the other storage techniques ($F = 4.52$, $df = 4$; fresh $P = 0.018$; leaves $P = 0.003$; plastic $P = 0.010$; other, unknown $P = 0.001$).

Statistical analysis suggested that starch pH had little effect on mould numbers. Mould numbers were highest in sago starch with pH < 4, but there was no statistical significance between this low pH category and the other categories.

Analysis of the effect of a_w on yeast or mould numbers could not be done using analysis of variance, as there was at least one group with fewer than two cases for both yeasts and moulds.

5.3.1.2 Identification and prevalence of mould genera

Moulds were identified to genus level or beyond where possible. The identity and number of cfu/g (within the limitations of the methodology) of each mould species isolated from the 71 sago samples are listed in Appendix 4.4. Table 5.01 demonstrates the prevalence of the fungal genera identified in sago starch. The genus *Penicillium* Link was present in over 60% of sago samples tested. Other genera of moulds to occur in numerous sago samples were *Scytalidium* Pesante (30 %) and *Aspergillus* Link (24 %). Numerous genera, including *Aureobasidium* Viala and Boyer, *Acremonium* Link, *Fusarium* Link, *Cladosporium* Link, *Trichoderma* Pers. and *Cylindrocarpon* Wollenw. were isolated from 10% or more of samples.

Table 5.01: Prevalence of individual mould genera from sago samples collected from the East Sepik Province (S1 and S2) and the Western Province (W1,W2 and W3). Refer to Section 4.2.1.1 for collection details.

Genus	S1 (11)	W1 (16)	S2 (15)	W2 (16)	W3 (12)	Total (70)	Prevalence (%)
Penicillium	6	10	7	11	9	43	61
Scytalidium	5	6	7	0	3	21	30
Aspergillus	7	3	2	3	2	17	24
Aureobasidium	2	0	4	9	0	15	21
Acremonium	3	2	4	4	1	14	20
Geotrichum-like	1	4	7	1	1	14	20
Fusarium	4	3	0	4	2	13	19
Cladosporium	3	3	4	0	2	12	17
Trichoderma	1	2	2	4	1	10	14
Cylindrocarpon	0	5	0	0	2	7	10
Paecilomyces	0	3	1	1	0	5	7
Curvularia	1	0	1	0	1	3	4
Aphanocladium-like	0	0	0	2	1	3	4
Scopulariopsis	0	0	0	1	1	2	3
Gliomastix	1	1	0	0	0	2	3
Phialophora	0	1	0	0	1	2	3
Zythia	1	0	0	0	0	1	1
Pestalotia	1	0	0	0	0	1	1
Chrysosporium	1	0	0	0	0	1	1
Moniliella	1	0	0	0	0	1	1
Stachybotrys	0	1	0	0	0	1	1
Helicostylum	0	1	0	0	0	1	1
Talaromyces	0	1	0	0	0	1	1
Humicola	0	0	1	0	0	1	1
Absidia	0	0	0	1	0	1	1
Cephalosporiopsis	0	0	0	1	0	1	1
Neurospora	0	0	0	1	0	1	1

Legend: S1: Samples collected from the East Sepik Province, November 2002
S2: Samples collected from the East Sepik Province, June 2003
W1: Samples collected From the Western Province, March 2003
W2: Samples collected From the Western Province, April 2004
W3: Samples collected From the Western Province, April 2005

Although both *Aureobasibium* and *Geotrichum* Link are yeasts, they commonly exist in a filamentous state, so were enumerated with the moulds. *Aureobasibium* spp. were isolated from approximately 21% of samples. *Geotrichum* and *Geotrichum*-like organisms were isolated from 20% of samples surveyed using DRBC agar (refer to Section 5.3.3 for enumeration of *Geotrichum* spp. using *Geotrichum* selective agar).

Other genera isolated from sago starch that could to be identified to genus level include: *Paecilomyces* Bain.; *Curvularia* Boedijn; *Scopulariopsis* Bain.; *Gliomastix* Guéguen; *Phialophora* Medlar; *Zythia* Fr.; *Pestalotia* de Not.; *Chrysosporium* Corda; *Moniliella* Stolk and Dakin; *Stachybotrys* Corda; *Helicostylum* Bain.; *Talaromyces* Benjamin; *Humicola* Traaen; *Absidia* van Tieghem; *Cephalosporiopsis* Moreau; and *Neurospora* Shear and Dodge.

Statistical analysis was conducted using Fisher's exact test to determine whether there was an association between the presence/absence of the most commonly occurring moulds (those with a prevalence of $\geq 10\%$) and storage duration, storage technique, starch pH, starch a_w and the province from which the sago sample was collected. In general, there was no association between any of these factors and the presence/absence of any of the moulds analysed. The only exceptions were for *Geotrichum* spp. and *Geotrichum* like fungi, which were not so commonly isolated from the Western Province ($P = 0.037$, one sided) and *Cylindrocarpon* spp., which was isolated only from the Western Province ($P = 0.033$, one sided).

5.3.2 Enumeration of mucoraceous moulds

Growth was observed on some SMA, but no mucoraceous moulds were isolated that were not also isolated from DRBC agar.

5.3.3 Enumeration of *Geotrichum* species

Numbers of *Geotrichum* spp. were generally low, being below the limit of detection (1.0×10^2 cfu/g) for the majority of sago samples. *Geotrichum* spp. were detected in nine sago samples using *Geotrichum* selective agar; four of which were from the East Sepik Province and five from the Western Province. The highest count was 7.4×10^4 cfu/g. The numbers of colony forming units of *Geotrichum* spp. per gram of sago starch are listed in Appendix 4.2.

5.3.4 Enumeration of actinomycetes

No actinomycetes were isolated from sago starch using actinomycete agar.

5.3.5 Mycoflora of implicated sago samples

5.3.5.1 Fungal enumeration of implicated sago samples

Table 5.02 demonstrates the number of yeasts and moulds isolated from the two implicated samples. The predominant genera of filamentous fungi isolated from W0605-01 were *Trichoderma* and *Penicillium*. At least five different species of penicillia were isolated. *Penicillium* was also one of the most abundant genera in sample W1105-01, although different species were isolated to those from W0605-01. Other abundant genera in W1105-01 were *Fusarium* and *Aureobasidium*. The identities of the moulds from each sample are listed in Appendix 4.4.

Table 5.02: Results of fungal enumeration of sago samples implicated in SHD (cfu/g).

Organism	**W0605-01**	**W1106-01**
Total yeasts (pH 6.5)	8.4×10^7	6.5×10^5
Total yeasts (pH 4.5)	4.0×10^7	3.9×10^5
Total moulds (pH 6.5)	1.2×10^7	1.4×10^5
Total moulds (pH 4.5)	1.0×10^7	6.2×10^4
Mucoraceous moulds	Confluent growth of *Trichoderma* spp.	1.0×10^2
Geotrichum	$< 1.0 \times 10^2$	$< 1.0 \times 10^2$
Actinomycetes	$< 1.0 \times 10^2$	$< 1.0 \times 10^2$

5.3.6 Ergosterol analysis of sago starch

Ergosterol was detected in all but two sago samples analysed. The lower limit for detection was 0.10 µg /g of sago starch. Appendix 4.3 lists ergosterol levels in sago starch.

A Pearson correlation test was conducted between yeast and ergosterol and mould and ergosterol. The correlation at the P = 0.001 level was 0.742 for yeast and 0.629 for moulds.

An ANOVA was conducted to determine whether correlations between ergosterol and storage duration, storage technique, pH, and a_w existed (using the categories

outlined in Table 4.01). Samples greater than five weeks old had significantly higher ergosterol levels than all the fresher categories ($F = 0.221$, $df = 4$; < 1 week: $P = 0.025$; 1–3 weeks $P = 0.019$; 3–5 weeks $P = 0.042$). There was negligible association between ergosterol levels and storage technique, the only statistically significant difference ($P = 0.05$) occurred between sago starch stored in leaves compared to sago in the other and unknown category ($F = 1.71$, $df = 4$, $P = 0.029$). Analysis could not be conducted for pH or a_w due to the fact that for both parameters at least one group had fewer than two cases.

5.4 Discussion

Yeasts and moulds were commonly isolated from sago starch. However, the human health implications of the presence of yeasts may differ greatly from the presence of moulds. Yeasts are rarely pathogenic to humans. Notable exceptions are *Geotrichum candidum*, *Cryptococcus neoformans* and *Candida* spp. which have been associated with septicaemia and disseminated infection, predominantly in immunocompromised individuals (Sheehy, 1976; Kassamali *et al.*, 1987; Badenhorst *et al.*, 1991; Enoch *et al.*, 2006). Yeasts also occasionally play a role in gastrointestinal illness, with the overgrowth of some yeasts in the intestine resulting in diarrhoea (Talwar *et al.*, 1990).

In general, there appears to be very little evidence of yeasts being associated with foodborne illness. To the contrary, the use of yeast in food and beverage fermentation is well documented, having been used for thousands of years to improve food security and food safety. Yeasts play an important role in the fermentation of starch products worldwide (Campbell-Platt, 1994; Middelhoven, 2002; Annan *et al.*, 2003; Jespersen, 2003; Mugula *et al.*, 2003a), and it is likely that the yeasts in sago starch contribute to its fermentation. This study revealed high numbers of yeasts in sago starch, commonly reaching between 1.0×10^6 and 1.0×10^7 cfu/g. This range is comparable to yeast numbers recorded from fermented starch products such as kenkey, mawé, and Brazilian fermented cassava starch (Halm *et al.*, 1993; Rombouts and Nout, 1995; Lacerda *et al.*, 2005).

Analysis of yeast numbers in sago starch over time demonstrated no significant difference over the first five weeks, but significantly lower numbers in sago stored for five weeks or more. As sago ages it typically dries out, and may become more aerated. Thus, fermentation decreases or ceases, and yeast numbers decrease correspondingly.

It is difficult to draw conclusions on the effect of storage on yeast numbers. The finding that woven containers had significantly more yeast than fresh sago is likely due to the age difference of the two categories. Samples categorised as fresh sago were collected on the day of extraction, so fermentation had not progressed to completion. The differences between the 'other and unknown' storage category compared to sago stored in woven containers or in leaves is difficult to interpret given the undefined nature of the category. In Chapter 9 the influence of two of the storage techniques categorised as 'other', namely smoked and in earthenware pots, on microbial numbers is addressed.

Yeast numbers were significantly lower at pH < 4 than they were at pH 4.00–4.99. Although yeasts are typically acid tolerant, generally having a pH minimum of between 1.5 and 3.5, and an optimum in the slightly acidic range (Lund and Eklund, 2000), it would be expected that viable numbers be lower nearer the limits for growth.

As opposed to yeasts, which appear to make up part of the natural microbial flora required for healthy fermentation in sago starch, filamentous fungi probably play little useful role in the fermentative activity as they are classically aerobic organisms. Although some exceptions occur in the usefulness of filamentous fungi in the fermentation of various traditional foods (e.g. *Rhizopus* spp. in tempeh), the diversity of genera and variability in mould numbers between samples noted in this study suggests they are opportunistic contaminants of sago starch. Analysis of the data suggests that mould numbers increase with storage duration. Sago less than one week old had significantly less moulds than sago three to five weeks old, and sago greater than five weeks old.

As previously mentioned, it is thought that the conditions become less favourable for fermentation in older sago. Fermentation itself probably does not affect growth and survival of filamentous fungi, but conditions that are favourable for fermentation (high moisture, anaerobic conditions) ensure low mould numbers. However, as sago is removed from the bundle for consumption, the remaining sago is more likely to be exposed to air and to contamination from humans and the environment. Thus, one would expect lower fungal numbers to be found in the early stages of storage.

Sago starch stored in bags or baskets made of natural woven fibre had significantly higher mould numbers than sago stored by other techniques. The small holes in the bags and baskets favour colonisation by moulds in two main ways. They allow the flow of air into the sago starch, making it a more hospitable environment for moulds, and they allow airborne spores to colonise the starch. Moreover, bags and baskets are often reused, so they may act as a source of inoculum. Sago starch that is wrapped tightly in leaves or packed tightly in a plastic bag is more likely to remain anaerobic and have a barrier to airborne contamination by filamentous fungi.

The statistical difference between mould numbers in woven containers compared to the 'other and unknown' category was highly significant. Many of the samples in the 'other' component were stored in pots and covered with water. The majority of the 'unknown' samples were collected in the Sepik Province, where storage in pots is most common. It is expected that this storage technique would be the most successful at inhibiting mould growth, providing both a good physical barrier to spore contamination, and highly anaerobic conditions. Due to the undefined nature of the category 'other and unknown' it can only be speculated at this stage that the Sepik method of storage in pots and immersion in water is optimal for prevention of mould growth. This theory will be further explored in Chapter 9. As with yeast numbers, the lower number of moulds in fresh (un-stored) sago is indicative of lack of opportunity for the fungi to grow and sporulate.

Analysis of the effect of a_w on fungal numbers was not possible, because there were insufficient sago samples in the low a_w categories. Although the category boundaries could have been changed to enable statistical analysis, all categories would have been well inside the accepted range of growth for yeasts and moulds, which can

tolerate a_w as low as 0.88 and 0.80 respectively (Farkas, 1997). It seems the a_w of sago starch is never low enough to limit fungal growth.

Penicillium spp. and *Aspergillus* spp. were among the most commonly isolated moulds in sago starch, present in 61% and 24% of samples respectively. *Fusarium* spp. were present in approximately 19% of samples tested. These genera are ubiquitous in distribution and are the dominant fungi associated with food contamination and mycotoxin production (Hocking and Pitt, 2003). Other genera isolated from sago starch have also been associated with mycotoxin production, including *Trichoderma, Cylindrocarpon, Stachybotrys* and *Talaromyces* (Frisvad and Thrane, 2000; Bennett and Klich, 2003). While the presence of mycotoxigenic genera does not necessarily equate to the presence of mycotoxins, such contamination is still undesirable and constitutes a potential health risk.

Use of designated agar to isolate mucoraceous moulds and actinomycetes largely proved to be unsuccessful. Dichloran rose-bengal chloramphenicol agar is designed to inhibit mucoraceous moulds such as *Rhizopus* and *Mucor* (Bridson, 1998). Consequently, SMA was utilised to ensure the results weren't biased against mucoraceous moulds. However, plates usually had little or no fungal growth, and no moulds were isolated from SMA that were not isolated and enumerated using DRBC agar. There was considerable bacterial growth on actinomycete agar, but no actinomycetes were isolated. It is possible that actinomycetes were out-competed on the agar, making enumeration impossible. A more selective agar that inhibits growth of non-actinomycete bacteria would be advantageous in future studies.

The use of *Geotrichum* selective agar also was of limited use, leading to the isolation of *Geotrichum* spp. from nine samples. By comparison, *Geotrichum* spp. and *Geotrichum*-like isolates were isolated from 14 samples using DRBC agar. Interestingly, of the nine samples positive for *Geotrichum* spp. using *Geotrichum* selective agar, five were from the Western Province, and four from the East Sepik Province. This finding contradicts the statistical analysis based on DRBC agar isolations that found *Geotrichum* spp. more likely to be isolated from the East Sepik Province than the Western Province.

At the genus level, there was some commonality of fungi isolated from the two sago samples that were implicated in SHD outbreaks. *Penicillium* was one of the most commonly isolated genera in both samples, although the species differed in the two samples. Five different species of penicillia were identified from the W0605-01 sample, and three from the W1105-01 sample, suggesting that old sago starch is a suitable substrate for this organism. Given the similarity of appearance of many of the penicillia on DRBC agar, it is likely that some species of this genus were overlooked. It is possible that there may be some species of *Penicillium* that were not identified but which were present in both implicated samples, although the same could be said for any similar looking colonies present in high numbers.

Despite the diversity of penicillia, the predominant isolate on the DRBC plates from sample W0605-01 was *Trichoderma* spp. However, this genus was not isolated from sample W1105-01. Although the predominance of *Trichoderma* spp. in sample W0605-01 cannot be overlooked, one should be careful in determining the significance of the finding. This genus is well known for its antagonism towards other moulds in the form of antibiotics, mycoparasitism, and competition for nutrients and space (Solfrizzo *et al.*, 1994; Rocha-Ramirez *et al.*, 2002).

Due to the logistical constraints in PNG, particularly in rural areas where outbreaks tend to occur, the samples had to be stored for some time prior to their arrival in Australia. Sample W1105-01 had been stored for in excess of one month prior to reaching JCU. Both samples were refrigerated during most of this interim period, once they arrived at a laboratory with refrigeration facilities. Nonetheless, the fungal community could have undergone considerable change during this period. Thus, results of fungal culture from the implicated samples should be interpreted with due caution.

Due to the limited nature of previous mycological studies on sago starch, comparisons are of limited use. In their limited study of sago sourced from markets, Amoa *et al.* (1999) isolated *Aspergillus* spp. from all six samples in which fungal identification was conducted, but *Penicillium* spp. were not isolated. It would seem that the techniques used to identify moulds were limited in the study of Amoa and co-workers, as only two genera were reported. Studies on implicated sago samples

by Donovan *et al.* (1976) and Stace *et al.* (unpublished) also revealed only limited mould genera. Donovan and co-workers (1976) reported only single colonies of *Paecilomyces lilacinus* and *Tilletiopsis minor*, although shortcomings in sample size and isolation techniques were noted by the authors. Stace and co-workers (unpublished) identified *Trichoderma harzianum* and *Gliocladium virens* as the only moulds present in "significant numbers". They did not report the actual mould number and gave no indication of other moulds present. Given the competitive nature of *Trichoderma* spp. (Gams and Bissett, 1998), other moulds present might have been of some importance.

Ergosterol analysis was used as an indicator of fungal biomass. This method overcomes some of the shortcomings inherent in culture and enumeration of moulds. The use of colony forming units as an estimate of fungal biomass is based on the flawed assumption that all filamentous moulds sporulate with the same ease and intensity. Ergosterol is the predominant sterol in most fungi, and is generally absent from other organisms (Seitz *et al.*, 1979). Thus it is a robust indicator of fungal biomass compared to culture and enumeration, which can be heavily biased by rate of spore production and hyphal fragmentation (Schnurer, 1993; Marin *et al.*, 2005), and possibly also the viability of the mould on artificial media.

The mean value for ergosterol content in sago starch was 5.23 µg/g, and the median value 1.81 µg/ml. The mean value is considerably higher than the median due to five samples with very high ergosterol contents, in excess of 20 µg/g. High ergosterol levels correlated reasonably with fungal numbers, with yeasts having a correlation coefficient of 0.742 and moulds 0.629. All of the samples with ergosterol levels above 20 µg/g had high yeast numbers ($\geq 3.8 \times 10^5$ cfu/g) and four of the five samples had high mould numbers (one sample with 6.5×10^2 cfu/g, all other samples $\geq 3.8 \times 10^5$ cfu/g). Thus despite the limitations of culture and enumeration of fungi, and particularly moulds, there appears to be some correlation between fungal enumeration and ergosterol levels.

It is interesting to note that the implicated sample that was analysed for ergosterol, W0605-01, had both the highest ergosterol level and the highest number of moulds (cfu/g) of all the samples tested. Unfortunately ergosterol analysis was not conducted

on the other implicated sample, W1105-01, as insufficient sample was made available for this analysis. Nonetheless, the number of moulds isolated was reasonably high, at 1.4×10^5 cfu/g.

The mean value of ergosterol in sago samples (5.23 µg/g) was comparable to that found in other starch substrates. Kos *et al.* (2004) reported levels of 0.88 to 3.6 µg/g in mould infected corn kernels. Abramson and co-workers (1998) found the mean ergosterol level in wheat naturally infected with *Fusarium* head blight to be 14.25 µg/g and in experimentally infected wheat the mean was 16.87 µg/g of ergosterol. Thus the median ergosterol value in sago starch is slightly higher than infected corn, but lower than infected wheat. However, it should be remembered that the majority of sago samples analysed were not considered 'infected': they were samples suitable for consumption. The implicated sago sample, having an ergosterol value of 41.48 µg/g, was well above the median levels of infected wheat and corn.

The main limitation of ergosterol analysis in this study is that the result is influenced by the presence of both yeasts and moulds. Most, if not all, yeasts present in sago are likely to be a natural part of the microbial flora that have no ill effects on human health. However, moulds are indicative of unwanted contamination, and could potentially have adverse effects on human health. Nonetheless, analysis of ergosterol levels in sago starch revealed some interesting trends. Most importantly, sago samples stored for five or more weeks had significantly higher ergosterol levels than samples less than one week old, samples one to three weeks old, and samples three to five weeks old. This finding corresponds reasonably well with the number of moulds isolated from sago starch, which were significantly higher in samples greater than five weeks old compared to samples less than one week old and samples one to three weeks old. To the contrary, yeast numbers were lower in samples older than five weeks compared to samples aged one to three weeks. Thus, high ergosterol levels, particularly in old sago starch, are probably indicative of mould growth as opposed to yeast growth.

There was little correlation between storage technique and ergosterol levels, despite the correlation between sago stored in woven containers and mould numbers. For reasons already discussed, sago stored in woven baskets is prone to contamination.

However, the lack of correlation between ergosterol levels and sago stored in woven containers suggests that while spore numbers might be high (hence the high enumeration), growth of moulds does not necessarily occur inside the bundle. If the sago is left undisturbed or kept moist, mould growth is likely to be minimised inside the bundle. However, mould growth is likely to be high on the outer edge of the bundle, and after continued disturbance. As such, the starch may become unfit for human consumption from a food quality and food safety perspective. Indeed, the isolation of filamentous fungi from sago starch, particularly genera such as *Penicillium* and *Aspergillus*, necessitates a thorough investigation of commonly occurring mycotoxins.

CHAPTER 6: THE PRESENCE OF COMMON MYCOTOXINS AND MYCOTOXIGENIC FUNGI IN SAGO STARCH

6.1 Introduction

In recent years considerable effort has been dedicated to mycotoxin research, and not without reason. It is now recognised that mycotoxins have been a major factor in contamination of food over the past one thousand years, and probably for many preceding thousands of years (Beardall and Miller, 1994; Peraica *et al.*, 1999; Etzel, 2002). In recent times, human diseases such as Shoshin-kakke (yellow rice toxicity) and Alimentary Toxic Aleukia, along with numerous animal diseases, have been linked to the consumption of mycotoxin-contaminated foods. Despite their association with human and animal illness, there remained a paucity of information pertaining to fungal toxins until the 1960s, when the term mycotoxin was coined (CAST, 2003) in the aftermath of the discovery of aflatoxins and their role in 'turkey X disease'.

Despite the large number of mycotoxins that have been identified in recent years, for many their role in disease, if any, remains unknown. Nonetheless, exposure to mycotoxins has been associated with rapid death, tumour formation, liver disease and immunosuppression (Bennett and Klich, 2003; CAST, 2003). Issues such as vitamin deficiency, caloric deprivation and infection can influence the severity of mycotoxicosis. As a consequence of the aforementioned problems and lower production and storage standards, mycotoxicosis is more common in the developing world (Bennett and Klich, 2003).

The presence of mycotoxins in human food continues to be a significant problem, predominantly as a health issue but also as a trade barrier. Over 100 countries have specific limits for mycotoxins in food or feed, with a wide variety of toxins regulated in Europe (van Egmond, 2004). Currently in Australia the only mycotoxins that are

regulated in human foods are the aflatoxins, with the presence of ergot also regulated (Food Standards Australia New Zealand, 2000). Such regulations are generally absent in developing countries, including PNG. On a worldwide basis, it seems likely that the number of mycotoxins regulated and stringency of the limits will increase in the near future (Moss, 2000), although such changes are likely to occur initially in developed nations.

Despite the recent research on mycotoxins, their chronic effect on human health and longevity remains largely unknown. It is generally accepted that exposure to mycotoxins is more likely when food handling and storage methods are poor, malnutrition is prevalent, and regulations to protect consumers are scarce (Bennett and Klich, 2003). Consequently, mycotoxins in the diet of people in developing countries are likely to contribute to chronic ill health (Moss, 2000) and could be expected to have a greater adverse effect on health than they do in the developed world.

Many surveys have been conducted on foods from tropical regions, including surveys of foods produced and consumed in PNG. In the surveys conducted to date in PNG, a variety of foods, including sago starch, have been tested for a few mycotoxins and other naturally occurring hepatotoxins (Cunningham and Le Page, 1972; Wookey, 1972; Amoa *et al.*, 1999). Despite limitations in sample size and scope of mycotoxins, foods such as sago starch and peanuts have tested positive for aflatoxins (Wookey, 1972; Amoa *et al.*, 1999). Given the documented presence of mycotoxins in foods produced in PNG, and speculation that mycotoxins are involved in SHD (Taufa, 1974; Donovan *et al.*, 1977), a thorough investigation of sago starch for various mycotoxins was merited.

In selecting which mycotoxins should be screened for, the mycoflora of sago starch was considered (see Chapter 5 and Appendix 4), along with the role of some classical mycotoxins associated with human health issues and trade restrictions. As a result, aflatoxin B_1, aflatoxin B_2, aflatoxin G_1 aflatoxin G_2, ochratoxin A, cyclopiazonic acid, sterigmatocystin, citrinin and zearalenone were screened in sago starch. Selected fungal isolates were screened for their ability to produce selected toxins in

pure culture, such as ochratoxin A, cyclopiazonic acid, sterigmatocystin, citrinin, penicillic acid and patulin.

6.2 Materials and Methods

6.2.1 Sample Collection

Sago samples were collected from the Western Province and the East Sepik Province, as outlined in Section 4.2.1.1. Samples were collected from the East Sepik Province in November 2002 and June 2003, and from the Western Province in March 2003. Samples collected from subsequent expeditions into the Western Province in April 2004 and April 2005 were not analysed for mycotoxins. The two sago samples that were implicated in outbreaks of sago haemolytic disease in the Western Province, W0605-01 and W1105-01, were analysed for the presence of mycotoxins.

6.2.2 Screening sago starch for mycotoxins

6.2.2.1 Extraction process

Following collection, sago samples were stored at -20 °C until analysis. Thawed samples were sieved through a 2 mm square mesh to remove lumps and homogenise. A single extraction technique suitable for the screening of multiple mycotoxins was used, based on that of Blaney *et al.* (1984). A representative 25 g sub-sample of sago starch was extracted using 90 ml of acetonitrile (HPLC grade, EM Science, Kilsyth, Victoria), 10 ml of 4% potassium chloride (Ajax Chemicals, Sydney, New South Wales) and 2 ml of 5 N hydrochloric acid (Ajax Chemicals, Sydney, New South Wales). The mixture was sonicated for 2 min using a sonicating waterbath (Consolidated Ultrasonics Asia Pty Ltd), and then 50 ml was filtered through Whatman GFA filter paper (Crown Scientific, Acacia Ridge, Queensland). The filtrate was placed in a separating funnel with 50 ml of distilled water. Fifty millilitres of dichloromethane (high purity, EM Science, Kilsyth, Victoria) was added, and shaken by hand for 1 min. The lower fraction was filtered through anhydrous granular sodium sulphate (Ajax Chemicals, Sydney, New South Wales).

A further 50 ml of dichloromethane was added to the upper fraction and shaken by hand for one min. The resulting lower fraction was filtered through anhydrous granular sodium sulphate. The combined lower fraction was collected and taken to dryness with gentle heat and a stream of air.

6.2.2.2 Thin layer chromatography

Sago samples were tested for the presence of aflatoxin B_1, aflatoxin B_2, aflatoxin G_1, aflatoxin G_2, ochratoxin A, cyclopiazonic acid and sterigmatocystin using two-dimensional thin layer chromatography (TLC). Citrinin and zearalenone were tested using one-dimensional TLC. Standards for all toxins were obtained from Sigma Aldrich Pty Ltd (Castle Hill, New South Wales).

The dried extract was resuspended in 200 µl of dichloromethane, and 10 µl was applied near a corner of a 10 cm × 10 cm thin layer chromatographic aluminium sheet, pre-coated with 0.2 mm silica gel 60 (Merck 1.05553 silica plates, Alltech Associates Pty Ltd, Baulkham Hills, New South Wales). The two dimensional plates were run in the first dimension using 95:5 chloroform/methanol (EM Science, Kilsyth, Victoria) and in the second dimension using 5:4:1 toluene/ethyl acetate/formic acid (TEF) (EM Science, Kilsyth, Victoria). The one dimensional plates were run in TEF only.

6.2.2.3 Visualisation of toxins and limits of detection

Following chromatography, plates were observed for the presence of mycotoxins. A Spectroline CC-80 ultraviolet fluorescence cabinet (Spectronics Corporation, New York) was used for all visualisation under ultraviolet (UV) light, at either 254 nm (short wavelength) or 365 nm (long wavelength).

Aflatoxins B_1 and B_2 were visible as blue spots and aflatoxins G_1 and G_2 as green-blue spots under long wavelength UV light.

Ochratoxin A was visible as a blue-green spot under UV light. Spraying the spot with alcoholic bicarbonate solution (Appendix 2.3.1.1) intensified the fluorescence of the spot, and changed the colour from blue/green to bright blue (Scott, 1990).

Cyclopiazonic acid was visualised under ambient light as a purple streaked spot by swabbing the appropriate area of the plate with freshly prepared Erhlich's solution (Appendix 2.3.1.2) and gently heating (Blaney, 1989).

Sterigmatocystin could be observed in high concentrations as a faint 'red brick' coloured spot under ambient light. It was confirmed by swabbing the appropriate area with 20% potassium hydroxide (Ajax Chemicals, Sydney, New South Wales) dissolved in methanol (EM Science, Kilsyth, Victoria) and then examining for bright yellow fluorescence under long wave UV light (Scott, 1990).

Citrinin was visualised as a yellow-green fluorescent spot under long wave UV light. At high concentrations the spot was streaked. The presence of citrinin was confirmed by spotting the presumptive citrinin with boron trifluoride-methanol complex (Merck Pty Ltd, Kilsyth, Victoria), resulting in a colour change from fluorescent yellow to fluorescent blue when observed under long wave UV light.

Zearalenone was visible as a pale blue spot, which was more intense under short wave UV light than under long wave UV.

The ratios of chromatographic mobility of each mycotoxin relative to the solvent front (R_f) and the limits of detection in parts per million (ppm, equivalent to µg/g) are listed in Table 6.01. Citrinin was visible as a pronounced streak at R_f 0.09 to 0.53, with the greatest intensity at approximately 0.45.

Table 6.01: The R_f and detection limits for mycotoxin screening in sago starch.

Mycotoxin	R_f $CHCl_3$/MeOH	R_f T:E:F	Detection limit (ppm)
Aflatoxin B_1	0.62	0.22	0.005
Aflatoxin B_2	0.56	0.20	0.001
Aflatoxin G_1	0.48	0.15	0.005
Aflatoxin G_2	0.40	0.12	0.001
Ochratoxin A	0.17	0.6	0.025
Cyclopiazonic acid	0.06 - 0.24	0.21 - 0.36	0.500
Sterigmatocystin	0.8	0.65	0.100
Citrinin	NA	0.09 - 0.53	0.005
Zearalenone	NA	0.17	0.100

NA: Not applicable

6.2.3 Screening pure cultures of fungi isolated from sago starch for mycotoxins

Twenty-one strains of fungi isolated from sago starch were tested for production of ochratoxin A, cyclopiazonic acid, sterigmatocystin, citrinin, penicillic acid and patulin. All isolates belonged to the genera *Penicillium* and *Aspergillus*, with the exception of one isolate of *Fusarium semitectum*. Isolates were grown in pure culture using wheat grain as a substrate (Appendix 2.4.1.6) held at 25 °C for 4 weeks. The culture material (10 g) was extracted using the same method as outlined in Section 6.2.2.1, with the addition of a hexane extraction to remove unwanted oils from the wheat substrate. After initial extraction in acidified acetonitrile, 50 ml of water was added to the 50 ml of acetonitrile extract, then 50 ml of hexane (HPLC grade, Lomb Scientific Archerfield, Queensland) was added and shaken by hand in a separatory funnel for 1 min. The hexane extract was then discarded and the remaining acidified acetonitrile extract partitioned with dichloromenthane, as outlined above.

As many of the isolates tested in this study belonged to the genus *Penicillium*, cultures were screened for production of the additional mycotoxins patulin and penicillic acid. Standards for both toxins were obtained from Sigma Aldrich Pty Ltd (Castle Hill, New South Wales).

All mycotoxins were detected using two dimensional TLC, as outlined in Section 6.2.2.2. Visualisation techniques, R_f values and detection limits for ochratoxin,

cyclopiazonic acid and sterigmatocystin were the same as those outlined in Section 6.2.2.3. The R_f values of citrinin in two dimensional TLC were between 0.00 and 0.09 in the first dimension (1-D), and 0.09 and 0.53 in the second dimension (2-D). The greatest intensity was at 0.45 in the second dimension (intensity was uniform between 0.00 and 0.09 in the first dimension).

Patulin and penicillic acid were visualized under ambient light after being spotted with anisaldehyde reagent (Engel and Teuber, 1984) (Appendix 2.3.1.3). Patulin reference standard was evident as a red-orange spot with R_f values of 0.33 (1-D) and 0.37 (2-D). Penicillic acid standard appeared as a green spot with R_f values of 0.33 (1-D) and 0.41 (2-D). The limit of detection for both patulin and penicillic acid was 5 ppm.

Citrinin concentrations produced in pure culture were determined by comparing the fluorescent intensity of extracts to known standards on TLC.

6.3 Results

6.3.1 Sample collection

There was considerable overlap in samples analysed for microbial contamination (Chapters 5 and 6) and those analysed for mycotoxins. However, some samples that underwent microbial analysis were not tested for mycotoxins. Consequently, information pertaining to the age and storage of each sample analysed for mycotoxins is listed in Appendix 5.

6.3.2 Presence of mycotoxins in sago starch

Fifty-one samples were tested for the presence of aflatoxin B_1, aflatoxin B_2, aflatoxin G_1, aflatoxin G_2, ochratoxin A, cyclopiazonic acid, sterigmatocystin, citrinin and zearalenone. All samples were negative for all mycotoxins tested. Two samples, W0303-07 and W0303-12, showed the characteristic purple-mauve colour upon treatment with Ehrlich's solution just above the R_f of the standard of cyclopiazonic

acid, which might have been CPA imine (Cole and Cox, 1981), or another related compound. Figure 6.01 shows the spot in question for sample W0303-12.

The two sago samples that were implicated in episodes of SHD, W0605-01 and W1105-01, were analysed for the above mycotoxins, and for patulin and penicillic acid. Both samples were negative for all mycotoxins. A compound similar to that observed in samples W0303-07 and W0303-12, speculated to be CPA imine, was observed in one implicated sample (W0605-01).

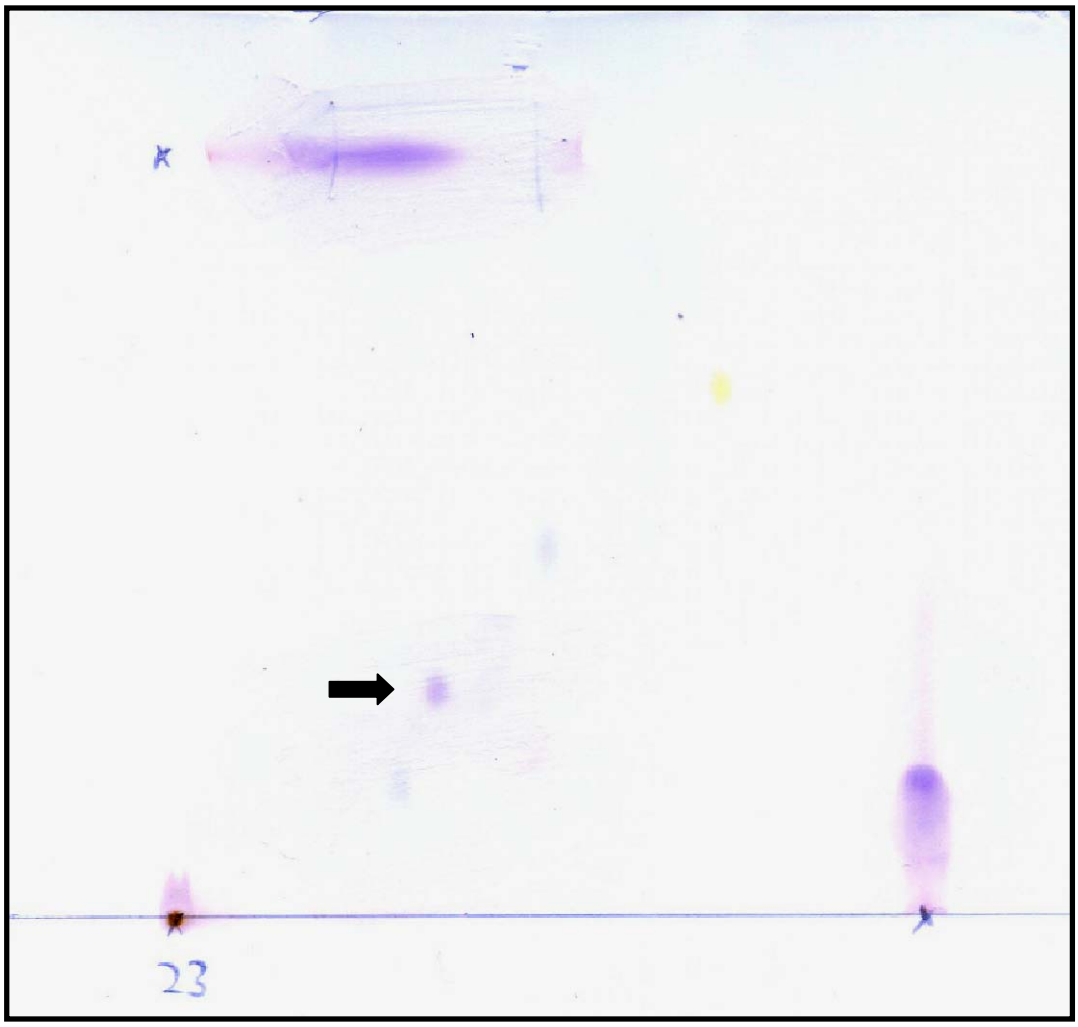


Figure 6.01: Thin layer chromatography plate of sample W0303-12 and CPA standards after treatment with Ehrlich's solution. The arrow marks the area of purple just above CPA in the first dimension, which is possibly CPA imine.

6.3.3 Production of mycotoxins by fungi in pure culture isolated from sago starch

All 21 fungal isolates tested were negative for the production of ochratoxin, cyclopiazonic acid, penicillic acid and patulin under the test conditions. However, 14 isolates were positive for citrinin production, and 1 isolate was positive for sterigmatocystin. Citrinin concentrations were estimated by visual comparison of intensity of spots with that of standards of known concentrations. The comparison was performed at levels within one order of magnitude of the detection limits, and estimates were repeated and checked between two operators to improve precision. Table 6.02 lists the concentrations of citrinin and sterigmatocystin produced by each isolate.

Table 6.02: Citrinin and sterigmatocystin concentrations produced in pure culture by fungi isolated from sago starch.

Isolate	Identification	Citrinin (ppm)	Sterigmatocystin (ppm)
W1-1101	*P. steckii**	2,000	< 0.1
W1-1109	*P. steckii**	4,200	< 0.1
W1-1301	*P. steckii**	1,000	< 0.1
W1-1501	*P. steckii**	1,700	< 0.1
S2-0503	*P. steckii**	4,200	< 0.1
S2-1305	*P. steckii**	2,000	< 0.1
S2-1806	*P. steckii**	800	< 0.1
S1-0201	*P. brevicompactum*	2,000	< 0.1
W1-1602	*P. brevicompactum*	4,200	< 0.1
W2-0303	*P. brevicompactum*	4,200	< 0.1
W2-0703	*P. brevicompactum*	4,200	< 0.1
W2-1401	*P. brevicompactum*	4,200	< 0.1
S2-1602	*Penicillium* sp.	4,200	< 0.1
W2-0101	*Penicillium sp.*	< 0.005	< 0.1
W2-1003	*Penicillium sp.*	< 0.005	< 0.1
W1-1106	*A. flavipes*	200	< 0.1
S2-0705	*A. flavipes*	< 0.005	< 0.1
S1-0302	*Aspergillus* sp.	< 0.005	< 0.1
S1-0802	*Aspergillus versicolor*	< 0.005	500
W1-0904	*Aspergillus sp.*	< 0.005	< 0.1
S1-0406	*Fusarium semitectum*	< 0.005	< 0.1

Legend: *P. = Penicillium*; *A. = Aspergillus*,
*: Maybe more accurately described as *P. citrinum*-affinity (see Section 6.4)
Refer to Appendix 4.4 the approximate enumeration of each isolate in sago starch

6.4 Discussion

Despite evidence of fungal contamination of sago starch (see Chapter 5), none of the common mycotoxins screened for in this study was present in sago starch. There are a number of factors that contribute to the lack of mycotoxins in sago starch. First, only a small percentage of fungal species produce these common mycotoxins. Although many of the commonly isolated genera of fungi from sago are associated with mycotoxin production, mycotoxin synthesis is often species specific. For example, only 3 of approximately 100 species of the genus *Aspergillus* are known to produce aflatoxins (Hocking and Pitt, 2003). Moreover, the synthesis of any given mycotoxin is dependent not only on the species of fungi, but also the strain (Sweeney and Dobson, 1998).

Secondly, mycotoxigenic fungi do not produce toxic secondary metabolites under all conditions. There are many environmental factors that influence toxin synthesis including temperature, water activity, pH and nutrient availability (Sweeney and Dobson, 1998; Kozlovskii *et al.*, 2000; Pitt *et al.*, 2000). Optimal requirements for mycotoxin production are likely to be dependent on a combination of parameters, but to date most studies have concentrated on the effect of just one or two factors.

It is difficult to generalise about nutrient requirements for optimal mycotoxin production. Davis *et al.* (1967) found that zinc, iron and magnesium were required for optimal aflatoxin yield, but even low levels of manganese resulted in a reduction of aflatoxin. Lillehoj *et al.* (1974) found the addition of low levels of copper, manganese, cadmium, chromium and lead increased aflatoxin yield, but high levels had an inhibitory effect. The addition of zinc also increased aflatoxin yield. Kozlovskii *et al.* (2000) found citrinin production to increase greatly in the presence of manganese and zinc ions. Nutrient requirements for mycotoxin production vary depending upon fungal strain, the mycotoxin, the substrate, and other growth conditions, thus total consensus among such studies is unlikely, and direct comparisons are complex.

Metal analysis of sago starch was conducted on three samples in this study (see Appendix 1.2). It is worth noting that sample W0605-01 (implicated in SHD) had

noticeably higher levels of aluminium, barium, copper, iron, manganese and zinc than the other two samples that were tested. Increased levels of a combination of such metals might create a more favourable environment for the production of mycotoxins. Although no known mycotoxins were detected in the implicated samples, it is hypothesised that other currently undescribed mycotoxins might be produced in sago samples associated with SHD (see Chapter 8).

From a human perspective, sago starch is generally considered a good source of dietary carbohydrates, but is otherwise nutritionally poor (Vaughan and Geissler, 1997). One may speculate that the low level of trace elements in sago starch might be one factor that contributes to the general absence of classical mycotoxins.

Another factor that might contribute to the lack of mycotoxins in sago starch is fermentation (see Chapter 9 for details of fermentation of sago starch). The fermentation of sago starch may prevent or reduce the incidence of mycotoxins by both indirect and direct means. Indirectly, the anaerobic environment that favours fermentation reduces the incidence of filamentous fungi (see Chapter 5). Directly, both mould and lactic acid fermentations have been shown to reduce the toxicity and/or concentration of mycotoxins (Nakazato *et al.*, 1990; Nout, 1994). Moreover, the anaerobic environment could result in decreased mycotoxin synthesis, as low oxygen availability has previously been shown to limit mycotoxin production (Landers *et al.*, 1967; Keller *et al.*, 1997; Watanabe *et al.*, 2004).

In sago starch, filamentous fungi are contaminants that are not considered to play a major role in starch fermentation. Thus, they are unlikely to contribute to mycotoxin detoxification. However, lactic acid bacteria and yeasts are common in sago starch (see Chapter 9), and potentially play a role in mycotoxin detoxification. In lactic acid fermentations at pH $\leq$ 4.0, aflatoxin B_1 can be converted to the less toxic aflatoxin B_{2a} (Nout, 1994). Mokoena and co-workers (2005) demonstrated that zearalenone and fumonisin B_1 concentrations decreased significantly in fermented maize after three or more days, although there was no decrease in toxicity. The yeast *Saccharomyces cerevisiae* has been shown to degrade patulin during fermentative growth (Moss and Long, 2002). The effect of lactic acid fermentations on mycotoxins was identified as a high priority research area by Motarjemi and Nout

(1996). Although some work has been conducted in this area since that time, as already noted, further work could be highly beneficial for consumers of traditional fermented foods in developing countries.

The storage duration of sago starch is likely to influence mycotoxin levels. In this study the vast majority of samples were aged one month or less. Mycotoxins, by definition, are secondary metabolites, which are usually produced as fungi mature (Hocking and Pitt, 2003). The absence of mycotoxins isolated in this study might be due, in part, to the relatively short storage duration. If sago starch is suitably stored, the starch will support vigorous fermentation for at least four weeks (see Chapter 9), preventing mould growth. In poorly stored or old sago, the starch dries out, becomes aerated and allows growth of filamentous fungi (see Chapter 5). As the fungi mature, the toxigenic strains, if present, might then produce mycotoxins.

Selected factors that affect fungal growth in sago starch were addressed in Chapter 5. Considerable research conducted on the environmental and ecological factors associated with the bulk storage of agricultural commodities suggests there is a diverse range of factors that interact to influence fungal growth (Magan and Lacey, 1988; Dunkel, 1992; Magan *et al.*, 2003). As a result, heterogeneous growth is likely. Although sago starch is not traditionally stored in large quantities, there is still likely to be substantial variation within a bag or bundle of sago, particularly in terms of oxygen availability. The heterogeneous nature of mycotoxin production within food commodities has been recognised in recent years and appropriate sampling plans developed (Miraglia *et al.*, 2005; Whitaker, 2006). While all efforts were made to adhere to the principles of such sampling plans throughout this study, the negative results of this study cannot categorically rule out mycotoxin production in sago starch.

Nonetheless, the findings of the present study are somewhat at odds with the preliminary study conducted by Amoa *et al.* (1999). Amoa and co-workers detected aflatoxins from four of five moist sago samples and all three dried samples analysed. However, levels were low, never exceeding 15 ppb; the upper limit of allowable aflatoxin contamination in Australian foods (Food Standards Australia New Zealand, 2000). Despite *Aspergillus* spp. being commonly isolated from sago samples in

Amoa's study, it seems surprising that aflatoxins would be present in moist sago starch stored for less than one month, as was reported to be the case. Unfortunately, no effort was made to enumerate or further identify the *Aspergillus* isolates involved, and no information was given as to which aflatoxins were present.

The screening of two sago samples associated with outbreaks of SHD for classical mycotoxins revealed that the selected mycotoxins were absent from both samples. The lack of aflatoxins, ochratoxin and zearalenone detected in sago samples associated with SHD is in keeping with the findings of Donovan *et al.* (1977), who analysed one such sago sample but was unable to detect aflatoxin, ochratoxin, zearalenone or byssochlamic acid.

Notwithstanding the lack of known mycotoxins in sago starch observed in this study, there is potential for the production of mycotoxins. The suspected presence of CPA imine in one implicated sago sample suggests that the presence of CPA in sago starch could have implications for human health. Cyclopiazonic acid is produced by a several species of the genera *Penicillium* and *Aspergillus* (Bryden *et al.*, 2004). *Penicillium* spp. were isolated from the relevant sago sample associated with SHD (W0605-01) and from one of the "edible" sago samples positive for suspected CPA imine (W0303-12). However no *Penicillium* or *Aspergillus* species were isolated from sample W0303-07 (see Appendix 4.4), the other sample positive for CPA imine. Toxins can persist after the associated fungi have lost viability (CAST, 2003), thus the lack of *Penicillium* spp. or *Aspergillus* spp. in sample W0303-07 may be indicative of microbial succession, or storage conditions unfavourable to the growth of such fungi following sample collection.

A high number of fungal isolates tested (14 of 21) produced citrinin in pure culture, in many cases at concentrations in excess of 4000 ppm. In addition, 1 of the 21 isolates produced sterigmatocystin, at a concentration of approximately 500 ppm. Of the 14 isolates that tested positive for citrinin production, 7 were strains of *Penicillium steckii*, and 5 were strains of *Penicillium brevicompactum*. While *P. steckii* has been reported to produce citrinin (Cole and Cox, 1981), evidence of citrinin production in *P. brevicompactum* is scarce. Moreover, there remains some debate over the ability of *P. steckii* to produce citrinin, with some suggestion that

citrinin is not produced by the species (Pitt, 1979). Thus, the citrinin producing *P. steckii* might more accurately be considered *P. citrinum*-affinity until definitive identification can be conducted on all such isolates. Despite the isolation of numerous fungal strains from sago starch capable of citrinin synthesis, and the one strain capable of sterigmatocystin synthesis, neither mycotoxin was detected in sago starch. It seems that a factor, or a combination of factors such as those previously discussed, inhibits production of these mycotoxins in sago starch.

It is difficult to determine the true prevalence of citrinin in foods. Frisvad and Thrane (2000) stated that citrinin rarely has been isolated from food commodities, While Xu *et al.* (2006) suggested citrinin lacked stability in foodstuffs. Contrary to these findings, Bennett and Klich (2003) stated that citrinin is regularly associated with human foods. Regardless of previous findings, these results demonstrate that there is the potential for citrinin production in sago starch. The implications of exposure to citrinin in the human diet are at this stage largely unknown, but could be serious.

Citrinin is generally considered to be one of the minor mycotoxins, being less toxic than many of the others, and usually acting in synergism with them. Nonetheless, it has been associated with numerous diseases, including yellow rice disease and Balkan Endemic Nephropathy in humans, and various diseases in animals (Saito *et al.*, 1971; Pfohl-Leszkowicz *et al.*, 2002; Bennett and Klich, 2003; CAST, 2003). It is nephrotoxic to all animals tested in laboratory experiments, and carcinogenic (Bennett and Klich, 2003; CAST, 2003). It has also been suggested that extracts of a citrinin producing strain of *P. citrinin* might cause haemolytic anaemia in mice (Lura *et al.*, 2004).

Trivedi and co-workers have shown that heating citrinin can markedly change its structure and toxicity. Heating citrinin to 140 ºC under semi-moist conditions detoxifies the compound (Kitabatake *et al.*, 1991). However, heating citrinin to around 90 –100 ºC for 30 min can result in the formation of citrinin H1 (Trivedi *et al.*, 1993). This compound was shown by the authors to be 10 times more toxic to HeLa cells than citrinin. The significance of their finding in relation to this study is twofold. Firstly, the extraction of mycotoxins involved the gentle heating of liquid under a stream of air. Although all care was taken to not overheat the extracts, it is

plausible to speculate that citrinin started to decompose in this process, resulting in false negative results. The effect would be greatest in the sago starch screening, where levels of citrinin would have been low comparative to growth of fungal isolates in pure culture. Secondly, if citrinin is present in sago starch, it might be transformed into the more toxic citrinin H1 during the cooking process, becoming a greater health threat to sago consumers.

Interestingly, a high incidence of renal disease has been noted by health professionals in the Western Province of PNG (Dr. Addy Sitther, pers. com.). The aetiology of such a disease cannot be determined without a thorough epidemiological study. However, chronic exposure to citrinin, citrinin H1 and/or other mycotoxins through the consumption of stored sago starch and other foods might contribute.

CHAPTER 7: HAEMOLYTIC ACTIVITY OF MICROORGANISMS ISOLATED FROM SAGO STARCH AND METHODS FOR DETECTING SUCH ACTIVITY

7.1 Introduction

Sago haemolytic disease is characterised by the occurrence of acute intravascular haemolysis. Such a haemolytic crisis might result from one of numerous factors, including physical trauma, immune response, chemical agents and various natural toxins, including microbial toxins (McKenzie, 1996). Outbreaks of SHD have been linked with consumption of old sago, which suggests a pre-formed microbial toxin could be the cause.

Considerable research has been conducted on bacterial haemolysins and cytolysins, but much of this work pertains to their role in the pathogenesis of non-haemolytic diseases. Indeed, despite the large number of bacteria showing haemolytic activity *in vitro*, relatively few have been associated with haemolysis *in vivo* (see Section 2.6.2). Furthermore, there are no known reports in the literature of any of the classical foodborne pathogens causing acute intravascular haemolysis.

Despite the plethora of studies pertaining to bacterial haemolysins, comparatively few studies exist on the haemolytic nature of fungi or their associated metabolites. Various mycotoxins have been associated with haemorrhaging in domestic and laboratory animals (Schneider *et al.*, 1979; Abbas and Mirocha, 1988; Gunther *et al.*, 1989; Bucci *et al.*, 1996; Prathapkumar *et al.*, 1997; Park *et al.*, 1999; Javed *et al.*, 2005), and a link between a fungal metabolite and human haemorrhaging has been proposed (Vesper and Vesper, 2002). However, fungal metabolites have not yet been associated with diseases involving acute intravascular haemolysis.

The scarcity of known haemolysins of microbial origin causing acute intravascular haemolysis suggests that a novel compound might be the aetiological agent in SHD. Accordingly, in this study microorganisms isolated from sago starch, and sago starch *per se*, will be screened for haemolytic activity using blood agar. Methods will be developed to enable the extracts of microbes that are haemolytic on blood agar to be assayed for haemolytic activity in a semi-quantitative manner.

7.2 Materials and Methods

7.2.1 Initial screening using blood agar

7.2.1.1 Direct screening of micro-organisms isolated from sago starch on sheep blood agar

Sago samples were collected from the Western Province and East Sepik Province, as outlined in Section 4.2.1.1. Serial dilutions of sago starch were conducted in accordance with AS 1766.1.2-1991 (Standards Australia, 1991a). A 10 g ± 0.2 g sub-sample of sago starch was added to 90 ml of diluent (Appendix 2.1.1.1). The resulting 10^{-1} dilution was mixed for 2 min using a Seward Stomacher 400 (John Morris Pty Ltd, Bowen Hills, Queensland). Further dilutions were made as required using 9 ml aliquots of diluent. Appropriate dilutions were plated in duplicate using sheep blood agar (SBA), sheep blood agar with 100 mg of chloramphenicol (SBA-chlor), and sheep blood agar with 3% glucose and 100 mg of chloramphenicol (SBA-gluc), for bacteria, moulds and yeasts respectively (Appendices 2.1.1.4 and 2.4.1). The addition of glucose to blood agar was shown to improve detection of haemolysis in various species and strains of *Candida* (Manns *et al.*, 1994).

The SBA plates were incubated at 37 ºC for 24 hours, and subsequently refrigerated for 4 hours. Haemolytic bacteria were enumerated, and representatives of the different colony morphologies showing haemolytic activity were subcultured on SBA for confirmation of haemolytic activity and subsequent identification, preservation and further analysis of haemolytic activity. Gram stain, motility test and classical biochemical tests were used to presumptively identify bacteria (see

Appendix 2.1). Identification was confirmed using the Vitek® identification system (bioMérieux Australia Pty Ltd, Baulkham Hills, New South Wales). Isolates were preserved on Microbank alginate beads (bioMérieux Australia Pty Ltd, Baulkham Hills, New South Wales) stored at -70ºC.

The SBA-chlor plates were incubated at 30 ºC for up to 10 days. Plates were checked daily from day 5. Haemolytic moulds were enumerated, and representatives of the different colony morphologies showing haemolytic activity were subcultured for later identification, preservation, and further analysis of haemolytic activity.

The SBA-gluc plates were incubated at 30 ºC for 4 days. Plates were checked daily from day 2. Haemolytic yeasts were enumerated, and representatives of the different colony morphologies showing haemolytic activity were subcultured for later identification and further analysis of haemolytic activity.

Identification of moulds and yeasts was conducted as outlined in Section 5.2.1. Isolates of interest were sent to Centraalbureau voor Schimmelcultures at the Institute of the Royal Netherlands Academy of Arts and Sciences for definitive identification.

Once grown in pure culture on the appropriate blood agar derivative, all microbes were assigned a haemolytic value of 0+, 1+, 2+ or 3+. Table 7.01 defines this categorisation.

Table 7.01: Categories of haemolytic intensity for organisms isolated from sago starch.

Haemolytic value	**Observed haemolytic activity**
0+	No haemolysis
1+	Narrow zone of incomplete or partial haemolysis (α haemolysis)
2+	Narrow zone of complete haemolysis (β haemolysis) under, or extending only slightly beyond colony margin
3+	Zone of complete haemolysis (β haemolysis), extending 2 mm or more beyond colony margin

7.2.1.2 Screening of sterilised sago starch for haemolytic activity using blood agar

To determine whether there was a non-living chemical component of sago starch capable of causing haemolysis, 12 sago starch samples were screened for haemolytic activity using both SBA and human blood agar (HBA). Samples selected (listed in Appendix 6.2) covered various storage techniques and storage durations, and included one sample implicated in an outbreak of SHD, W0605-01. Sub-samples were sterilised by adding 2 ml of propylene oxide (Sigma Aldrich Pty Ltd, Castle Hill, New South Wales) to 20 g of sago starch, sealing in an airtight container and leaving overnight. A well was formed in SBA plates using a flame sterilised cork borer, and approximately 0.1 g of the sterilised sago starch was placed in the well. Plates were incubated at 30 ºC for 10 days, and examined for haemolysis every day from day 5.

7.2.1.3 Screening individual fungal isolates from sago starch for haemolytic activity using sheep blood agar and human blood agar

Direct plating of diluted sago starch onto blood agar plates proved to be unsuitable for the enumeration of haemolytic yeasts and moulds (see Section 7.3.1.1). Consequently, yeasts and moulds were also screened for haemolytic activity individually, in pure culture. After enumeration using DRBC agar, as outlined in Section 5.2.1, representative colonies of yeasts and moulds were subcultured onto SBA-gluc and SBA-chlor, respectively. Plates were incubated as outlined in Section 7.2.1.1.

Isolates were assigned a code indicative of province of origin and sample expedition (S1 or S2; W1, W2 or W3), the sago sample and the isolate number. Thus, W3-0201 would be given to the first isolate taken from the second sago sample collected on the third expedition to the Western Province.

Selected fungal isolates were later screened for haemolytic activity using both SBA-chlor and HBA with 100 mg/l of chloramphenicol (HBA-chlor). Plates were incubated at 30 ºC for 10 days. A range of isolates was selected to reflect the main genera isolated and the range of haemolytic activity as demonstrated previously on

SBA-chlor. All isolates able to be subcultured from the implicated sago samples (W0605-01 and W1105-01) were tested for haemolytic activity using both SBA-chlor and HBA-chlor.

Identification of moulds and yeasts was conducted as outlined in Section 5.2.1. Isolates of interest were sent to Centraalbureau voor Schimmelcultures at the Institute of the Royal Netherlands Academy of Arts and Sciences for definitive identification.

Strains from the four genera of filamentous fungi in which over 50% of isolates were either 2+ or 3+ haemolysers were selected for further study. Selection was on the basis of their haemolytic activity, frequency of isolation, and population size.

7.2.2 Development of a haemolytic assay for quantitative testing of microorganisms for haemolytic activity

7.2.2.1 Selection of appropriate growth conditions for bacteria, yeast and mould

Selected haemolytic isolates were grown in pure culture for later quantitative haemolytic analysis. For bacteria, two methods were trialled. In the first method, based on work by Elek and Levy (1954), bacteria were grown on 100 ml of SBA with a reduced agar content of 0.8%, set as a large slope in ‘medical flat’ bottles. Slopes were inoculated by flooding a cell suspension over the agar surface. Excess suspension was removed with a sterile disposable pipette. Slopes were incubated at 37 ºC for 48-72 hours.

In the second method, 200 ml of brain heart infusion broth (BHIB) (Appendix 2.1.1.7) was inoculated with a single colony. The broth culture was grown at 37 ºC for 48 hours in a shaking incubator (Bioline model BL 4720, Edwards Instrument Company, Narellen, New South Wales) set at 150 rpm.

The two methods used for bacteria, as outlined above, were used for yeasts, but incubation conditions differed for the yeasts. Cultures were grown at both 30 ºC and 37 ºC. Incubation times of 4 days and 10 days at both temperatures were tested.

Yeasts were also grown in a modified nutrient broth (Appendix 2.4.1.4), having the same constituents as blood agar, but without the agar or blood. The same incubation conditions as outlined above were tested. Furthermore, yeasts were grown on two formulations of sago agar (Appendix 2.4.1.5), which differed only in the composition of the yeast extract used. Filtenborg *et al.* (1990) noted that different brands of yeast extract greatly affected the production of mycotoxins by some *Penicillium*. The sago agar plates were inoculated in triplicate with a single colony on a bacterial loop spread over the entire surface of the agar plate. Plates were incubated at 30 ºC for 5 days.

Filamentous fungi were in grown on two formulations of sago agar (as above), with incubation durations of 2 weeks and 4 weeks assessed. In addition, three different grains, namely cracked corn, rice and wheat, were also tested for suitability. Initially, 20 g of grain in 100 ml conical flasks was used, but in later experiments 100 g of grain in 500 ml conical flasks was used (Appendix 2.4.1.6). Moist sterile grain was inoculated with spores from a pure culture of the appropriate mould. The conical flask was then gently shaken to evenly distribute the spores. Grain cultures were incubated at 30 ºC for 4 weeks. Cultures were shaken by hand every 4–5 days to ensure even growth of fungi through the grain.

7.2.2.2 Optimisation of extraction technique for maximum haemolytic activity

Various extraction techniques were used depending on the type of organism and its corresponding growth medium. Following incubation, large SBA slopes used for growth of bacteria and yeasts were frozen solid at -20 ºC and allowed to thaw. The resulting exudate was drawn off with a sterile pipette, and the agar slope refrozen and the process repeated (Elek and Levy, 1954). The resulting liquid was filtered using a 0.45 µl syringe filter (Sarstedt Australia Pty Ltd, Ingle Farm, South Australia) and stored at -20 ºC until required.

For bacterial and yeast cultures grown in broth, cultures were sonicated for 2 min (Bronwell Biosink II, Bronwell Scientific, Rochester USA). The broth culture was then centrifuged at 10,000 *g* (Heraeus Sepatech Suprafuge 22, Germany) for 15 min. The supernatant was collected and sodium chloride was added to a final

concentration of 0.85%. The resulting isotonic extract was filtered using a 0.45 µl syringe filter. Extracts were stored at -20 ºC until required.

For extractions of yeast and filamentous fungi cultures grown on sago agar, the agar was aseptically removed from the triplicate Petri plates and placed in a stomacher bag. One hundred millilitres of toxin diluent solution (TDS) (Appendix 2.4.2.1) was added and subsequently blended using a Seward Stomacher, as above. The blended agar–TDS mixture was sonicated for 2 min (as above). The mixture was then centrifuged at 10,000 *g* for 15 min. The supernatant was filtered using a 0.45 µl syringe filter, and stored at -20 ºC until required.

The pellet was resuspended in 100 ml of alcoholic TDS (Appendix 2.4.2.2) and thoroughly mixed for 10 min in a shaking incubator (as above) set at 25 ºC and 150 rpm. The mixture was then centrifuged and filtered (as above). To prevent ethanol from causing lysis of erythrocytes in the haemolytic assay, alcoholic TDS extracts were freeze dried (Dynavac Eng Pty Ltd, Victoria). The dried extracts were resuspended in 100 ml of sterile distilled water prior to use in the haemolytic assay.

Various methods were trialled for extraction of fungal cultures grown on grain. Initially, a method similar to that outlined above for cultures grown on sago agar was used. Following incubation, 10 g of grain was ground using a Waring commercial blender (John Morris Pty Ltd, Bowen Hills, Queensland). An aliquot of 50 ml of TDS was added to the grain and mixed in the blender. The mixture was sonicated for 2 min (as above) and then centrifuged at 10,000 *g* for 15 min (as above). The supernatant was filtered using Whatman No.1 filter paper (Crown Scientific, Acacia Ridge, Queensland), then filter sterilised (as above). The resulting liquid was stored at 4 ºC for up to 2 days prior to use in the haemolytic assay.

The resulting pellet was resuspended in 50 ml of alcoholic TDS and mixed thoroughly in a shaking incubator (as above). The mixture was centrifuged and filtered (as above). The supernatant was freeze-dried to remove the ethanol, and subsequently resuspended in 50 ml of sterile distilled water prior to use in the haemolytic assay.

The extraction of fungal cultures with alcohol was further refined and the use of methanol trialled. A 5 g sub-sample of culture material grown on wheat was combined with 20 ml of methanol. The mixture was blended using a Sorval Omni-mixer (Du Pont Denemours and Company, Newton, Connecticut), then sonicated for 2 min using a sonicating waterbath (Consolidated Ultrasonics Asia Pty Ltd). Following filtration using Whatman No.1 filter paper, the extract was taken to dryness under gentle heat and a stream of air. The extract was resuspended in a small volume of methanol prior to use in the haemolytic assay (see Section 7.2.2.3).

7.2.2.3 Optimisation of the haemolytic assay

Initially, a haemolytic assay based on the work of Rizzo *et al.* (1992) was trialled to test haemolytic activity of extracts. Various shortcomings (see Section 7.4) resulted in the development of a more suitable assay. Human blood was collected from the researcher's radial or ulnar vein by a certified phlebotomist, using sodium heparin vacuum blood collection tubes (Vacutainer®, BD Australia, North Ryde, New South Wales). To ensure consistency, only the researcher's blood was used in the assays: no other volunteers were sought to supply blood. The blood was centrifuged at 3,000 *g* for 5 min (Ependorf, Crown Scientific, Acacia Ridge, Queensland) and the plasma and buffy coat removed. Erythrocytes were washed in 20 volumes of phosphate buffered saline (PBS) and centrifuged at 3,000 *g* for 5 min. The supernatant was removed and cells were washed again in a further 20 volumes of PBS and subsequently centrifuged. A 2 ml aliquot of the washed, sedimented cells was diluted in 1.5 ml of PBS.

For liquid extracts, 150 µl of diluted erythrocytes was added to 1,050 µl of extract in triplicate test tubes. Triplicate tubes of 1,050 µl of TDS and 150 µl of diluted erythrocytes were used as the negative control. The positive control consisted of triplicate tubes of 1,000 µl of TDS, 150 µl of diluted erythrocytes and 50 µl of 10% Triton X-100 (BDH Chemicals, Kilsyth, Victoria), a non-ionic detergent. All tubes were incubated in a shaking water bath (model RW1812, Paton Industries Pty Ltd, Sepney, South Australia) set at 37 ºC and 150 rpm. After 30 min of incubation, a 150 µl sub-sample was removed from each test tube and placed in a 1.5 ml microcentrifuge tube. The microcentrifuge tubes were placed in the refrigerator for

10 min, and then centrifuged in a microcentrifuge (Ependorf, Crown Scientific, Acacia Ridge, Queensland) at 1,000 *g* for 5 min. A 70 µl sub-sample of supernatant was pipetted into a flat-bottomed 96 well plate (Sarstedt Australia Pty Ltd, Ingle Farm, South Australia). The 96 well plate was placed in a plate reader and read at 540 nm (Labsystems Multiscan EX plate reader with Genesis version 3 software, Pathtech Pty Ltd, Melbourne, Victoria).

The sub-sampling and subsequent absorbance readings were repeated after incubation periods of 60 min, 120 min and 240 min (in one assay, the final reading was taken at 210 min instead of 240 min). For some extracts, further readings were taken at 6, 8 and 23 or 24 hours. Readings were averaged for triplicate tubes and standard deviation determined. Results were graphed as a percentage haemolysis relative to the positive control.

For extracts that had been taken to dryness, as outlined in Section 7.2.2.2, extracts were resuspended for use in the haemolytic assay. Each dried extract was resuspended in an appropriate volume of methanol to obtain a concentration of culture material of approximately 20 g/ml. Fifty microlitres of resuspended extract (equivalent of 1 g culture material) was then placed into triplicate test tubes, and 1,000 µl of TDS added. Diluted erythrocytes (150 µl, as above) were added to each tube, and then incubated and subsequently sampled as outlined above. For some samples, the re-suspended extracts were further diluted in methanol prior to the addition of TDS to test the haemolytic activity in smaller quantities of culture material. The amount of culture material in each assay was documented. The volume of methanol in each tube in the assay remained 50 µl. For assays where the extracts were resuspended in 50 µl of methanol and added to each tube, the negative control consisted of 1,000 µl of TDS, 50 µL of methanol and 150 µl of erythrocyte suspension.

7.2.3 Application of assay to crude microbial extracts

The optimised haemolytic assay was used to determine the haemolytic activity of numerous bacteria, yeasts and filamentous fungi. Microorganisms were selected for

further study on the basis of their haemolytic activity on blood agar. A variety of culture conditions, as outlined in Section 7.2.2.1, were utilised for each isolate.

Haemolytic activity of filamentous moulds was compared to that of *Trichoderma reesei* Simmons ATCC 26921 and *Stachybotrys chartarum* ATCC 9182 (formerly *Stachybotrys atra* Corda). These two filamentous fungi were utilised as *T. reesei* has been associated with the production of the haemolytic polypeptide antibiotic paracelsin (Bruckner *et al.*, 1984), while many strains of *S. chartarum* have been shown to be haemolytic when grown on SBA (Vesper *et al.*, 1999).

7.3 Results

7.3.1 Initial screening using blood agar

7.3.1.1 Direct screening of microorganisms isolated from sago starch on sheep blood agar

Of the 30 samples tested, the majority of samples had less than 1.0×10^4 cfu/g of haemolytic bacteria. In only two samples were there 1.0×10^5 cfu/g or more haemolytic bacteria, one of which was sample W0605-01, a sample implicated in an outbreak of SHD. The vast majority of haemolytic bacteria were members of the *Bacillus* genus, mainly the *B. cereus* group, but also *B. pumilus* and *B. amyloliquefaciens* were present. The Vitek® system used to identify bacteria was unable to differentiate between *B. cereus* and other closely related species such as *Bacillus thuringensis*. Other haemolytic bacteria isolated were *Pseudomonas aeruginosa*, which was isolated from 4 of the 30 samples tested, and an α-haemolytic *Streptococcus* species, which was present in only 1 sample, but in high numbers. Appendix 6.1 shows the number of haemolytic bacteria isolated from sago starch, and the most commonly occurring species.

The spread plate method used to enumerate haemolytic bacteria did not lend itself to enumeration of haemolytic yeasts and moulds. Within a few days of incubation the mixed microbial flora caused vague haemolysis over the entire agar surface, making

interpretation for individual colonies difficult. Results were inconclusive and thus are not shown here. Refer to Appendix 4.4 for enumeration of haemolytic fungi.

7.3.1.2 Screening of sterilised sago starch for haemolytic activity using blood agar

Results of the haemolytic screening of sterile sago starch were inconclusive. There was no haemolytic activity noted on the SBA plates over 10 days, but some activity was noted on HBA plates. However, one of the two negative control plates had haemolysed by day 10. Furthermore, on those test plates that had haemolysed the haemolytic activity was not necessarily in the vicinity of the sago agar. In some cases, areas of blood agar around the sago starch remained non-haemolysed, but other areas of the plate were haemolysed. This pattern can be observed in Figure 7.01.

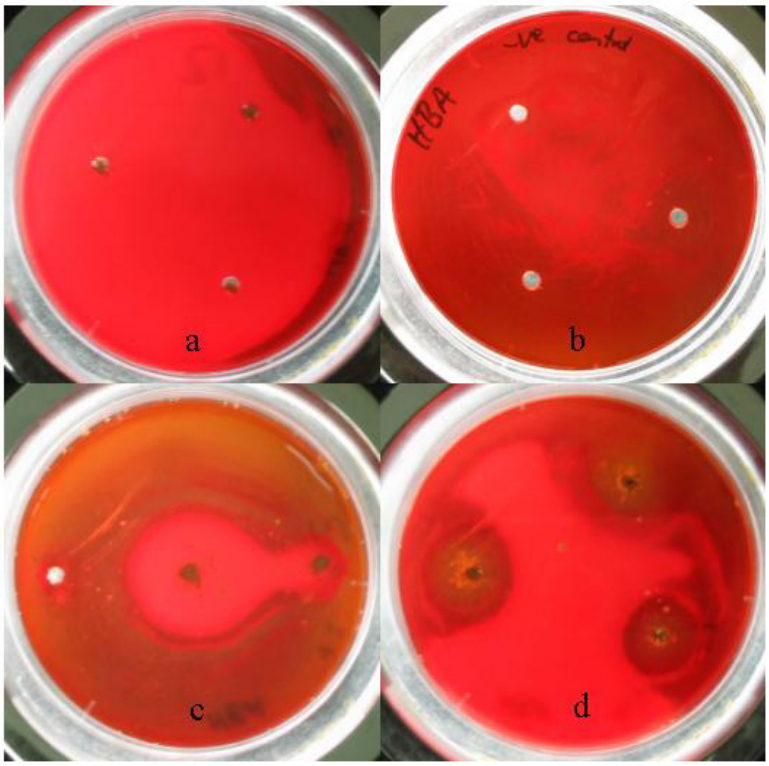


Figure 7.01: Observations of the screening of sago starch for haemolytic activity on HBA: (a) some haemolysis around periphery of plate, but no haemolysis in vicinity of sago starch; (b) negative control, in which no sago starch was added, showing widespread haemolysis over much of the plate; (c) haemolysis over much of the plate, but no haemolysis around two wells of sago starch; (d) haemolysis predominantly in the vicinity of wells containing sago starch.

7.3.1.3 Screening individual fungal isolates from sago starch for haemolytic activity using sheep blood agar and human blood agar

All haemolytic screening done immediately after fungal isolation was conducted using SBA derivatives. Results of haemolytic activity on SBA-chlor for each filamentous fungal isolate are shown in Appendix 4.4. Figure 7.02 shows the percentage 0+, 1+, 2+ and 3+ haemolytic isolates for the most commonly isolated fungal genera. It can be seen that the vast majority of *Penicillium* isolates were haemolytic to some degree, with 25% demonstrating pronounced (3+) haemolysis. Other genera to demonstrate noteworthy haemolysis were *Aspergillus, Trichoderma,* and to a lesser degree *Fusarium*. In excess of half the isolates of these genera were haemolytic on SBA-chlor, although none of the *Fusarium* isolates were 3+ haemolysers.

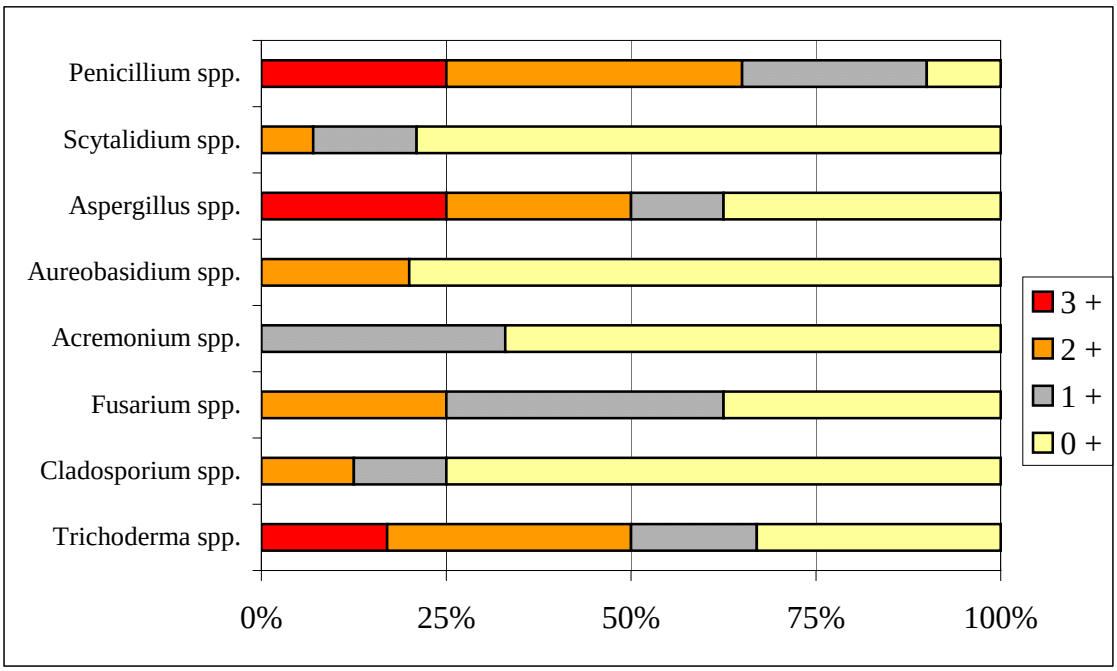


Figure 7.02: Percentage of isolates showing haemolytic activity on SBA-chlor for the most commonly occurring mould genera. Genera are listed in order of frequency of isolation from sago starch (i.e. *Penicillium* most commonly isolated, followed by *Scytalidium*, etc.).

Very few yeast isolates demonstrated clear haemolytic activity, although many exhibited large zones of partial haemolysis, causing a discolouration of the agar rather than clearing, similar in appearance to α haemolysis. Numerous yeasts isolated from sago starch that were at least partially haemolytic on SBA-gluc were selected for further investigation. Two of the isolates were identified as *Candida tropicalis* Castell. and *Geotrichum candidum* Link, while the other eight isolates were not identified.

A selection of fungal isolates was re-tested for haemolytic activity, using both SBA-chlor and HBA-chlor. Table 7.02 shows the haemolytic activity of the fungal isolates on the different agars. Generally, the haemolytic activity of any given isolate was consistent on the two different types of blood agar. However, there were some isolates that were non-haemolytic or only slightly haemolytic (1+) on one agar but demonstrated more pronounced haemolytic activity on the other agar. In most such cases, the increased haemolytic activity was on HBA-chlor.

All mould isolates able to be cultured from the two samples of sago starch associated with SHD outbreaks were tested using both SBA-chlor and HBA-chlor. In some instances, strains of the same species isolated from the same sago sample had differing haemolytic activities. Variability in haemolytic activity of a given strain on SBA-chlor and HBA-chlor was noted, as was the case for isolates from other sago samples (Table 7.02). The complete data set can be seen in Appendix 4.5.

Table 7.02: Comparison of haemolytic activity of selected fungal isolates using sheep blood agar (SBA-chlor) and human blood agar (HBA-chlor).

Genus/species	Code	SBA-chlor	HBA-chlor
Acremonium sp.	W2-0103	-	-
Aspergillus sp.	W2-0504	-	-
Aureobasidium sp.	S1- 0901	-	-
Cladosporium sp.	S2-0108	-	-
Cladosporium sp.	S2-1302	-	-
Fusarium sp.	W3-0202	-	-
Fusarium sp.	W5-0105	-	-
Geotrichum sp.	S2-0301	-	-
Geotrichum sp.	W2-1903	-	-
P. brevicompactum	S1- 0203	-	+
P. brevicompactum	W2-0501	+	+
P. brevicompactum	W4-0102b	+	+++
P. brevicompactum	W4-0128	++	++
Penicillium pinophilum	W4-0102a	++	++
P. steckii	W1-1109	-	+++
P. steckii	W1-1704	-	-
P. steckii	W1-2001	+	+++
P. steckii	W1-2002	+	+++
Penicillium sp.	W2-0301	+++	++
Penicillium sp.	W3-0602	-	-
Penicillium sp.	W5-0107	+++	-
Penicillium sp.	W5-0117	+++	+++
Penicillium sp.	W5-0136	+++	+
Phialophora sp.	W1-0901	-	-
Phialophora sp.	W3-0105	-	-
Scytalidium sp.	S1- 0204	-	-
Scytalidium sp.	W1-1201	-	-
Scytalidium sp.	S2-0401	-	-
Trichoderma harzianum complex	W2-0104	-	-
T. harzianum complex	W2-0105	+++	+++
Trichoderma harzianum	W2-1902	+++	-
Trichoderma virens	W4-0101	-	+++
T. virens	W4-0112	++	+++
T. virens	W4-0119	-	++

Legend: - equates to 0+,
+ equates to 1+,
++ equates to 2+
+++ equates to 3+ (as defined in Table 7.01)
P. = Penicillium; *T. = Trichoderma*.

7.3.2 Development of haemolytic assay

7.3.2.1 Selection of appropriate growth conditions for qualitatively testing microorganisms for haemolytic activity

The growth of bacterial isolates on SBA slopes proved to be unsuitable for later analysis using a haemolytic assay. The freeze-thaw extraction process resulted in lysis of red blood cells in the medium. Consequently, free haemoglobin present in the filtered bacterial extract affected the haemolytic assay. The growth of cultures in BHIB proved to be satisfactory and was subsequently used for the analysis of haemolytic activity in bacteria.

The growth of yeasts on SBA slopes for analysis of haemolytic activity also proved to be unsatisfactory (as above). Consequently, isolates tested for haemolytic activity using the quantitative assay were grown on the two sago agar formulations, in BHIB and in modified nutrient broth.

Preliminary investigations into the effect of growth substrate on haemolytic activity of fungi were conducted using one strain of yeast (*G. candidum*), three strains of *Penicillium*, and one strain of *Aspergillus* (Appendix 6.3). Cracked corn, rice, wheat and sago agar were tested using the assay described in Section 7.2.2. Cultures grown on wheat for 4 weeks produced the most consistent haemolytic activity across the filamentous fungi tested, whereas other substrates were variable depending on the fungal isolate. None of the substrates tested particularly favoured haemolytic activity in *G. candidum* (Appendix 6.3). In general cultures grown for 4 weeks were more haemolytic than cultures grown for 2 weeks (data not shown).

7.3.2.2 Optimal extraction of haemolytic component from microorganisms

Growth of bacteria and yeasts on blood agar slopes was incompatible with the haemolytic assay. More pronounced haemolytic activity was observed in yeasts grown in liquid culture than on solid growth medium (sago agar). Consequently, the extraction process for these organisms consisted of centrifugation and filtration of liquid cultures.

Early investigations of haemolytic activity were conducted using TDS and TDS/ethanol for extraction. The outcome of analysis suggested that extracts obtained using TDS/ethanol, then freeze-dried and resuspended in sterile distilled water, were no more haemolytic than extracts obtained using TDS. The observation of some haemolytic activity in filamentous fungi using TDS extraction led to the development of additional extraction techniques, as described in Section 8.2.1.

The extraction of fungal cultures in methanol, which was then taken to dryness with gentle heat under a stream of air, resulted in encouraging results for the fungal isolates on which it was performed. Methanol extracts that had been taken to dryness were resuspended in a small volume of methanol then diluted in TDS for use in the assay. The final concentration of methanol in the assay was approximately 4% (50 µl in a total volume of 1,200 µl), which had no adverse effect on erythrocytes.

All data presented in Sections 7.3.3.1 and 7.3.3.2 were obtained using centrifugation and filtration, while data presented in Section 7.3.3.3 was obtained using TDS extraction.

7.3.2.3 Optimisation of the haemolytic assay

Various modifications were made throughout the preliminary investigations to optimise the haemolytic assay for this application. Human blood was used instead of sheep blood. The assay involved the use of 9.5% erythrocytes (where washed, pelleted erythrocytes represents 100%), it was conducted in TDS rather than PBS, and sub-samples were refrigerated prior to centrifugation and measurement. A plate reader was used to read absorbance at 540 nm rather than reading the absorbance of individual tubes. Justification for these modifications will be discussed in Section 7.4.

7.3.3 Application of assay

7.3.3.1 Application of haemolytic assay to bacteria

A total of 15 bacterial isolates, 9 *B. cereus*, 2 *B. pumilus*, 1 *B. amyloliquefaciens* and 3 *P. aeruginosa* strains were tested for haemolytic activity in the assay. Figure 7.03 demonstrates the haemolytic activity of selected *Bacillus* isolates grown in BHIB, namely one strain of *B. cereus* group, one *B. pumilus* isolate and one *B. amyloliquefaciens* isolate. The extract from *B. cereus* caused complete lysis of erythrocytes within 30 min. Haemolytic activity increased with incubation time for the *B. amyloliquefaciens* extract, reaching 95% haemolysis after 4 hours incubation. The *B. pumilus* extract was non-haemolytic over the duration of the experiment, demonstrating essentially the same haemolytic activity as the negative control.

A further eight *B. cereus* group isolates was tested for haemolytic activity, with all but one having a similar level of haemolytic activity to that of the *B. cereus* group isolate shown in Figure 7.03. The other *B. cereus* group isolate and one additional *B. pumilus* isolate were non-haemolytic over 4 hours.

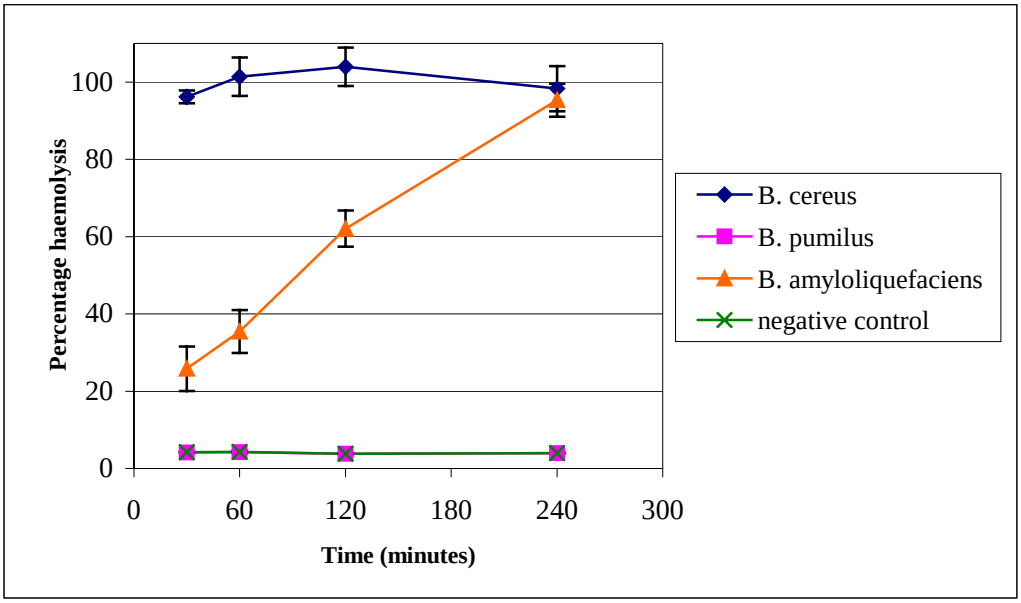


Figure 7.03: Haemolytic activity of selected *Bacillus* isolates over 4 hours using a semi-quantitative assay. Note that the *B. pumilus* extract was non-haemolytic, having the same level of activity as the negative control. Each tube contained the equivalent of 1,050 µl of liquid culture.

The haemolytic activity of the three *P. aeruginosa* extracts grown in BHIB was considerably less than that of *B. cereus* or *B. amyloliquefaciens*. After 4 hours, all *P. aeruginosa* extracts were less than 20% haemolytic. After 8 hours, one extract was approaching 40% haemolysis, with the other two extracts having less than 20% haemolysis (Figure 7.04). After 23 hours all three extracts had haemolysis values of between 40% and 90% (data not shown).

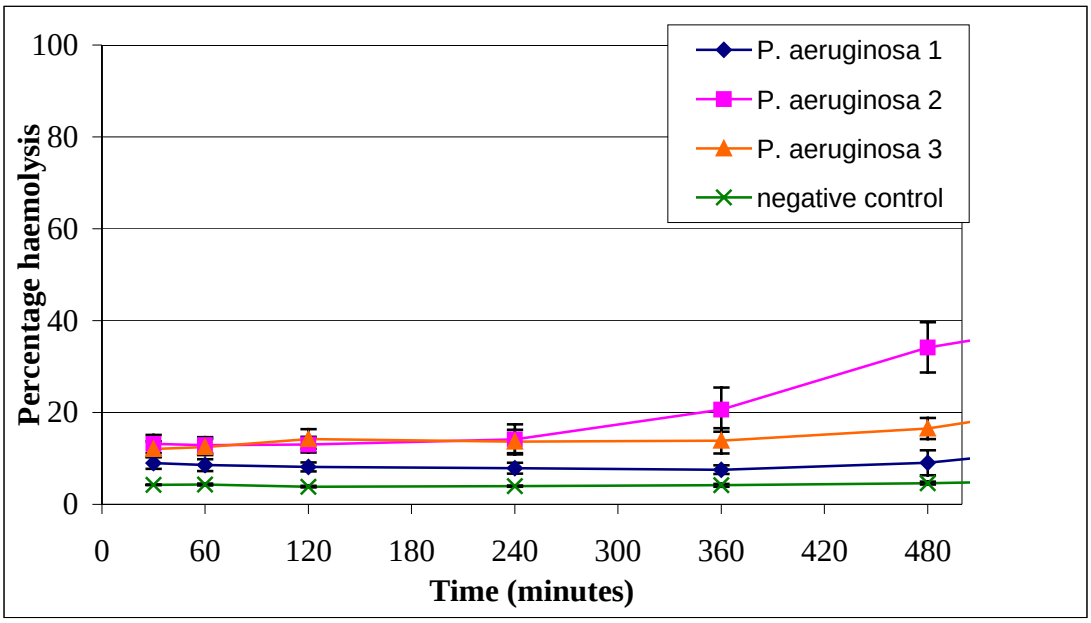


Figure7.04: Haemolytic activity of selected *P. aeruginosa* isolates over 8 hours using a quantitative assay. Each tube contained the equivalent of 1,050 µl of liquid culture.

7.3.3.1 Application of haemolytic assay to yeasts

Eight yeast isolates; one *G. candidum*, one *C. tropicalis*, and six unidentified isolates, were tested using the haemolytic assay. Despite the numerous combinations of growth conditions and extraction techniques, only limited haemolytic activity was observed in yeast extracts. Yeast extracts from cultures grown on solid media were generally non-haemolytic. No haemolytic activity was observed from yeast extracts of cultures grown in liquid culture for 4 days. However, haemolysis was observed in extracts of cultures grown for 10 days in liquid culture. There was little difference in the haemolytic activity of cultures grown in BHIB compared to those grown in

modified nutrient broth. Extracts of cultures grown at 37 ºC were more haemolytic than cultures grown at 30 ºC.

Figure 7.05 illustrates the haemolytic activity of extracts of selected yeasts grown in modified nutrient broth at 37 ºC for 10 days. It can be seen that the extracts had very little haemolytic activity over the first 4 hours. After 6 hours the extract of isolate W2-2204y was slightly haemolytic, and after 8 hours this extract and one other, from isolate W2-0404y, were clearly haemolytic. The extract from the third yeast isolate, W2-0303y, remained non-haemolytic at 8 hours. Other extracts of yeasts tested for haemolytic activity exhibited similar haemolytic activity to those isolates shown in Figure 7.05. None of the eight yeast extracts tested was significantly haemolytic over the first 6 hours, but most exhibited haemolytic activity at 8 hours. Neither of the identified yeasts, *G. candidum* and *C. tropicalis*, demonstrated greater than 50% haemolysis over 24 hours.

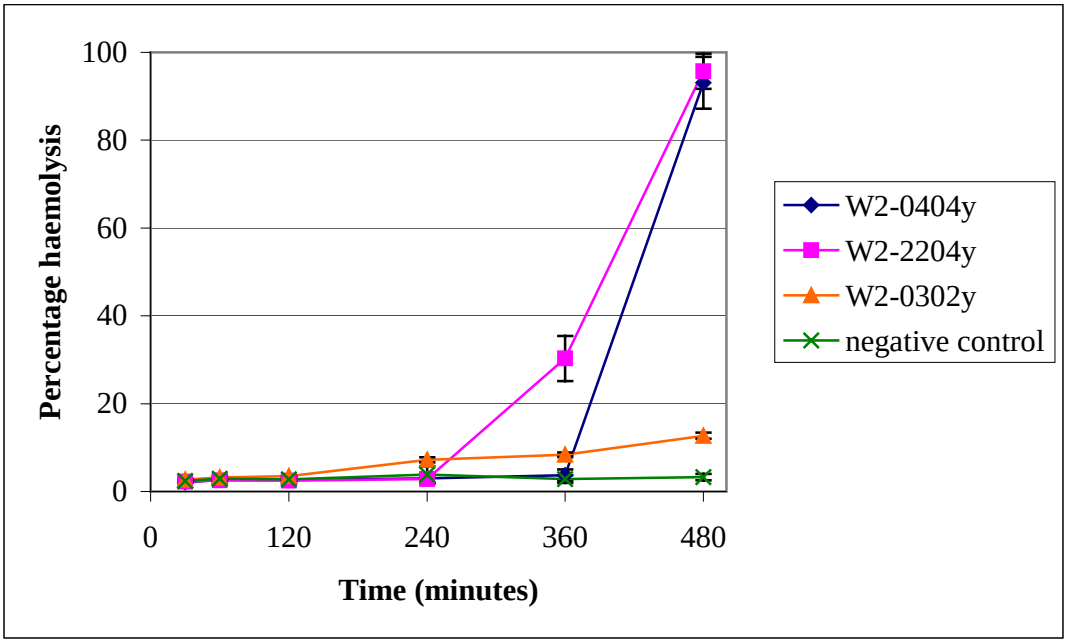


Figure 7.05: Haemolytic activity of selected yeast extracts (TDS) over eight hours. Each tube contained the equivalent of 1,050 µl of liquid culture.

7.3.3.2 Application of haemolytic assay to filamentous fungi

Following the optimisation of the assay, the suitability of the assay for testing fungal cultures grown on moist wheat was determined. Sterile moist wheat was incubated at

30 ºC for 4 weeks alongside a culture of *T. reesei* ATCC 26921 and *S. chartarum* ATCC 9182 grown on moist wheat. Following incubation, wheat was extracted with TDS, as outlined in Section 7.2.2.2. Figure 7.06 demonstrates that uninoculated wheat is not haemolytic, having a lower rate of haemolysis than the negative control. The two fungal strains had markedly different haemolytic activities. Over the first 4 hours, neither was haemolytic, but at 6 hours *S. chartarum* had caused complete haemolysis. *Trichoderma reesei* remained non-haemolytic for the duration of the experiment (8 hours).

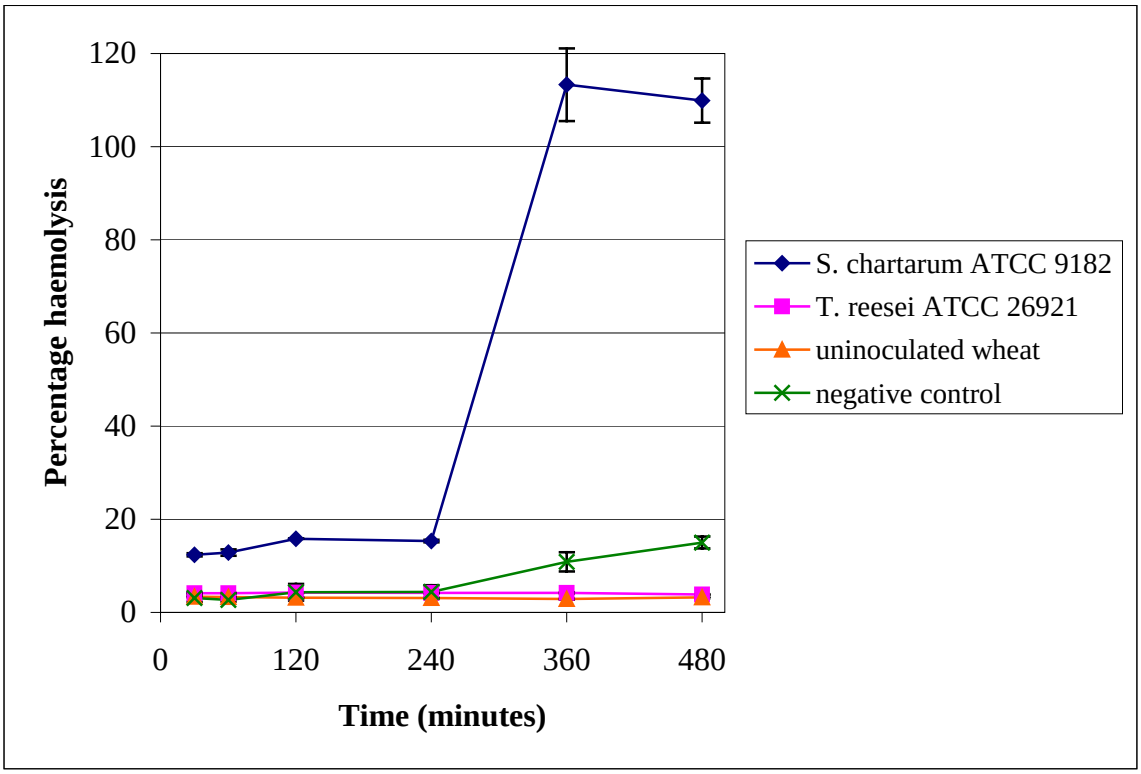
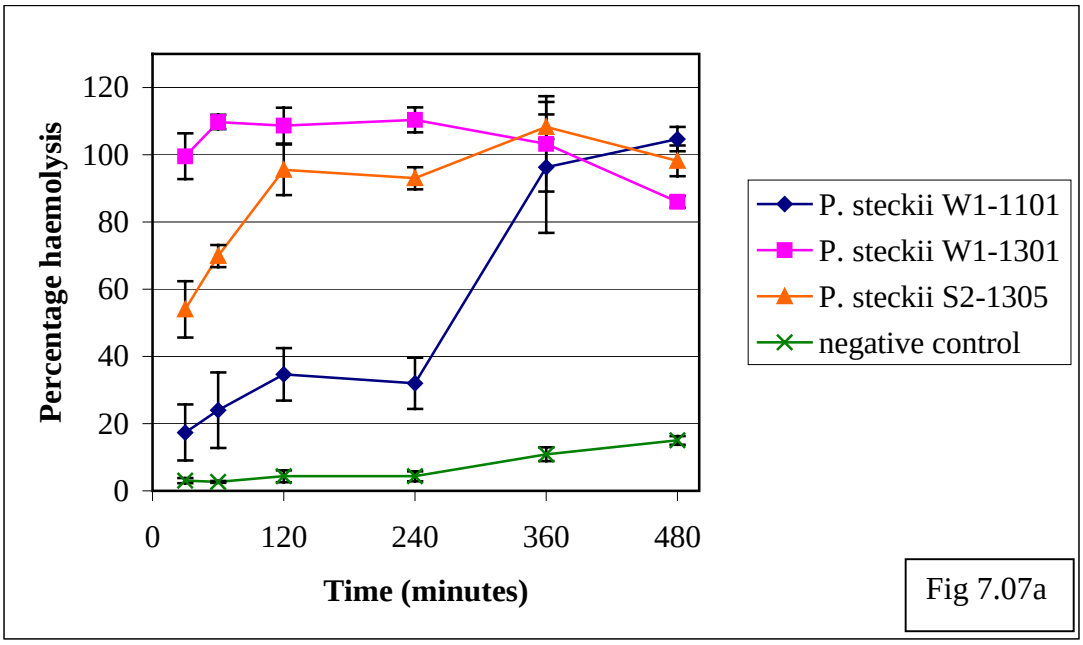


Figure 7.06: Haemolytic activity of uninoculated wheat, *S. chartarum* ATCC 9182, *T. reesei* ATCC 26921 (extracted in TDS), and a negative control. Each tube contained the equivalent of 0.175 g of culture material.

Initially, the haemolytic assay was applied to six isolates of filamentous fungi, namely three isolates of *P. steckii*, one *P. brevicompactum*, one *Aspergillus flavipes*, and one *F. semitectum*. Figure 7.07 demonstrates the haemolytic activity of the selected filamentous fungi. All three isolates of *P. steckii* were haemolytic after 6 hours, with two of the three isolates causing close to 100% haemolysis within 2 hours (Figure 7.07b). The other three isolates did not show the same degree of rapid haemolysis as *P. steckii* W1-1301 or *P. steckii* S2-1305. Nonetheless the

A. flavipes and *F. semitectum* isolates were similar in haemolytic activity to *P. steckii* W1-1101. The *P. brevicompactum* isolate was the least haemolytic of the six isolates, reaching a maximum of approximately 40 % haemolysis after 8 hours.



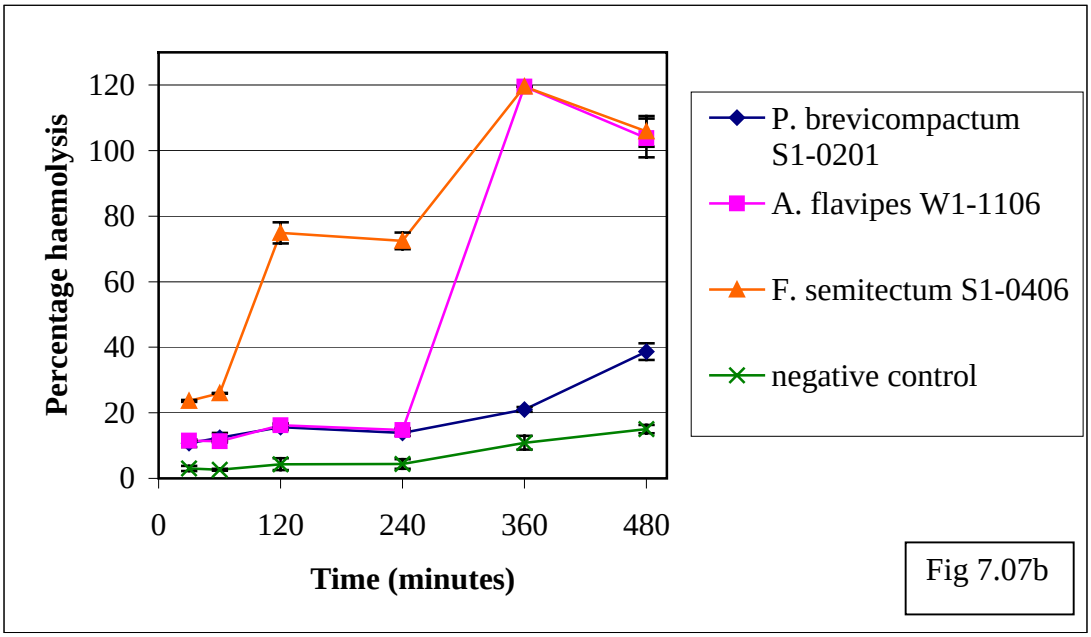


Figure 7.07: Haemolytic activity of fungi isolated from sago starch, extracted using TDS: (a) Three strains of *P. steckii*; (b) *P. brevicompactum*, *A. flavipes*, and *F. semitectum*. Each tube contained the equivalent of 0.175 g of culture material.

7.4 Discussion

The use of blood agar to test for haemolysis is valid as a method of screening, but it is difficult to quantify and compare the level of haemolytic activity. As such, the development of a suitable haemolytic assay was instrumental in determining the haemolytic activity of microbial isolates from sago starch. Many of the assays described in the literature are used to determine the cytolytic nature of toxins, more so than their haemolytic nature *per se*. Bernheimer (1988) suggested that erythrocytes are commonly used for testing cytolytic toxins because they are readily available, their lysis liberates haemoglobin (thus determination of degree of lysis can be measured spectrophotometrically), and because lytic agents for other types of mammalian cells are usually lytic for erythrocytes.

As previously discussed, relatively few organisms that are haemolytic *in vitro* are associated with acute haemolysis *in vivo* (Section 2.6). It would seem that in some cases at least, erythrocyte lysis could be a "spurious correlate", where lysis of other cells such as leukocytes provides the selective advantage (Rowe and Welch, 1994). For this study it was important to develop an assay that would allow high throughput and give some indication of the potency of the extract being tested.

Rowe and Welch (1994) suggested the amount of erythrocytes in an assay should be between 0.5 and 2.0% (where 100% is the concentration of cells in washed pellet). However, in the assay developed for the current study, a considerably higher concentration of erythrocytes was required. By using 9.5% erythrocytes, this assay was less sensitive than many in the published literature. The focus of this study was on finding potent haemolytic extracts, hence the loss in sensitivity provided the assay with a degree of selectivity. Moreover, the use of 9.5% erythrocytes in the assay better enabled the testing of pigmented extracts. Many of the crude fungal extracts were pigmented. Early trials using the assay described in Rizzo *et al.* (1992) were unsuccessful as some of the extracts had a similar absorbance at 540 nm as the positive control. One of the shortcomings of the assay developed by Rizzo and co-workers was that the final concentration of erythrocytes was only approximately 0.16%. By adding 9.5% erythrocytes to the assay, the positive control tube had a considerably higher absorbance than any of the extracts.

Two other amendments to the assay warrant justification. Toxin diluent solution was used in the optimised haemolytic assay. Many haemolytic assays use PBS or isotonic saline solution. However, the inclusion of gelatin in TDS acts as a stabilizing agent for some cytolytic toxins, preventing their denaturation. Furthermore, phospholipases require the addition of magnesium ions or calcium ions for maximal haemolytic activity (Bernheimer, 1988). Given the unknown nature of the haemolysins investigated in this study, the use of TDS was deemed necessary to ensure maximal haemolytic activity. The second amendment was the refrigeration of the sub-sample prior to centrifugation, as some cytolysins (i.e. phospholipases) require chilling to complete haemolytic activity *in vitro* (Bernheimer, 1988).

There were two apparent anomalies in the haemolytic assay used in this study that necessitate discussion. First, on occasions extracts produced greater than 100% haemolysis relative to the positive control. This trend was most pronounced in the assays of crude fungal extracts that were pigmented. Although the use of 9.5% erythrocytes in the assay enabled pigmented extracts to be tested, the pigmentation still had some influence on the overall absorbance reading. It stands to reason that a tube with a pigmented extract and all erythrocytes lysed would have a higher absorbance than the positive control consisting of lysed erythrocytes in a previously non-pigmented solution. Secondly, many of the extracts that produced 80–100% haemolysis in the early stages of the assay exhibited a gradual decrease in absorbance over subsequent hours. This is due to the partial conversion of haemoglobin to methaemoglobin, which can start occurring within 30 min of incubation (Bernheimer, 1988).

When assaying the fungal fractions for haemolytic activity, a small volume of methanol was used to facilitate re-suspension of the extract and to resuspend substances that were not very water soluble. The final concentration of methanol on addition of erythrocytes was approximately 4%. This appeared to have minimal effect on erythrocytes, with the negative control containing 4% methanol producing a very similar haemolysis curve to that of a negative control in the absence of methanol. However, low levels of ethanol have been shown to have a modulating effect on biological membranes in the presence of the mycotoxin T-2 (Schappert and

Khachatourians, 1984; Rizzo *et al.*, 1992). The possibility of methanol having a modulating effect cannot be discounted. For the purpose of this study, where the haemolytic activity of one fraction was being compared to another, all fractions were treated identically. Thus, the presence of methanol should not adversely affect the validity of the results.

Numerous studies have highlighted the species-specific nature of haemolysins. This observation has been made across haemolysins from bacterial, yeast and filamentous fungal origin (Salvin, 1951; Greenwood and Pickett, 1979; Bernheimer, 1988; DeLoach *et al.*, 1989). Consequentially, it was deemed important to test a variety of fungal genera for haemolytic activity on both SBA-chlor and HBA-chlor. Isolates selected for re-testing covered a range of previously recorded haemolytic activity, from 0+ to 3+ on SBA-chlor. Comparison of results on SBA-chlor in this experiment to results obtained immediately following isolation from sago starch (Appendix 4.4) show some differences. Some isolates showed a decreased ability to haemolyse erythrocytes depending on the number of times they were subcultured. This finding highlighted the need for appropriate storage and preservation of fungal isolates. Haemolytic activity was similar on SBA-chlor and HBA-chlor for most fungal isolates tested. Nevertheless, there were some notable exceptions, with numerous *Penicillium* and *Trichoderma* isolates being more haemolytic on HBA-chlor than SBA-chlor.

The use of animal blood in bioassays has advantages over the use of human blood, particularly in terms of biosafety, but it may come with a compromise in scientific rigour. Kothary and Kreger (1985) found mouse erythrocytes to be over 10,000 times more sensitive to a bacterial haemolysin than erythrocytes of numerous other animals, including sheep and humans. If such a discrepancy existed between sheep erythrocytes and human erythrocytes when subjected to haemolysins extracted in this study, the validity of the results could be questioned. The comparison of haemolytic activity on SBA-chlor and HBA-chlor in this study suggests a slight risk exists that a toxin capable of aggressively attacking human erythrocytes could be overlooked using sheep erythrocytes in the initial assays.

The screening of sterilised sago starch *per se*, and the isolation of haemolytic microorganisms from sago starch, is based on the assumption that a substance or organism capable of causing acute intravascular haemolysis *in vivo* will exhibit some degree of haemolysis *in vitro*. Sterilised sago incubated for 10 days on blood agar demonstrated variable results on HBA, but was consistently non-haemolytic on SBA (see Figure 7.01). Due to the inconclusive nature of these results, the haemolytic activity of sago starch is addressed in further detail in Chapter 8.

The absence of haemolytic activity on SBA is not sufficient evidence alone to discount the presence of phytotoxins from sago. The occurrence of naturally occurring substances with potentially deleterious health effects in some foods of plant origin is well documented e.g., cyanide production in cassava (Ruangkanchanasetr *et al.*, 1999; Bonmarin *et al.*, 2002; Oluwole *et al.*, 2002; Ngudi *et al.*, 2003), and favism in glucose-6-phosphate dehydrogenase deficient individuals (Lukens, 1993; McKenzie, 1996). There is scant evidence suggesting palm trees are associated with any such occurrences, although one study conducted in the early 1980s extracted a compound toxic to rats from the palmyra palm, *Borassus flabellifer* Linn. (Greig *et al.*, 1980). This palm is consumed in parts of Asia, but there is no evidence to suggest that it causes human illness. The general absence of plant mediated toxins in palms suggests that microbial toxins are a more likely cause of SHD.

Haemolytic microorganisms, mainly bacteria and fungi, were present in the majority of sago samples tested. The most commonly isolated genus of haemolytic bacteria from sago starch was *Bacillus*, with *P. aeruginosa* the only other haemolytic bacterium to be commonly isolated. *Bacillus cereus* has long been associated with episodes of food poisoning (see Section 2.4.2), is intensely haemolytic on blood agar, and was shown in this study to cause rapid haemolysis of erythrocytes in the haemolytic assay. In addition, it produces numerous toxins, many of which consist of haemolytic components (Jenson and Moir, 2003).

The role of *B. cereus* in food poisoning has been studied for over 50 years, and its production of diarrhoeal and emetic toxins are well documented (Granum, 1997; Jenson and Moir, 2003; Schoeni and Wong, 2005). These toxins generally result in

mild illness of short duration. Indeed fatalities attributed to *B. cereus* foodborne illness have been reported only rarely. Mahler *et al.* (1997) described the fatality of a previously healthy 17 year old due to fulminant liver failure attributed to consumption of food contaminated with *B. cereus* emetic toxin. While haemoglobinuria was noted in the patient, it was likely the result of thrombotic microangiopathy in small renal arteries, as noted on post-mortem. In a separate outbreak, five children within one family became ill one of whom, a seven year old, died due to liver failure within 13 hours of consuming a meal contaminated with *B. cereus* (Dierick *et al.*, 2005).

Lund and co-workers (2000b) isolated a new cytotoxin from *B. cereus* that was associated with a food poisoning outbreak in which several people developed bloody diarrhoea, and three patients died. The authors suggested the isolated cytotoxin caused necrotic enteritis, which would account for the bloody diarrhoea. There was no mention of acute intravascular haemolysis.

Bacillus cereus has been associated with acute intravascular haemolysis on rare occasions. Rodgers and co-workers (1980) reported a case of a patient with sickle cell anaemia and cholelithiasis who suffered *B. cereus* bacteraemia and haemolytic anaemia. The presence of sickle cell anaemia is of particular interest, given the association of some red blood cell disorders with predisposition to acute haemolytic crisis (Lukens, 1993; McKenzie, 1996). Such an association has been speculated in previous communications pertaining to SHD (Taufa, 1974; Donovan *et al.*, 1977).

Arnaout and co-workers (1999) also reported two cases of acute intravascular haemolysis associated with *B. cereus* septicaemia. Both cases were in severely immunocompromised patients. In one case gastrointestinal symptoms preceded haemolysis.

While *B. cereus* was commonly isolated from sago starch, numbers were generally below the threshold required to cause morbidity (approximately 10^5–10^7 cfu/g) . A low level of *B. cereus* contamination is common in a wide variety of processed and raw foods (Jenson and Moir, 2003), and for the most part cause no serious public

health concerns. Nonetheless, given the protean nature of *B. cereus*, its role in SHD cannot be discounted.

Other species of bacteria isolated are unlikely to contribute to SHD. *Bacillus pumilus* has been implicated occasionally in incidents of foodborne illness (Jenson and Moir, 2003). It can lyse sheep erythrocytes, although Hoult and Tuxford (1991) found it was considerably less haemolytic than *B. cereus*. The low level of haemolytic activity of *B. pumilus*, along with the infrequency of isolation, suggests it is unlikely to be involved in serious foodborne illness in sago starch. The same line of reasoning can be used to discount *B. amyloliquefaciens*.

Pseudomonas aeruginosa demonstrated very little haemolytic activity when grown in liquid media. Other researchers have documented the difficulty in obtaining haemolysins from *P. aeruginosa* in broth culture (Johnson and Boese-Marrazzo, 1980; Fujita *et al.*, 1988), but unfortunately such considerations were not taken into account prior to this study. Nonetheless, it would seem unlikely that this organism is involved in the aetiology of SHD, as it was only infrequently isolated from sago starch, generally in low numbers, and is not a known foodborne pathogen.

No attempt was made to culture the full range of haemolytic anaerobic bacteria in this study. However, attempts were made to culture *Clostridium* spp. when isolating bacteria of public health significance (see Section 4.3.3). Clostridia have commonly been associated with septicaemia and intravascular haemolysis (Lee, 1993; Pun and Wehner, 1996; Alvarez *et al.*, 1999; Jimenez *et al.*, 2002; Vaiopoulos *et al.*, 2004; Pirrotta *et al.*, 2005), but the genus was detected in less than 20% of sago samples tested, and only in very low numbers. Despite its role in foodborne illness, including pig bel (Lawrence and Walker, 1976; Hatheway, 1990; Murrell and Walker, 1991; Farrant *et al.*, 1996; Bates and Bodnaruk, 2003), it seems unlikely that *C. perfringens* is involved in the pathogenesis of SHD. Nonetheless, further study of the haemolytic nature of anaerobic bacteria is required before *Clostridium* spp. and other anaerobes can be fully discounted.

The screening of yeasts for haemolytic activity on glucose enriched SBA revealed very few isolates capable of β-haemolysis at 30 ºC. Manns *et al.* (1994) stated that

Candida albicans was only haemolytic when grown on glucose enriched agar at 37 ºC. On the basis of current information, SHD is likely to be the result of intoxication caused by a pre-formed toxin rather than by infection and subsequent toxin formation *in vivo* (see Chapter 3). The lower incubation temperature used was considered to more accurately reflect the growth conditions microorganisms would encounter growing on sago starch in PNG. Despite the tropical climate, temperatures rarely exceed 35 ºC in the shaded areas where sago is typically stored. Overnight, and during the cooler months, temperatures in the proximity of 30 ºC are typical.

The two identified yeast isolates, *G. candidum* and *C. tropicalis*, demonstrated reasonable haemolytic activity on glucose enriched blood agar, but had little haemolytic activity when tested in the quantitative assay. The other six unidentified isolates were mildly haemolytic, but typically such haemolysis was evident after long incubation times.

Even though early work on yeast haemolysins was published over 50 years ago (Salvin, 1951), to date little work has been completed in the field. Recent work has highlighted the need for glucose enriched agar for haemolytic activity (Manns *et al.*, 1994; Luo *et al.*, 2001). The early work of Salvin (1951) documented the different susceptibilities of erythrocytes of various animals. Sheep and human erythrocytes were considerably less susceptible than other species tested. Recent work by Samaranayake *et al.* (2005) looked into various virulence attributes of *Candida albicans*, including haemolysin production, but results were inconclusive.

At this stage the mode of haemolysis in yeasts, and the role it plays in pathogenesis, remain unknown. Further research into the field is justified, but is beyond the scope of this study. Notwithstanding the current lack of knowledge in the field of yeast haemolysins, it is unlikely that yeasts contribute to the aetiology of SHD. This supposition is based on the weak haemolytic activity of isolates from sago starch, as well as the lack of evidence linking yeasts to foodborne illness.

This study has isolated numerous filamentous fungal strains that demonstrate haemolytic activity on blood agar. The genera of most interest were *Penicillium*, *Aspergillus*, *Trichoderma*, and to a lesser degree *Fusarium* (Figure 7.02). While

considerable study has been conducted on asp-haemolysin from *A. fumigatus* (Section 2.6.3.3), comparatively little is known about compounds with haemolytic activity in other species of this genus, or in the other aforementioned fungal genera. Moreover, the evidence of fungal contamination of sago starch (Chapter 5), and the long speculated role of fungal metabolites in SHD (Taufa, 1974; Donovan *et al.*, 1977) dictate the need for further research. As such, isolates from the genera of filamentous fungi commonly exhibiting haemolytic activity were selected for further study, as addressed in Chapter 8.

CHAPTER 8: FURTHER STUDIES ON THE HAEMOLYTIC ACTIVITY OF SELECTED FUNGAL EXTRACTS AND SAGO STARCH SAMPLES

8.1 Introduction

Although relatively little is known about the nature of haemolysins of fungal origin, some studies have demonstrated filamentous fungi (Budzko and Negroni, 1975; Yokota *et al.*, 1977), yeasts (Salvin, 1951; Manns *et al.*, 1994), fungal extracts (Bruckner *et al.*, 1984) and mycotoxins (Gyongyossy-Issa *et al.*, 1985; DeLoach *et al.*, 1987; DeLoach *et al.*, 1989) to be haemolytic *in vitro*. The chemical structure of some haemolytic compounds of fungal origin has been determined, although most work has concentrated on compounds from macro-fungi (Faulstich *et al.*, 1983; Jaworski *et al.*, 1999; Sepcic *et al.*, 2003; Tateno and Goldstein, 2003). Currently little is known about the role, if any, of haemolytic compounds from fungi in disease pathogenesis.

Research papers have documented mycotoxins such as wortmanin (Abbas and Mirocha, 1988; Mirocha and Abbas, 1989) and apicidin (Park *et al.*, 1999) from *Fusarium* spp. causing haemorrhage in laboratory animals. There is much less information to substantiate a role for fungi in acute intravascular haemolysis, although evidence of haemoglobin in urine of laboratory animals has been documented. Bosch and Mirocha (1992) reported haematuria in rats fed rice culture of various different species of *Fusarium*, and Banotai *et al.* (1999) observed haematuria in mice fed the trichothecene deoxynivalenol (vomitoxin). Haematuria has also been noted in humans thought to have been exposed to *S. chartarum* (Vesper *et al.*, 1999). In addition, sporadic cases of immune mediated acute haemolytic crisis in humans after ingestion of the edible fungi *Paxillus involutas* have been documented (Winkelmann *et al.*, 1982; Winkelmann *et al.*, 1986).

Given the evidence to suggest that some fungal metabolites might be haemolytic, the objectives of this study were twofold: first, to use simple chromatographic techniques combined with a haemolytic assay to further characterise the active components produced by fungi from sago starch; and secondly, to assess if any of the active components bear similarities to known mycotoxins.

8.2 Materials and Methods

8.2.1 Extraction of the haemolytic component produced by filamentous fungi

Seven fungal isolates from sago starch were selected for further study from a larger number shown to have haemolytic activity on blood agar. Five of the isolates (three *P. steckii*, one *P. brevicompactum* and one *F. semitectum*) had been previously tested for haemolytic activity of their respective crude TDS extracts, and all but *P. brevicompactum* were found to be haemolytic within 6 hours (Figure 7.07) in the standard assay conditions. For *A. flavipes*, a different strain was used to that which was tested for haemolytic activity in the previous chapter, as the original strain was unable to be resurrected from storage. The seventh fungus, *T. virens* W4-0119, was isolated from one of the samples of sago associated with an outbreak of SHD. It had been shown to be haemolytic on HBA, but not on SBA (Table 7.02).

In addition to the seven fungal strains isolated from sago starch, the control organisms used in the previous study, *S. chartarum* ATCC 9182 and *T. reesei* ATCC 26921, were utilised throughout the current study. Sterile wheat, which had been incubated and extracted under the same conditions as the fungal cultures, was used as a negative control.

Appropriate methods of extraction were developed to enable the partial separation of the haemolytic component from other fungal metabolites. The method was based on those used for extraction of citrinin and other mycotoxins (Chapter 6), but with minor modifications. A 25 g sample of grain culture was macerated using a Sorvall Omni-mixer (Du Pont Denemours and Company, Connecticut, USA) and mixed with 90 ml of acetonitrile (HPLC grade, EM Science, Kilsyth, Victoria), 10 ml of 4%

potassium chloride (Ajax Chemicals, Sydney, New South Wales) and 2 ml of 5 M hydrochloric acid (Ajax Chemicals, Sydney, New South Wales). The mixture was sonicated for 2 min using a sonicating waterbath (Consolidated Ultrasonics Asia Pty Ltd), and then filtered through Whatman GFA filter paper (Crown Scientific, Acacia Ridge, Queensland). A 50 ml aliquot of filtrate (equivalent of 12.5 g of culture material) was placed in a separating funnel to which 50 ml of distilled water and 50 ml of hexane (HPLC grade, Lomb Scientific Archerfield, Queensland) were added. The separating funnel was shaken by hand for 1 min and allowed to settle, with the lower fraction subsequently collected and temporarily set aside. The upper (hexane-rich) fraction was collected and the lower fraction was re-extracted using a further 50 ml of hexane. Both hexane-rich fractions were combined, filtered through Whatman phase separating paper (Crown Scientific, Acacia Ridge, Queensland) and taken to dryness with gentle heating under a stream of air. The resulting hexane-soluble fraction (extract A) was stored in aluminium foil-capped test tubes at -20 ºC until required.

The remaining acetonitrile/water/KCl/HCl fraction was then returned to the separating funnel and extracted with two successive 50 ml aliquots of dichloromethane. This partitioned the acetonitrile between water-rich and dichloromethane-rich fractions. The dichloromethane-rich fractions were filtered through anhydrous granular sodium sulphate (Ajax Chemicals, Sydney, New South Wales) and taken to dryness with gentle heating under a stream of air. The resulting dichloromethane/acetonitrile-soluble fraction (extract B) was stored in aluminium foil-capped test tubes at -20 ºC until required.

Prior to the haemolytic assay, the hexane and dichloromethane fractions were resuspended in their corresponding solvent. The equivalents of approximately 0.25 g and 0.10 g of culture material were dispensed into respective sets of triplicate tubes and taken to dryness under a stream of air. The dried extracts were resuspended in 50 µl of methanol and assayed for haemolytic activity as described in the previous chapter (Section 7.2.2). Negative controls for the haemolytic assay consisted of tubes that had an equivalent quantity of hexane or dichloromethane added and taken to dryness under a stream of air. The negative control tubes then had 50 µl of methanol added, as previously described (Section 7.2.2.3).

8.2.2 Separation of haemolytic components of fungal culture extracts using preparative layer chromatography

Preparative layer chromatography (PLC) was used to further separate the haemolytic component(s) of the fungal extracts. The stored hexane-rich extracts of the seven fungal strains isolated from sago starch, the two positive control organisms and the negative control (as above) were redissolved in an appropriate volume of hexane. Two hundred microlitres (equivalent to 4.4 g culture material) was spotted onto a 20 cm × 20 cm preparative chromatography plate (Analtech Silica Gel G 1000 micron, Banksia Scientific Company Pty Ltd, Brisbane, Queensland). Each extract was spotted along the width of the plate, leaving margins of approximately 2 cm at each end. Within both margins 5 µl of extract from *P. steckii** S2-1305 was spotted as a marker for citrinin, which the isolate produced (see Section 6.4).

Plates were developed in 5:4:1 TEF (EM Science, Kilsyth, Victoria). Following chromatography, plates were observed in ambient light, and briefly under shortwave (254 nm) and longwave (365 nm) UV light (Spectroline CC-80 ultraviolet fluorescence cabinet, Spectronics Corporation, New York). The plates were divided into two sections on the basis of how far the citrinin band moved (visible in the two 5 µl spots of *P. steckii** S2-1305). Using a pencil, a line was drawn from the leading edge of citrinin in one margin to the leading edge of citrinin in the other margin. The silica from the lower segment (from the origin to equal with the leading edge of the citrinin band) and the upper segment (from just beyond the leading edge of citrinin to the solvent front) was scraped off the glass plate using a scalpel blade. The margins of the plates were not included in the upper and lower segments, to avoid the possibility of *P. steckii** S2-1305 contaminating the extracts. Each segment was placed in 50 ml disposable centrifuge tubes (Sarstedt Australia Pty Ltd, Ingle Farm, South Australia) with 20 ml methanol. The methanol suspension was thoroughly mixed by hand, and then filtered through Whatman No. 2 filter paper (Crown Scientific, Acacia Ridge, Queensland). The resulting extract was taken to dryness with gentle heat under a stream of air, and stored at -20 ºC until required. Dried extracts of the PLC segments were redissolved in a known volume of methanol, diluted as required, and used in the haemolytic assay.

When assaying the separate sections, the amount of culture material tested was calculated on the assumption that all the active component(s) applied to the plate went into a single segment.

8.2.3 Further separation of haemolytic component from *Penicillium steckii* W1-1301

In an attempt to further separate the haemolytic component of filamentous fungi, one isolate, *P. steckii* W1-1301, was selected for further investigation. The separation of components on PLC was refined, and the use of microfine silica separatory columns was trialled.

The hexane-rich extract of *P. steckii* W1-1301 was fractionated using PLC. The equivalent of 3 g of culture material was dissolved in 200 µl of hexane and spotted along the length of the plate as previously described (Section 8.2.2), but without the 5 µl spots of *P. steckii* (S2-1305). Plates were run in TEF, as above, and observed in ambient and UV light (as above). To visualise non-fluorescent bands, a 5 µl aliquot of the redissolved extract was spotted along a 5 cm × 20 cm aluminium TLC sheet pre-coated with 0.2 mm silica gel 60 (Merck 1.05553 silica plates, Alltech Associates Pty Ltd, Baulkham Hills, New South Wales). A similar procedure was applied using TLC plates incorporating an indicator that fluoresced at 254 nm (Merck 1.05554 silica plates, supplier as above). Following chromatography, the chromatographic sheets were first examined under long and short wave UV and any fluorescent bands marked. Next, plates with fluorescent indicator were examined under short wave UV, where UV-absorbing bands became visible as dark (non-fluorescing) bands and were marked. Plates were then exposed to iodine vapour (Sigma Aldrich Pty Ltd, Castle Hill, New South Wales) by placing a few crystals of iodine in a sealed jar with the plate for 15 min. Alternatively, the chromatographic sheet was swabbed with methanol/sulphuric acid (4:1) and charred at 100 ºC for 30 min. These various methods of visualisation were used to guide removal of bands from PLC plates, based on the assumption that the ratio of chromatographic mobility relative to the solvent front (R_f value) would be the same on the PLC plate as it was on the TLC sheet.

Based on the visualisation of various bands, segments were removed from the PLC plate. The silica was removed from the glass plate and placed in 50 ml centrifuge tubes (Sarstedt Australia Pty Ltd, Ingle Farm, South Australia), to which 20 ml of methanol was added. Tubes were mixed thoroughly by hand for 2 min and then centrifuged at 3,000 *g* (Ependorf, Crown Scientific, Acacia Ridge, Queensland). The supernatant was collected, and taken to dryness and stored as above (Section 8.2.2).

Further separation of the haemolytic component(s) in extract A and extract B (from Section 8.2.2) was also attempted using normal phase and reverse phase separatory columns respectively. A normal phase column (Sep-Pak® silica cartridge, Waters Australia, Rydalmere, New South Wales) was primed with 4 ml of hexane. Extract A was redissolved in hexane, and 2 ml (the equivalent of 9 g of culture material) was subsequently added to the primed column. The column was eluted with one 4 ml wash of hexane, followed by two 4 ml washes of hexane/dichloromethane (75:25) and three 4 ml washes of hexane/dichloromethane (50:50). The resulting six fractions were taken to dryness at 30 ºC under a stream of nitrogen, and stored at -20 ºC until required.

The reverse phase columns (C-18 column, 6.0 ml, Alltech Associates Pty Ltd, Baulkham Hills, New South Wales) were primed with methanol/water (50:50). Extract B was redissolved in ethanol/water (50:50) and 2 ml (the equivalent of 9 g of culture material) was added to the primed column. The column was eluted with two 6 ml washes of methanol/water (50:50), followed by two washes of 75:25 methanol/water, and finally two washes of 100% methanol. The resulting six fractions were taken to dryness at 30 ºC under a stream of nitrogen, and stored at -20 ºC until required.

All fractions were redissolved in methanol and diluted appropriately for use in the haemolytic assay. When assaying separate fractions of one fungal extract, the amount of culture material in each tube was calculated on the assumption that all of the active component(s) went into a single fraction.

8.2.4 Testing for the presence of haemolytic activity in sago starch using hexane extraction and quantitative assay

Twelve sago starch samples were tested for haemolytic activity using the quantitative haemolytic assay. Starch samples were extracted as described for fungal cultures (Section 8.2.1) and the resulting hexane-rich extracts were tested for haemolytic activity using the previously described assay (Section 7.2.2). Due to the inconclusive results of haemolytic screening of sago starch on BA (Section 7.3.1.2), the same 12 samples, plus one additional sample implicated in an outbreak of SHD (W1105-01), were analysed using the quantitative assay.

Sago starch extract was resuspended and dispensed prior to the haemolytic assay as previously described for culture material (Section 8.2.1). Minor modifications were made on account of the expected low level of haemolytic activity. First, the equivalent of 1.0 g and 0.25 g of material was used, as opposed to 0.25 g and 0.10 g used for culture material. Secondly, the assay was conducted over 6 hours rather than 4 hours.

8.3 Results

8.3.1 Extraction of the haemolytic component produced by filamentous fungi

Figures 8.01 to 8.10 show comparisons between the haemolytic activity of hexane-rich extracts and dichloromethane-rich extracts for the various controls, and the seven fungal strains isolated from sago starch.

Figure 8.01 demonstrates that wheat used as the culture medium had very little if any haemolytic activity. Low levels of haemolytic activity were evident in the dichloromethane-rich extract in which 0.25 g of wheat was assayed, reaching approximately 40% after 4 hours of incubation. Both hexane-rich extracts and the dichloromethane-rich extract with 0.10 g of material were all non-haemolytic over 4 hours, producing very similar haemolysis in the assay as negative controls.

The positive control *S. chartarum* produced strong haemolytic activity, with 0.1 g culture equivalent in the hexane-rich fraction reaching 80% after 4 hours (Figure 8.02). The dichloromethane-rich fraction was less than half as active, reaching 80% after 4 hours with 0.25 g of culture equivalent. The *T. reesei* positive control produced a similar pattern and level of activity, also greater in the hexane-rich fraction (Figure 8.03).

Two *P. steckii* isolates from sago produced at least twice the haemolytic activity of the positive control isolates (Figures 8.04 and 8.05). Hexane-rich extracts of isolates W1-1101 and W1-1301, containing 0.1 g culture equivalent, reached 100% activity after only 1 hour and dichloromethane-rich extracts of 0.1 g equivalent exceeded 80% haemolysis after 4 hours.

Strong haemolytic activity was also observed in the other fungal strains tested (Figures 8.06 to 8.10), with all but *T. virens* W4-0119 causing approximately 80% haemolysis or higher within 30 min of incubation with erythrocytes. Hexane fractions were more rapidly haemolytic than dichloromethane fractions except in *P. steckii* S2-1305 (Figure 8.06) and *F. semitectum* S2-0207 (Figure 8.09). In the case of these two isolates there was very little difference in the haemolytic activity of hexane-rich and dichloromethane-rich extracts for 0.25 g culture equivalent, but there was more activity in the 0.1 g equivalent dichloromethane-rich extract than the corresponding hexane-rich extract.

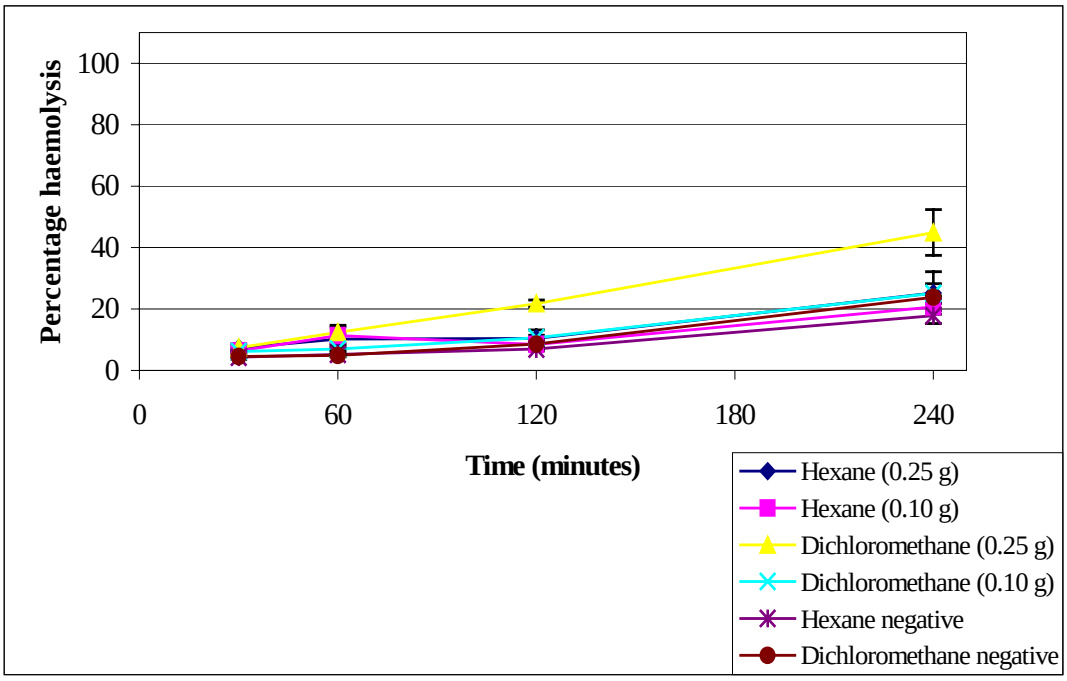


Figure 8.01: Haemolytic activity of hexane-rich and dichloromethane-rich extracts of sterile wheat (weight extracted in parenthesis), and extraction solvents alone (negative controls).

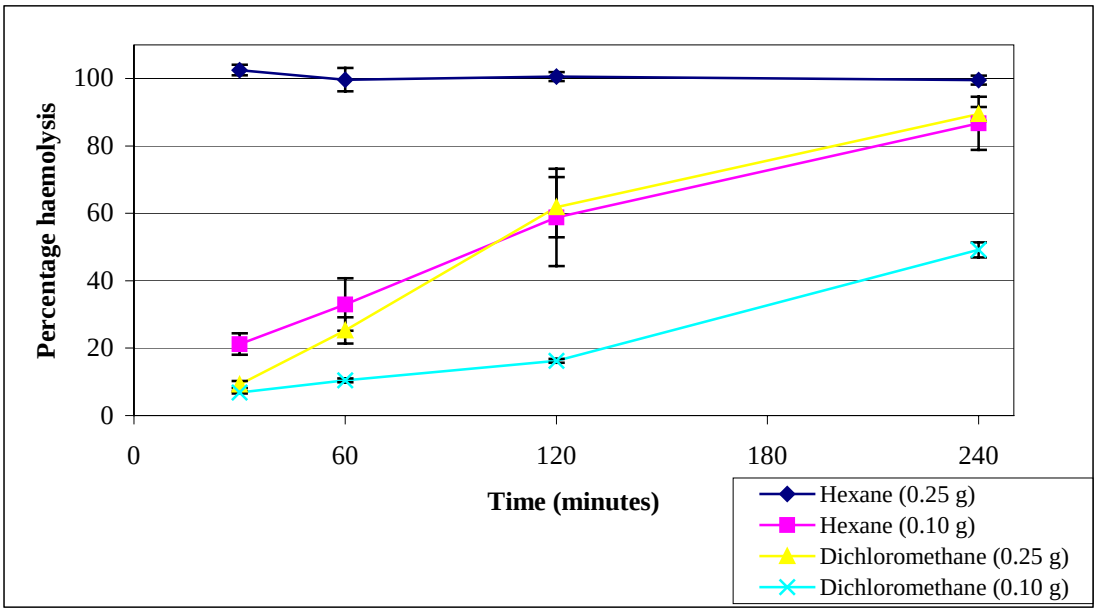


Figure 8.02: Haemolytic activity of hexane-rich and dichloromethane-rich extracts of *S. chartarum* ATCC 9182 culture (positive control organism).

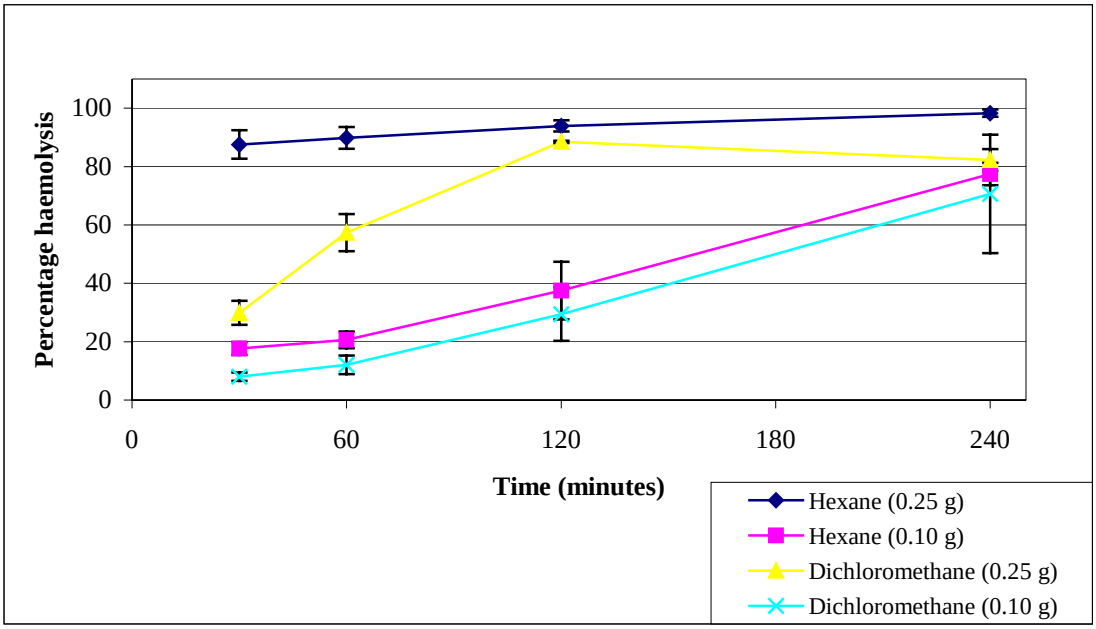


Figure 8.03: Haemolytic activity of hexane-rich and dichloromethane-rich extracts of *T. reesei* ATCC 26921 culture (positive control organism).

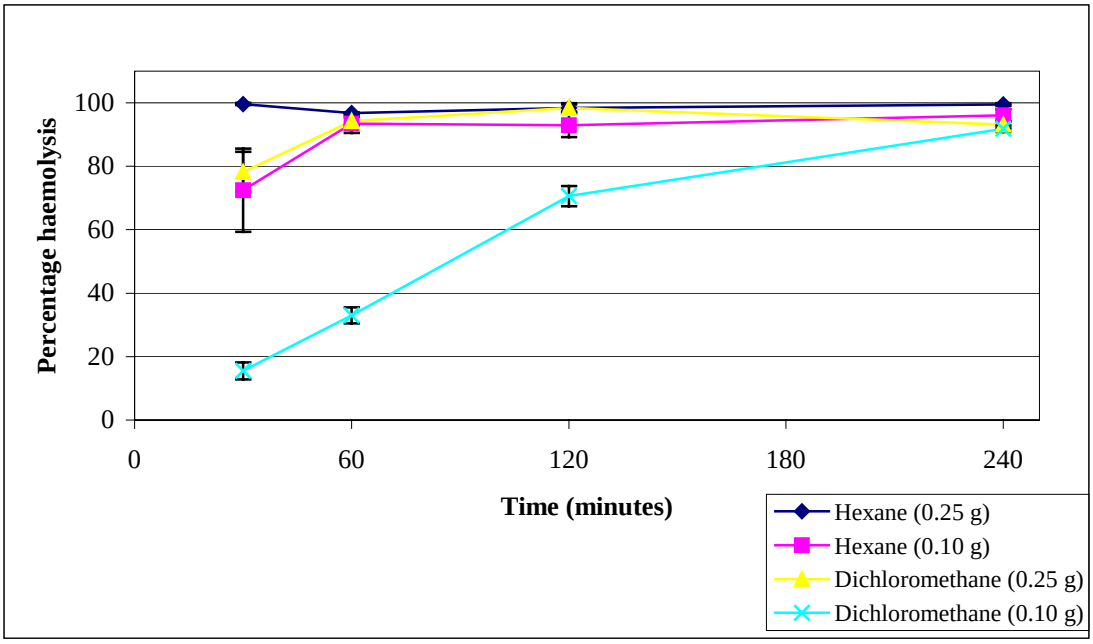


Figure 8.04: Haemolytic activity of hexane-rich and dichloromethane-rich extracts of *P. steckii* W1-1101.

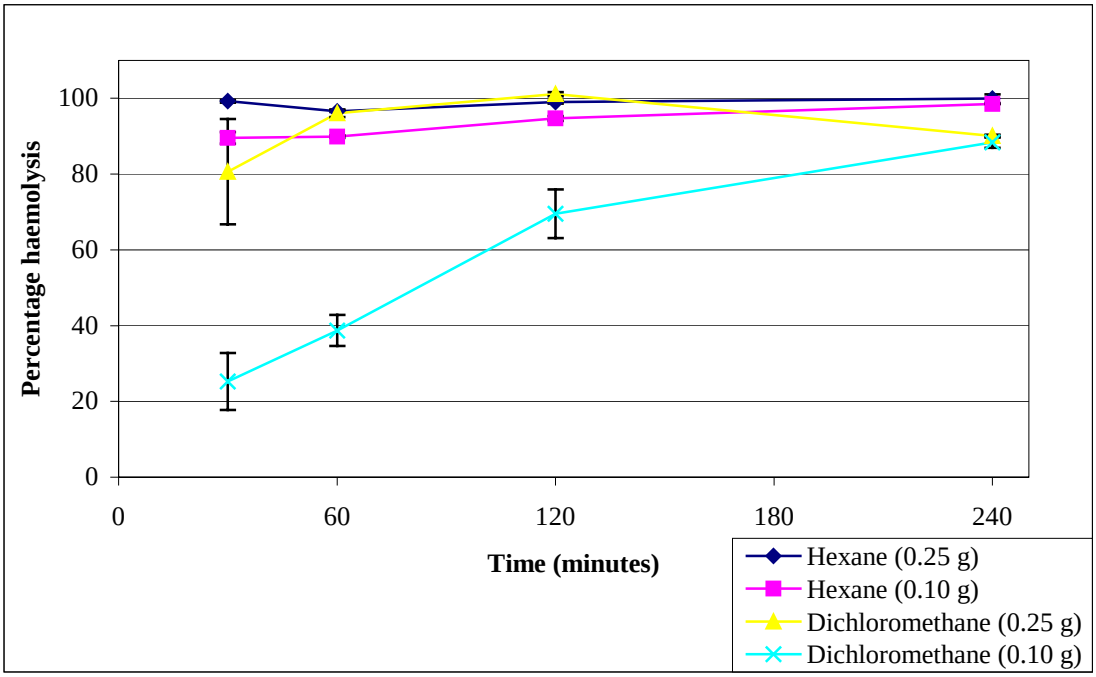


Figure 8.05: Haemolytic activity of hexane-rich and dichloromethane-rich extracts of *P. steckii* W1-1301.

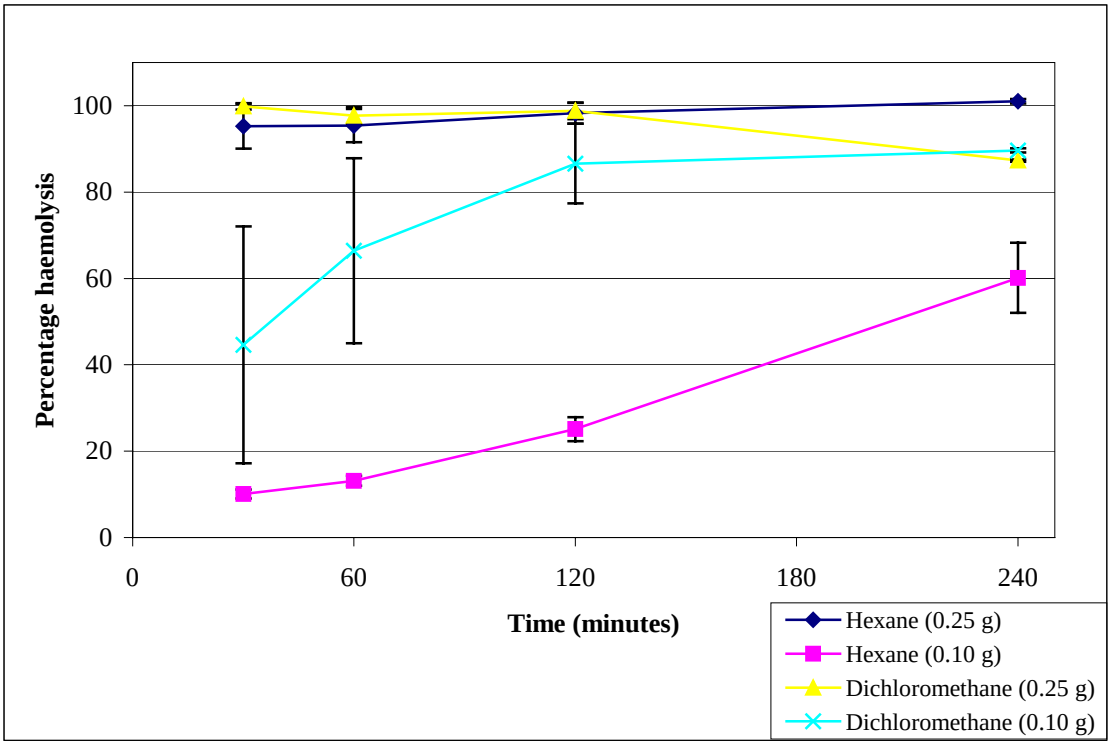


Figure 8.06: Haemolytic activity of hexane-rich and dichloromethane-rich extracts of *P. steckii* S2-1305.

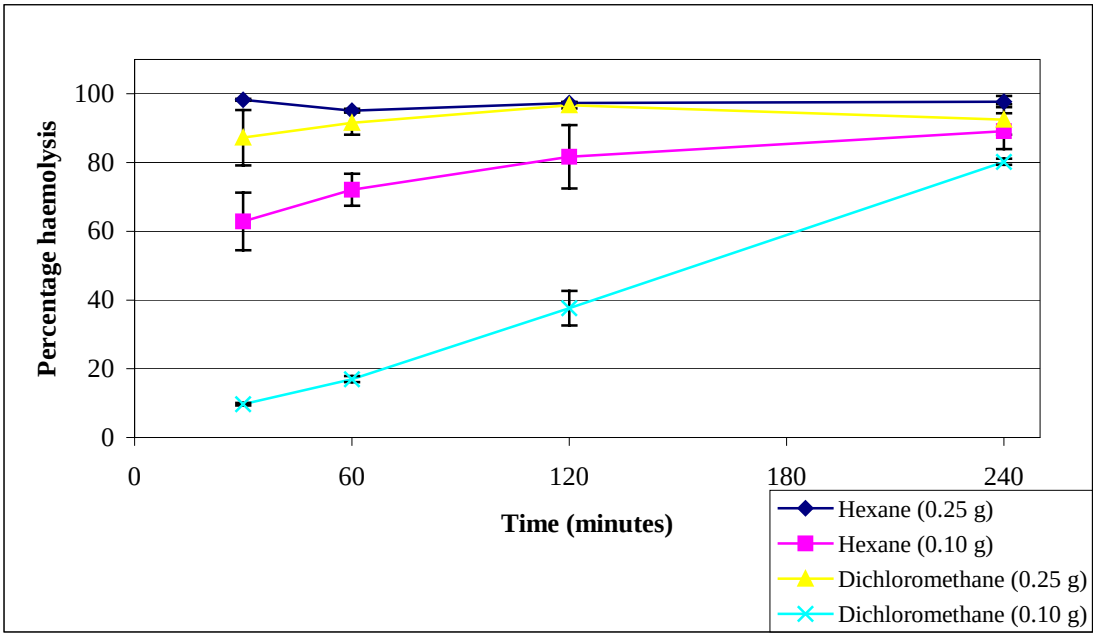


Figure 8.07: Haemolytic activity of hexane-rich and dichloromethane-rich extracts of *P. brevicompactum* S1-0201.

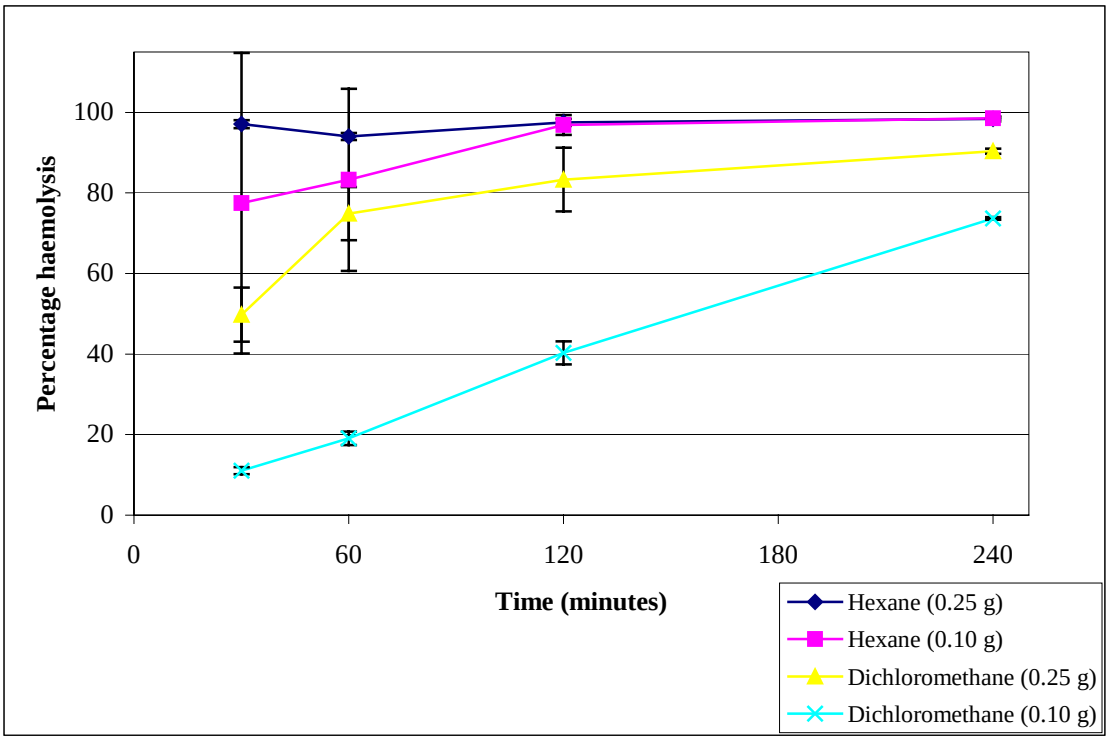


Figure 8.08: Haemolytic activity of hexane-rich and dichloromethane-rich extracts of *A. flavipes* S2-0207.

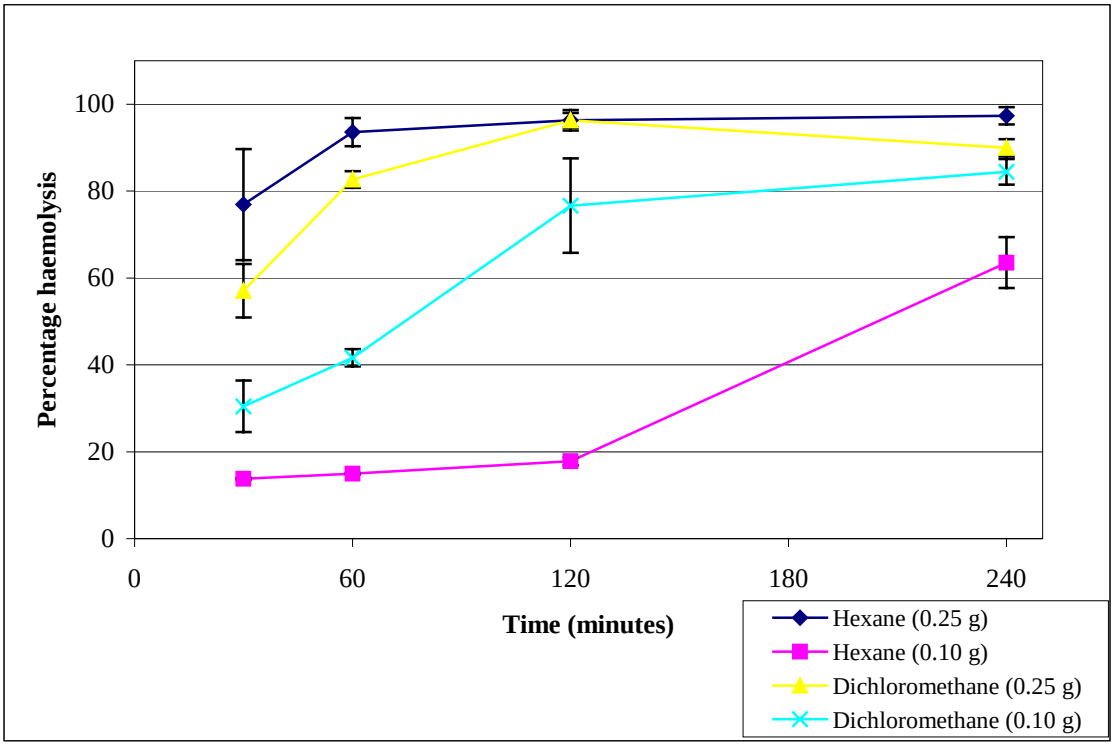


Figure 8.09: Haemolytic activity of hexane-rich and dichloromethane-rich extracts of *F. semitectum* S2-0207.

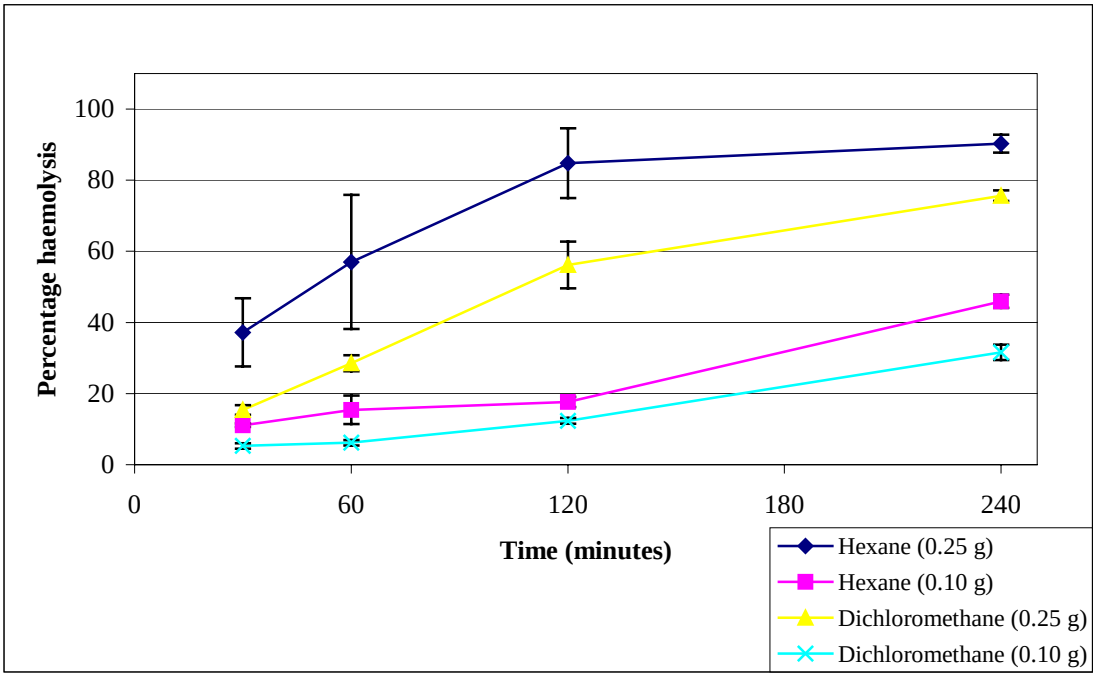


Figure 8.10: Haemolytic activity of hexane-rich and dichloromethane-rich extracts of *T. virens* W4-0119, from implicated sago.

Direct comparisons of haemolytic activity of TDS extracts (Section 7.3.3.3) with hexane and dichloromethane extracts were not possible, as different quantities of culture material were used in the TDS assays. However, extrapolation suggested that for equivalent quantities of culture material, hexane and dichloromethane extracts had comparable haemolytic activity to the TDS extracts. Further studies were conducted using solvent extraction rather than TDS in an attempt to garner more information about the chemical properties of the haemolytic compound(s). Moreover, the evaporation of solvents such as hexane and dichloromethane can be carried out with relative ease compared to TDS, and the dried extract resuspended for use in the haemolytic assay.

8.3.2 Separation of haemolytic components of fungal culture extracts using preparative layer chromatography

Due to their greater haemolytic activity in most isolates, hexane-rich extracts were selected for further partitioning. Figures 8.11 to 8.20 illustrate the haemolytic activity of the two fractions of the hexane extract separated using PLC. Considerable haemolytic activity was observed in the lower fraction of the sterile wheat (negative control) (Figure 8.11). The implications of this finding will be discussed in Section 8.4.

In all isolates, there was substantially more haemolytic activity in the upper section (above citrinin) of the PLC plate than in the lower section (citrinin and components below) (Figures 8.12 to 8.20). The isolates with most activity in the previous experiments (Section 8.3.1), *P. steckii* W1-1101 and *P. steckii* W1-1301, were also highly active in this experiment. On a weight basis, there appeared less activity in each of these two fractions than in the parent hexane-rich extracts, which could indicate either splitting of activity between upper and lower sections, or simply failure to recover all activity from the PLC plates. In support of the former possibility was the activity in the lower fractions of these two *P. steckii* isolates. The *S. chartarum* control also had some activity in the lower band.

The haemolytic activity of *T. virens* W4-0119 was notably slower to express than for the other fungal strains isolated from sago starch, taking 4 hours to reach

approximately 80% haemolysis. All other strains originating from sago had reached this level of haemolysis after 2 hours. However, the haemolytic activity of *T. virens* W4-0119 was still comparable to the positive control strain of the same genus, *T. reesei* ATCC 26921.

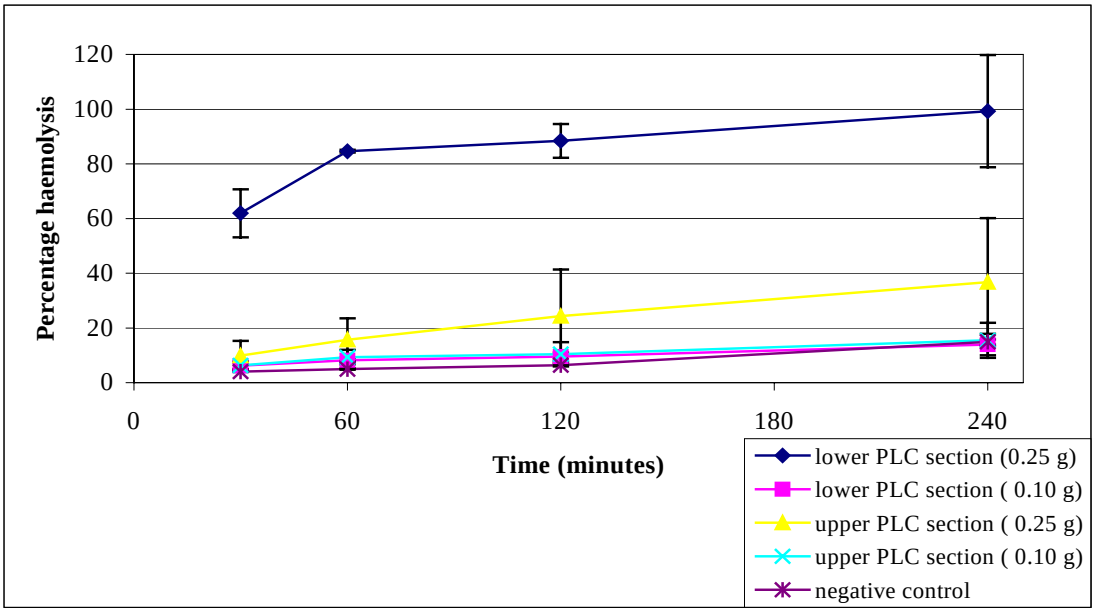


Figure 8.11: Haemolytic activity in the hexane extract of uninoculated wheat after separation into two fractions on a PLC plate: lower section from origin up to and including citrinin (R_f 0–0.55); upper section above this (R_f 0.55–1.0).

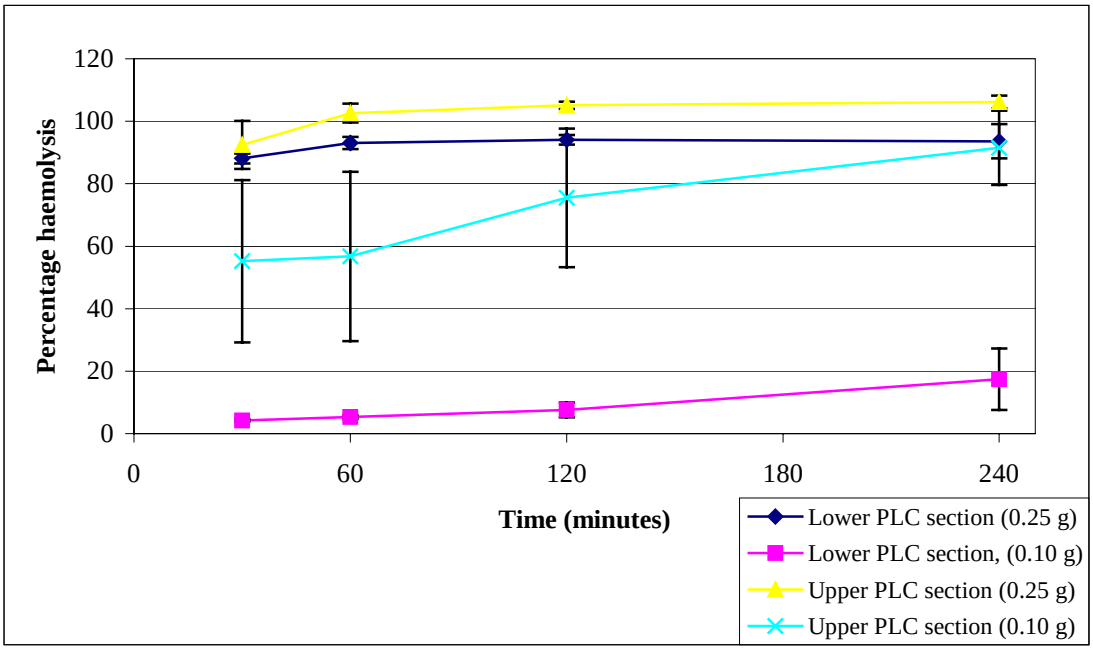


Figure 8.12: Haemolytic activity in the hexane extract of *S. chartarum* ATCC 9182 after separation into two fractions on a PLC plate: lower section from origin up to and including citrinin (R_f 0–0.55); upper section above this (R_f 0.55–1.0).

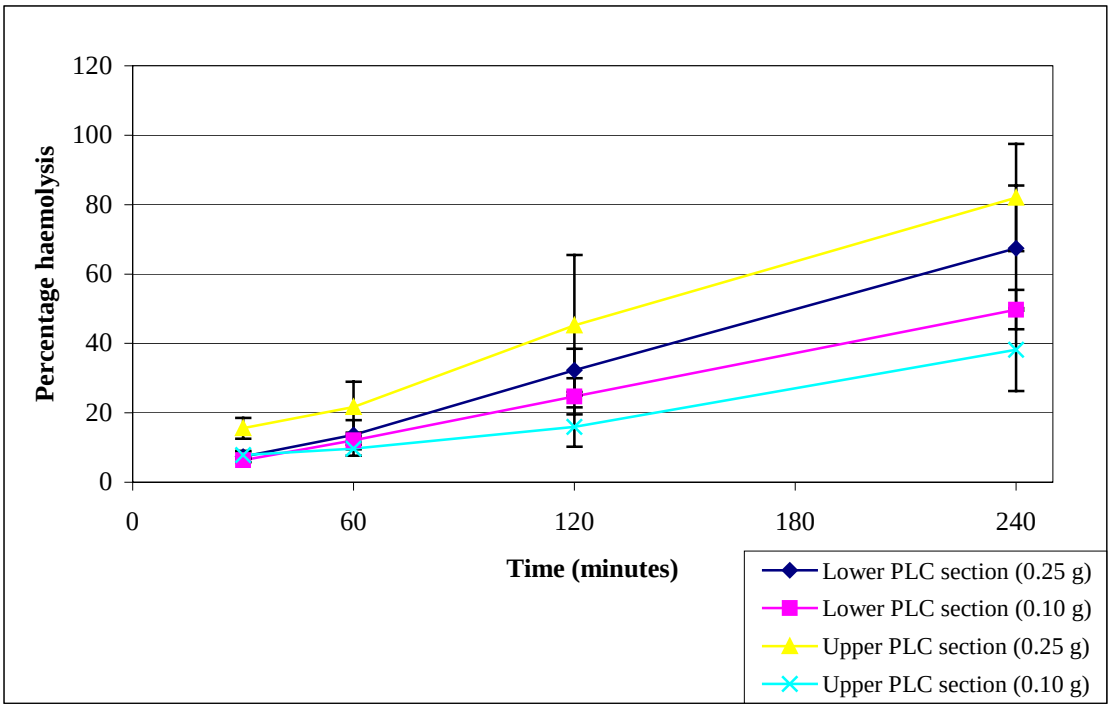


Figure 8.13: Haemolytic activity in the hexane extract of *T. reesei* ATCC 26921 after separation into two fractions on a PLC plate: lower section from origin up to and including citrinin (R_f 0–0.55); upper section above this (R_f 0.55–1.0).

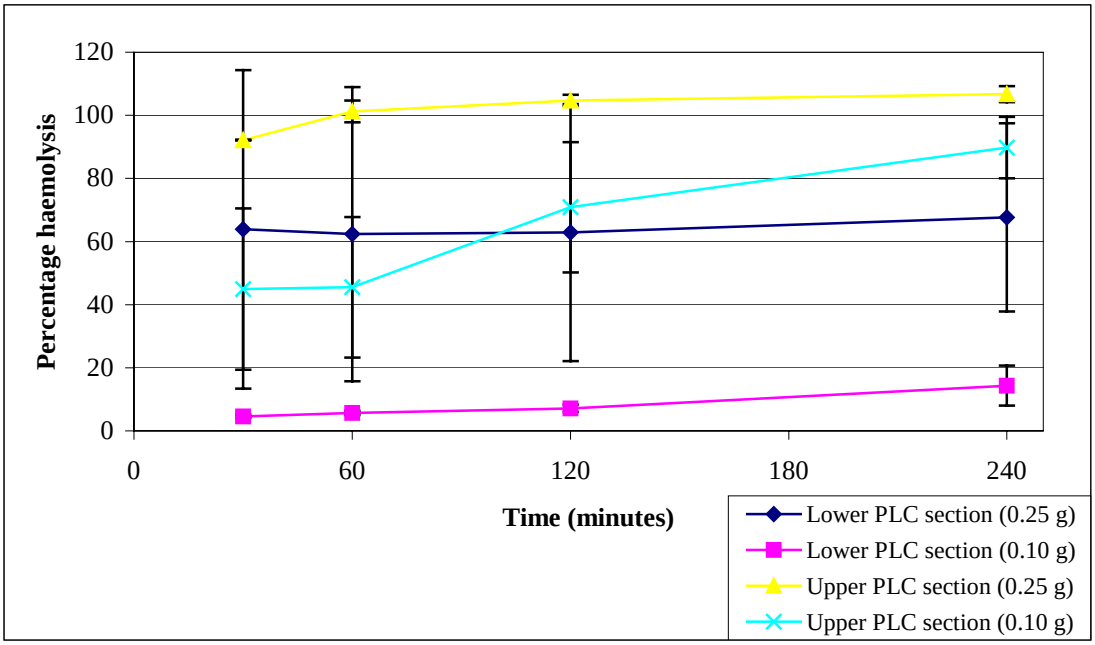


Figure 8.14: Haemolytic activity in the hexane extract of *P. steckii* W1-1101 after separation into two fractions on a PLC plate: lower section from origin up to and including citrinin (R_f 0–0.55); upper section above this (R_f 0.55–1.0).

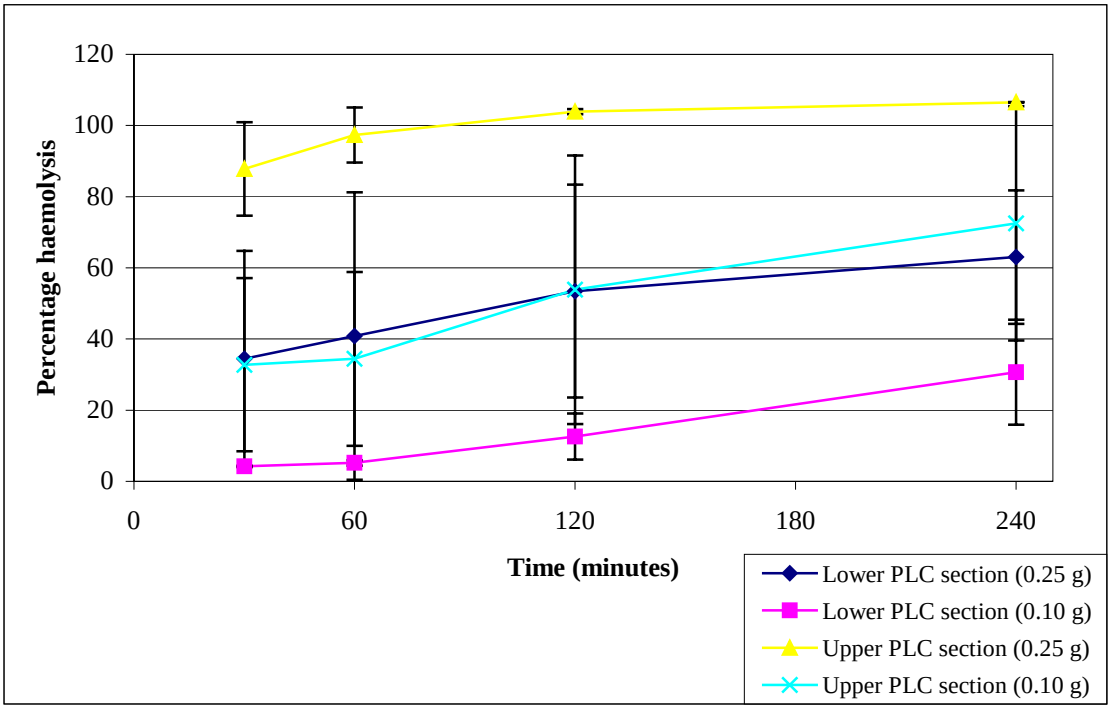


Figure 8.15: Haemolytic activity in the hexane extract of *P. steckii* W1-1301 after separation into two fractions on a PLC plate: lower section from origin up to and including citrinin (R_f 0–0.55); upper section above this (R_f 0.55–1.0).

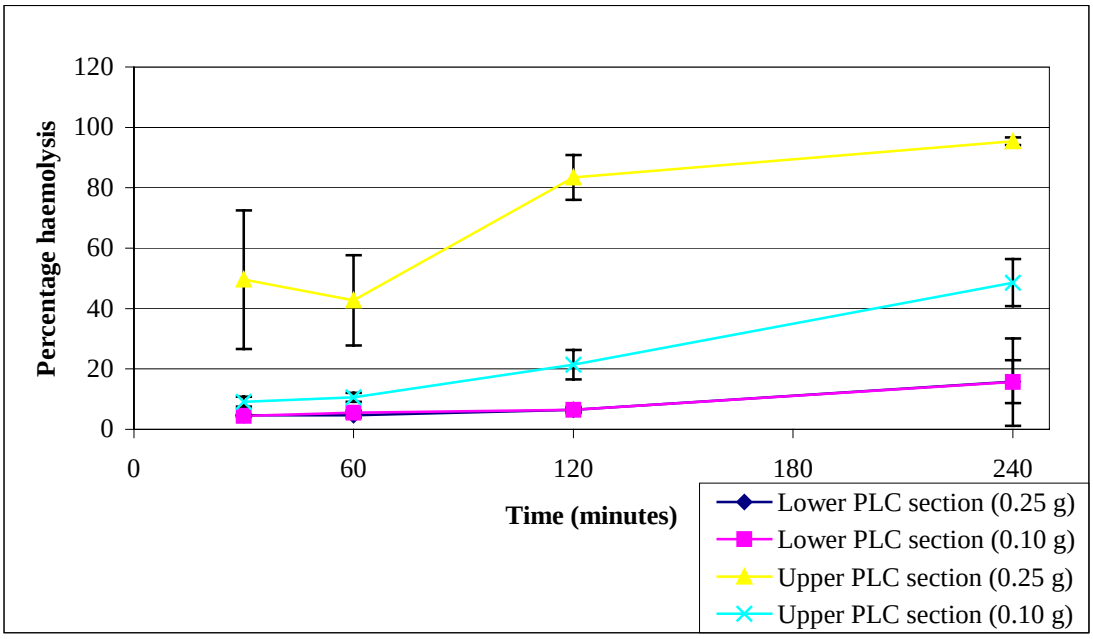


Figure 8.16: Haemolytic activity in the hexane extract of *P. steckii* S2-1305 after separation into two fractions on a PLC plate: lower section from origin up to and including citrinin (R_f 0–0.55); upper section above this (R_f 0.55–1.0).

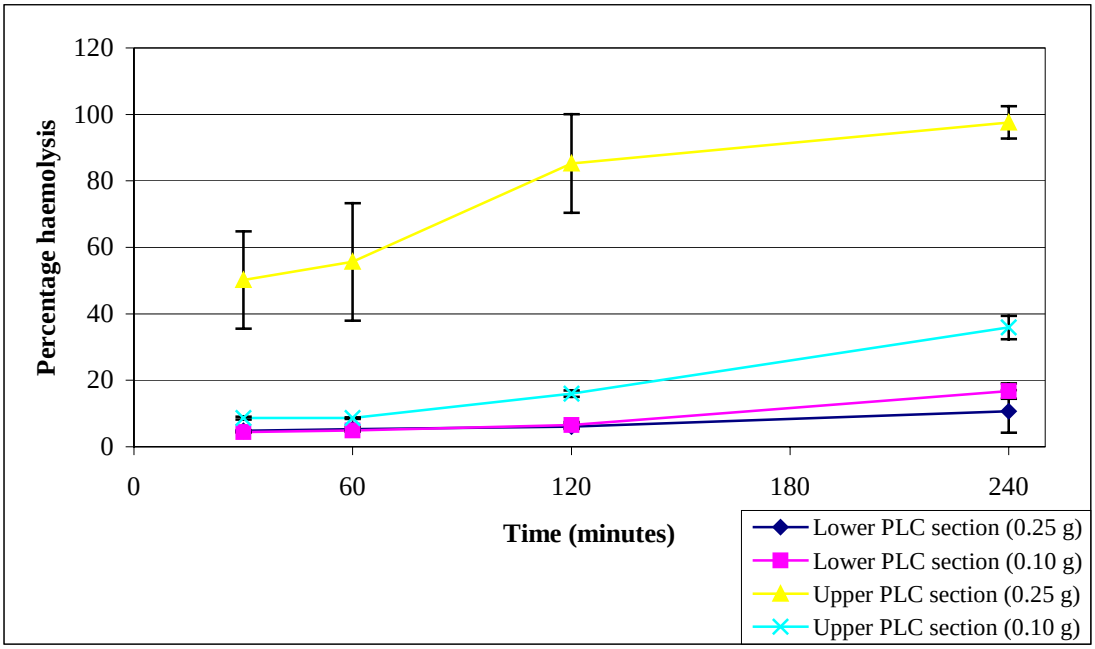


Figure 8.17: Haemolytic activity in the hexane extract of *P. brevicompactum* S1-1201 after separation into two fractions on a PLC plate: lower section from origin up to and including citrinin (R_f 0–0.55); upper section above this (R_f 0.55–1.0).

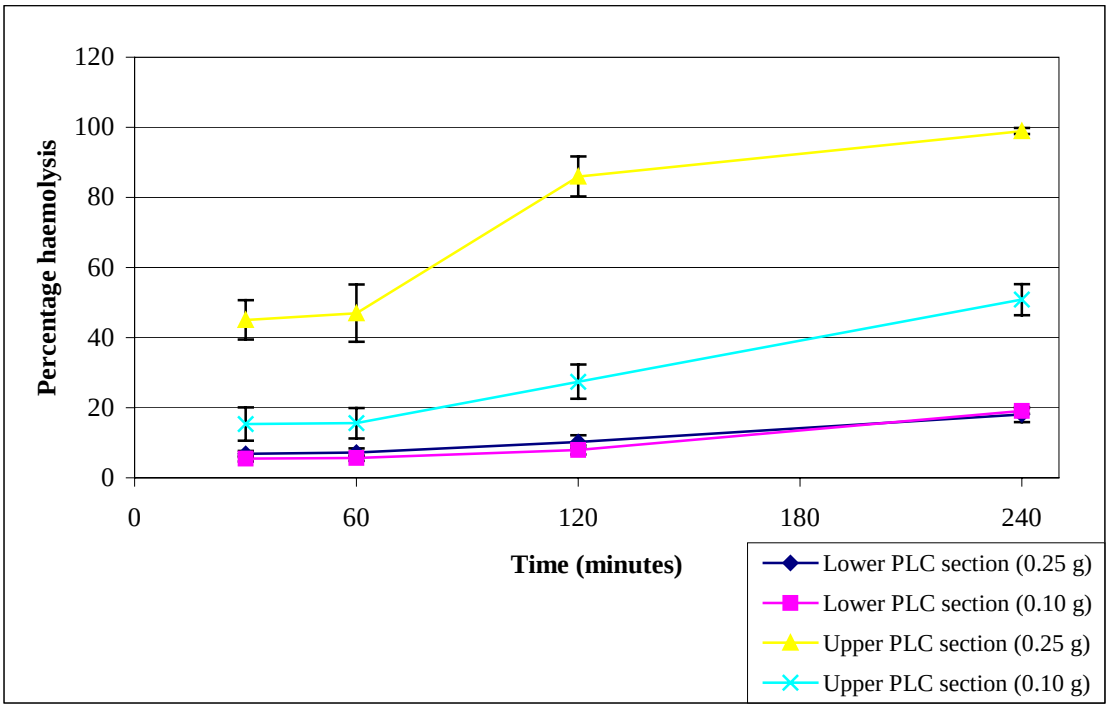


Figure 8.18 Haemolytic activity in the hexane extract of *A. flavipes* S2-0207 after separation into two fractions on a PLC plate: lower section from origin up to and including citrinin (R_f 0–0.55); upper section above this (R_f 0.55–1.0).

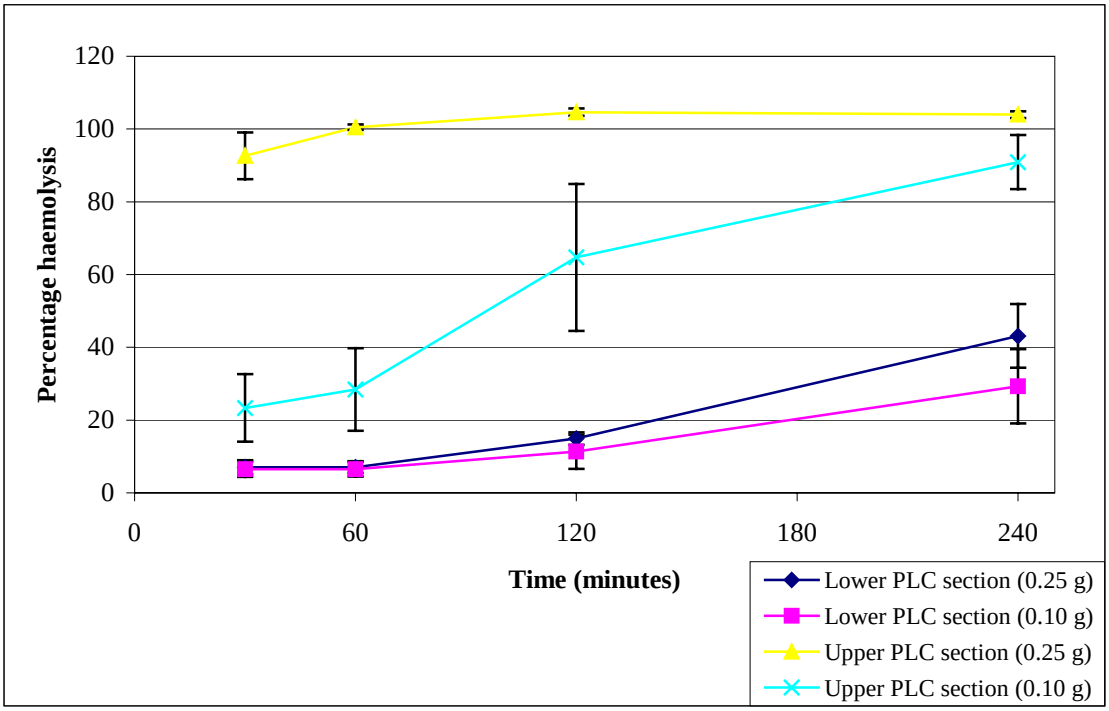


Figure 8.19: Haemolytic activity in the hexane extract of *F. semitectum* S1-0406 after separation into two fractions on a PLC plate: lower section from origin up to and including citrinin (R_f 0–0.55); upper section above this (R_f 0.55–1.0).

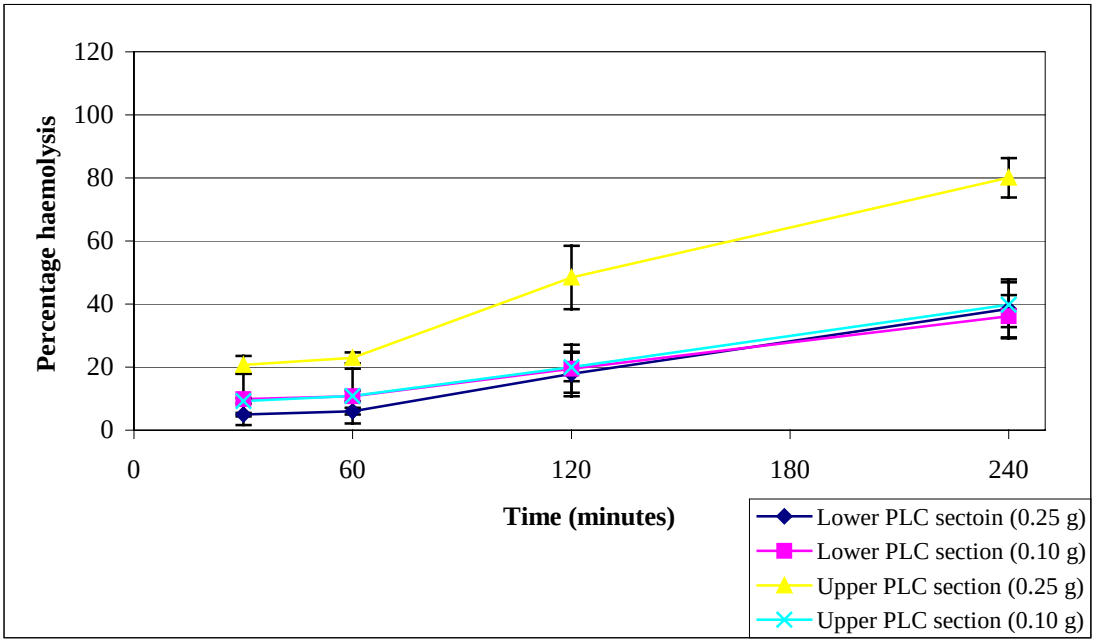


Figure 8.20: Haemolytic activity in the hexane extract of *T. virens* W4-0119 after separation into two fractions on a PLC plate: lower section from origin up to and including citrinin (R_f 0–0.55); upper section above this (R_f 0.55–1.0).

8.3.3 Further separation of haemolytic component(s) of *Penicillium steckii* W1-1301

Penicillium steckii W1-1301 was selected for further separation of haemolytic component(s). Selection of this isolate was on the basis that it represented a species commonly isolated from sago starch, and the majority of the haemolytic activity was in the hexane-rich fraction. Furthermore, when standard error was taken into account, there was clearly greater haemolytic activity in 0.25 g of the upper PLC section than in the corresponding lower PLC section (Figure 8.15).

A separate *P. steckii* W1-1301 extract to that used in Section 8.3.2 was used in this study. Culture, extraction and storage conditions were identical to those used for extracts in the previous study (see Chapter 7 and Section 8.2.1).

Following development of the PLC plate in TEF, three clear fluorescent yellow-green bands were visible under long wave UV light (Figure 8.21), along with one additional pale blue band too faint to be visualised in a photograph (R_f = 0.85). The large yellow fluorescent band within segment one was citrinin, with the R_f matching that of citrinin run in TEF on TLC (Chapter 6). It is noted that citrinin was far more soluble in the dichloromethane-rich fraction than in the hexane-rich fraction, but there was sufficient in the hexane-rich extract to use as a marker. Segment 1 encompassed all of the citrinin and a single fluorescent yellow-green band above it. Segment 2 consisted of a second fluorescent yellow-green band approximately 20 mm wide, which migrated 112 to 132 mm from the origin (R_f = 0.70 to 0.82). Segment 3 consisted of the area immediately above segment 2 to the solvent front (R_f = 0.82 to 1.0), including the pale blue band with R_f = 0.85. The silica in each of the segments was removed and extracted as described in Section 8.2.2.

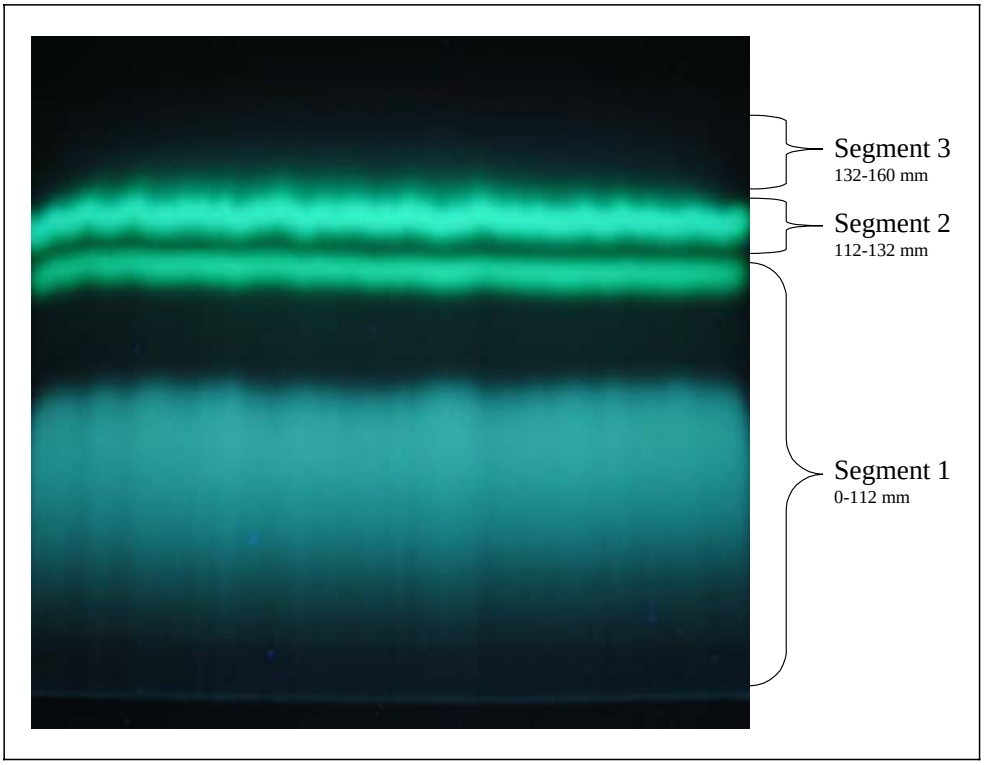


Figure 8.21: Preparatory layer chromatography plate of *P. steckii* W1-1301 hexane fraction photographed under long wave UV light. Three segments, as labelled to the right of the plate, were removed from the plate for analysis of haemolytic activity. The large area of fluorescence within segment 1 is citrinin.

Following the extraction of each segment of the PLC plate, extracts were assayed for haemolytic activity (Figure 8.22). More pronounced and rapid haemolytic activity was observed in segments 2 and 3 than in segment 1. The haemolytic activity was very similar in segments 2 and 3, with all of the comparable tubes having similar haemolysis curves. Tubes containing the equivalent of 0.45 g of culture material caused 80% haemolysis within 1 hour. The degree and rate of haemolysis decreased with decreasing concentration of culture material, to the point where 0.02 g of culture equivalent material was only slightly haemolytic after 4 hours. Nonetheless, even 0.04 g of culture equivalent produced in excess of 70% haemolysis after 4 hours incubation.

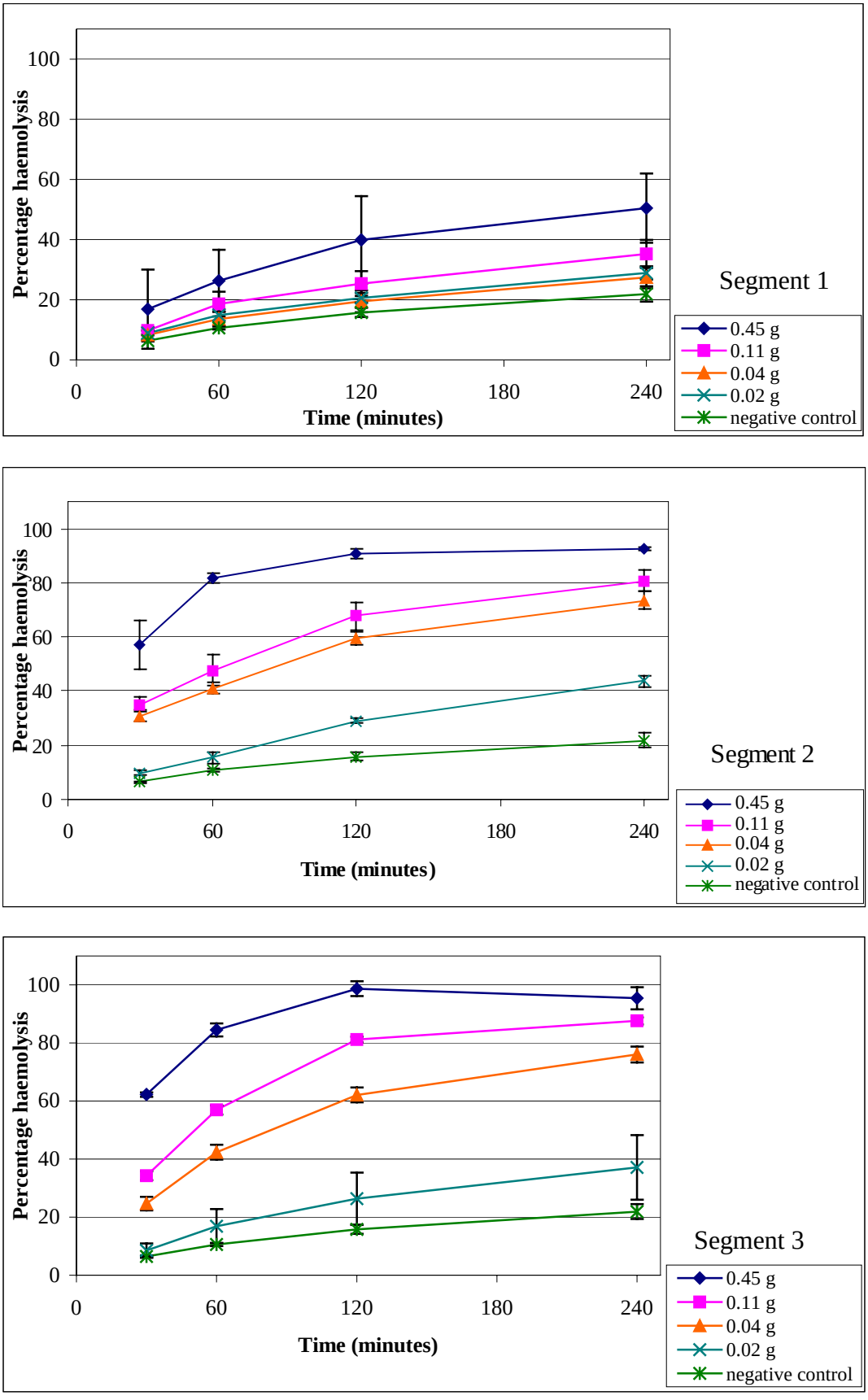


Figure 8.22: Graphs showing the haemolytic activity of the three segments from the PLC plate of *P. steckii* W1-1301.

The TLC plates incorporating fluorescent indicator showed only a single definite UV-absorbing band, which was citrinin. The use of iodine to visualise non-fluorescent compounds on TLC run in parallel with PLC revealed some material that was otherwise: invisible under ambient light; non-fluorescent under long-wave UV light; and non-absorbing (or absorbing very little) of UV light on TLC plates incorporating fluorescent indicator. Prior to exposure to iodine, the TLC plate was observed under UV light and visible bands were marked with pencil. Figure 8.23 shows where the most concentrated band of citrinin was (marked 'cit'), as well as the two clearly visible fluorescent bands above citrinin (marked 'y' and 'x'). Encompassing the upper margin of 'fluorescent band x', and extending further from the origin was a band that was clearly visible following treatment with iodine. The citrinin band was the only other definite band visible after iodine treatment.

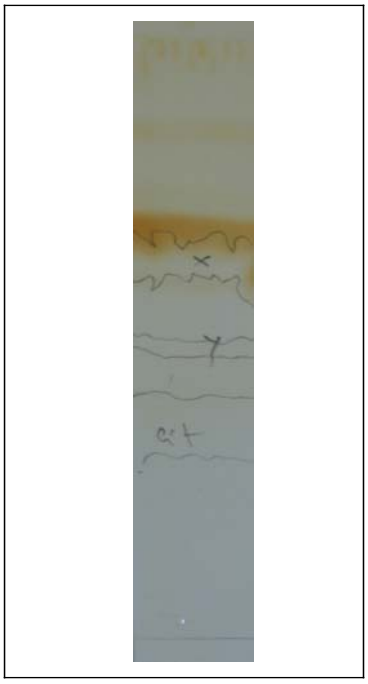


Figure 8.23: *P. steckii* W1-1301 TLC plate exposed to iodine. The markings in pencil illustrate where citrinin was most concentrated (cit), and where the two highly visible fluorescent bands were ('y' and 'x'). The iodine-stained band just above but overlapping fluorescent band 'x' appears to be associated with the main haemolytic agent.

Microfine silica separatory columns were used with only limited success for *P. steckii* W1-1301. Use of the normal phase sep-pak resulted in the separation of most of the haemolytic activity into fraction 3 (the second 75:25 hexane/dichloromethane wash), fraction 4 (the first 50:50 hexane/dichloromethane wash) and fraction 5 (the second 50:50 hexane/dichloromethane wash). There was little difference in haemolytic activity between these three fractions (data not shown). Using reverse phase separatory columns, the vast majority of haemolytic activity came out in fraction 5 (the first 100% methanol wash), suggesting very non-polar behaviour and strong hexane affinity. This approach was not pursued as little separation was achieved and more promising results were obtained using PLC. However, it is possible that it could work with further refinement and different non-polar solvents.

8.3.4 Testing for the presence of haemolytic activity in sago starch using hexane extraction and quantitative assay

Figure 8.24 illustrates the haemolytic activity of four sago samples, two of which (W0605-01 and W1105-01) were associated with outbreaks of SHD. In general, low levels of haemolytic activity were detected in sago starch. The two sago starch samples associated with SHD outbreaks were less haemolytic than the two other samples shown, although standard errors were very high. The remaining nine samples tested had levels of haemolytic activity lower than that of sago sample W0303-02 (data not shown). Using Pearson's correlation coefficient (two sided) (SPSS Australasia Pty Ltd, North Sydney, New South Wales) there was no correlation between age of sample and level of haemolytic activity ($n = 12$, $r = 0.671$, $P = 0.137$).

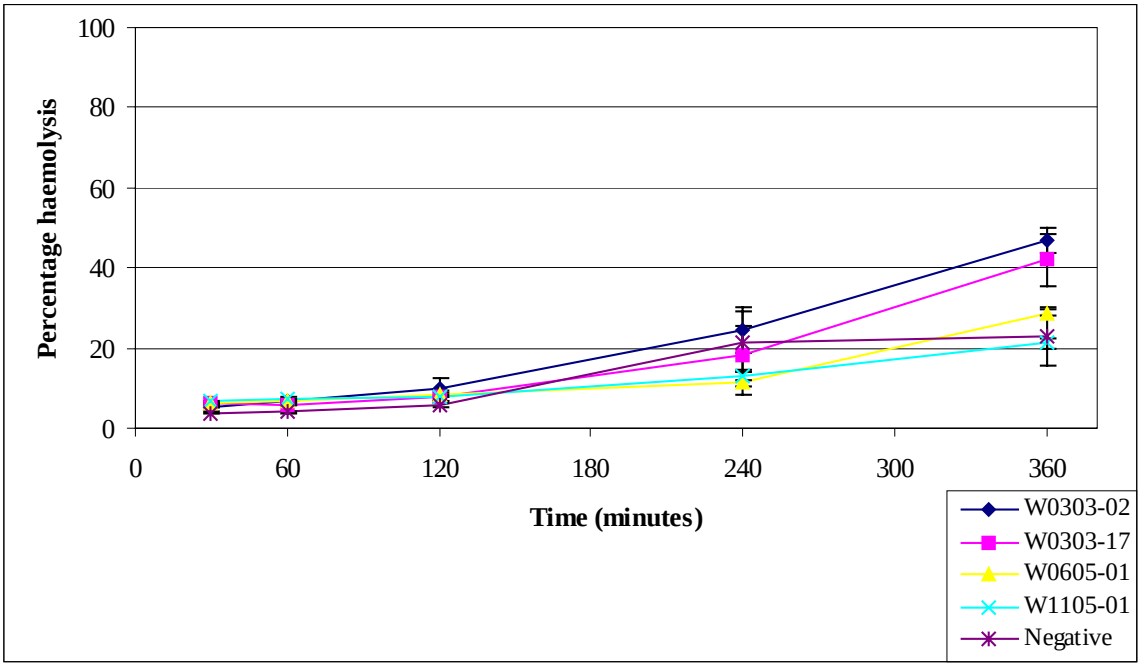


Figure 8.24: Haemolytic activity of hexane-rich extracts of sago starch (1.0 g equivalent).

8.4 Discussion

Although only preliminary fractionation of extracts has been conducted, this study provides an insight in to the nature of the haemolytic compound(s) produced by selected species of filamentous fungi, namely *P. steckii*, *P. brevicompactum*, *A. flavipes*, *F. semitectum* and *T. virens*. First, the results from the previous chapter suggest that the haemolytic agent can be extracted into TDS (Figure 7.07), and thus can be solubilized in aqueous media, or at least induced to form a colloidal suspension in aqueous media. Many natural products with a low solubility in water are able to form colloidal suspensions in aqueous media (Silberberg, 2000).

Secondly, the haemolytic compound(s) readily extract(s) into mildly acidified acetonitrile/water (and alcohol), but preferentially partitions from acidified acetonitrile/water (1:1) into hexane. This is unusual compared to most mycotoxins, but the behaviour should be interpreted with caution, as there are exceptions to the following generalities. Nonetheless, it suggests that the haemolytic component is characteristic of substances such as long chain aliphatic or aromatic compounds that are either primarily hydrocarbons or that lack reactive functional groups capable of

proton donation (Morrison and Boyd, 1992). Such substances include steroids and terpenes, oils, fats, waxes and glycerides, as well as many chlorinated hydrocarbons. In contrast, the behaviour is not characteristic of polar compounds such as proteins and highly hydroxylated compounds such as carbohydrates and trichothecene mycotoxins. Alkaline nitrogen-containing compounds (alkaloids) are also highly polar in dilute aqueous acid, and would not partition into hexane. Acidic compounds (organic acids and phenols) like citrinin are dissociated in aqueous acid, but are unlikely to partition into hexane unless the acid moiety is attached to a large, otherwise non-polar molecule.

It should be noted that partitioning between different solvent mixtures is always competitive, with the ratio present in the preferred solvent after a single partition commonly ranging from 60 to 90%. This was demonstrated in this study by the presence of some citrinin in the hexane-rich extract, although most remained in the acetonitrile:water.

Thirdly, the observation that the haemolytic component(s) of *P. steckii,* (PS-1), do(es) not absorb sufficient UV light to quench the fluorescent indicator used in TLC plates suggests one of two things. It may be that PS-1 is present in only very small amounts, or it may be that it does not have a conjugated unsaturated bond system (Silverstein *et al.*, 2005). As a definite band was observed after iodine treatment, the later explanation appears most likely.

The haemolytic activity of an extract of uninoculated wheat, used as a control in this study, warrants discussion. Figure 8.01 shows the haemolytic activity of hexane and dichloromethane extracts of wheat. In general, extracts were non-haemolytic, with the exception of the 0.25 g dichloromethane extract. This extract caused approximately 40% haemolysis after 4 hours, and may have been due to residual dichloromethane in the wheat extract. Many of the 0.25 g dichloromethane extracts were haemolytic within the first 30 min of incubation. Over the first 60 min, the negative control for dichloromethane was similar to all other controls. Thus, rapid haemolysis in fungal dichloromethane extracts is likely to be indicative of the true haemolytic ability of the fungus. To err on the side of caution, greater emphasis can be placed on the haemolytic activity of hexane fungal extracts. Throughout the

experiment the hexane extracts of sterile wheat were comparable to the negative controls (as defined in Section 8.2.1).

An anomaly also exists in the haemolytic activity of the lower section of the hexane extract of uninoculated wheat separated using PLC (Figure 8.11). As the hexane fraction had previously been shown to be non-haemolytic (Figure 8.01), it is difficult to explain this result other than experimental error. Again, this has little bearing on subsequent results, as most of the haemolytic activity of fungal cultures was in the upper section of the PLC plates. The upper PLC section of the uninoculated wheat hexane extract had considerably lower levels of haemolytic activity, although 0.25 g of extract was slightly haemolytic, reaching approximately 40% over 4 hours.

With the above experimental constraints in mind, the results obtained in this study nonetheless demonstrate clear haemolytic activity in fungal extracts. This investigation lacked the time and resources to pursue the complete characterisation and identification of PS-1 and similar compounds in the other fungal isolates. However, it is possible to compare the features of PS-1 (and to a lesser degree extracts from other fungal isolates) with known haemolytic and/or haemorrhagic fungal metabolites.

Many of the haemolytic agents isolated from fungi are proteins and peptides. A haemolytic polypeptide of the peptaibol class, paracelsin, has been isolated from *T. reesei* ATCC 26921 (Bruckner *et al.*, 1984). Indeed, it is on the basis of this discovery that the organism was selected as a positive control in this study. Paracelsin has since been demonstrated to be a group of at least eight very similar peptaibols, each with a molecular mass of approximately 1,900 Da (Pocsfalvi *et al.*, 1997). Peptaibols are characterised by their short chain length, C-terminal alcohol residues and the presence of unusual amino acids (Whitmore and Wallace, 2004). Their amphipathic nature enables many of them to form ion channels in lipid bilayer membranes, resulting in antimicrobial activity (Chugh and Wallace, 2001). Various peptaibols are known to be haemolytic (Bruckner *et al.*, 1984; Jaworski *et al.*, 1999).

In comparison, stachylysin, the haemolytic compound isolated from *S. chartarum*, is a protein with a molecular weight of slightly less than 12,000 Da. Despite the

considerably larger size relative to peptaibols, there may be some similarity in haemolytic activity. Stachylysin is a highly polar protein that may be able to integrate into the erythrocyte membrane. Pores form in the centre of membrane depressions in erythrocytes treated with the haemolysin (Vesper *et al.*, 2001). Pore forming haemolysins are also common in bacteria (Section 2.6).

Another known haemolytic fungal metabolite, asp-hemolysin, is produced by *Aspergillus fumigatus*. It is also largely proteinaceous in nature with some carbohydrate present, and a molecular weight estimated as 30 kDa (Yokota *et al.*, 1977). Asp-hemolysin had a UV absorption spectrum with maximum of 280 nm. It completely lost haemolytic activity in 40 min at 50 ºC.

As is common with other peptides and proteins, the peptaibols, stachylsin and asp-hemolysin all have strong affinity with water:methanol, and were originally purified by gel filtration in these solvents on sephadex columns. This degree of affinity with water:methanol, and lack of affinity for hexane is characteristic of proteins and peptides, so it seems highly unlikely that any compounds in these classes would have the hexane-affinity displayed by PS-1. As is illustrated by asp-hemolysin, proteins also tend to be unstable in heat, whereas PS-1 appeared to maintain full activity during various processes of solvent extraction and heating to 100 °C for up to 30 min to remove solvents.

Wortmannin is a haemorrhagic mycotoxin that has been shown to cause haemoglobinuria in laboratory rats (Gunther *et al.*, 1989). It is a steroid-like compound produced by *P. wortmannii, P. funiculosum, Myrothecium roridum, F. oxysporum, F. sambucinum* and *F. avenaceum* (Mirocha and Abbas, 1989). It was reported by Abbas and Mirocha (1988) to be insoluble in water, yet sparingly soluble in hexane. They did report that after extraction from fungal cultures in ethyl acetate, it was dissolved in acetonitrile, and hexane was used to remove lipids, while wortmannin remained in the acetonitrile extract.

Wortmannin has a conjugated unsaturated bond system including carbonyl groups, with UV absorption maxima at 210, 254 and 292 nm (Abbas and Mirocha, 1988). In combination, these factors tend to rule out wortmannin as the identity of PS-1.

However, a less polar derivative of wortmannin is a possibility, and it would be constructive to assay wortmannin in the *Penicillium* species isolated from sago, in case it has some involvement in the SHD syndrome.

Several trichothecene mycotoxins are produced by some *Fusarium* species, and have been associated with human disease syndromes such as Alimentary Toxic Aleukia in which haemorrhage is one of the reported signs (Section 2.5.2.6). Examples of trichothecenes produced by the fusaria include T-2 toxin and diacetoxyscipenol, the latter of which has been reportedly produced by *F. semitectum* (Molto *et al.*, 1997), one of the haemolytic fungi isolated from sago. Trichothecenes are highly polar hydroxylated compounds (Cole and Cox, 1981; Kenji, 1983), which have absolutely no hexane affinity. However, the hexane-rich fraction of *F. semitectum* (and other fungal isolates) was highly active in the haemolytic assay (Figure 8.09), enabling trichothecenes to be discounted as the haemolytic agent in fungal isolates from sago starch.

Another compound demonstrated to possess haemolytic activity against mouse erythrocytes is miliacin, which is produced by the marine fungus *Chaetomium olivaceum* (Smetanina *et al.*, 2001), but is apparently also frequently encountered in higher plants (Olifson *et al.*, 1991). It is a pentacyclic triterpenoid (3β-methoxyolean-18-ene) with a molecular weight of 440 Da, a single double bond, and a single methoxy group substituent. From the structure, it appears quite likely that this compound would have more characteristics in common with PS-1 than other compounds discussed above. While this might provide a useful lead, there is currently no suggestion that it is produced by any of the haemolytic fungi from sago. Moreover, Olifson *et al.* (1991) stated that the compound was an important growth promoter in cattle with no toxic properties. Although haemolysins of microbial origin have repeatedly been shown to have varying degrees of activity depending on the target species, it is unlikely that a compound that has no long term effects on one species of mammal (namely cattle) would cause such a severe haemolytic crisis in humans.

The range of compounds of fungal origin shown to have haemolytic activity appears to be very broad, and points to the need for a careful and systematic approach

towards the characterisation of PS-1 and other haemolytic compounds associated with fungi isolated from sago starch. Methods that warrant further consideration include: the use of specific reagents for visualising lipids and proteins on TLC; further separation and purification by chromatography, guided by the established haemolytic assay; HPLC separation with detection systems not reliant on fluorescence and UV absorption; liquid chromatography mass spectrometry (LC-MS) or gas chromatography mass spectrometry (GC-MS); and nuclear magnetic resonance (NMR) structural studies.

Very little haemolytic activity was observed in the haemolytic assay of any of the 12 sago samples tested following extraction in hexane. If the fungal compound(s) that have been investigated in the current study were associated with SHD, one would expect the same compound(s) to be detectable in the implicated samples of sago starch. Indeed, it is plausible that the haemolytic activities of fungal isolates from sago starch are in no way associated with SHD.

Nonetheless, the lack of haemolytic activity in sago starch does not definitively preclude such compounds from being associated with the illness. As discussed in Section 6.4 the growth, and thus mycotoxin production, of filamentous fungi in stored commodities is usually very heterogeneous. Consequently, testing the remaining portion of implicated sago samples could foreseeably result in false negative results. While the portion consumed by the people who later became ill with SHD obviously had a high concentration of the causative compound(s), other portions of the same sago sample might only have low concentrations, if present at all.

Finally, the legitimacy of the sago sample received in Australia cannot be ensured. Samples were sent to JCU, arriving some weeks after the two outbreaks for which samples were analysed. Although not highly likely, it is possible that under the commotion of severe illness amongst many family members, a different (but perhaps similar) sago sample was collected from the village. The differences only need be minor, and it could be the case that sago starch derived from the same palm but stored in a different location or in a different container could have a vastly different microbiota to the sample associated with illness. The effect of selected storage

methods and storage duration on the microbial ecology of sago starch is addressed in the following chapter.

In conclusion, further studies addressing the purification, and determining the chemical properties, of haemolytic compounds from haemolytic fungi is required before a link with SHD can be proved or disproved.

CHAPTER 9: MICROBIAL ECOLOGY OF SAGO STARCH

9.1 Introduction

The microbial ecology of foods varies greatly, but the importance of a sound understanding of such issues is unvarying. The complex interaction of chemical and environmental factors determines which organisms can or cannot grow (Montville, 1997). While much attention is given to the growth and survival of pathogenic and toxigenic organisms in foods, the microbial activity in foods has broader implications. Such activity can result in increased nutritional value, detoxification of natural toxins, inhibition of pathogenic microorganisms, changes to organoleptic qualities and improved preservation (Nout and Rombouts, 1992; Battcock and Azam-Ali, 1998; Hoover, 2000).

Many studies have been conducted on naturally fermented foods, with numerous overviews available (Nout and Rombouts, 1992; Campbell-Platt, 1994; Steinkraus, 1994; Beuchat, 1997; Battcock and Azam-Ali, 1998). To date, the microbial ecology of sago starch remains largely unknown. One preliminary study by Omoloso (1999) noted the presence of LAB and low pH, reported to be indicative of “a form of fermentation”.

The microbial dynamics of sago starch fermentation need to be understood, particularly the extent of fermentative activity and its role in preservation. Furthermore, the function of other biologically important microorganisms in sago starch warrants investigation. This study deals with the presence of selected biologically important microorganisms, and endeavours to determine what effect they have on nutrition and preservation of sago starch.

9.2 Materials and Methods

9.2.1 Nitrogen fixation

9.2.1.1 Analysis of nitrogen fixation and isolation of nitrogen fixing bacteria from the pith and rhizoplane of the sago palm

The pith and roots of five sago palms were tested for nitrogen fixation activity and the presence of nitrogen fixing bacteria. The palms tested were growing in a swamp near Balimo village, Western Province. Pith samples were taken after the bark of the trees was removed with a machete and the site sterilized with 70% ethanol (Recochem Inc, Lytton, Queensland). A sterile hand auger (19 mm) was used to take triplicate core samples at approximately 1.5 m above ground level. The surface roots (top 50 mm of soil) of the sago palms were removed first with a sterile spade and excised with sterile scissors and forceps. Triplicate samples were taken from each tree with the spade and other implements being sterilized between operations with 70% ethanol. All samples were placed in individual 100 ml sterile containers (Sarstedt Australia, Technology Park, South Australia).

Samples were taken to a field laboratory where processing was commenced within two hours of sampling. One gram of pith was weighed (0.33 g was taken from three replicate samples) and ground in a sterile mortar in 9 ml of diluent (Appendix 2.1.1.1). Serial dilutions (10^{-1}–10^{-7}) were prepared in diluent. Then, 1 ml aliquots of each dilution were placed in triplicate gas-tight tubes containing 9 ml Endophyte (EN) medium (Appendix 2.5.1.1) (Elbeltagy *et al.*, 2001). Samples were enumerated using the MPN technique (Standards Australia, 1991d).

A 1 g sample of palm root was weighed (0.33 g was taken from three replicate samples) and the roots were washed free of loosely attached soil by agitating the root pieces in four changes of sterile, distilled water in a sterile specimen container. The washed roots were then ground in a sterile mortar in 9 ml of diluent and diluted to 10^{-7}, as above. Aliquots (1 ml) of each dilution were placed in triplicate gas-tight tubes of *Burkholderia* (BAz) medium (Appendix 2.5.1.2), Diazotroph (NFb) medium (Appendix 2.5.1.3) and glucose (LG) medium (Appendix 2.5.1.4) (Dobereiner, 1980;

Cavalcante and Dobereiner, 1988; Estrada-De Los Santos *et al.*, 2001). Samples were enumerated using the MPN technique (Standards Australia, 1991d).

All tubes were incubated at ambient temperature (25–30 ºC) for 8 days then observed for evidence of growth (cloudy appearance or pellicle formation). Positive tubes were scored as presumptive nitrogen fixers and taken for later confirmation using gas chromatography (GC). For confirmation, 1 ml of headspace gas was removed from presumptive positive tubes and replaced with 1 ml of acetylene using a Hamilton gas syringe (Hamilton Company, Reno, Nevada). Tubes were incubated for a further 24 hours at 30 ºC. Following incubation, a 1 ml sample of headspace gas was taken and injected into a 6 ml vacuum blood collection tube (Vacutainer®, BD Australia, North Ryde, New South Wales) to which 50 µl of propane was subsequently added. All gas was supplied by BOC Australia (Townsville, Queensland).

To isolate nitrogen fixing bacteria, 5 µl was taken from presumptive positive tubes and plated onto the corresponding solid medium. Plates were incubated for 6 days at 25 ºC. Cultures were subcultured onto nitrogen free agar (Appendix 2.1.1.3) and pure cultures obtained. They were then established in an overnight broth culture incubated at 30 ºC, grown in glucose-thioglycollate (GT) medium (Appendix 2.5.1.5) supplemented with 1.0 g/l of casamino acids. A 250 µl aliquot of the overnight culture was inoculated into a Vacutainer® tubes (as above) containing 5 ml of GT medium, as outlined by Neilson and Sparell (1976). Cultures were incubated for 12 hours at ~25 ºC under a stream of nitrogen gas bubbled through the broth, then sealed and incubated for a further 4 days at 30 ºC. Following incubation, 1 ml of headspace gas was taken and injected into a 6 ml Vacutainer® tube (as above) to which 50 µl of propane was subsequently added.

Isolates that did not grow anaerobically were grown on nitrogen free agar slopes supplemented with 0.02 g/l of yeast extract (Gyaneshwar *et al.*, 2001), incubated at 30 ºC for 4 days. Bottles were sealed using a Suba seal (19 mm septum size, Sigma Aldrich Pty Ltd, Castle Hill, New South Wales) and 2 ml of acetylene was added. After 6 hours at 30 ºC, a 1 ml sample of headspace was taken and delivered to a Vacutainer® tube (as above) with 50 µl of propane.

All gas samples were analysed by GC for the presence of ethylene. Nitrogenase readily reduces acetylene to ethylene, making acetylene reduction a convenient method for the evaluation of biological nitrogen fixation (Turner and Gibson, 1980). Gas chromatography was conducted using a Hewlett Packard HP6890 series GC system fitted with a J&W 1134332 GS-GASPRO column (Aligent Technologies, Forest Hill, Victoria) and a flame ionisation detector set at 300 ºC. The oven temperature was isothermal at 50 ºC and the injector temperature was 105 ºC. Helium was used as the carrier gas. Under these conditions the retention time was 0.580 min for ethylene and 0.788 min for acetylene. Ethylene standards of known concentration were assayed using the GC to enable the determination of ethylene levels in the samples. All gas for chromatography work was supplied by BOC Australia (Brisbane, Queensland).

9.2.1.2 Analysis of nitrogen fixation in sago starch and isolation of nitrogen fixing bacteria from sago starch

Ten sago samples were tested for nitrogen fixation. Duplicate 5 g samples of sago starch were weighed into Universal tubes and sealed with a Suba seal. One of the duplicate tubes was gassed with nitrogen for 2 hours, while the other tube was left ungassed. All tubes were incubated at 30 ºC for 24 hours, and then 2 ml of acetylene was added to each tube. Tubes were incubated for a further 30 ºC for 24 hours. Following incubation 2 ml of headspace gas was removed and placed in a Suba sealed universal to which 50 µl of propane was added. Acetylene reduction assays were conducted using GC, as outlined in Section 9.2.1.1. Sago samples were dried at 45 ºC for 4 days to determine the dry weight. Results are presented as the rate of ethylene production on a sago dry weight basis.

Selected samples of sago starch were also tested for the presence of nitrogen fixing bacteria using bacterial isolation techniques. A 10 g (± 0.1 g) test portion was diluted in 90 ml diluent and mixed for 2 min (Seward Stomacher 400, John Morris Pty Ltd, Bowen Hills, Queensland). Serial dilutions were made using 9 ml aliquots of PBS (Appendix 2.1.1.13). The resulting dilutions (10^{-1}–10^{-6}) were plated in duplicate on NFb agar and nitrogen free agar (Appendix 2.1.1.3) and incubated at 30 ºC for

7 days. Presumptive nitrogen fixing bacteria were estimated by counting mucoid colonies on duplicate mannitol nitrogen free media.

Selected colonies were picked off the NFb agar and nitrogen free agar for identification and confirmation of nitrogen fixation using acetylene reduction. Additionally, selected bacterial strains belonging to the family *Enterobacteriaceae* that were isolated when testing sago starch for the presence of *E. sakazakii* (Chapter 4) were tested. Pure cultures were grown, gas samples obtained, and GC used to test for acetylene reduction as outlined in Section 9.2.1.1. Positive isolates were identified to species level using Vitek® II (bioMérieux Australia Pty Ltd, Baulkham Hills, New South Wales).

9.2.2 Determination of nitrogen levels in sago starch

Fifty one samples of sago starch were tested for nitrogen levels using the method outlined by Sweeney (1989). The samples tested for nitrogen levels were the same as those tested for the presence of mycotoxins, but did not include the two samples collected late in the project that were associated with outbreaks of sago haemolytic disease. Details of village of origin, storage technique and storage duration for each sago sample can be seen in Appendix 5.

Sub-samples of sago starch were weighed (to 0.001 g) and pelletised, then analysed using an Elementar RapidN analyser (Elementar Analysensysteme GmbH, Hanau, Germany). The lower limit for accurate detection of nitrogen using this method was 0.02%.

Dry matter content of samples was determined by heating at 105 ºC under an atmosphere of nitrogen using an automated Leco thermogravimetric analyser (Leco Corporation, St Joseph, Michigan).

9.2.3 Analysis of sago starch for vitamin B_{12}

Six samples of sago starch, collected from the Western Province in April 2004, were tested for vitamin B_{12} (cobalamin) by the Royal Perth Hospital biochemistry laboratory (NATA accredited laboratory 14671). Cobalamin was extracted using acid/heat and assayed by a microbiological method, using *Euglena gracilis* as the test organism (method reference RPH.BI.VITM.0070).

From three of the six samples tested for cobalamin, bacterial isolates were tested for presumptive Vitamin B_{12} synthesis. A 10 g sample of sago starch was taken and diluted in 90 ml diluent. Subsequent serial dilutions were made in 9 ml aliquots of physiologic saline (Appendix 2.1.1.10), to a dilution of 10^{-6}. Duplicate plates of standard plate count agar (Appendix 2.1.1.9) were spread plated with 100 µl of the appropriate dilutions (10^{-3}–10^{-6}) and incubated at 30 ºC for 3 days. Representative bacterial colonies were isolated and characterised by observing colony morphology, Gram reaction, catalase, and oxidase activity (Appendix 2.1.1).

Resulting isolates were screened for their ability to synthesize vitamin B_{12}. Colonies grown on nutrient agar (Appendix 2.1.1.11) were picked off and placed in physiologic saline until Macfarland standard 5-6 was obtained. A 100 µl aliquot was inoculated into 10 ml of B_{12} synthetic medium (anonymous, 1984) (Appendix 2.5.2.1) and incubated for up to 8 days at 30 ºC. Those tubes showing growth were used to inoculate fresh B_{12} synthetic medium and incubated as previously. This process was continued until six subculture cycles had been completed. Bacteria that still produced turbidity after six growth cycles were considered presumptively positive for vitamin B_{12} synthesis (Keuth and Bisping, 1993). Isolates were also grown in basal vitamin testing media (Appendix 2.5.2.2) under anaerobic conditions to determine whether anaerobic conditions were essential for any isolates to synthesise vitamin B_{12} (Lockhead and Burton, 1957; Kamata *et al.*, 1991). Isolates were subcultured three times under anaerobic incubation, at 30 ºC for 8 days on each occasion.

9.2.4 Comparison of the effect of selected traditional storage techniques on microbial communities

The effect of selected traditional storage techniques on microbial communities was determined by testing sago stored wrapped in sago leaves, smoked, and stored in earthenware pots with additional water. Nine large bundles of sago starch, each weighing 17–20 kg, were purchased from the Balimo market. Each bundle was 3 days old or less. The sago was brought to Australia on the day of purchase, and the following day sampling commenced. Prior to sampling, three sago bundles were opened, and sago from each bundle placed in an earthenware pot. Each pot was filled to three-quarters full with sago (~5 kg moist sago starch), and then sterile distilled water was added until the water level was about 5 cm above the sago starch. Pots were loosely covered with foil and stored at room temperature.

A further three samples were placed over a small fire made primarily of dry palm fronds. Once the flames had subsided the bundles were briefly placed directly onto the hot coals and turned regularly. The bundles were removed after approximately 5 min on the fire. The remaining three bundles remained as they were at the time of purchase: tightly wrapped in sago leaves.

All three storage methods were sampled weekly for 4 weeks, and then once more 7 weeks after commencement of the experiment. Over the course of the experiment total viable bacteria, LAB, yeasts, moulds, mucoraceous fungi and haemolytic organisms were enumerated. The pH was also determined. Total viable bacteria were enumerated using the method outlined in Section 4.2.7. Yeasts and moulds were enumerated using the method outlined in Section 5.2.1, and mucoraceous fungi enumerated using the method outlined in Section 5.2.2. Haemolytic microorganisms (bacteria, yeasts and moulds) were enumerated using the methods outlined in Section 7.2.1. The pH was determined using the method outlined in Section 4.2.1.2.

Lactic acid bacteria were enumerated using de Man Rogosa Sharpe (MRS) agar (Appendix 2.5.3.1). A 10 g sample of sago starch was diluted in 90 ml of diluent (Appendix 2.1.1.1). The sample was mixed for 2 min using a Seward Stomacher 400 (John Morris Pty Ltd, Bowen Hills, Queensland). Subsequent serial dilutions were

made in one-quarter strength Ringer's solution (Appendix 2.5.3.2). One hundred microlitres of the appropriate dilutions were spread onto duplicate MRS agar plates using a glass spreader. Plates were incubated at 30 ºC in an increased carbon dioxide environment (CO_2Gen, Oxoid Ltd., Thebarton, South Australia) for 48 hours.

9.2.5 Survival of bacterial pathogens in sago starch

9.2.5.1 Survival of seeded pathogens in sago starch

Type strains of selected potentially pathogenic bacteria were used to determine their survival in sago starch. Sago starch was seeded with *B. cereus* ATCC 10876, *E. coli* ATCC 25922, *L. monocytogenes* ATCC 19115, *Salmonella* sp. ACM 3762, and *S. aureus* ATCC 25923. The experiment was conducted on both fresh and previously stored sago starch. The fresh sago was obtained from Balimo, Western Province. The starch was extracted from the sago palm one day prior to transportation to Australia. Following collection, the starch was stored at 4 ºC. The previously stored sago starch was collected from the Wewak region, East Sepik Province. It had been stored for 2 weeks in a plastic bag prior to collection. Both samples were subsequently stored and transported as outlined in Section 4.2.1.1. Experimentation commenced one day after arrival in Australia.

Cultures of bacteria were grown in nutrient broth (Appendix 2.1.1.12) incubated at 37 ºC for approximately 24 hours. Cultures were then diluted (using diluent) to a dilution of approximately 5×10^5 cfu/ml (assuming the overnight culture contained between 10^8 and 10^9 cfu/ml). Ten millilitres of this dilution was then added to 500 g of sago starch, resulting in approximately 10^4 cfu/g of each pathogen. The sago was mixed thoroughly using a hand-held electric eggbeater. The beater blades were sterilised with 70% ethanol prior to use. To decrease the likelihood of competition between bacterial pathogens having an effect on their survival, three separate batches of sago starch were inoculated. One batch was inoculated with *E. coli* and *Salmonella* sp., one batch with *B. cereus* and *S. aureus*, and the third batch with *L. monocytogenes*. For each batch tests were conducted in triplicate, resulting in a total of nine sago starch samples, all in sealed plastic containers. The sago was incubated at 30 ºC for the duration of the experiment.

Standard methods of enumeration, as outlined in Chapter 4, were used to determine the survival of *E. coli* (Section 4.2.4.2), *B. cereus* (Section 4.2.2) and *S. aureus* (Section 4.2.6). Standard methods for the detection of *Salmonella* spp. (Section 4.2.4.3) and *L. monocytogenes* (Section 4.2.5) were modified to allow estimation of the most probable number (Standards Australia, 1991d). For enumeration of *Salmonella* spp., 10 g (± 0.2 g) of sago starch was diluted in 90 ml of BPW (Appendix 2.1.4.8), and mixed for 2 min using a Seward Stomacher, as previously. Two subsequent serial dilutions were made in 9 ml of BPW. Triplicate 9 ml tubes of BPW were then inoculated with 1 ml of each of the serial dilutions, resulting in a total of nine tubes. The BPW tubes were incubated at 37 ºC for 18–20 hours. One millilitre was taken from each pre-enrichment tube and placed in RV broth (Appendix 2.1.4.10), then incubated at 37 ºC for 18–20 hours. A 5 µl nichrome loop was used to take a loopful of RV broth and streak onto XLD agar (Appendix 2.1.4.11). The XLD plates were examined after 18–24 hours at 37 ºC for typical colonies. Colonies were confirmed as *Salmonella* using polyvalent 'O' and polyvalent 'H' antiserum (Oxoid Ltd, Thebarton, South Australia).

For *L. monocytogenes* enumeration, 10 g (± 0.2 g) of sago starch was diluted in 90 ml of half Fraser broth (Appendix 2.1.5.1) and mixed for 2 min, as previously. Two subsequent serial dilutions were made in half Fraser broth. Triplicate 9 ml tubes of half Fraser broth were then inoculated with 1 ml of each of the serial dilutions, resulting in a total of nine tubes. The half Fraser broth tubes were incubated at 30 ºC for 24 hours. One hundred microlitres was taken from each half Fraser broth and placed in 9 ml of Fraser broth (Appendix 2.1.5.2), then incubated at 37 ºC for 48 hours. A 5 µl loopful of broth was streaked onto Oxford agar (Appendix 2.1.5.3). The Oxford plates were examined after 24–48 hours at 37 ºC for typical colonies. Colonies were confirmed using the CAMP test (Sutherland *et al.*, 2003).

The lower limit of detection for all organisms except *S. aureus* was 30 organisms/g in the fresh sago and 3 organisms/g in the previously stored sago. The limit of detection for *S. aureus* was 100 cfu/g in both fresh and previously stored sago.

Samples were tested twice weekly for survival of pathogens in fresh sago, and weekly for pathogen survival in old sago. The pH and a_w of the fresh sago was determined at the time of sampling using the methods outlined in Section 4.2.1.2 and Section 4.2.1.3 respectively. These two parameters were not tested in the previously stored sago.

9.2.6 The fermentation of sago starch

9.2.6.1 Enumeration of lactic acid bacteria and acid production in sago starch

Lactic acid bacteria were enumerated from 12 sago samples collected from the Western Province in April 2005. Sub-samples of 10 g (± 0.2 g) were aseptically weighed out and diluted in 90 ml of one-quarter strength Ringer's solution. The sample was mixed for 2 min using a Seward Stomacher, as previously. Further dilutions were made in MRS broth (Appendix 2.5.3.3), and then 100 µl of the appropriate dilutions were spread plated in duplicate onto MRS agar supplemented with cycloheximide, sodium nitrate and polymyxin B (MRS-SCP) (Appendix 2.5.3.4). The addition of the aforementioned supplements was based on similar modifications made by Coventry e*t al.* (1997) to minimise the growth of Gram negative bacteria and fungi. Plates were incubated anaerobically (AnaeroGen, Oxoid Ltd., Thebarton, South Australia) at 30 ºC for 48 hours.

For acid analysis, 5 g of starch was mixed with 10 ml of distilled water (as for pH analysis, Section 4.2.1.2). A 3 ml aliquot of the resulting suspension was taken and 50 µl of toluene (Sigma Aldrich Pty Ltd, Castle Hill, New South Wales) added to stop further microbial activity. Samples were stored at -20 ºC until analysis was conducted. The volatile fatty acids (VFAs) tested for were acetic acid, propionic acid, iso-butyric acid, n-butyric acid, iso-valeric acid, n-valeric acid, n-caproic acid and n-heptonoic acid. The organic acids tested for were pyruvic acid and lactic acid.

Samples were thawed and vacuum distilled prior to acid analysis. The organic acids were converted to their more volatile methyl esters before distillation using acidified methanol. This was done by adding 2 ml of methanol and 200 µl of concentrated sulphuric acid to 1 ml of thawed sample. Samples were left at room temperature

overnight. One millilitre of the mixture was then pipetted into a Thunberg tube. For VFAs, 900 µl of sample and 100 µl of 1 M sulphuric acid were pipetted into the Thunberg tube. The ground glass fitting was coated with vacuum grease and the collection trap attached. All samples were frozen in liquid nitrogen and then evacuated using a vacuum pump. The bulb of the Thunberg tube was then placed in liquid nitrogen, and the tube containing the frozen sample held at room temperature. Distillation was allowed to occur until completion (~1 hour). Standards of known concentrations of all acids analysed were processed identically to samples, and used for quantification.

Analysis of samples was conducted using GC. An Agilent 6890 gas chromatograph fitted with a J&W 0.53 mm × 30 m DB-FFAP column with a 1 am coating (Aligent Technologies, Forest Hill, Victoria) and a flame ionisation detector set at 250 ºC. The oven temperature was initially 90 ºC for 5 min, and subsequently increased 10 ºC/min to 190 ºC, where it was held for 2 min. Samples were run alongside standards that were treated identically to samples. The injector temperature was 250 ºC and nitrogen was used as the carrier gas. Standards were analysed for later quantification of samples.

Regression analysis of LAB numbers, pH and acid content was conducted using SPSS 12.0.1 for Windows (SPSS Australasia Pty Ltd, North Sydney, New South Wales).

9.2.6.2 The microbiota and acid production in laboratory fermented sago starch

Fresh sago was obtained from the Balimo region of the Western Province and transported to Australia, as described in Section 9.2.5.1. Triplicate samples weighing ~1.5 kg each were tightly packed into sterilised plastic containers with airtight lids and incubated at 30 ºC. Samples were tested regularly, initially on a daily basis, but less frequently after the first 6 days. The triplicate samples were tested for pH using the method described in Section 4.2.1.2, and the enumeration of various organisms was conducted using previously described methods: LAB (Section 9.2.6.1), yeasts and moulds (Section 5.2.1), faecal coliforms and *E. coli* (Section 4.2.4.2), *S. aureus* (Section 4.2.6) and *B. cereus* (Section 4.2.2). Samples were also tested for the

presence of *Salmonella* spp. and *L. monocytogenes* using the methods outlined in Section 4.2.4.3 and Section 4.2.5 respectively.

Acid analysis was conducted using the method outlined in Section 9.2.6.1.

9.2.7 The role of lactic acid bacteria in the preservation of sago starch

9.2.7.1 Inhibition of selected bacterial strains by lactic acid bacteria

Selected LAB isolated from sago starch were tested for their ability to inhibit the growth of selected indicator bacteria. Organisms tested were *E. coli* ATCC 25922, *Enterobacter aerogenes* ATCC 13048, *Salmonella salford* ACM 3762, *B. cereus* ATCC 10876, *L. monocytogenes* ATCC 19115 and *S. aureus* ATCC 25923. The most commonly occurring strains of LAB, based on colonial morphology, isolated from sago starch (Section 9.2.6.1) were subcultured in MSR-SCP broth (Appendix 2.5.3.4) and incubated in an enriched CO_2 atmosphere (CO_2Gen, Oxoid Ltd., Thebarton, South Australia) at 30 ºC for 48 hours. A 1 µl aliquot was spotted onto a segment of a MRS-SCP agar plate (eight aliquots tested per 90 mm Petri plate) and incubated anaerobically (AnaeroGen, Oxoid Ltd, Thebarton, South Australia) at 30 ºC for 48 hours. This resulted in eight single colonies of LAB evenly separated on the MRS-SCP agar. Six replicates were produced. Plates were then overlayed with 15 ml of molten nutrient agar (Appendix 2.1.1.11) tempered to 45 ºC, containing 300 µl of overnight broth of one of the indicator bacteria. Once set, the overlaid plates were incubated at 37 ºC for 24 hours. The same process was followed for the other five indicator organisms, thus utilising the six replicates of MRS-SCP agar. Plates were observed for zones of inhibition in the upper layer, where the indicator organism was growing.

9.2.7.2 The role of bacteriophages in the inhibition of indicator organisms

To determine whether bacteriophages were involved in the inhibition of indicator bacteria, a method based on that of Kekessy and Piguet (1970) was employed. Lactic acid bacteria were grown in broth and then on agar as outlined in Section 9.2.7.1. Following anaerobic incubation at 30 ºC for 48 hours the agar was aseptically

detached from the edge of the Petri plate and inverted. The inverted agar was then covered with 10 ml of molten nutrient agar containing 200 µl of overnight broth of the appropriate indicator organism (as above). Plates were incubated at 37 ºC for 24 hours and observed for zones of inhibition. The same process was repeated for the remaining five indicator organisms. The use of this method ensured there was no direct contact between the lactic acid bacteria and the indicator strain. Since bacteriophages are non-diffusing, the presence of zones of inhibition eliminated bacteriophages as the cause (Kekessy and Piguet, 1970; Tagg and McGiven, 1971).

9.2.7.3 Testing the sensitivity of inhibiting substances to enzymatic treatments

Eight strains of LAB exhibiting inhibition of indicator organisms were selected for further study. The experiment outlined in Section 9.2.7.1 was repeated, with the addition of selected enzymes to determine whether the substance causing inhibition was sensitive to such treatments. One microlitre of 200 mg/ml solutions of pepsin, trypsin and α-glucosidase was placed next to the LAB colony prior to being overlaid with the indicator species (Ohmomo *et al.*, 2000). Plates were incubated as outlined above.

9.2.7.4 The production of organic acids and volatile fatty acids in selected isolates of lactic acid bacteria

Four LAB isolates were selected to test for the production of VFAs and organic acids. Triplicate inhibition assays as outlined in Section 9.2.7.1 were set up, using only *E. coli* ATCC 25922 as the indicator organism. Following completion of the experiment, 1 g of agar from within the zone of inhibition, but avoiding the LAB colony, was macerated in 9 ml of distilled water. A negative control of sterile MRS-SCP agar overlaid with un-inoculated nutrient agar and incubated alongside the samples was also analysed. Samples were immediately distilled and then tested for the presence of VFAs and organic acids, as outlined in Section 9.2.6.3. Results were generated by comparing acid concentrations in agar surrounding LAB growth with levels in un-inoculated agar.

9.3 Results

9.3.1 Isolation of nitrogen fixing bacteria from sago palms

9.3.1.1 Nitrogen fixation and isolation of nitrogen fixing bacteria from the pith and rhizoplane of the sago palm

The MPN results suggest that nitrogen fixation occurs in the rhizoplane of sago palms, but not in the pith. There was evidence of nitrogen fixation in the rhizoplane of all five palms using LG medium, with numbers of nitrogen fixing bacteria up to 2.4×10^5 organisms/g. Lower numbers of nitrogen fixing bacteria were enumerated in the rhizoplane using BAz and NFb media. In the pith of the sago palm, nitrogen fixing bacteria were detected in one of the five samples tested, but only in very low numbers (Table 9.01).

Table 9.01: Estimated number of nitrogen fixing bacteria isolated from sago palms using the MPN technique and confirmation using GC.

Sago palm	**Rhizoplane (organisms/g)**			**Pith (organisms/g)**
	BAz	**LG**	**NFb**	**EN**
1	< 0.03	2.4×10^5	2.3×10^1	< 0.03
2	< 0.03	2.4×10^3	2.3×10^1	3.6
3	2.3×10^1	4.3×10^1	2.4×10^2	< 0.03
4	< 0.03	1.5×10^2	9.1	< 0.03
5	< 0.03	4.3×10^2	2.3×10^1	< 0.03

Numerous bacterial strains were isolated from the rhizoplane, including members of the genera *Serratia*, *Pseudomonas*, *Enterobacter*, *Flavimonas* and *Photobacterium*. None of the isolates exhibited nitrogenase activity (acetylene reduction) in pure culture.

9.3.1.2 Analysis of nitrogen fixation in sago starch and isolation of nitrogen fixing bacteria from sago starch

The level of nitrogenase activity as determined by acetylene reduction was variable in sago starch. In 2 of the 10 samples tested no nitrogenase activity was detected, and

in a further 3 samples activity was very low. In the remaining five samples nitrogenase activity ranged from a minimum of 0.025 nM g^{-1} $hour^{-1}$ to a maximum of 0.558 nM g^{-1} $hour^{-1}$ (Table 9.02).

Table 9.02: Rate of nitrogen fixation (nM of ethylene production) in sago starch and enumeration of presumptive nitrogen fixing bacteria from selected sago samples. No distinction was made between aerobic and anaerobic nitrogen fixation.

Sago sample	**Approximate storage duration**	**Nitrogen fixation rate nM g^{-1} $hour^{-1}$**	**Presumptive nitrogen fixers (organisms/g)**
W0404-01	3 weeks	0.000	1.1×10^6
W0404-03	3 weeks	0.000–0.002	2.1×10^6
W0404-06	1 month	0.012–0.023	3.2×10^7
W0404-07	unknown	0.002–0.003	Not enum.
W0404-08	2 months	0.000–0.010	Not enum.
W0404-09	2-3 months	0.000	Not enum.
W0404-10	2 months	0.335–0.494	Not enum.
W0404-13	3 months	0.029–0.558	1.1×10^7
W0404-14	1 week	0.218–0.430	5.9×10^8
W0404-15	1 month	0.025–0.119	NA

Not enum: samples not enumerated.

Only two bacterial strains isolated from sago starch were positive for nitrogenase activity in pure culture. These species were identified as *Enterobacter cloacae* and *Raoultella ornithinolytica*.

9.3.2 Determination of nitrogen levels in sago starch

Nitrogen levels in sago starch were very low. Under the conditions of detection, nitrogen levels of ≤ 0.02% nitrogen could not be accurately quantified. Thus, 37 of the 51 samples tested were below the lower limit of detection. Of the 14 samples that could be interpreted with confidence, 1 sample had a nitrogen content of 0.20%. All other samples had nitrogen contents of 0.10% or less. Results for all samples tested are shown in Appendix 7.1.

9.3.3 Analysis of sago starch for vitamin B_{12} and isolation of B_{12} synthesisers

Vitamin B_{12} was detected in all five samples tested. Results are shown in Table 9.03.

Table 9.03: Vitamin B_{12} levels in five samples of sago starch.

Sago sample	Age of sample	Vitamin B_{12} (ppb)
W0404-01	3 weeks	0.34 ± 0.07
W0404-07	unknown	1.27 ± 0.26
W0404-10	2 months	1.15 ± 0.59
W0404-13	3 months	1.23 ± 0.30
W0404-14	1 week	0.77 ± 0.13
W0404-15	1 month	1.71 ± 0.43

Vitamin B_{12} synthesising bacteria were isolated from one of the three samples tested (W0404-13). One isolate was identified as *Citrobacter freundii*, and the other isolate also belonged to the family *Enterobacteriaceae*, with affinities to *Erwinia* and *Pantoea*.

9.3.4 Comparison of the effect of selected traditional storage techniques on microbial communities

The effect of three traditional storage techniques on numbers of total viable bacteria, LAB, yeasts, moulds, mucoraceous fungi and haemolytic organisms in sago starch was studied over seven weeks. No mucoraceous fungi were isolated from sago stored under any of the three conditions tested. The enumeration of haemolytic microorganisms proved to be difficult in mixed culture, as was found to be the case in other experiments (see Section 7.3.1.1). Haemolytic bacteria were noted in some samples, but numbers were low (generally 10^2–10^3 cfu/g).

Figure 9.01 shows the number of total viable bacteria isolated from sago starch stored using three different traditional techniques. The smoked sago starch initially had considerably lower total bacterial numbers, but after 1 week numbers were comparable to the other two storage methods. After 6 weeks total bacterial numbers decreased slightly in smoked sago and in sago stored in earthenware pots with water, while bacterial numbers in sago stored wrapped in sago leaves remained high.

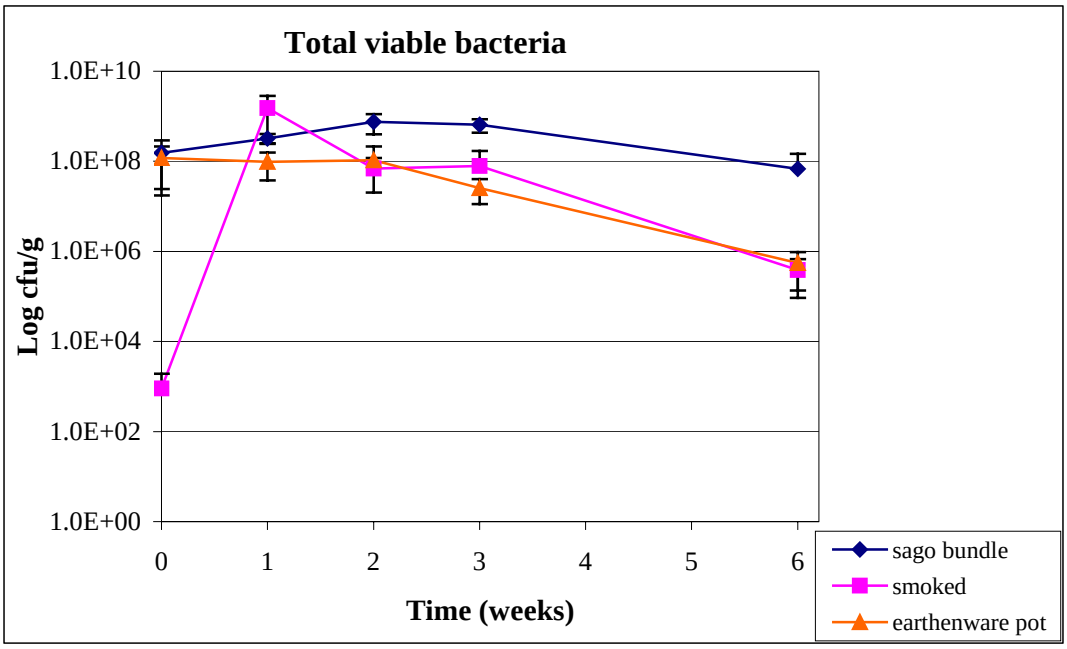


Figure 9.01: Number of total viable bacteria (cfu/g) over 6 weeks in sago stored wrapped in sago leaves (sago bundle), smoked sago and sago stored in an earthenware pot covered with water.

A similar trend (as observed for total viable bacteria) was evident for LAB (Figure 9.02). Initially, numbers of LAB were low in the smoked sago, but after 1 week all storage techniques gave rise to comparable numbers. Numbers of LAB remained comparable in all storage techniques over the duration of the experiment.

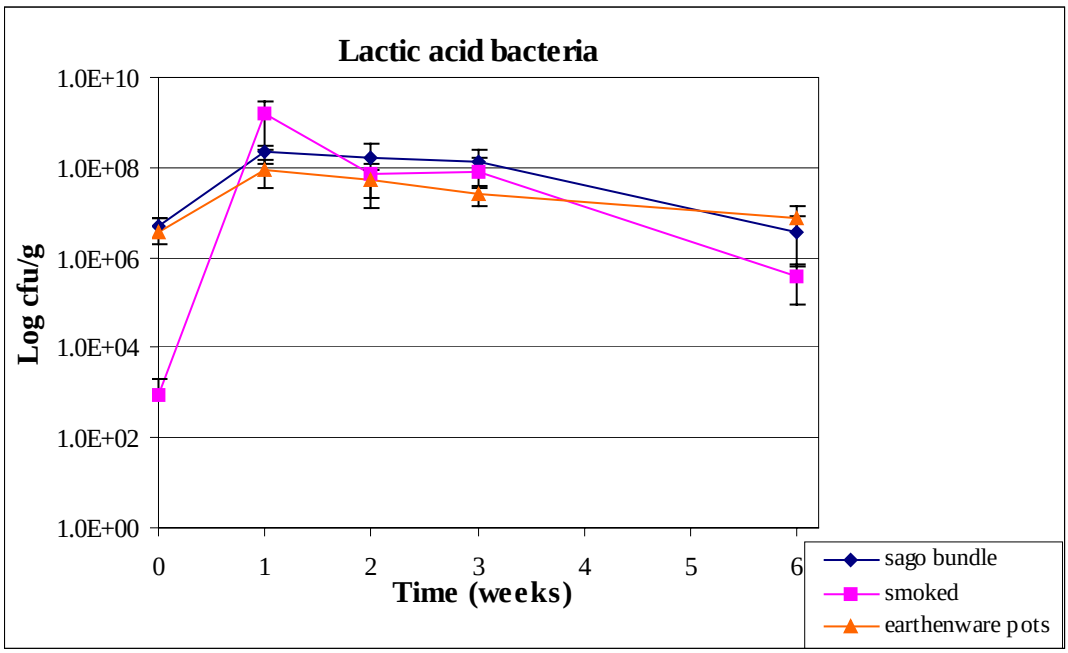


Figure 9.02: Number of LAB (cfu/g) over 6 weeks in sago stored wrapped in sago leaves (sago bundle), smoked sago and sago stored in an earthenware pot covered with water.

As for total bacteria and LAB, yeast numbers were initially low in the smoked sago, but had increased after 1 week (Figure 9.03). There were few differences among yeast numbers in any of the storage methods between 1 week and the conclusion of the experiment.

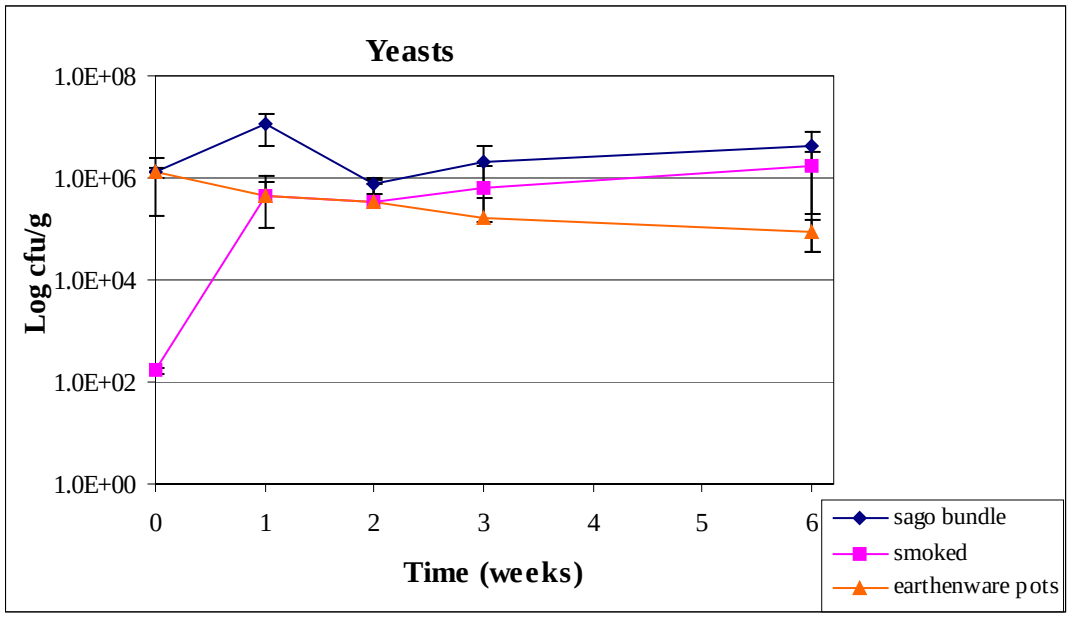


Figure 9.03: Number of yeasts (cfu/g) over 6 weeks in sago stored wrapped in sago leaves (sago bundle), smoked sago and sago stored in an earthenware pot covered with water.

The trend observed for moulds was different to the trends observed for other microorganisms. Initially, mould numbers were similar (between 10^2 and 10^4 cfu/g) for all storage techniques (Figure 9.04). However, over the following 2 weeks mould numbers increased in sago wrapped in leaves and smoked sago, but decreased in sago stored in earthenware pots with water. In the latter storage method, mould numbers remained low at week 3, but by week 6 had increased by approximately three log cycles to have a similar level of growth as sago stored under the other two methods.

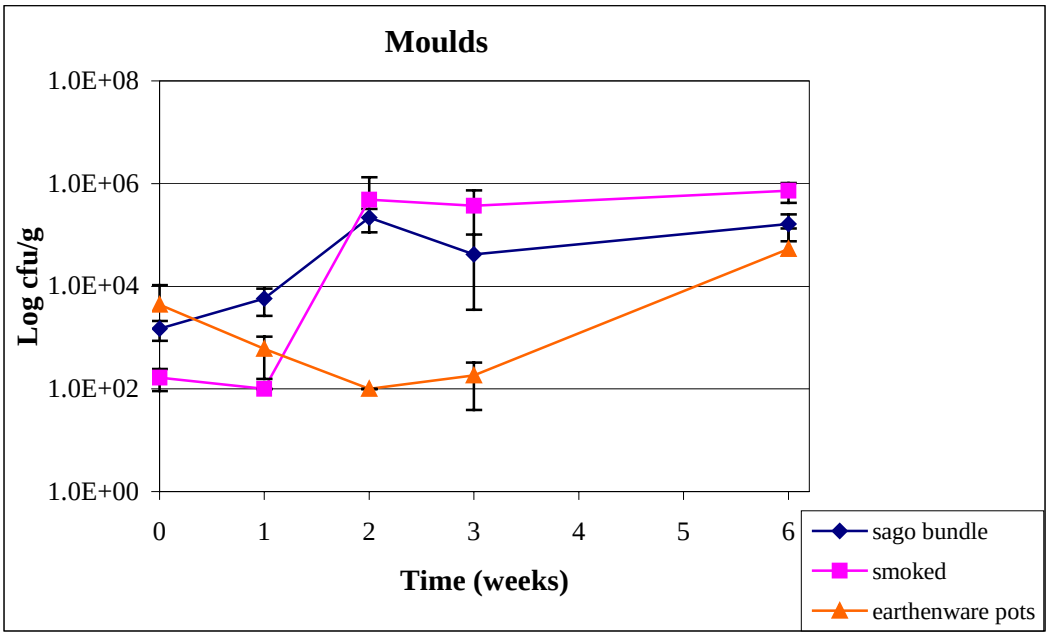


Figure 9.04: Number of moulds (cfu/g) over 6 weeks in sago stored wrapped in sago leaves (sago bundle), smoked sago and sago stored in an earthenware pot covered with water.

9.3.5 Survival of bacterial pathogens in sago starch

The MPN method was used to determine the survival of all bacterial pathogens in sago starch except *S. aureus*. Consequently, in the cases where the most probable number was outside the upper or lower limit of detection, it is not possible to determine mean or standard deviation of the triplicate samples. Table 9.04 show the results of pathogen survival in both fresh and previously stored sago as accurately as possible given the aforementioned limitation.

In fresh sago there was a decrease in pathogen numbers throughout the course of the experiment. *Escherichia coli* was able to survive in sago starch for the duration of the experiment, with numbers remaining above the upper limit of detection for the first 10 days. Numbers gradually decreased on days 14 and 21. *Salmonella* sp. was still detectable at very low levels on day 7, but was not detectable thereafter.
Bacillus cereus did not survive well in sago starch: numbers were low on the day of inoculation and remained low on day 3. It could not be detected from day 7 onwards. Despite high numbers of *S. aureus* being detected on the day of inoculation, the organism was not detectable on day 3 of the experiment, or subsequent days.
Listeria monocytogenes survived in low numbers until day 3, but was not detected from day 7 onwards.

None of the organisms tested survived well in previously stored sago. Both *E. coli* and *S. aureus* were detected in low numbers after 2 weeks, despite being undetectable after 1 week. *Salmonella* sp. and *L. monocytogenes* were not detected after the first day. *Bacillus cereus* was not detected on the day of inoculation, but was detected in very low numbers over the following 2 weeks. None of the pathogens was detected in previously stored sago starch at day 21.

Table 9.04: Estimated bacterial counts per gram of sago for various bacterial pathogens in sago starch over three weeks. Sago starch was seeded with approximately 1.0×10^4 cfu/g of each pathogen on day 0.

Fresh sago	**Day 0**	**Day 3**	**Day 7**	**Day 10**	**Day 14**	**Day 21**
E. coli	$>1.1 \times 10^4$	$>1.1 \times 10^4$	$>1.1 \times 10^4$	$>1.1 \times 10^4$	$\sim 8.9 \times 10^3$	6.0×10^2
Salmonella sp.	$\sim 3.9 \times 10^3$	2.9×10^2	$<3.6 \times 10^1$	ND	ND	ND
B. cereus	$<3.6 \times 10^1$	$<3.6 \times 10^1$	ND	ND	ND	ND
S. aureus	2.1×10^5	ND	ND	ND	ND	ND
L. monocytogenes	$\sim 8.1 \times 10^3$	$<2.3 \times 10^2$	ND	ND	ND	ND
Stored sago	**Day 0**		**Day 7**		**Day 14**	**Day 21**
E. coli	1.2×10^1		ND		1.4×10^2	ND
Salmonella sp.	1.0×10^1		ND		ND	ND
B. cereus	ND		1.3×10^1		< 4	ND
S. aureus	1.5×10^3		ND		1.0×10^2	ND
L. monocytogenes	3.1×10^2		ND		ND	ND

Legend: > All triplicate samples were above the upper limit of detection
~ One of the triplicate samples was above the upper limit of detection, but the other two samples were within the detection range
< One or two of the samples were below the detectable limit and the remaining samples were within the detectable range
ND Not detected. All triplicate samples below the lower limit of detection

The water activity of fresh sago starch remained high throughout the course of the experiment, with the lowest recorded value being 0.997. The pH steadily decreased as the experiment progressed. At the commencement of the experiment the sago starch was pH 6.60, but the mean pH dropped to 4.64 ± 0.31 on day 21.

9.3.6 The fermentation of sago starch

9.3.6.1 Enumeration of lactic acid bacteria and acid production in sago starch

Table 9.05 shows LAB numbers, pH and acid concentrations for the 12 samples of sago starch tested. Lactic acid bacteria were isolated from all 12 samples. Numbers ranged from 3.9×10^4 cfu/g to 6.7×10^7 cfu/g. Acids were also detected in all samples. Acetic acid and propionic acid were present in all 12 samples, while lactic acid was detected in 6 of the samples. Only one sample was positive for iso-butyric acid, and two samples were positive for iso-valeric acid. The values given in Table 9.05 are for the combined iso- and n- forms of these two acids, but more strongly reflect the n- form. Butyric acid was detected in nine samples, in most cases at higher levels than lactic acid. Valeric acid was detected in six samples, but always at low levels. No pyruvic acid was detected in any of the sago samples.

Table 9.05: Enumeration of LAB, pH values and detection of various acids in sago starch.

Sago sample	**pH**	**LAB cfu/g**	**Acid analysis (mM/kg sago starch)**				
			Acetic	**Propionic**	**Butyric**	**Valeric**	**Lactic**
W0405-01	4.18	6.7×10^7	18.54	2.79	4.29	0.16	4.09
W0405-02	4.98	1.3×10^7	10.75	7.39	1.04	0.00	0.00
W0405-03	4.94	4.8×10^5	4.52	2.28	11.63	0.35	0.46
W0405-04	4.29	2.0×10^7	8.82	11.59	8.46	0.83	1.69
W0405-05	5.15	1.9×10^7	2.71	3.83	1.66	0.23	0.00
W0405-06	6.16	3.9×10^4	2.25	0.69	0.00	0.00	0.00
W0405-07	6.62	7.3×10^6	1.82	0.56	0.00	0.00	0.00
W0405-08	6.40	5.8×10^5	5.86	1.22	1.52	0.00	0.00
W0405-09	4.63	5.7×10^6	3.74	3.35	18.73	0.88	0.00
W0405-10	4.60	3.2×10^6	7.80	3.98	1.32	0.09	0.35
W0405-11	6.81	3.6×10^4	2.06	0.35	0.00	0.00	0.45
W0405-12	4.18	1.2×10^7	19.34	1.36	0.07	0.00	3.84

Regression analysis was conducted to determine whether any correlations existed between pH, total acid concentration and LAB numbers. Using Pearson's correlation coefficient (two sided), a strong correlation was observed between starch pH and log total acid (n = 12, r^2 = 0.846, P = 0.000). Weaker correlations were observed between log LAB and pH (n = 12, r^2 = 0.489, P = 0.01) and log LAB and total acid (n = 12, r^2 = 0.424, P = 0.02)

9.3.6.2 The microbiota and acid analysis of actively fermenting sago starch

The incubation of fresh sago starch at 30 ºC in airtight containers resulted in a steady decrease in pH (Appendix 7.2), and an initial rise in LAB and yeast numbers (Figure 9.05). There were approximately 10^5 cfu/g of LAB at the commencement of the experiment, rising to approximately 10^7 cfu/g after 24 hours of incubation. Numbers remained constant at just below 10^7 cfu/g until day 23. No LAB were detected on day 30 (< 10^2 cfu/g). A similar trend was noted in yeasts, where numbers increased two log cycles (10^6–10^8 cfu/g) within the first 2 days of incubation. Yeast numbers were not as stable as LAB numbers, but remained high (~10^7 cfu/g) up to day 23 of the experiment. On day 30, the final day of the experiment, yeast numbers had decreased to around 10^5 cfu/g.

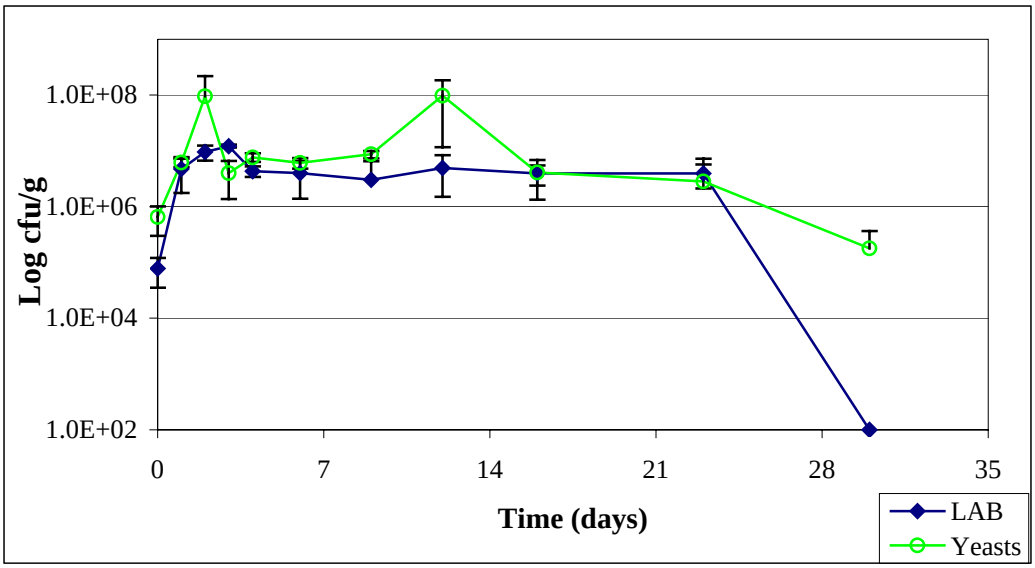


Figure 9.05: Colony forming units of LAB and yeasts in actively fermenting sago starch.

The fermenting sago starch was tested for the presence of various bacterial pathogens and filamentous fungi. *Bacillus cereus*, *L. monocytogenes*, *Salmonella* spp. and *S. aureus* were not detected. Faecal coliforms and *E. coli* were present in high numbers for much of the experiment. During the first 2 weeks numbers of both faecal coliforms and *E. coli* were over the upper limit of detection, 1.1×10^4 organisms/g. On day 16, *E. coli* was present in very low numbers only. On days 23 and 30 it was not detected. Numbers of faecal coliforms also decreased late in the experiment, and were not detectable on day 30. Numbers of filamentous fungi remained low throughout the experiment, never exceeding 5.0×10^2 cfu/g (Table 9.06).

Table 9.06: Faecal coliforms, *E. coli* and filamentous fungi isolated from triplicate samples of fermenting sago starch over 30 days.

Day	Faecal coliforms	*E. coli*	Filamentous fungi
0	$>1.1 \times 10^4$	$>1.1 \times 10^4$	$2.8 \times 10^2 \pm 1.3 \times 10^2$
3	$>1.1 \times 10^4$	$>1.1 \times 10^4$	$< 2.0 \times 10^2$
6	$>1.1 \times 10^4$	$>1.1 \times 10^4$	$< 2.0 \times 10^2$
9	$>1.1 \times 10^4$	$>1.1 \times 10^4$	$< 1.5 \times 10^2$
12	$>1.1 \times 10^4$	$>1.1 \times 10^4$	$2.5 \times 10^2 \pm 1.5 \times 10^2$
16	$>1.1 \times 10^4$	$< 4.0 \times 10^1$	$< 4.5 \times 10^2$
23	$< 4.3 \times 10^1$	ND	$< 5.0 \times 10^2$
30	ND	ND	$< 2.5 \times 10^2$

Refer to Table 9.04 for definitions of symbols used.

Concentrations of propionic acid, iso-butyric acid, iso-valeric acid, n-valeric acid and pyruvic acid were low in fermenting sago. The maximum level of propionic acid was detected at day 12 (7.83 mM/kg), decreasing slightly to 5.09 mM/kg by day 30. For the other minor acids, concentrations reached their maximum at either day 23 (pyruvic acid, 3.33. mM/kg), or day 30 (iso-butyric acid, 1.71 mM/kg; iso-valeric acid, 2.36 mM/kg; n-valeric acid, 6.40 mM/kg). Levels of acetic acid, n-butyric acid and lactic acid were also low in the first week of fermentation, but increased substantially late in the experiment (Figure 9.06).

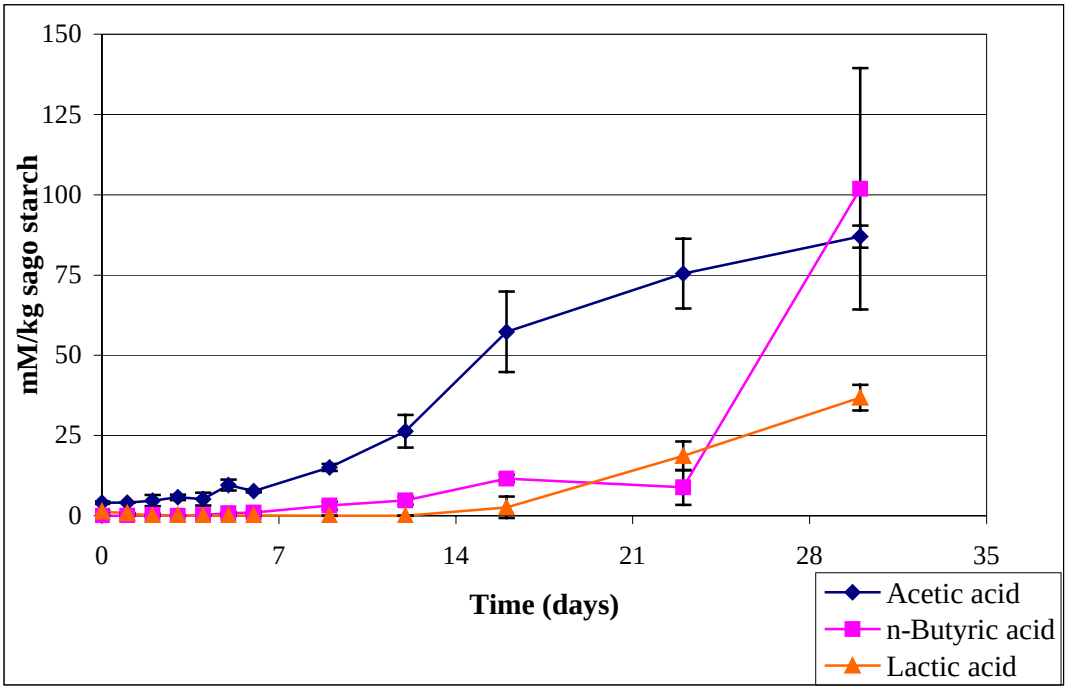


Figure 9.06: Levels of acetic acid, n-butyric acid and lactic acid in fermenting sago starch.

At the commencement of the experiment, the pH of the sago starch was 4.93 ± 0.06. The pH increased slightly in the early stages of the experiment, and was 5.47 ± 0.63 on day 4. From that point onwards the pH decreased, reaching 3.07 ± 0.15 on day 30 (Appendix 7.2). A correlation was observed between log total acid concentration and pH of sago starch (n = 11, r^2 = 0.920, P = 0.000).

9.3.7 The role of lactic acid bacteria in the preservation of sago starch

9.3.7.1 Inhibition of selected bacterial strains by lactic acid bacteria

Of the 50 LAB isolates tested for their ability to inhibit the six selected indicator organisms, 43 isolates inhibited the growth of all six. A further three LAB isolated inhibited five of the six indicator organisms.

9.3.7.2 The role of bacteriophages in the inhibition of indicator organisms

The experiment in which agar was inverted prior to being overlaid with molten agar containing the indicator organism produced the same results as above, with 43

isolates inhibiting all indicator organisms. The retention of antimicrobial activity discounts phage-mediated lysis as the cause of inhibition. The results for this experiment can be seen in Appendix 7.3.

9.3.7.3 The sensitivity of inhibiting substances to enzymatic treatments

The inhibitory substances produced by the eight selected LAB strains were not sensitive to pepsin, trypsin or α-glucosidase. The resistance of the inhibitory substances to protease activity indicates that the substances in question are not proteinaceous.

9.3.7.4 The production of organic acids and volatile fatty acids in selected isolates of lactic acid bacteria

Four LAB isolates that had shown inhibition of indicator organisms were tested for production of acid in pure culture. None of the four isolates produced iso-butyric acid, iso-valeric acid or n-valeric acid. Only one of the four isolates produced propionic acid and n-butyric acid. All four isolates produced pyruvic acid, acetic acid and lactic acid (Table 9.07).

Table 9.07: Production of various acids by selected LAB isolated from sago starch grown in pure culture.

Origin of LAB isolates	**Acid analysis (mM/g agar)**				
	Acetic acid	**Propionic acid**	**n-Butyric acid**	**Pyruvic acid**	**Lactic acid**
W0405-01	41.24 + 8.17	0.00	0.00	3.59 + 0.44	179.42 + 13.24
W0405-05	24.14 + 19.31	1.44 + 3.42	1.99 + 3.45	4.39 + 1.58	112.31 + 5.06
W0405-06	44.25 + 25.36	0.00	0.00	8.48 + 0.65	88.29 + 0.95
W0405-08	23.97 + 7.25	0.00	0.00	5.94 + 0.31	138.05 + 0.71

9.4 Discussion

As with many foods of plant origin, the factors that contribute to the nutritional status of sago starch and its suitability for consumption are complex. It is difficult to determine the extent of nitrogen fixation associated with the growth of *M. sagu*, and in stored sago starch. The use of LG medium was successful in the enumeration of

nitrogen fixing bacteria associated with the rhizoplane of *M. sagu*. This medium was used by Cavalcante and Dobereiner (1988) for the isolation of a newly described bacteria associated with sugar cane. Using the MPN technique and GC confirmation, the maximum number of nitrogen fixing bacteria isolated in this study in the rhizoplane was 2.4×10^5 organisms/g. The isolation and identification of these organisms revealed the presence of *Enterobacter, Pseudomonas* and *Serratia*, among others. Strains of these three genera have been reported to be diazotrophic (Sprent and Sprent, 1990; Gyaneshwar *et al.*, 2001), although no nitrogenase activity (acetylene reduction) was observed in pure cultures of these isolates in this study. This result is perhaps not surprising considering that growth of an organism on 'nitrogen free' medium does not guarantee that it is nitrogen fixing and that few isolates were tested for nitrogenase activity.

According to Klucas (1991), high number of diazotrophs are required for significant nitrogen fixation to occur, and in the tropics approximately 10^7 diazotrophs/g of soil or root material is common (Odu, 1977; Klucas, 1991). On the basis of the low numbers of diazotrophic bacteria enumerated using MPN, and the lack of nitrogenase activity in pure cultures, it seems unlikely that nitrogen fixation contributes greatly to the growth rate and starch yield of *M. sagu*, or to the nutrient levels in the soil of sago stands.

Analysis of the rate of nitrogenase activity in sago starch suggests that nitrogen fixation occurs in sago starch. Four of the 10 samples had maximum rates of ethylene production of 0.119 nM g^{-1} $hour^{-1}$ or higher. This is comparable to nitrogenase activity of rhizosphere soil associated with tropical grasses, but considerably lower than the activity in roots of various non-legumes (Day, 1977; Sprent and Sprent, 1990; Elbeltagy *et al.*, 2001). The nitrogen fixers found in sago starch presumably originated from soil and/or foliage, bearing in mind that there was no substantial evidence of endophytic colonization of palm pith.

Numbers of presumptive nitrogen fixing bacteria were also indicative of nitrogen fixation in sago starch, being $> 10^6$/g in all five samples for which enumeration was conducted. However, it should be noted that enumeration was on the basis of growth on nitrogen free media. In the past the use of this method alone has resulted in claims

of nitrogen fixation by a variety of bacteria that might not be justified (Odu, 1977). It is likely that the confirmation of isolates identified as presumptive nitrogen fixing bacteria would have resulted in lower numbers of nitrogen fixing bacteria in sago starch.

Nitrogenase activity was detected in two species of bacteria isolated from sago starch, namely *Enterobacter cloacae* and *Raoultella ornithinolytica*. The diazotrophic nature of *E. cloacae* is well documented, both as a free living diazotroph and in association with plants (Pedersen *et al.*, 1978; Rennie, 1981; Wright and Weaver, 1981; Silvester and Musgrave, 1991). The *Raoultella* genus was recently proposed for a cluster of *Klebsiella* spp. that grow at 10 ºC and utilise L-sorbose as a carbon source (Drancourt *et al.*, 2001). Various species of *Klebsiella* are known diazotrophs, but no reports exist of the diazotrophic nature of *R. ornithinolytica* (formerly *K. ornithinolytica*).

The presence of diazotrophic bacteria in sago starch is consistent with the isolation of such organisms from high carbohydrate substrates. Indeed, Silvester and Musgrave (1991) stated that non-photosynthetic diazotrophs occupy an ecological niche in substrates containing ample respirable carbon and reduced oxygen tension. It would seem that fermenting sago starch provides these conditions. Molybdenum is also required for nitrogen fixation (Evans and Burris, 1992) and was detected in adequate amounts for fixation in all five samples of sago starch tested (data not shown).

Despite the presence of confirmed diazotrophic bacteria in some samples of sago starch, nitrogen levels of the 51 sago samples analysed were low. The fixation of nitrogen in sago starch may be significant in terms of providing a nitrogen source for other microbes, thus influencing the microbial ecology of sago starch. In order to support their growth on carbon bases, microbes require approximately one atom of nitrogen for every 10 atoms of carbon utilized (Alexander, 1977). Nitrogen fixation is unlikely to have a major effect on human dietary intake as most, if not all, of the nitrogen added to the sago starch by way of fixation activity is utilized by other microbes as they ferment the vast amounts of carbon available.

Vitamin B_{12} was detected in sago starch at levels of approximately 10–20 ng/g. Further investigations into the bioavailability of the sago-derived vitamin B_{12}, as described by Watanabe *et al.* (2002), were not conducted in this study. If the vitamin B_{12} present were in a bioavailable form, it would make sago starch a good source of the vitamin, having levels comparable to many other food sources including traditionally fermented tempeh (Areekul *et al.*, 1990; Stabler and Allen, 2004).

The role microorganisms play in the nutritional value of sago starch remains largely unresolved, although their role would appear to be minor. However, this study demonstrated the important role of microorganisms in storage and fermentation of sago starch. A comparison of three selected traditional storage techniques for their influence on microbial communities showed high levels of LAB in all sago samples. Smoked sago yielded considerably lower numbers of LAB and other microbes, such as total viable bacteria, yeasts and moulds, at the commencement of the experiment. While decreased numbers of LAB suggest reduced fermentative activity, the decrease in numbers of moulds and total viable bacteria are likely to result in improved food safety. Storage of sago starch that reduces mould and bacterial numbers should be encouraged, as this reduces potentially mycotoxigenic moulds (classical mycotoxins as discussed in Chapter 6, and novel haemolytic compounds in Chapter 8) and high levels of faecal contamination (Chapter 4). It was noted that mould numbers were higher (and total bacterial numbers comparable), in smoked sago than in the other storage methods from week 2 to week 6 of storage. However, heat differentially influences the survival of microbes (Pflug and Gould, 2000). The initial decrease in numbers of LAB following heat treatment undoubtedly influenced the successional events occurring in the smoked sago. Conclusions made on the basis of changes in numbers are tentative as they are based on an imperfect measurement taken at relatively broad time intervals when considered in terms of a microbe's life events. No information is available from such measures on the changed nature of microbial interspecies dynamics or the altered pattern of metabolic pathways which follow from various storage treatments.

The smoking of sago starch just prior to storage, as replicated in the present experiment, was observed on Misima Island, in the Milne Bay Province. In other parts of PNG where sago samples were collected, sago bundles were often observed

to be stored long-term above the cooking fire. These fires smoulder every day for most of the day, which means that the sago is constantly smoked. This potentially results in a reduction of microbial growth through heat inhibition, reduced a_w and the presence of phenols in the smoke (Davidson, 1997). For logistical reasons the constant smoking of sago starch could not be replicated in the laboratory environment. Given the initial reduction of microbial numbers immediately following the smoking of the sago in this experiment, it is proposed that the continual smoking of sago as a storage method would provide increased food safety from a microbiological perspective.

The storage of sago starch wrapped in leaves, and stored in earthenware pots in which the sago is covered with water, yielded similar numbers of total viable bacteria, LAB and yeasts throughout the experiment. The similar number of LAB suggests that the storage of tightly packed moist sago in leaves is equally as conducive to fermentation as the storage of sago in pots and covered with water. However, the latter method has the advantage of inhibiting mould growth. At the commencement of the experiment, mould numbers were very similar across treatments, as prior to the experiment all sago had been stored using the same method (wrapped in leaves). However mould numbers declined for the following 2 weeks in the water-covered earthenware pots, and at week 2 moulds were undetectable ($\leq 10^2$ cfu/g). Numbers remained low at week 3.

For the following 3 weeks, until week 6, the water evaporated in the earthenware pots, causing the sago to dry out. As a consequence mould numbers increased to be comparable with that of sago wrapped in leaves. In the Sepik region, where sago starch is commonly stored in earthenware pots, sago starch is always kept well watered, with regular changes to the water (see Chapter 3). It would seem that the main advantages of storing sago in an earthenware pot is that it is easy to maintain a moist, anaerobic environment conducive to fermentation but unfavourable to mould growth, and it provides a physical barrier to airborne spores. It is well known that oxygen is usually necessary for fungal growth (Filtenborg *et al.*, 2000), and that most filamentous fungi grow poorly under anaerobic conditions (Pitt and Hocking, 1999). This was demonstrated again in the two-phased experiment in earthenware pots.

Thus, providing water is regularly reapplied to the sago starch, storage in earthenware pots is the storage method of choice for long-term storage of sago.

Statistical analysis of results presented in Chapter 4 demonstrated a relationship between age of sago starch and the presence of bacterial pathogens. Significantly lower numbers of *B. cereus*, *E. coli*, total coliforms and total viable bacteria were isolated in older sago starch than fresher starch, suggesting that the growth and survival of some bacterial pathogens and indicator species is inhibited in sago starch. The seeding of both fresh and old sago starch with bacterial pathogens shows conclusively that bacterial pathogens such as *B. cereus, L. monocytogenes, Salmonella* sp. and *S. aureus* do not survive beyond 1 week in sago starch, with most undergoing a rapid decrease in population numbers. However *E. coli* survives in high numbers in fresh sago starch over the first 10 days of the experiment.

Results of other studies pertaining to the presence and survival of *Bacillus* spp. in fermented foods vary, suggesting a number of factors contribute, not the least being the substrate. Oguntoyinbo and Oni (2004) commonly isolated *B. cereus* from fermented vegetable protein, often in high numbers. Coulin *et al.* (2006), on the other hand, noted a decrease in *Bacillus* spp. as acid accumulated in fermenting cassava. The nature of the substrate is likely to influence the characteristics of the fermentation, thus influencing the development of microbial communities.

The minimum pH for growth of *B. cereus* in culture is 4.5–5.0 (Thomas *et al.*, 1993; Lund and Eklund, 2000). At the commencement of the experiment investigating the survival of bacterial pathogens in fresh sago starch, the pH of the sago starch was 6.60. Despite this value being well within the accepted pH range of *B. cereus*, survival of this organism was poor, suggesting other factors were contributing to the inhibition. In the presence of 0.1 M organic acids (acetate, formate or lactate) the minimum pH for growth of *B. cereus* was found to be between 5.6 and 6.1 (Wong and Chen, 1988). On the basis of results obtained in this study, it seems unlikely that organic acid concentrations reach 0.1 M within 1 week of fermentation (Figure 9.06).

The inhibition of *B. cereus* in sago starch can be attributed to a combination of factors. The sub-optimal pH, together with the presence of organic acids and

competitive constraints due to LAB are likely to be the main factors limiting growth and sporulation (Wong and Chen, 1988; Rossland *et al.*, 2003; Rossland *et al.*, 2005). Other factors that have not been thoroughly investigated in this study, such as hydrogen peroxide and bacteriocin production by LAB, may also contribute to the inhibition of *B. cereus*.

Similar factors are likely to contribute to the inhibition of other bacterial pathogens, including *L. monocytogenes, Salmonella* spp. and *S. aureus*. All of these pathogens are acid tolerant, capable of growth at pH 4.0 or less (Lund and Eklund, 2000). Considerable work has been conducted on the inhibitory effects of bacteriocins on *L. monocytogenes* (Wan *et al.*, 1997; Morgan *et al.*, 2001; Loessner *et al.*, 2003; Yamazaki *et al.*, 2003), however factors such as organic acid production and competition for nutrients have also been shown to inhibit *L. monocytogenes*. (Amezquita and Brashears, 2002; Nilsson *et al.*, 2005). The minimum pH for growth of *Salmonella* sp. is dependant on the acidulant. For example, the minimum pH for growth was 5.40 in the presence of acetic acid (Chung and Goerpfert, 1970). Furthermore, organic acids are said to be more effective bactericidal agents under anaerobic conditions than under aerobic conditions (Jay *et al.*, 2003). *Staphylococcus aureus* is generally considered a poor competitor unless it outnumbers other organisms in the environment (Stewart, 2003). Furthermore, at pH extremes, growth is very slow. As with other bacteria, organic acids are more bactericidal than other acids at a given pH (Baird-Parker, 2000b). Thus, in actively fermenting sago starch one would expect poor survival of such pathogens.

Faecal coliforms and *E. coli* were better able to survive in sago starch than the aforementioned pathogenic bacteria. Survival for approximately 2 weeks was observed in both the pathogen survival in fresh sago experiment (using a laboratory strain of *E. coli*, see Section 9.3.4) and the progressively fermenting sago starch (using naturally occurring endogenous *E. coli*, see Section 9.3.6.2). Interestingly, the laboratory strain of *E. coli* did not survive in previously fermented sago starch (Section 9.3.4). In essence, the use of a laboratory strain and endogenous *E. coli* show a similar trend, that is, the organism survives the early stages of fermentation.

Numerous studies have observed the presence of coliforms in traditionally fermented products. Some studies have shown a rapid rate of decline for coliforms under certain conditions (Nout *et al.*, 1989; Masha *et al.*, 1998; Mugula *et al.*, 2003b), but survival of coliforms has also been documented. Over 4 days of fermentation of 'bushera', a non-alcoholic sorghum beverage, coliform numbers initially increased, followed by progressively decreasing numbers until day 4, when they were no longer detected (Muyanja *et al.*, 2003). Nout and Rombouts (2000) provide examples of Enterobacteriaceae numbers in both sauerkraut and fermented cucumbers, in which the bacteria are present in initially high numbers, and are detectable for the first 10 days of fermentation. Masha (1998) suggested the inhibition of coliforms coincides with the drop in pH to around 4.0. A number of factors contribute to the survival rate of *E. coli* in sago starch, with low pH likely to play a role.

Tsav-Wua *et al*. (2004) noted a considerably lower number of coliforms in laboratory modified fermented cassava flour compared to the traditionally fermented product. Exposure to contamination during processing and poor water quality were suggested as reasons for comparatively high numbers of coliforms in traditionally fermented 'kpor umilin'. The same factors are likely to account for faecal contamination in sago starch (See Chapter 10).

The investigation into the natural microbiota of actively fermenting sago revealed a similar trend to that observed in the pathogen survival experiments. No *B. cereus*, *L. monocytogenes*, *Salmonella* spp. or *S. aureus* were detected, but naturally occurring *E. coli* and faecal coliforms were present in high numbers for the first 12 days. By day 23 numbers of *E. coli* and faecal coliforms were greatly reduced. Throughout the experiment numbers of filamentous moulds remained low. This experiment was conducted in sealed plastic containers. These containers ensured the sago remained moist, providing a favourable environment for fermentation, and provided a physical barrier to the entry of fungal spores, preventing additional colonisation.

Following an initial increase of two log cycles, LAB numbers in fermenting sago remained constant over the following 3 weeks at approximately 10^7 cfu/g. Numbers of LAB were high at the commencement of the experiment (~ 10^5 cfu/g) indicating

that fermentation had commenced, despite the sago starch being refrigerated within hours of completion of the extraction process. Indeed, field observations showed that gas production is evident within 2 hours of packing sago into plastic bags. The production of sago starch is a time consuming process, often taking a whole day. During this time the starch extracted early in proceedings is settling to the bottom of a canoe (or other suitable receptacle), covered in water. The anaerobic conditions, along with the tropical climate, provide an ideal environment for fermentation to commence. The presence of high numbers of yeasts (almost 10^6 cfu/g) and a low initial pH were also indicative of prior fermentation. The pH of sago starch continued to decrease throughout the experiment despite the reasonably stable LAB and yeast populations, to a final value of approximately pH 3.

The enumeration of LAB, and acid analysis, in 12 sago samples collected in the Western Province was also indicative of fermentation. The natural fermentation of sago starch is the likely reason for the poor survival of pathogens in sago starch. Numerous studies have demonstrated the inhibitory effects of LAB on spoilage and food poisoning organisms (Attaie *et al.*, 1987; Ashenafi and Busse, 1989; Harris *et al.*, 1989; Olasupo *et al.*, 1997; Amezquita and Brashears, 2002; Rossland *et al.*, 2003; Inatsu *et al.*, 2004; Rossland *et al.*, 2005). Lactic acid bacteria produce a variety of antimicrobial substances and create conditions unfavourable for the growth of pathogens and spoilage organisms. Due to the numerous modes of inhibition and the synergy of some such modes, it is often difficult to differentiate the effect of one factor from another (Hoover, 2000).

Of the 50 LAB isolated from sago starch that were tested for antimicrobial activity, more than 85% exhibited inhibition of all indicator organisms. The addition of catalase in the MRS agar was used to minimise inhibition caused by hydrogen peroxide. The role of bacteriophages was excluded for all 50 isolates (Section 9.3.7.2). Inhibition of indicator organisms due to bacteriocins production was considered unlikely in the eight isolates tested due to the resistance of the inhibitory substance to three important proteolytic treatments (Section 9.3.7.4).

Nonetheless, the role of bacteriophages and bacteriocins in the inhibition of bacterial pathogens in sago starch cannot be ruled out on the basis of this study alone, with its

limited testing protocol. While bacteriophages have been associated with LAB, they are typically considered an impediment to the fermentation process (Johnson and Steele, 1997; Lu *et al.*, 2003). There is little evidence to suggest that bacteriophages associated with LAB selectively infect or inhibit the growth of pathogenic organisms during fermentation.

In contrast, bacteriocins produced by LAB have been shown to inhibit the growth of various Gram positive bacteria, particularly *L. monocytogenes* (Harris *et al.*, 1989; Lewus *et al.*, 1991; Wan *et al.*, 1997; Morgan *et al.*, 2001; Benkerroum *et al.*, 2002). Using a screening method similar to that used in this study, Coventry *et al.* (1997) detected bacteriocin production in just 0.2% of approximately 6.6×10^5 LAB colonies from dairy and meat sources. Given that only eight LAB isolates were tested for bacteriocin production in this study, it is not surprising that none was positive. Furthermore, LAB strains showing non-selective inhibition were selected for further study. Bacteriocins, by definition, usually act against "closely related" bacteria (Montville and Winkowski, 1997), thus the activity of LAB-derived bacteriocins is generally confined to other Gram positive organisms.

Organic acids, including VFAs, play an important role in inhibiting pathogenic bacteria in sago starch. The ability of organic acids to inhibit the growth of spoilage organisms and pathogens is well documented (Davidson, 1997; Lund and Eklund, 2000). All four LAB isolates tested in pure culture for acid production were capable of producing considerably higher concentration of lactic acid than acetic acid. While such a finding is not surprising in LAB, it is contrary to the observations of acid concentrations in sago starch. In the 12 samples of sago starch analysed, and in the laboratory-fermented sago, acetic acid and butyric acid concentrations were higher than lactic acid concentrations. This suggests that conditions in fermenting sago starch favour acetic acid and butyric acid production over lactic acid production Organisms that produce lactic acid as the sole product of fermentation are termed homofermentative, whereas heterofermentative organisms are able to produce a variety of end products, including acetic acid. However, even some of the homofermentative LAB are facultatively heterofermentative under non-optimal growth conditions (Montville, 1997; Hoover, 2000). No effort was made to

characterise the carbohydrates present in sago starch, but it seems that conditions that favour heterofermentative activity prevail.

In the laboratory-fermented sago starch *E. coli* numbers decreased dramatically between day 12 and day 16. During this time the acetic acid concentration increased two-fold, from approximately 25 mM/kg to over 50 mM/kg, but there was no significant change in pH (3.95 ± 0.05 to 3.79 ± 0.16). Interestingly, in the pathogen survival experiment *E. coli* numbers decreased significantly between days 14 and 21, although the pH remained above 4 (Section 9.3.4). The accepted pH minimum for growth of *E. coli* is approximately 4.0 for non-pathogenic strains (Lund and Eklund, 2000). The increase in acetic acid concentration after approximately two weeks of fermentation, and to a lesser degree the subsequent drop in pH to below 4.0, may inhibit the growth of *E. coli*. The production of comparatively high concentrations of acetic acid in sago starch relative to the other acids analysed undoubtedly influence the growth and survival of other organisms also. Acetic acid has been shown to effectively inhibit the growth of a variety of pathogenic bacteria, including *Bacillus* spp., *Clostridium* spp., *L. monocytogenes*, *Salmonella* spp. and *S. aureus*. Indeed, very few groups of bacteria are known to tolerate acetic acid (Davidson, 1997). However, in the case of sago starch the role of other acids, such as butyric acid, should not be discounted.

The rapid decrease in LAB numbers between day 23 and day 30 was unexpected, but might be explained by the rapid increase in butyric acid at the same time. Inhibition of coliform bacteria by butyric acid has been demonstrated at concentrations of between 5-11 mM (Wolin, 1969; Sun *et al.*, 1998). Information pertaining to the effect of high concentrations of butyric acid on LAB is scarce, although a high tolerance is probable. Nonetheless, concentrations of 100 mM/kg, as was the case on day 30 of the experiment, could be sufficient to inhibit LAB.

It is difficult to determine the role the individual acids play in inhibition of different groups of organisms. Ultimately, the cumulative production of all acids results in a decrease in pH, which is likely to be one of the most significant factors in pathogen inhibition. A correlation between starch pH and log total acid in the sago samples tested, and in the laboratory-fermented sago starch, was to be expected due to the

direct relationship between -log [H] and pH. Indeed, in both instances the correlation was very strong, which suggests that no other acids (additional to those analysed) significantly influence sago starch pH.

This study has made apparent the need for further research into the fermentation of sago starch. To date no attempt has been made to identify the major fermentative organisms. Moreover, the exact role that yeasts play in the fermentation of sago starch has not been addressed thus far. The analysis of acids in sago starch has addressed macro-phenomena, but the conditions prevailing in micro-niches and their influence on pathogen survival need to be determined. A thorough study of LAB for bacteriocin production, and a greater understanding of the role of the various acids present in sago starch, is warranted.

Sago starch fermentation is spontaneous, with minimal input and intervention from humans beyond appropriate storage. Further study could contribute to improved storage duration, and increased food safety and food security for consumers of sago starch. Until a greater understanding of the fermentation of sago starch is gained, storage methods that minimise airborne contamination and favour rapid, thorough and extended fermentation should be encouraged. Furthermore, allowing the sago to ferment for approximately one week prior to consumption will greatly reduce the risk of foodborne illness associated with bacterial pathogens. Further fermentation prior to consumption would decrease the risk associated with faecal contamination and pathogenic *E. coli*, however the extended storage time could result in increased fungal contamination. These findings, along with other findings of the study, will be used to form recommendations for safe sago starch production and storage in the following chapter.

CHAPTER 10: HACCP ANALYSIS OF SAGO STARCH AS A FOOD

10.1 Introduction

The HACCP system was originally developed for NASA to provide astronauts with microbiologically safe foods on space missions (Jouve, 2000). The underlying principle behind HACCP is that by understanding how a food product becomes unsafe, measures can be developed to prevent or detect shortcomings, thus circumventing contaminated foods reaching the consumer. The system was first presented to the scientific community in 1971, but initial lack of description resulted in slow uptake of the system in the food industry (Bernard, 1997). The formation of the National Advisory Committee on Microbiological Criteria for Foods (NACMCF) in 1988 has been largely responsible for the further development of HACCP. The numerous publications since the inception of NACMCF are indicative of the continued modifications and simplifications being made to facilitate the increased relevance and successful uptake of the HACCP system (NACMCF, 1992; NACMCF, 1994; NACMCF, 1998).

The NACMCF describes HACCP as a "systematic approach to the identification, assessment of risk and severity, and control of the microbiological, chemical and physical hazards associated with each segment of the food system from production to consumption" (NACMCF, 1994). The reasonably recent and specific origins of HACCP, along with the needs of the main sponsoring agencies, dictate that the system is most commonly applied in modern, industrialised food processing plants. Nonetheless, HACCP is applicable to all sections of the food industry ranging from basic agriculture through to processing and consumer use (Bernard, 1997). The system has been applied to home and small-scale food production, and in recent years to food production in the developing world (WHO, 1993; Ehiri, 1995; Moy *et al.*, 1997; Ehiri and Prowse, 1999; WHO, 1999; FAO, 2001; Motarjemi, 2002).

The importance of sago starch as a staple food in areas of PNG has been well established (Chapters 2 and 3). Given the occurrence of SHD and the high incidence of gastrointestinal illness in PNG (Chapter 2), application of HACCP to the production and storage process is warranted. The identification of critical control points (CCPs) will lead to the improved safety of sago starch as a staple food source in rural PNG. The HACCP analysis will be based largely on the findings of the sociological survey (Chapter 3), along with findings from the other research chapters of this thesis (Chapters 4-9). In making this analysis, every effort will be made to ensure outcomes are relevant and applicable to sago starch consumption in the context of the rural communities where sago is consumed most commonly.

10.2 Materials and Methods

Hazard analysis and critical control point application consists of seven principles. Despite minor discrepancies in the wording, the fundamentals of the seven principles remain relatively uniform. The fundamentals of these seven principles are as follows (NACMCF, 1992; NACMCF, 1998):

1. Conduct a hazard analysis. Prepare a list of steps in the process where significant hazards occur and describe the preventative measures;
2. Identify the critical control points in the process;
3. Establish critical limits for the preventative measures associated with each identified critical control point;
4. Establish critical control point monitoring requirements. Establish procedures for using the results of monitoring to adjust the process and maintain control;
5. Establish corrective action to be taken when monitoring indicates that there is a deviation from the established critical limit;
6. Establish effective record keeping procedures that document the HACCP system;
7. Establish procedures for the verification that the HACCP system is working correctly.

The International Commission on Microbiological Specifications for Foods (ICMSF) recognised two types of CCPs: CCP 1 to control a hazard, and CCP 2 for hazard minimisation (ICMSF, 1986). Although this differentiation of CCPs has not been adopted by the NACMCF, the principle is still advocated by some authors (Forsythe, 2000; Jay *et al.*, 2005), and lends itself to application in the developing world where absolute control of hazards might not be feasible.

The NACMCF outlines numerous steps that should be followed prior to commencement of HACCP analysis. These steps include assembling an appropriate HACCP team, describing the food, identifying the intended use of the food, and the development and verification of a flow diagram to describe the process. The reader is referred to these documents for further detail of the initial steps, and the seven principles of HACCP (NACMCF, 1992; NACMCF, 1998).

The requisite preliminary steps and principles of HACCP were applied to sago starch production and storage as applicable.

10.3 Results

10.3.1 Preliminary steps prior to HACCP analysis

10.3.1.1 Description of food and intended use

Sago starch *per se* and its role as a staple dietary carbohydrate has been described in detail elsewhere (Chapter 2). In brief, sago starch is extracted from the sago palm, *M. sagu*. The flour is very high in carbohydrates, but generally low in vitamins and minerals. The starch is removed from the macerated pith of the sago palm using water, resulting in moist starch that ferments naturally during storage. Sago starch is stored in a variety of receptacles, including clay pots, woven bags and baskets made of bush materials, tree leaves (commonly sago leaves but various other leaves can be used), plastic containers and second hand plastic bags. The starch is generally stored until it is all consumed, which in most households is less than one month, but can be

two to three months. It is always stored at ambient temperature, and is generally not well protected from airborne contamination or vermin.

Dependency on sago starch is greatest in lowland and coastal areas of PNG. In many of the lowland areas of the country, where it is difficult to grow other carbohydrate crops, the dependency on sago starch is very high, and it may form the basis of every meal consumed. In general, everyone above weaning age consumes sago, including the young and the elderly. Given the health indicators of PNG (Section 2.3), it is likely that many immunocompromised individuals eat sago starch.

10.3.1.2 Flow diagram of sago production and storage

A simplified flow diagram describing the process of starch production and storage is shown below (Figure 10.01). For further details of the starch production and storage process refer to Section 2.1.2.

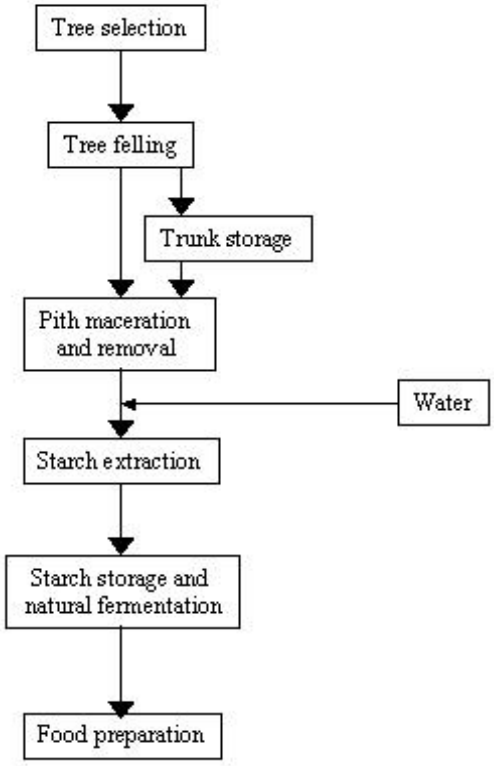


Figure 10.01: Flow diagram illustrating the major steps in the sago extraction and storage process in rural PNG.

10.3.2 Application of HACCP

10.3.2.1 Hazard analysis, control measures and critical control points

With the exception of tree felling, where no food safety hazards were identified, potential hazards were identified, appropriate control measures determined and CCPs established for each step in the sago making process (Table 10.01). Many of the CCPs identified could not be activated in the environment in which the work was conducted. Consequently, CCPs were characterised as notional or practicable, based on their suitability to application in village-based production of sago starch in PNG. Notional CCPs were identified in all six steps where hazards were identified, namely tree selection, trunk storage, pith maceration and removal, starch extraction, starch storage and fermentation, and food preparation. A total of six practicable CCPs were identified, occurring in all of the aforementioned stages of the process except starch extraction. Two of the six practicable CCPs were in the storage and fermentation stage, suggesting that this stage is particularly important in determining the food safety of sago starch. All CCPs identified were a CCP 2.

The basis of selection of CCPs that could be implemented realistically in rural PNG (the practicable CCPs) will be addressed in detail in Section 10.4.

Table 10.01: Initial steps of HACCP application: identification of steps involved in the process, the associated hazards, appropriate control measures and corresponding CCPs.

Step	Hazards	Control measures	CCPs Notional	CCPs Practicable
Tree selection	(a) Growth of palm in contaminated soil: faecal & chemical	Minimise contamination through adequate sanitation, appropriate waste disposal & sound environmental practises of industry; select the tree for processing in a location that has not been recently contaminated		CCP 2 (#1)
Tree felling	No hazards identified			
Trunk storage	(a) Enteric pathogens (trunks in water) (b) Environmental & zoonotic pathogens (trunks on land)	(a) Improved sanitation (b) Do not store trunks for long duration; minimise animal access to sago logs	CCP 2	 CCP 2 (#2)
Pith maceration and removal	(a) Environmental pathogens (b) Pathogens of human origin (c) Inoculation of macerated pith with dirty utensils	(a) Minimise contact of sago pith with soil (b) Wash hands with soap & water; minimise body contact with pith (c) Thoroughly clean utensils prior to use	CCP 2 CCP 2	 CCP 2 (#3)
Starch extraction	(a) Contaminated water (b) Environmental pathogens (c) Pathogens of human origin	(a) Use clean water: minimal faecal contamination (b) Minimise contact of pith/starch with soil; use clean receptacles (c) Wash hands with soap & water; minimise body contact with pith/starch	CCP 2 CCP 2 CCP 2	
Starch storage and fermentation	(a) Survival & multiplication of pathogens (b) Contamination via air- & soil- borne fungal spores (c) Exposure to vermin (insects, rodents) (d) Compromised storage conditions with time resulting in fungal growth	(a) Use clean water (if used); minimise environmental contamination; rapid and prolonged fermentation (b) Storage under airtight conditions away from main sources of contamination (c) Storage under conditions unfavourable to infestation (d) Rapid and prolonged fermentation; limit storage duration	CCP 2 CCP 2	 CCP 2 (#4) CCP 2 (#5) CCP 2 (#5) CCP 2 (#4)
Food preparation	(a) Survival of pathogens in sago (b) Re-contamination by handling, utensils, environment (c) Growth of pathogens in cooked sago if not consumed within 4 hrs	(a) Adequate cooking (b) Wash hands and use clean utensils (c) Consume sago soon after cooking	 CCP 2 CCP 2	CCP 2 (#6)

10.3.2.2 Critical limits, monitoring procedures and corrective actions

Following the determination of the critical control points, critical limits, monitoring procedures and corrective actions were established for all practicable CCPs (Table 10.02). The establishment of verification and documentation procedures was beyond the practical extent of the HACCP analysis of village based sago production and storage process.

Table 10.02: Critical limits, monitoring procedures and corrective actions for practicable CCPs of the sago production and storage process.

Practicable CCP	**Critical limits**	**Monitoring procedures**	**Corrective action**
#1: Tree selection	No evidence of human or animal faecal deposits; no evidence of recent sago extraction activity nearby	Observation	Select another tree
#2: Trunk storage on land (dry)	Limit dry trunk storage duration to no longer than 2 days	Time keeping	Discard if visible fungal contamination
#3: Clean utensils	No residual sago pith in recess of macerating utensils; utensils must be free of dirt, grime, etc.	Observation	Re-clean prior to use
#4: Fermentation	Storage conditions favouring fermentation: anaerobic, moisture retaining; fermentation for 1 week to decrease incidence of bacterial pathogens; storage for no longer than 5 weeks to limit fungal growth	Observation Odour Time keeping	Thoroughly cook & consume immediately if fermentation ceases
#5: Airtight storage	Use of clay pots, well sealed clean plastic bags or other air-tight containers for storage; do not store in close proximity of potential sources of contamination	Observation Odour	Discard portions that are visibly contaminated with fungi
#6 Cooking	Time, temperature "Until thoroughly cooked"	Tactile Visual inspection	Keep cooking

10.4 Discussion

The goal of HACCP in food production in the developing world is ultimately the same as that of the developed world, to improve food safety. However, an increased level of pragmatism is required when applying such a system in the developing world. In this analysis, 17 CCPs were identified, of which only 6 were deemed capable of implementation. All of the CCPs were considered to be CCP 2, being more likely to minimise a hazard than to control it. The establishment of critical limits was based, where applicable, on research findings described in previous chapters. Only those control/minimisation measures that were deemed likely to be adhered to under the current social and economic climate of rural and remote PNG were nominated as CCPs of significance in the short term. These will be discussed.

Various control measures need to be employed in the first step of sago starch processing. As discussed in Chapter 3, disposal of human waste is usually by way of pit toilets in villages. However, when people are making sago away from their village, bush disposal is the norm. When selecting an appropriate tree for processing, the area should be checked for evidence of recent faecal contamination, be it of human or other animal origin. If faecal matter is present the preferable course of action would be to select a different palm. When bush disposal of human faecal waste is necessary, it should occur as far away from the felled tree as possible. Guidelines for campers in Australia state such activity should occur a minimum distance of 100 m from camping sites and water (anonymous, ; anonymous). A similar distance from sago making sites would be appropriate. Ideally, disposal should occur in the vicinity of immature palms, thus decreasing the likelihood of contaminating an area to be used for sago processing in the near future. In terms of sago starch processing, it is important that individuals maintain, as much as possible, appropriate levels of personal hygiene consistent with food preparation.

The majority of hazards associated with sago starch consumption are microbiological in nature, however chemical contamination is also a consideration. There is much consternation in areas of the Western Province over possible contamination from upstream industry. In the past, ecological damage has been attributed to activities Ok Tedi Mining Limited (Hyndman, 1988). Heavy metal analysis of just three samples

of sago starch, one of which was implicated in an outbreak of SHD, revealed no detrimental impact on sago starch (See Appendix 1.2). It is difficult to say conclusively that heavy metal toxicity is not a health issue for people in the region on the basis of just three samples, however it seems unlikely that it contributes directly to SHD.

The second CCP identified in the processing of sago starch is in the trunk storage step. The majority of respondents to the sociology survey store trunks on an occasional basis (Section 3.3.1.3), but the practice is by no means obligatory. It generally occurs when people do not have enough time to process the entire tree in one day. Trunks are stored in water (rivers, lakes, lagoons, etc) or on land. Although no data are currently available, it seems likely that the storage of the logs in water might encourage fermentation, thus preserving the sago starch within the log. However, thorough fermentation may not ensue, and there is a risk of contamination with faecal pathogens. Improved sanitation would decrease the risk of such contamination. As improved sanitation is outside the scope of this study, the storage of trunks in water was deemed a notional CCP, and will not be addressed in further detail.

Due to aerobic conditions and exposure to air-borne spores, fungal contamination of trunks stored on land is likely, as is contamination from environmental and enteric bacterial pathogens. Sago processors are to be encouraged to complete the maceration and extraction of starch from trunks on the day of tree felling whenever possible. On occasions when this cannot be done, the processing should be completed the following day. By reducing the duration of trunk storage, there is a decreased likelihood of microbial contaminants reaching high numbers, and decreased opportunity for animals to access the trunk.

The sociological survey (Chapter 3) revealed that in the Western Province, where SHD has been more frequently observed, 45% of respondents never cleaned utensils used for pith maceration. In the East Sepik Province, 22% of respondents never cleaned their utensils. In addition to these figures, numerous respondents in both provinces only occasionally cleaned equipment, and the extent of cleaning was often rudimentary. Moreover, adzes and similar equipment were often observed during

sample collection expeditions that had not been cleaned between usage, and supported visible fungal growth. Growth and survival of bacteria on extraction tools is also likely. Thus, there is the potential for inoculation of fresh sago with moulds and undesirable bacteria unless remnants of sago pith are removed and the tools rinsed after use. Sago producers should be encouraged to ensure all the equipment they use has been washed (CCP 2 [#3]).

Two practicable CCPs were identified in the starch storage and fermentation step of sago processing, namely rapid and prolonged fermentation and storage under appropriate conditions. Both can be applied to address two separate hazards.

The inhibition of selected bacterial pathogens in fermenting sago starch was demonstrated in Chapter 9. Survival of *Salmonella* sp., *B. cereus*, *S. aureus* and *L. monocytogenes* was much reduced by day 7. On this basis it might be prudent to allow sago starch to ferment for one week to reduce the likelihood of gastroenteritis. Active fermentation is likely to also inhibit the growth of filamentous fungi in sago starch. Although no direct studies investigating the survival of filamentous fungi in sago starch have been conducted, the anaerobic conditions encountered have been hypothesised as a contributing factor to their low numbers in fresh, fermenting sago starch (Chapter 5). While the influence of oxygen availability varies between fungal species, Pitt and Hocking (1997) stated that most episodes of food spoilage attributable to filamentous fungi occur under aerobic conditions. Moreover, some studies have demonstrated decreased mycotoxin production at low oxygen concentrations (Landers *et al.*, 1967; Keller *et al.*, 1997; Watanabe *et al.*, 2004), thus even if filamentous fungi are present in actively fermenting anaerobic/microaerophilic sago starch, the risk to health may be decreased. Statistical analysis demonstrated higher fungal numbers and ergosterol levels in sago starch five weeks or older compared to starch less than one week or one to three weeks old (Chapter 5). Given the possibility that filamentous fungi play a role in SHD, and the isolation of citrinin producing fungal strains from sago starch (Chapter 6), the consumption of starch over five weeks old is to be discouraged.

In cases where there is a need to store sago starch for long periods (over one month), storage under conditions of reduced oxygen tension is likely to have two main

beneficial effects. First, it will favour the rapid fermentation of sago starch, thus reducing the likelihood of survival of bacterial pathogens. Secondly, it will greatly decrease access of spores to the sago starch, thus reducing the level of fungal (and to a lesser degree, bacterial) contamination. Previous studies demonstrated reduced numbers of filamentous fungi in sago stored in earthenware (clay) pots, relative to smoked sago or sago wrapped in leaves, particularly under moist conditions after two to three weeks of storage (Section 9.3.4). Although densely packed sago starch wrapped in leaves has a comparable rate of fermentative activity to that of starch stored in pots (based on LAB numbers, Section 9.3.4), it seemed evident that filamentous fungi germinated in the matrix of the leaf covering, penetrated into the sago mass and flourished and sporulated there.

The use of clay pots and other water tight containers as storage vessels for sago starch is common practice in the Sepik region, but none of the respondents of the sociological survey in the Western Province reported their use. As such, the introduction of clay pots and similar vessels into the Western Province might not be embraced, especially if coupled with the increased manual burden of making them or financial burden of purchasing them. The advantage of clay pots over other large watertight containers is yet to be determined, but is likely to be minimal. The porous nature of clay might create a desirable microaerophilic environment, rather than an anaerobic environment (as is likely to be the case in modern synthetic non-porous containers). Additionally, the porous nature might facilitate evaporative cooling of the container, reducing the temperature of the stored sago slightly. Whether these factors have any effect, desirable or otherwise, on microbial communities is unknown at this stage.

On the basis of current knowledge, it is recommended that for long-term storage sago starch be stored in airtight, watertight containers with a lid. Indeed, buckets and large cooking pots were commonly recorded as methods of storage in the East Sepik Province (Section 3.3.1.4), although a small number of respondents stated that sago did not keep well in pots (data not shown). Given the relative affordability of plastic buckets, their use as long-term storage containers might be encouraged throughout sago eating areas of PNG. Until further studies are completed on the use of non-porous, manufactured storage vessels, it is suggested that enough water be added to

the sago to submerge the starch, and the water replaced with fresh water regularly (every one to two days), as is done in the Sepik region for sago starch stored in clay pots. If lids are fitted loosely to buckets and pots it should minimise airborne contamination, but allow some oxygen exchange, as is likely to occur through clay pots.

Safe, long-term storage of sago starch is of particular importance in times of drought. In this eventuality, when there is insufficient water to process sago starch, only a small volume of water is required to maintain good fermentation and freshness of stored sago starch relative to extraction of new starch. During times of drought, when fresh running water is in scarce supply, the use of boiled (and subsequently cooled) water to maintain moist conditions in long-term stored sago is recommended.

In the absence of watertight containers, strong, clean plastic bags might be suitable for long-term sago storage. This possibility warrants further investigation. Currently plastic bags are often used for sago storage, but are typically filled to the point that there is a large surface area of sago starch exposed to the environment at the opening of the bag. If bags were filled to only two thirds full, they could be sealed to greatly reduce airborne contamination. Large ‘zip-lock’ plastic bags would be ideal for this sort of storage, but currently they are not widely available in rural areas of PNG. It is not feasible to keep the starch submerged in water when stored in a plastic bag, but water could be added as required to maintain a moist environment that favours fermentation. A further advantage of plastic containers and sealed plastic bags for sago storage is that they provide a barrier to soilborne contamination and vermin. If future studies reveal that microaerophilic conditions are desirable for sago fermentation, storage in plastic bags could be modified appropriately.

The use of tree leaves to wrap sago starch (sago bundles) is widespread in PNG, particularly in the Western Province. The likelihood of fungal contamination is considered greatest when bundles are partially used. Conditions become more aerobic, contamination from humans is more likely, and the barrier that the tightly wrapped leaves provide to vermin, airborne and soilborne microbes is compromised. Although long-term storage of sago starch wrapped in tree leaves is not recommended, it is recognised that it will continue to occur in the foreseeable future.

If sago starch wrapped in leaves is stored for long durations, it should be stored away from direct sunlight, as this will dry the sago starch and make conditions less favourable for fermentation. Consumers should be encouraged to keep the bundle intact for as long as possible, then to eat the starch as quickly as possible once opened.

The use of woven bags and baskets for sago storage that allow contamination with spores and encourage aerobic conditions should be discouraged. Furthermore, to decrease the likelihood of environmental contamination sago starch should not be stored in direct contact with the ground. When pervious storage vessels are used, sago should be stored hanging from a wall or ceiling.

Thorough cooking of sago starch acts as the final CCP. Cooking greatly decreases microbial numbers, thereby reducing the likelihood of gastroenteritis. However, some pathogenic bacteria are known to produce heat stable toxins (Nataro and Kaper, 1998; Balaban and Rasooly, 2000; Granum and Baird-Parker, 2000). Furthermore, many of the known mycotoxins are heat stable, with cases of human mycotoxicosis following consumption of heat treated foods documented (Bhat *et al.*, 1989; Lye *et al.*, 1995). Although there is no evidence to suggest that any of the common mycotoxins are frequently present in sago starch (Chapter 6), the possibility of novel mycotoxins in sago starch cannot be discounted (Chapter 8).

Two species of bacteria that are known to produce heat stable toxins, namely *B. cereus* (Granum and Baird-Parker, 2000) and *S. aureus* (Balaban and Rasooly, 2000), have been isolated from sago starch (Chapter 4). In addition, outbreaks of SHD follow the consumption of cooked sago, suggesting a heat stable toxin is involved in the aetiology of the disease. Consequently, cooking sago starch can only minimise the risk of foodborne illness, not control it. As such, the cooking process is a CCP 2, albeit a very important one.

Sago starch is rarely stored after cooking in PNG, although precooked sago is sometime sold in markets as a ‘fast food’. Where possible sago should be consumed immediately following cooking to decrease the likelihood of re-contamination.

By just concentrating on the control measures in this proposed HACCP plan that were deemed practicable, rather than all identified CCPs, there remain steps in the process whereby contamination can occur. Two important examples of hazards that could be controlled in many other circumstances are the use of non-potable water in the sago extraction step and lack of attention to hand washing.

In the rural environment where this study was conducted, there is no possibility of obtaining totally safe water supplies due to the lack of infrastructure. As previously stated, only 40% of the population in PNG have access to safe drinking water (PNG Ministry of Health, 2000). One would expect the majority of those people live in urban centres, in which case considerably less than 40% of the rural population would have access to safe water. Consequently, in most villages potable water is in short supply, if present at all. Under current rural living conditions it would be unsustainable to use safe drinking water for starch extraction. As such, people making sago are encouraged to obtain their water as far from sources of sewage or animal contamination as possible. Where possible the water source should be free flowing and clear. Similarly, the lack of clean water combined with very infrequent use of soap, largely due to low incomes, means thorough hand washing is unlikely to occur in the current socio-economic climate.

It is envisaged that future education will function to reduce contamination levels in sago starch. More informed site selection should reduce contamination as a result of recent human and animal activity and inform on the best location in which to dig temporary wells. Knowledge of microbial contamination processes will allow those engaged in extraction to be more careful about possible routes of microbial contamination. Subsequent steps in the process, namely fermentation and cooking, should reduce the likelihood of illness to acceptable levels.

In making such recommendations, the benefit(s) of environmental contamination must be kept in mind. It is through environmental contamination that the organisms responsible for the fermentation process are likely to originate. More research is required pertaining to the fermentation of sago starch, including the source of inoculum, and the worthiness of “backslopping”, the process of inoculating freshly extracted sago starch with a small quantity of fermenting starch. This technique is

used in various other indigenous fermented foods (Nout *et al.*, 1989; Nout, 1991; Masha *et al.*, 1998).

The development of a HACCP plan is a constantly evolving process. As knowledge of the aetiology of SHD increases, and further work on the fermentation of sago starch is conducted, there may be a need to modify the plan. Moreover, with time it is hoped that there will be improved socio-economic conditions in rural areas of PNG, resulting in greater access to clean water and improved personal hygiene, coupled with appropriate education. Such an outcome would also lead to the requirement of a revised and modified HACCP plan. In the interim, one must not become too quixotic in the search for solutions. The strategy adopted here is to reduce the overall levels of contamination in the various stages of sago starch production and preparation, thus making food as safe as possible. Such a strategy is based on sound food hygiene principles (Baird-Parker, 2000a). Nonetheless, in developing world scenarios such as rural PNG, it should not occur at the expense of food availability.

This HACCP plan will form the basis of educational pamphlets and posters that will be distributed in sago consuming areas of PNG. The aim of the educational material is to raise awareness of safer production practices. It is important that sago consumers do not become alarmed by the perceived dangers of sago consumption, as this could ultimately lead to a decrease in consumption, putting further pressure on food supply in a country with a highly regional and rapidly increasing population.

CHAPTER 11: GENERAL DISCUSSION

Food security continues to be a problem worldwide, particularly in developing countries. Papua New Guinea is no exception, with general health and nutritional statistics painting a bleak picture. Child health indicators and socio-economic conditions dictate that the importance of adequate food supply is greatest in areas of the country heavily dependent on sago starch (Mueller and Smith, 1999; Mueller, 2001; Mueller *et al.*, 2001; Temu and Saweri, 2001).

Compounding the problem of adequate food security and nutrition in rural lowland areas of PNG is the issue of food safety, and in particular the occurrence of SHD. Despite the best efforts of a select few researchers, the disease remains an enigma. The paucity of knowledge pertaining to the illness can be attributed to two main factors. First, logistical constraints in lowland rural PNG make disease surveillance and aetiological studies very difficult. Throughout the course of this study only two samples of sago starch associated with outbreaks of SHD have been obtained, and in both instances the samples did not arrive in Australia for some weeks after the occurrence of the disease. As such, meaningful microbiological studies of implicated samples are difficult to conduct, and must be interpreted with caution. Secondly, documented cases of SHD are sporadic and infrequent (particularly in the years leading up to this study). Moreover, access to medical assistance in lowland PNG is limited, particularly in the Western Province. As well as having serious implications for patients suffering the illness, lack of medical assistance makes it difficult to collect relevant epidemiological data.

In addition to SHD, the incidence of gastrointestinal illness in PNG, as with other developing countries, is high (WHO, 2004). While it is recognised that provision of safe drinking water is of the utmost importance in decreasing the incidence of gastrointestinal illness, foods are also a key factor (see Chapter 1). Basic principles of food microbiology suggest that foods produced at the village level, where access to safe water required for food processing is limited and the importance of personal hygiene in food production not widely known, are a likely cause of mild foodborne illness.

On account of the long held belief that mycotoxins might play a role in the aetiology of SHD (Taufa, 1974; Donovan *et al.*, 1977), and a need for improved food safety and food security in rural and remote PNG, a broad study of the microbiology of sago starch was undertaken, with the aim of improving the safety of sago starch as a food source.

The presence of common bacterial foodborne pathogens suggests that sago starch is a likely cause of mild foodborne illness, although the regular practice of thorough cooking substantially decreases the health risk. Of greatest public health concern is the evidence of high levels of faecal contamination in sago starch (Chapter 4). Sociological data and field observations (Chapter 3) suggest that much of this contamination originates from the water used during extraction of the sago starch from the pith of the palm. Although clean water sources are often difficult to obtain in areas of sago extraction, such a finding highlights the need for sago producers to consider carefully their water source for starch extraction.

Results of the mycological survey and ergosterol analysis were indicative of universal fungal contamination of sago starch (Chapter 5). From a food safety and food spoilage perspective, the presence of filamentous fungi is of greater pertinence than the presence of yeasts. The presence of filamentous fungi in sago starch may lead to the starch being discarded — a practice that should not be discouraged but may at times contribute to food security problems — and could lead to the presence of mycotoxins in the starch. The high prevalence of *Penicillium* spp. in sago samples considered suitable for human consumption, and in samples associated with SHD, is noteworthy. Various experts have made the observation that the aspergilli are more commonly associated with commodity spoilage/mycotoxin production in warm sub-tropical and tropical environments, and penicillia often associated with cool temperate climates (Hocking, 1997; Moss, 2000; Hocking and Pitt, 2003). The frequent isolation of *Penicillium* spp. from sago starch in PNG is contrary to such a generalization.

The recurrent isolation of penicillia from sago starch is also of importance due to the toxigenic potential of the genus. The genus *Penicillium* produces a broader range of

mycotoxins than any other genus (Pitt, 1997). While this study found scant evidence of common mycotoxins contaminating sago starch, many *Penicillium* isolates were demonstrated to be capable of citrinin production in pure culture, along with a single *Aspergillus* isolate. Citrinin has been proposed as a possible potentiating agent in other mycotoxicoses (Section 2.5.2.5), and such a role should not be discounted in SHD until more is known about the aetiology of the disease. Also of interest is the recent suggestion by Lura and co-workers that citrinin might cause mild haemolytic anaemia in mice (Lura *et al.*, 2004).

Despite the absence of common mycotoxins in sago starch *per se*, one cannot conclude that mycotoxins do not contribute to SHD. History suggests that many mycotoxins have been discovered following investigations of diseases of unknown aetiology. Other microbial toxins have also been identified and characterised only following disease outbreaks. Thus, an investigation of microbial isolates from sago starch for novel toxic metabolites was justified. Such a task is a monumental one, particularly in the absence of food samples that have been associated with illness, as was the case throughout most of this study. Consequently, the current investigation was based on the assumption that a compound producing *in vivo* haemolysis (as is the case in SHD) would be haemolytic *in vitro*. While it is recognised that such an assumption does not hold true in many episodes of acute intravascular haemolysis of known aetiology, and indeed very few microbial haemolysins have been associated with acute intravascular haemolysis (see Section 2.6), a sound method of searching for potentially toxic compounds capable of causing SHD was required.

This study found sago starch to be a reservoir of haemolytic organisms. By concentrating on the haemolytic activity of filamentous fungi (Chapter 8) the study found numerous fungal genera to be haemolytic *in vitro*. Those isolates demonstrating pronounced haemolytic activity, which were commonly isolated from sago starch, warrant further investigation. Many such isolates belong to the genus *Penicillium*, a well known mycotoxigenic genus that was present in the two samples of implicated sago starch, as well as approximately 60% of sago samples 'fit for consumption'. Other genera of fungi, such as *Aspergillus*, *Fusarium* and *Trichoderma*, along with selected bacterial isolates, are also of some interest as a possible aetiological agent of SHD.

The haemolytic assay developed in this study is readily applicable to activity driven fractionation, which will enable active compound(s) to be separated and ultimately identified in future studies. When the active fraction can be appropriately purified *in vitro* studies into the haemolytic mode of action on human erythrocytes should be conducted to help characterise the compound(s), and more importantly provide an insight into the aetiology of the disease. The use of an animal model to determine whether the compound(s) can elucidate symptoms consistent with SHD would also be of great value.

Determination of the aetiological agent of SHD was not achieved in this study, however the broad nature of the work ensured recommendations aimed at improving the microbial safety of sago starch could be made. Such recommendations were based largely on the information garnered in the sociological study (Chapter 3), a sound understanding of the microbial ecology of sago starch (Chapter 9), and the application of a HACCP plan (Chapter 10). Of particular interest is the finding that sago starch is a naturally fermented product. The fermentation of sago starch is probably the single most important factor influencing the development of microbial communities, and thus food safety of the product. Active fermentation was shown to inhibit the growth and survival of selected bacterial pathogens, although the survival of both laboratory and naturally occurring strains of *E. coli* in sago starch is of some public health concern. Another benefit of actively fermenting sago starch is that conditions favourable for fermentation are generally not favourable for the growth of filamentous fungi. Although the level of risk that filamentous fungi constitute to sago consumers can not be definitively stated on the basis of the current study, storage methods and durations that moderate or prevent the growth of such organisms should still be recommended.

The findings of this research suggest that outbreaks of SHD continue to occur despite there being no reports of the illness in the literature since the 1970s. Hospital records and personal communications provide evidence of numerous outbreaks in the past five years, with the severity of the illness, a lack of adequate treatment and logistical constraints within lowland PNG combining to ensure a high mortality rate. Accordingly, there remains a desperate need for further studies into the aetiology of

the illness, and further preventative measures that can be adopted. Such research, along with much needed agronomic studies, will ensure that sago starch continues to be an ecologically sustainable, economically viable and culturally valuable food source in lowland PNG.

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APPENDIX 1: SOCIOLOGICAL SURVEY

Appendix 1.1 Example of Sociology Survey

An example of the survey used to gain knowledge of the sociological aspects of sago production and consumption in provided on pages 304–313.

Sociology Questionnaire

The purpose of this document is to collect information on the production and storage of sago starch in various regions of Papua New Guinea. By collating this data it is hoped that the best techniques for storage and preparation can be determined. In many cases, some of the questions asked may not be applicable to a given village.

A. IDENTIFIERS

Date:___/___/04 Sample Number:_____ Age of sample:_____

Village:_____District:_____

Province:_____

B. SAGO CULTIVATION AND MANAGEMENT

1. How many types of sago palms are found in this area? _____

2. Do you plant sago suckers and harvest them, or let the sago palms grow naturally?

☐ Plant suckers ☐ naturally

3.Who is responsible for planting sago palms?

a) Males b) Females c) Both males and females

4. Management of sago strands:

4.1 Who is responsible for clearing?

a) Males b) Females c) Both males and females

4.2 Who is responsible for cutting new growth?

a) Males b) Females c) Both males and females

5. Are either of the following tasks considered unenjoyable and difficult?

☐ Palm felling ☐ Starch extraction and processing

6. Explain how knowledge and skills of sago planting and management get passed on to younger generations?

……
……
……..

7. Explain how skills of extracting sago are passed on from older to younger people.

……
……
……

C. HARVESTING

8. What time of year do you usually cut down the sago palm and produce sago starch?

☐ Wet season ☐ Dry season ☐ As required

9. How do people know when a palm is ready for harvest?

☐ Before flowering has begun ☐ during flowering ☐ After flowers die

10. The sago palm is cut down by:

☐ Males ☐ Females ☐ Both males and females

11. After cutting the sago palm down, do you store the sago logs before processing the sago?

☐ Yes ☐ No (Go to Question 14)

12. After felling the sago palm, how long do you store the sago log before extracting the starch:

☐ 1 day ☐ 2 days ☐ 3 days ☐ 4 days ☐ 5 days

☐ Other If other, state how long ……

13. If the logs are stored before the sago is processed, what method is used:

☐ Underwater ☐ Dry ☐ Other

If other, please specify how logs are stored
……
……
……

14. Do you have to transport the log from where it is cut down to where it is processed?

☐ Yes ☐ No

15. Where does the water used in the sago making process come from?

☐ River ☐ Lagoon ☐ Sago swamp ☐ Lake

☐ Rainwater ☐ Bore water

16. How far away from the village is the sago processed?

☐ At or near village ☐ At or near camping place ☐ At or near sago swamp

17. How is human waste disposed of?

☐ Pit toilets ☐ Bush disposal ☐ toilets over water ☐ Other

If other, please specify
……..……

18. How far from the sago processing site is the human waste disposed of?

☐ <100 m ☐ 100-500 m ☐ > 500 m ☐ assessor's estimate

19. Do any animals have access to the sago starch while it settles during processing?

☐ Pigs ☐ Poultry ☐ Other animals

20. The sago extraction process is done by:

☐ Male children	☐ Single men	☐ Fathers	☐ Grandfathers
☐ Female children	☐ Single females	☐ Mothers	☐ Grandmothers

21. What colour is the sago starch usually at time of processing?

☐ Red	☐ White	☐ Lime	☐ Black	☐ Orange

☐ Other If other, state what colour it is …….

22. In the starch making process, how is the pith extracted:

☐ Rasp	☐ milled	☐ hand sticks	☐ sago beater

23. Is the equipment used cleaned afterward:

☐ Yes ☐ No (Go to Question 25)

24. If the equipment is cleaned, state what method or methods of cleaning are used (mark all that are applicable)?

Excess sago waste and dirt removed by hand	
Excess sago waste and dirt removed by rag/cloth	
Equipment washed in water used during the sago processing	
Equipment washed in fresh water	
Other cleaning methods (please specify)	

D. DRYING AND STORAGE

25. When the starch has settled during processing, how soon after is the starch dried and stored?

☐ As soon as Possible ☐ Later on the same day ☐ The next day

26. Is the sago starch dried before storage?

☐ Yes ☐ No (Go to Question 31)

27. If the sago is dried, how long is the starch left to dry before being stored?

☐ < 1 day ☐ 1 day ☐ 2 days ☐ > 2 days

28. How is the sago dried?

☐ Sun ☐ Solar drier ☐ Smoked ☐ Other

If other, please specify ……..

29. How is the sago starch stored?

☐ Wrapped in leaves ☐ In plastic ☐ Underwater ☐ Underground

☐ With additives ☐ Other

29.1 If other, please specify how the sago is stored

……...……..……...
……
……
……

30. If sago starch is stored with additives, please describe what is used as an additive and what affect it has.

……
……
……
……
……

31 If the sago starch is stored underwater, what is it stored in?

☐ Earthen vessel ☐ wrapped in leaves ☐ In plastic ☐ Other

32. If leaves are used to store sago in, what sort of leaves are used?

☐ Sago leaves ☐ Banana leaves ☐ Pandanus leaves ☐ Fern leaves

☐ Other If other, please give name and brief description of the plant

……
……
……

33. Is sago consumed immediately after processing?

☐ Yes ☐ No

34. If the sago is stored, how long is it usually stored for?

☐ < 2 weeks ☐ 2-4 weeks ☐ > 4 weeks

35. Do you ever throw away old sago, or do you keep it until it is all eaten?

☐ Thrown out ☐ All eaten

36. What characteristics do you use to determine whether the sago is fit to eat (mark all that are applicable. Or none if not applicable)?

☐ Colour ☐ Smell ☐ Taste ☐ Texture

☐ Other If other, please specify ……

37. If bad sago is identified by colour change, what colour does the sago become?

☐ Grey ☐ Off white ☐ Green ☐ Black ☐ Pink

☐ Other If other, state colour…….

E. PREPARATION AND CONSUMPTION

38. Sago starch is typically fed to which age groups (mark all boxes that are applicable)

☐ Breastfed infants ☐ 2-5 years ☐ 5-60 years ☐ Elderly (grandparent)

39. How significant is sago as a food source?

☐ Staple food ☐ Minor food ☐ Ceremonial only

☐ Other Specify……..

40. How is sago starch usually prepared before eating?

	Method 1	Method 2	Method 3
Boiled			
Wrapped			
Frying pan			
Earth oven			
Other			
Name given			

41. Has there been any change in the amount of sago consumed?

☐ Yes ☐ No

Explain:

……

……

……

42. Have there been any known or suspected cases of illness resulting in the consumption of sago?

☐ Yes ☐ No

42.1 If yes, what is the severity of the illness?

☐ mild, e.g stomach cramps ☐ moderate e.g vomiting, diarrhoea ☐ severe e.g hospital or health centre visit

F. SOCIAL AND CULTURAL SIGNIFICANCE OF SAGO

43. Access and control of sago stands
(Fill in the appropriate response)

Resource	Access (Males, Females or Both)	Control/ Ownership (Males, Females or Both)
Land		
Swamps and water ways		
Sago land		
Trees and tree resources		
Sago stands		
Processed sago		

44. Who inherits sago stands?

☐ Males ☐ Females ☐ Both

45. Does sago have any cultural significance in your society?

☐ Yes ☐ No

If yes explain

……

……

……

46. Does sago consumption play a role in ceremonial feasts?

☐ Yes ☐ No

If yes, explain:

……

……

……

47. Does your society have any myths and beliefs related to the way sago is processed, stored and prepared for consumption?

☐ Yes ☐ No

If yes, explain:

……

……

48. Problems of sago cultivation, management, processing and storage

Problem	Existence		If yes explain details of problem
	Yes	No	
Planting			
Management			
Distance to sago lands			
Felling and Processing			
Quality of processed sago			
Storage			
Consumption			
Other issues			

49. Any comments that you may have are welcome.

Appendix 1.2 Metal Analysis of Sago Starch

Metal analysis was conducted on one sample implicated in SHD, and also on the two samples that were 'fit for consumption'. Metal analysis was done by Dr. Yi Hu at the Advanced Analytical Centre, James Cook University, Townsville.

Table A1.01: Metal analysis of three samples of sago starch: W0605-01 (from Suki, implicated in an outbreak of SHD), W0405-09 (from Ali, one month old), and W0405-13 (from Balimo, fresh).

	Concentration (mg/kg)		
Element	**W0605-01**	**W0405-09**	**W0405-13**
aluminium	456 + 7	1.18 + 0.02	3.70 + 0.09
antimony	< 0.0262	< 0.0181	< 0.0195
arsenic	< 0.524	< 0.362	< 0.391
barium	1.79 + 0.03	0.393 + 0.014	0.774 + 0.035
beryllium	< 0.0524	< 0.0362	< 0.0391
cadmium	< 0.0262	< 0.0181	< 0.0195
chromium	0.561 + 0.020	0.168 + 0.010	0.228 + 0.116
cobalt	< 0.0524	< 0.0362	< 0.0391
copper	1.32 + 0.02	0.114 + 0.005	0.527 + 0.016
iron	204 + 4	4.84 + 0.22	5.81 + 0.51
lead	0.068 + 0.0026	0.304 + 0.006	< 0.0195
manganese	21.3 + 0.5	5.36 + 0.04	3.43 + 0.09
mercury	< 0.262	< 0.181	< 0.195
molybdenum	0.497 + 0.013	0.286 + 0.005	0.352 + 0.012
nickel	0.275 + 0.005	< 0.0362	< 0.0391
selenium	< 0.524	< 0.362	< 0.391
silver	< 0.0262	< 0.0181	< 0.0195
thallium	< 0.0262	< 0.0181	< 0.0195
zinc	5.64 + 0.11	< 1.81	< 1.95

APPENDIX 2: MEDIA AND REAGENTS

Unless otherwise stated, chemicals for all the media listed are available from one or both of the following suppliers:

- Crown Scientific, Townsville, Qld
- Sigma Aldrich Pty Ltd, Castle Hill, N.S.W.

Appendix 2.1 Bacterial Isolation and Confirmation Media and Reagents

Appendix 2.1.1 Commonly used media and reagents

2.1.1.1 Diluent

Bacteriological peptone (Oxoid Ltd., Thebarton, S.A.)	1 g
Distilled water	1 l

Dissolve peptone in water and adjust to pH 7.0 ± 0.1.
Autoclave at 121 ^{o}C for 15 min, then aliquot required volume.

2.1.1.2 Gram stain

Crystal violet solution
Grams iodine
Safranin

Prepare slide and allow to air dry. Fix in 95% methanol for 1 min. Apply alcoholic crystal violet for 1 min, and rinse off with iodine solution. Allow iodine solution to remain on slide for 1 min. Gently rinse with tap water and counterstain with safranin for 20 sec. Air dry and view under oil immersion at 1,000 × magnification.

2.1.1.3 Nitrogen free agar

Mannitol	10 g
K_2HPO_4	0.8 g
KH_2PO_4	0.2 g
$MgSO_4.7H_2O$	0.5 g
$FeCl_3.6H_2O$	0.1 g
$Na_2MoO_4.2H_20$	0.005 g
Agar	15 g
Distilled water	1 l

Combine all ingredients and dissolve. Adjust to pH 6.7 ± 0.1.
Sterilise by autoclaving at 121 ºC for 15 min.

Maltose or galactose can be used in place of mannitol.

2.1.1.4 Sheep blood agar

Blood agar base (Oxoid Ltd., Thebarton, S.A.)	40 g
sheep blood	50 ml
Distilled water	1 l

Dissolve agar base in distilled water.
Sterilise by autoclaving at 121 ºC for 15 min. Allow agar to cool to approximately 50 ºC, aseptically add the blood, mix and pour into Petri plates.

2.1.1.5 Catalase reagent

Hydrogen peroxide	1 ml
Distilled water	9 ml

Store at 4 ºC protected from light for up to 2 weeks

2.1.1.6 Oxidase reagent

NNNN'-tetramethyl-p-phenylenedamine	0.1 g
Ascorbic acid	0.01 g
Distilled water	10 ml

Reagent must be stored protected from light. Make fresh reagent daily.

2.1.1.7 Brain heart infusion broth (BHIB)

BHIB base (Oxoid Ltd., Thebarton, S.A.)	37 g
Distilled water	1 l

Dissolve broth base in distilled water. Dispense into appropriate container and sterilise by autoclaving at 121 ºC for 15 min.

2.1.1.8 Tryptone soy agar (TSA)

TSA base (Oxoid Ltd., Thebarton, S.A.)	40 g
Distilled water	1 l

Dissolve broth base in distilled water. Dispense into appropriate container and sterilise by autoclaving at 121 ºC for 15 min.

2.1.1.9 Standard plate count agar

Standard plate count agar base (Oxoid Ltd., Thebarton, S.A.)	23.5 g
Distilled water	1 l

Dissolve agar base in distilled water and adjust to pH 7.0 (± 0.2). Dispense into 100 ml aliquots and sterilise by autoclaving at 121 ºC for 15 min. Allow to cool in water bath to ~ 45 ºC prior to use.

2.1.1.10 Physiologic saline

NaCl	8.5 g
Distilled water	1 l

Dissolve salt in distilled water. Adjust to pH 7.0 ± 0.2. Sterilise by autoclaving at 121 ºC for 15 min.

2.1.1.11 Nutrient agar

Nutrient agar base (Oxoid Ltd., Thebarton, S.A.)	28 g
Distilled water	1 l

Dissolve agar in distilled water. Adjust to pH 7.4 ± 0.2. Sterilise by autoclaving at 121 ºC for 15 min.

2.1.1.12 Nutrient broth

Nutrient broth base (Oxoid Ltd., Thebarton, S.A.)	13 g
Distilled water	1 l

Dissolve broth base in distilled water. Adjust to pH 7.4 ± 0.2. Dispense into appropriate volumes and sterilise by autoclaving at 121 ºC for 15 min.

2.1.1.13 Phosphate Buffered Saline (PBS)

10 × PBS	
NaCl	80 g
KH_2PO_4	2 g
Na_2HPO_4	11.5 g
dH_2O	1 l

Dissolve ingredients and adjust to pH 7.2. Sterilise by autoclaving at 121 ºC for 15 min. Dilute 100 ml of 10 × buffer in 900 ml of sterile distilled water for working concentration.

Appendix 2.1.2 *Bacillus cereus* isolation media and confirmation stain

2.1.2.1 Polymyxin pyruvate egg-yolk mannitol bromothymol blue agar (PEMBA)

PEMBA agar base (Oxoid Ltd., Thebarton, S.A.)	20.5 g
Egg yolk emulsion (Oxoid Ltd., Thebarton, S.A.)	25 ml
Polymyxin B sulphate (Oxoid Ltd., Thebarton, S.A.)	50000 IU
Distilled water	475 ml

Suspend PEMBA base in distilled water and adjust to pH 7.2 ± 0.2. Place in water bath to dissolve. Autoclave at 121 ºC for 15 mins, then cool to approximately 50 ºC in a waterbath. Aseptically add polymyxin B sulphate and egg yolk emulsion. Mix and dispense into Petri plates.

2.1.2.2 Tryptone soy polymyxin broth

Tryptone soy broth (Oxoid Ltd., Thebarton, S.A.)	30 g
Polymyxin B sulphate (Oxoid Ltd., Thebarton, S.A.)	50 000 IU
Distilled water	1 l

Dissolve tryptone soy broth in distilled water and adjust to pH 7.3 ± 0.2. Autoclave at 121 ºC for 15 mins. Dispense in 9 ml aliquots.
Dissolve polymyxin B sulphate in 3.3 ml sterile distilled water. Before use add 0.1 ml polymyxin B sulphate solution.

2.1.2.3 Holbrook and Anderson spore stain

5 % aqueous malachite green
0.3 % sudan black in 70 % ethanol
0.5 % safranin in 20 % ethanol

Prepare a fixed slide of 24-48 hour culture and flood with malachite green. Heat the slide until it steams. Do not allow stain to boil or slide to dry. Allow stain to heat for at least 2 min, then gently wash slide and blot dry. Flood slide with sudan black and stain for 15 min. Wash the slide with xylene for 5 seconds and blot dry. Counterstain with safranin for 20 seconds, wash, dry and observe under 1,000 × magnification.

Appendix 2.1.3 *Clostridium* spp. isolation and confirmation media

2.1.3.1 Cooked meat media

Cooked meat medium (Oxoid Ltd., Thebarton, S.A.)	1 g
Neomycin sulphate (Sigma Aldrich Pty Ltd, Castle Hill, NSW)	1.43 mg
Distilled water	10 ml

Weigh 1 g of cooked meat pellets into glass universal bottles. Add 10 ml of distilled water to each. Adjust to pH 7.2 ± 0.2. Autoclave at 115 ºC for 15 min.
Allow to cool, then add 0.25 ml of 5.7 g/l stock solution of filter sterilised neomycin sulphate dissolved in water.
Use media immediately after it has cooled, or boil in water bath for 15 min then allow to cool immediately prior to use to remove dissolved oxygen.

2.1.3.2 Tryptose sulphite cycloserine (TSC) agar

Perfringens agar base (Oxoid Ltd, Thebarton, S.A.)	23 g
D-cycloserine (Oxoid Ltd, Thebarton, S.A.)	
Egg yolk emulsion (Oxoid Ltd, Thebarton, S.A.)	25 ml
Distilled water	500 ml

Suspend perfringens agar base in distilled water and adjust to pH 7.6 ± 0.2. Place in water bath to dissolve. Autoclave at 121 ^{o}C for 15 mins, then cool to approximately 50 ºC in a waterbath. Add 2 ml of sterile distilled water to D-cycloserine. Aseptically add D-cycloserine and egg yolk emulsion. Mix and dispense into Petri plates.

2.1.3.3 Saccharolytic clostridia agar

1 M KH_2PO_4 (pH 7.5)	30 ml
1 M $MgSO_4$	1 ml
Solution M	0.5 ml
0.2 M $FeSO_4$ in 0.1 M H_2SO_4	0.2 ml
Tryptone (Oxoid Ltd, Thebarton, S.A.)	10 g
Yeast Extract (Oxoid Ltd, Thebarton, S.A.)	6 g
Sodium thioglycolate	0.5 g
Glucose	16 g
Agar	15 g
Distilled water	970 ml

Solution M	
$MnCl_2$	10 mM
$CaCl_2$	30 mM
$CoCl_2$	5 mM
Na_2MoO_4	5 mM

Dissolve all ingredients and adjust to pH 7.0 ± 0.2.
Autoclave at 121 ºC for 15 min.
Cool and pour into Petri plates.

2.1.3.4 Lactose gelatine medium

Tryptose (Oxoid Ltd., Thebarton, S.A.)	15 g
Yeast extract (Oxoid Ltd., Thebarton, S.A.)	10 g
Lactose	10
Na_2HPO_4	5 g
Phenol red	0.005 g
Gelatin	120 g
Distilled water	1 l

Suspend all ingredients except lactose and phenol red in distilled water. Adjust to pH 7.5 (± 0.2), and dissolve in boiling water bath. Add lactose and phenol red, then dispense in 10 ml aliquots in universal bottles.
Sterilise by autoclaving at 121 ºC for 15 min

2.1.3.5 Nitrate motility medium

Lab-lemco	3 g
Peptone (Oxoid Ltd., Thebarton, S.A.)	5 g
K nitrate	5 g
Na_2HPO_4	2.5 g
Galactose	5 g
Glycerol	5 g
Agar	3 g
Distilled water	1 l

Suspend all ingredients in distilled water and adjust to pH 7.3 (± 0.2). Dissolve in boiling water bath. Dispense in 10 ml aliquots in universal bottles. Sterilise by autoclaving at 121 ºC for 15 min

Appendix 2.1.4 *Enterobacteriacea*e isolation and confirmation media

2.1.4.1 Lauryl tryptose broth

Lauryl tryptose broth (Oxoid Ltd., Thebarton, S.A.)	35.6 g
Distilled water	1 l

Dissolve LT broth in distilled water and allow to dissolve. Adjust to pH 6.8 ± 0.2, dispense 9 ml volumes into universal bottle with inverted Durham tube.
Autoclave at 121 ºC for 15 min.

2.1.4.2 Eosin methylene blue agar

Eosin methylene blue agar base (Oxoid Ltd., Thebarton, S.A.)	37.5 g
Distilled water	1 l

Dissolve EMB base in water and allow to dissolve. Adjust to pH 6.8 ± 0.2. Autoclave at 121 ºC for 15 min. Allow to cool to approximately 60 ºC and shake thoroughly to oxygenate the media. Dispense into Petri plates in laminar flow cabinet.

2.1.4.3 EC broth

EC broth (Oxoid Ltd., Thebarton, S.A.)	37 g
Distilled water	1 l

Dissolve EC broth in distilled water and allow to dissolve. Adjust to pH 6.9 ± 0.2, dispense 9 ml volumes into universal bottle with inverted Durham tube. Autoclave at 121 ºC for 15 min.

2.1.4.4 Tryptone water

Tryptone (Oxoid Ltd., Thebarton, S.A.)	20 g
NaCl	5 g
distilled water	1 l

Dissolve tryptone and NaCl in distilled water and allow to dissolve. Adjust to pH 7.0 (± 0.2) and dispense 9 ml volumes into universal bottles.
Autoclave at 121 º C for 15 min.

2.1.4.5 Kovac's solution

Para-dimethyl-aminobenzaldehyde	5 g
Amyl alcohol	75 ml
Conc. HCL	25 ml

Place para-dimethyl-aminobenzaldehyde in a suitable bottle wrapped in foil. Gradually add amyl alcohol/HCL and dissolve.

2.1.4.6 Citrate slopes

Simmons citrate agar base (BBL, Becton Dickinson, North Ryde, NSW)	24.2 g
Distilled water	1 l

Dissolve agar base in distilled water and adjust to pH 6.9 (± 0.2). Dispense into Bijoux bottles. Sterilise by autoclaving at 121 ºC for 15 min and set as slopes.

2.1.4.7 Urease slopes

Urea agar base (Oxoid Ltd., Thebarton, S.A.)	24 g
Urea	20 g
Distilled water	1 l

Dissolve agar base in 950 ml distilled water and adjust to pH 6.8 (± 0.2). Sterilise by autoclaving at 115 ºC for 15 min. Dissolve urea in 50 ml distilled water, filter sterilise and add to agar. Aseptically dispense into Bijoux bottles and set as a slope.

2.1.4.8 Buffered peptone water (BPW)

BPW base (Oxoid Ltd., Thebarton, S.A.)	20 g
Distilled water	1 l

Dissolve BPW base in distilled water. Adjust to pH 7.2 (± 0.2). Autoclave at 121 ºC for 15 min.

2.1.4.9 Mannitol selenite cystine (MSC) enrichment broth

Selenite (mannitol) broth base (Oxoid Ltd., Thebarton, S.A.)	19 g
Selenite (Oxoid Ltd., Thebarton, S.A.)	4 g
Cystine	100 mg
Distilled water	1 l

Dissolve selenite in distilled water. Add the broth base and warm to dissolve. Adjust pH to 7.1 (± 0.2). Aseptically dispense 10 ml into sterile universal bottles. Sterilise by placing in boiling water bath for 10 min.

Prepare stock solution of L-cystine by dissolving 100 mg in 15 ml of 1M NaOH and make up to 100 ml with sterile distilled water.

Add 0.1 ml of L-cystine stock solution to each 10 ml broth immediately prior to use.

2.1.4.10 Rappaport-Vassiliadis (RV) enrichment broth

RV broth base (Oxoid Ltd., Thebarton, S.A.)	30 g
Distilled water	1 l

Dissolve RV broth in distilled water and heat gently to dissolve. Dispense 10 ml aliquots into universal bottles. Autoclave at 115 ºC for 15 min.

2.1.4.11 Xylose lysine desoxycholate (XLD) agar

XLD agar base (Oxoid Ltd., Thebarton, S.A.)	53 g
Distilled water	1 l

Add agar base to distilled water and adjust to pH 7.4 (± 0.2). Place in boiling water bath, agitate frequently and boil until dissolved. Do not autoclave. Allow to cool, mix thoroughly and poor into Petri plates in laminar flow cabinet.

2.1.4.12 Bismuth sulphite agar (BSA)

BSA base (Oxoid Ltd., Thebarton, S.A.)	20 g
Distilled water	500 ml

Dissolve agar base in distilled water and adjust to pH 7.6 (± 0.2). Place in boiling water bath, agitate frequently and boil for 1 min. Do not autoclave. Allow to cool, mix thoroughly and poor thickly (~20 ml) into Petri plates in laminar flow cabinet. Store in dark for a maximum of 1 day.

2.1.4.13 Lysine decarboxylase broth

Peptone	5 g
Yeast extract	3 g
Glucose	1 g

Bromcresol purple (0.2 % solution)	10 ml
L-lysine	5 g
Distilled water	1 l

Dissolve peptone, yeast extract and glucose in 950 ml of distilled water. Adjust to pH 6.7 (± 0.1). Add indicator and sterilise by autoclaving at 115 ºC for 20 min. Dissolve L-lysine in 50 ml of distilled water and filter sterilise. Add to decarboxylase broth and dispense into sterile Bijoux bottles, filling to capacity.

2.1.4.14 Ornithine (ONPG) broth

Peptone water

Peptone	10 g
NaCl	5 g
Distilled water	1 l

Dissolve ingredients and adjust to pH 7.5 (± 0.2). Dispense into 1.5 ml aliquots and sterilise by autoclaving at 121 ºC for 15 min.

ONPG stock

o-nitrophenyl-β-D-galacto-pyranoside	0.6 g
Na_2HPO_4	0.142 g
Distilled water	100 ml

Dissolve ingredients and adjust to pH 7.5 (± 0.2). Filter sterilise. Store in refrigerator in the dark.

ONPG broth

Aseptically add 0.5 ml or ONPG broth to peptone water prior to use

2.1.4.15 Violet red bile lactose (VRBL) agar

VRBL agar base (Oxoid Ltd., Thebarton, S.A.)	38 g
Distilled water	1 l

Suspend agar base in distilled water and adjust to pH 7.4 (± 0.2). Dissolve in boiling water bath and allow to boil for 2 min. Do not autoclave.
Pour into Petri plates in laminar flow cabinet.

2.1.4.16 α-Glucosidase reagent

PNP- α-glucosidase	0.05 g
PBS	10 ml

Dissolve PNP- α-glucosidase in PBS. Adjust pH to7.0.

2.1.4.17 Sorbitol fermentation agar

Tryptone	10 g
Yeast extract	3 g
Bromocresol purple	0.01 g
Sorbitol	10 g
Agar	12 g

Distilled water	1 l

Dissolve ingredients and adjust to pH 7.4 (± 0.2). Dispense into 10 ml aliquots in screw-capped test tube and sterilise by autoclaving at 121 ºC for 15 min.

Appendix 2.1.5 *Listeria* isolation and confirmation media

2.1.5.1 Half Fraser broth

Fraser broth base (Oxoid Ltd., Thebarton, S.A.)	14.35 g
Half strength Fraser supplement (Oxoid Ltd., Thebarton, S.A.)	1 vial
Distilled water	225 ml

Dissolve broth base in distilled water. Sterilise by autoclaving at 121 ºC for 15 min. Just prior to use resuspend I vial of supplement in 4 ml 50% ethanol and add to broth.

2.1.5.2 Full Fraser broth

Fraser broth base (Oxoid Ltd., Thebarton, S.A.)	28.7 g
Full strength Fraser supplement (Oxoid Ltd., Thebarton, S.A.)	1 vial
Distilled water	500 ml

Dissolve broth base in distilled water. Sterilise by autoclaving at 121 ºC for 15 min. Just prior to use resuspend I vial of supplement in 5 ml 50% ethanol and add to broth. Aliquot 10 ml into universal bottles.

2.1.5.3 Oxford agar (Listeria selective agar Oxford formulation)

Oxford agar base (Oxoid Ltd., Thebarton, S.A.)	27.75 g
Oxford selective supplement (Oxoid Ltd., Thebarton, S.A.)	1 vial
Distilled water	500 ml

Dissolve agar base in distilled water and adjust to pH 7.0 (± 0.2).
Sterilise by autoclaving at 121 ºC for 15 min. Cool to ~ 50 ºC. Resuspend the contents of the selective supplement with 5 ml of 50% ethanol. Aseptically add to the agar, mix and pour into Petri plates in laminar flow cabinet.

2.1.5.4 PALCAM agar

PALCAM agar base (Oxoid Ltd., Thebarton, S.A.)	34.5 g
PALCAM selective supplement (Oxoid Ltd., Thebarton, S.A.)	1 vial
Distilled water	500 ml

Dissolve agar base in distilled water and adjust to pH 7.2 (± 0.2).
Sterilise by autoclaving at 121 ºC for 15 min. Cool to ~ 50 ºC. Resuspend the contents of the selective supplement with 2 ml sterile distilled water. Aseptically add to the agar, mix and pour into Petri plates in laminar flow cabinet.

Appendix 2.1.6 *Staphylococcus* isolation media

2.1.6.1 Baird-Parker agar

Baird-Parker agar base (Oxoid Ltd., Thebarton, S.A.)	63 g
egg yolk tellurite emulsion (Oxoid Ltd., Thebarton, S.A.)	50 ml
distilled water	1 l

Dissolve agar base in distilled water and adjust to pH 6.8 (± 0.2).
Sterilise by autoclaving at 121 ºC for 15 min. Cool to ~ 50 ºC.
Aseptically add 50 ml egg yolk tellurite emulsion and mix. Pour into Petri plates in laminar flow cabinet.

2.1.6.2 Rabbit plasma (coagulase test)

Sterile rabbit plasma (bioMériuex Australia Pty Ltd, Baulkham Hills, N.S.W.)

Resuspend according to manufacturers instructions.

Add equal volumes of a 24 hour broth culture to a 1/5 dilution of rabbit plasma. Incubate at 37 ºC, checking for coagulation hourly for four hours, then after 24 hours.

Appendix 2.2 Fungal Isolation and Confirmation Media

Appendix 2.2.1 Isolation media

2.2.1.1 Dichloran rose-bengal chloramphenicol (DRBC) agar

DRBC agar base (Oxoid Ltd., Thebarton, S.A.)	31.5 g
Chloramphenicol	100 mg
Distilled water	1 l

Dissolve agar base in distilled water and adjust to pH 5.6 (± 0.2).
Dissolve chloramphenicol in 1 ml acetone and add to agar.
Sterilise by autoclaving at 121 ºC for 15 min. Cool to ~ 50 ºC. Pour into Petri plates in laminar flow cabinet.

2.2.1.2 Potato carrot agar

Sliced potato	20 g
Sliced carrot	20 g
Chloramphenicol	100 mg
Agar	15 g
Tap water	1 l

Add potato and carrot to approximately 200 ml of tap water. Bring to the boil and allow to simmer for 2 min. Strain liquid (discard solids) then make up to 1 L with additional tap water. Add agar and dissolve in boiling water bath.
Dissolve chloramphenicol in 1 ml of acetone and add to agar.

Sterilise by autoclaving at 121 ºC for 15 min. Cool to ~ 50 ºC. Pour into Petri plates in laminar flow cabinet.

2.2.1.3 Czapek yeast autolysate (CYA) agar

K_2HPO_4	1.0 g
Czapek concentrate	10 ml
Yeast autolysate or extract	5 g
Sucrose	30 g
Agar	15 g
Distilled water	1 l

Combine all ingredients except sucrose and dissolve by boiling. Add sucrose and sterilise by autoclaving at 121 ºC for 15 min. Cool to ~ 50 ºC. Pour into Petri plates in laminar flow cabinet.

Czapek concentrate

$NaNO_3$	30 g
KCL	5 g
$MgSO_4.7H_2O$	5 g
$FeSO_4.7H_2O$	0.1 g
Distilled water	100 ml

2.2.1.4 Malt extract agar (MEA)

Malt extract	20 g
Peptone	1 g
Glucose	20 g
Agar	15 g
Distilled water	1 l

Combine all ingredients except glucose and dissolve by boiling. Add glucose and sterilise by autoclaving at 121 ºC for 15 min. Cool to ~ 50 ºC. Pour into Petri plates in laminar flow cabinet.

2.2.1.5 Glycerol nitrate (GN25) agar

K_2HPO_4	0.75 g
Czapek concentrate (See Appendix 2.2.1.3)	7.5 ml
Yeast autolysate or extract	3.7 g
Glycerol, analytical grade	250 g
Agar	12 g
Distilled water	750 ml

Combine all ingredients and dissolve by boiling. Sterilise by autoclaving at 121 ºC for 15 min. Cool to ~ 50 ºC. Pour into Petri plates in laminar flow cabinet.

2.2.1.6 Synthetic Mucor agar

Glucose	40 g
Asparagine	2 g
KH_2PO_4	0.5 g

$MgSO_4$	0.25 g
Thiamine	0.5 mg
Chloramphenicol	100 mg
Agar	15 g
Distilled water	1 L

Combine ingredients and dissolve in boiling water bath. Dissolve chloramphenicol in 1 ml of acetone and add to agar.
Sterilise by autoclaving at 121 ºC for 15 min. Cool to ~ 50 ºC. Pour into Petri plates in laminar flow cabinet.

2.2.1.7 *Geotrichum* selective agar

Potato dextrose agar (Oxoid Ltd., Thebarton, S.A.)	39 g
Lactic acid	1.25 ml
Thiobendazole	8 g
Iprodione	1.0 ml
Rose bengal	0.05 g
Chloramphenicol	100 mg
Distilled water	1 l

Adjust to approximately pH 8 before autoclaving. Aseptically pH after autoclaving to pH 6.6

Iprodione in the form of Roval (250 g/L). Final concentration of iprodione in medium 250 ppm.

2.2.1.8 Actinomycete agar

Asparagine	0.50 g
K_2HPO_4	0.50 g
Glucose	10 g
Agar	15 g
Distilled water	1 l

Combine all ingredients except glucose and dissolve by boiling. Add glucose and sterilise by autoclaving at 121 ºC for 15 min. Cool to ~ 50 ºC. Pour into Petri plates in laminar flow cabinet.

2.2.1.9 One-quarter strength Czapek Dox agar

$NaNO_3$	0.75 g
K_2HPO_4	0.25 g
$MgSO_4.7H_2O$	0.125 g
KCl	0.125 g
$FeSO_4.7H_2O$	0.0025 g
Sucrose	7.5 g
Agar	15 g
Distilled water	1 l

Combine all ingredients except sucrose. Dissolve in boiling water bath. Add sucrose then sterilise by autoclaving at 121 ºC for 15 min. Cool to ~ 50 ºC. Pour into Petri plates.

Appendix 2.2.2 Reagents for ergosterol analysis

2.2.2.1 Saponification solution

Dissolve 5 g potassium hydroxide in 25 ml ethanol and 35 ml methanol

2.2.2.2 Ergosterol standard

Prepare stock standard of 0.5mg/ml of ergosterol in ethanol. Prepare working standard of 25µg/ml by evaporating 100µl of stock solution to dryness using nitrogen and dissolving in 2ml hexane. Standards should be refrigerated, and kept for no longer than two weeks.

Appendix 2.3 Reagents Used for Visualisation and Confirmation of Mycotoxins

Appendix 2.3.1 Visualisation and confirmation reagents

2.3.1.1 Alcoholic bicarbonate solution

$NaHCO_3$	6.0 g
Alcohol (methanol or ethanol)	20 ml
Distilled water	100 ml

Combine and dissolve.

2.3.1.2 Ehrlich's solution

p-dimethylaminobenzaldehyde	0.3 g
5 M HCl	10 ml

Combine and dissolve.

2.3.1.3 Anisaldehyde solution

p-anisaldehyde	0.5 ml
Concentrated sulphuric acid	5 ml
Glacial acetic acid	85 ml

Combine ingredients.

Appendix 2.4 Media and Reagents for Haemolytic Analysis of Microorganisms

Appendix 2.4.1 Growth media

2.4.1.1 Human blood agar

Blood agar base (Oxoid Ltd., Thebarton, SA)	40 g
Human blood	50 ml
Distilled water	1 l

Dissolve agar base in distilled water.
Sterilise by autoclaving at 121 ºC for 15 min. Allow agar to cool to approximately 50 ºC, aseptically add the blood, mix and pour into Petri plates.

2.4.1.2 Blood agar with chloramphenicol

To the appropriate blood agar (human blood agar, as above, or sheep blood agar, Appendix 2.1.1.4) add 100 mg/l of chloramphenicol (Sigma Aldrich Pty Ltd, Castle Hill, NSW) dissolved in 1 ml of 50:50 acetone-water prior to autoclaving.

2.4.1.3 Blood agar with glucose

Make blood agar with chloramphenicol as above, but dissolve ingredients in 950 ml. After sterilisation allow to cool to 50 ºC, then add 30 g filter sterilised glucose dissolved in 50 ml distilled water.

2.4.1.4 Modified nutrient broth

Proteose peptone	15 g
Liver digest	2.5 g
Yeast extract	5 g
Sodium chloride	5 g
Distilled water	1 l

Dissolve ingredients in distilled water and dispense in appropriate volumes.
Sterilise by autoclaving at 121 ºC for 15 min.

2.4.1.5 Sago agar for secondary metabolite production

Difco sago agar

Yeast extract (Difco: BioScientific, Gymea, NSW)	7 g
Sago starch	50 g
$MgSO_4.7H_2O$	0.5 g
$CuSO_4$	0.005 g
$ZnSO_4$	0.001g
Agar	15 g
Distilled water	1 l

Dissolve all ingredients except sago starch in distilled water and adjust to pH 6.5 (± 0.2). Add dried sago starch and sterilise by autoclaving at 121 ºC for 15 min.

Sigma sago agar

As above, but the yeast extract listed above is replaced with:

Yeast extract (Sigma Aldrich, Castle Hill, NSW)	7 g

2.4.1.6 Grain media

Selected grain (either cracked corn, rice or wheat)	20 g
Sterile distilled water	15 ml

Place grain in a 100 ml conical flask and add 10 ml of distilled water. Stopper with a cottonwool plug and cover plug with aluminium foil. Sterilise by autoclaving at 121 ºC for 15 min. After sterilisation aseptically add remaining 5 ml of sterile water to the grain and mix.

Up-scaled grain media

Selected grain (either cracked corn, rice or wheat)	100 g
Sterile distilled water	60 ml

Place grain in a 500 ml conical flask and add 40 ml of distilled water. Stopper with a cottonwool plug and cover plug with aluminium foil. Sterilise by autoclaving at 121 ºC for 15 min. After sterilisation aseptically add remaining 20 ml of sterile water to the grain and mix.

Appendix 2.4.2 Extraction solutions

2.4.2.1 Toxin diluent solution (TDS)

10 × *toxin diluent solution*

100 mM Tris, pH7.2	12.1 g
1.5 M NaCl	87.68 g
100 mM $MgCl_2$	9.52 g
100 mM $CaCl_2$	11.1 g
Distilled water	1 l

Dissolve ingredients in distilled water and adjust to pH 7.0. Sterilise by autoclaving at 121ºC for 15 min. For working concentration TDS, dissolve 2 g of gelatin in 900 ml of distilled water and autoclave for 121 ºC for 15 min. Aseptically add 100 ml of 10 × TDS and mix.

2.4.2.2 Alcoholic toxin diluent solution

Dissolve 2 g of gelatin in 200 ml of distilled water and autoclave for 121 ºC for 15 min. Aseptically add 700 ml of methanol and 100 ml of 10 × TDS and mix.

Appendix 2.5 Media and Reagents Used in Microbial Ecology Investigations

Appendix 2.5.1 Media used in nitrogen fixation studies

2.5.1.1 Endophyte (EN) medium

Solution A

K_2HPO_4	0.8 g
KH_2PO_4	0.2 g
NaCl	0.1 g
$Na_2MoO_4.2\ H_20$	0.025 g
FeNaEDTA	0.028 g
Yeast extract	0.1 g
Mannitol	3.0 g
Sucrose	5.0 g
Lactic acid	0.24 g
Malic acid	1.7 g
Agar	2.0 g
Distilled water	900 ml

Solution B

$MgSO_4.\ 7H_2O$	0.2 g
$CaCl_2$	0.06 g
Distilled water	100 ml

Solution C

p-aminobenzoic acid	0.0005 g
biotin	0.001 g
Distilled water	2 ml

Dissolve solution A in 900 ml distilled water and solution B in 100 ml distilled water. Adjust pH of solution A to 7.0. Autoclave separately at 121 ºC for 15 min. Allow to cool and combine. Dissolve solution C in 2 ml of distilled water. Filter sterilise and add to media.

Add agar (15 g/l) to solution A for pure growth culture.

2.5.1.2 *Burkholderia* (BAz) medium

Azelaic acid	2.0 g
K_2HPO_4	0.4 g
KH_2PO_4	0.4 g
$MgSO_4.\ 7\ H_20$	0.2 g
$CaCl_2$	0.02 g
$Na_2MoO_4.2\ H_20$	0.002 g
FeNaEDTA	0.06 g
Bromothymol blue (dissolved in ethanol)	0.075 g
Agar	2.3 g
Distilled water	1 l

Dissolve all ingredients in distilled water. Adjust to pH 5.7 using KOH. Dispense and autoclave at 121 ºC for 15 min.

2.5.1.3 Diazotroph (NFb) medium

Malic acid	0.5 g
Sucrose	0.5 g
K_2HPO_4	0.5 g
$MgSO_4$. 7 H_20	0.2 g
NaCl	0.1 g
$CaCl_2$	0.02 g
Na_2MoO_4.2 H_20	0.002 g
$MnSO_4$. H_20	0.01 g
FeNaEDTA	0.06 g
Biotin	0.1 mg
Bromothymol blue (dissolved in ethanol)	0.075 g
Agar	1.75 g
Distilled water	1 l

Dissolve all ingredients except K_2HPO_4, FeNaEDTA and agar in distilled water and adjust to pH 6.8. Add agar and autoclave at 121 ºC for 15 min. Dissolve K_2HPO_4 and FeNaEDTA in 10 ml of distilled water and sterilise separately. Combine and dispense into appropriate volumes.

Add agar (15 g/l) for pure growth culture.

2.5.1.4 Glucose (LG) medium

Glucose	7.5 g
K_2HPO_4	0.2 g
KH_2PO_4	0.6 g
$MgSO_4$. 7 H_20	0.2 g
$CaCl_2$	0.02 g
Na_2MoO_4.2 H_20	0.002 g
$FeCl_3$. 6 H_20	0.01 g
Bromothymol blue (dissolved in ethanol)	0.075 g
Agar	1.8 g
Distilled water	1 l

Dissolve all ingredients in distilled water and adjust to pH 6.0. Sterilise by autoclaving at 121 ºC for 15 min. Dispense in appropriate volumes.
Add agar (15 g/l) for pure growth culture.

2.5.1.5. Glucose-thioglycollate (GT) medium

Glucose	10 g
Sodium thioglycollate	0.5 g
K_2HPO_4	6.3 g
NaH_2PO_4	1.7 g
Na_2MoO_4.2 H_20	0.008 g
FeNaEDTA	0.06 g
Resazurin	0.001 g

Dissolve all ingredients except FeNaEDTA in distilled water and adjust to pH 7.0. Sterilise by autoclaving at 121 ºC for 15 min. Dissolve FeNaEDTA in 5 ml of distilled water, filter sterilise and add to media.

Add Casamino acids (1.0 g/l) and agar (15 g/l) for pure culture growth.

Appendix 2.5.2 Media used in vitamin B_{12} analysis

2.5.2.1 B_{12} synthetic medium

Solution A

Glucose	10 g
Casein hydrolysate (vitamin free)	3 g
CH_3COONa	5 g
KH_2PO_4	1 g
K_2HPO_4	1 g
$MgSO_4.\ 7H_2O$	0.4 g
$MnSO_4.\ 4\ H_2O$	0.02 g
NaCl	0.02 g
FeNaEDTA	0.03 g
$CoCl_2.\ 6H_2O$	0.008 g
Distilled water	950 ml

Solution B

4-aminobenzoic acid	200 µg
d-biotin	10 µg
Calcium D^+ pantothenate	200 µg
Folic acid	100 µg
Pyridoxine HCl	4 µg
Niacin	2 µg
Riboflavin	2 µg
Thiamine HCl	2 µg
Distilled water	50 ml

Dissolve all ingredients for solution A in 950 ml of distilled water and adjust to pH 6.5. Sterilise by autoclaving at 121 ºC for 15 min. Dissolve al ingredients for solution B in 50 ml of distilled water. Filter sterilise solution B and add to solution A once it has cooled. Dispense appropriately.

2.5.2.2 Basal vitamin test medium

Glucose	1 g
Casamino acids	1 g
KH_2PO_4	1 g
KNO_3	0.5 g
$MgSO_4.\ 7H_2O$	0.4 g
$CaCl_2$	0.1 g
NaCl	0.1 g
FeNaEDTA	0.03 g
$CoCl_2.\ 6H_2O$	0.008 g

Sodium thioglycollate	0.5 g
Resazurin	0.001 g
Distilled water	1 l

Dissolve all ingredients except glucose and casamino acids in 980 ml of distilled water and adjust to pH 6.8. Sterilise by autoclaving at 121 ºC for 15 min. Dissolve remaining ingredients in 20 ml distilled water, filter sterilise and add to cooled media. Dispense appropriately.

Modified from the following:
Lockhead A G and Burton M O (1957) Qualitative studies of soil microorganisms. XIV. Specific vitamin requirements of the predominant bacterial flora. *Canadian Journal of Microbiology* 3: 35-42
Kamata S, Nakamura H, Kakiichi N, Komine K, Ito O, Hayashi M, Otsuka H and Uchida K (1991) Cobalt salt-induced increase in vitamin B_{12} production by *Escherichia coli* in anaerobic fermentation. *Animal Science and Technology (Japan)* 62: 411-416

Appendix 2.5.3 Media used in storage technique and pathogen survival studies

2.5.3.1 de Man Rogosa Sharpe (MRS) agar

MRS agar base (Oxoid Ltd., Thebarton, SA)	62 g
Distilled water	1 l

Dissolve agar in distilled water and adjust to pH 6.2 ± 0.2. Sterilise by autoclaving at 121 ºC for 15 min. Allow to cool and dispense into Petri plates.

2.5.3.2 One quarter strength Ringer's solution

Full strength Ringer's solution

NaCl	6.0 g
KCl	0.075 g
$CaCl_2$	0.1 g
$NaHCO_3$	0.1 g
Distilled water	1 l

Dissolve ingredients in distilled water and dispense in appropriate volumes. Sterilise by autoclaving at 121 ºC for 15 min. For one-quarter strength Ringer's solution, dilute 250 ml of full strength Ringer's solution in 750 ml sterile distilled water and aseptically dispense as required

2.5.3.3 de Man Rogosa Sharpe broth

MRS broth base (Oxoid Ltd., Thebarton, SA)	52 g
Distilled water	1 l

Dissolve broth in distilled water and adjust to pH 6.2 ± 0.2. Dispense into required volumes and sterilise by autoclaving at 121 ºC for 15 min.

2.5.3.4 MRS-SCP agar and MRS-SCP broth

Make MRS agar (Appendix 2.4.3.1) or MRS broth (Appendix 2.4.3.3), with the addition of 50 mg/l of cycloheximide. Sterilize as above and allow to cool. Dissolve 1 g potassium sorbate and 200 mg of sodium nitrate in 10 ml of distilled water. Filter sterilise, and add to agar required medium. Dispense as appropriate.

Catalase can be added at a concentration of 37,000 U/l following sterilisation.

APPENDIX 3: ISOLATION AND ENUMERATION OF BACTERIA OF PUBLIC HEALTH SIGNIFICANCE FROM SAGO STARCH

Appendix 3.1 Sample Information for Sago Starch Tested for the Presence of Bacterial Foodborne Pathogens

Table A3.01: Village of origin, storage method and storage duration of sago starch samples analysed for bacterial pathogens.

Code	Village	Region & Province	Storage	Storage duration	pH	a_w
S1102-01	Biwat	Angoram, ESP	banana leaves	2 months	NT	NT
S1102-02	Magendo 4	Angoram, ESP	freshly made	NA	NT	NT
S1102-03	Keram	Angoram, ESP	banana leaves	3 weeks	NT	NT
S1102-04	Ombos	Angoram, ESP	basket	$\leq$ 6 weeks	NT	NT
S1102-05	Reugian camp	Angoram, ESP	freshly made	NA	NT	NT
S1102-06	Taway	Angoram, ESP	freshly made	NA	NT	NT
S1102-07	Taway	Angoram, ESP	clay pot	3 weeks	NT	NT
S1102-08	Kaindi	Wewak, ESP	unknown	1 day	NT	NT
S1102-09	Makung	Wewak, ESP	unknown	1 day	NT	NT
S1102-10	Making	Wewak, ESP	smoked	1 month	NT	NT
M1102-01	Misima	Misima Is, MBP	leaves & smoked	2 days	NT	NT
W0303-01	Ali	Balimo, WP	sago leaves	2 months	3.72	0.993
W0303-02	Ali	Balimo, WP	sago leaves	3 months	5.00	0.996
W0303-03	Ali	Balimo, WP	sago leaves	3 months	5.60	0.996
W0303-06	Kaniya	Balimo, WP	sago leaves	3 weeks	3.84	0.995
W0303-07	Dimissi	Morehead, WP	leaves	3 weeks	4.45	0.994
W0303-09	Rouku	Morehead, WP	rice bag	1 month	3.87	0.993
W0303-11	Kuria	Bamu, WP	woven basket	3 weeks	6.90	0.994
W0303-12	Alagi	Bamu, WP	rice bag	3 weeks	5.45	0.994
W0303-13	Gawi	Bamu, WP	sago leaves	1 month	7.06	0.994
W0303-14	Gawi	Bamu, WP	sago leaves	1 year	6.53	0.932
W0303-15	Saiwase	Mapodo, WP	rice bag	4 weeks	5.54	0.996
W0303-16	Kaenewe	Mapado, WP	sago leaves	1 month	5.38	0.994
W0303-17	Madila	Mapado, WP	sago leaves	2 months	4.42	0.993
W0303-18	Ugu	Mapado, WP	sago leaves	1 week	4.28	0.996
W0303-19	Kini	Balimo, WP	sago leaves	1 day	7.03	0.994
W0303-20	Kini	Balimo, WP	sago leaves	3 days	6.24	0.996
S0603-01	Yame	Maprik, ESP	banana leaves	6 days	5.32	0.998
S0603-02	Kukal	Maprik, ESP	large plastic bag	11 days	4.50	0.999
S0603-03	Hamahup 2	Maprik, ESP	banana leaves & palm frond	7 days	6.04	1.000
S0603-04	Srankwandu	Wosera, ESP	plastic bag	5 days	4.17	0.999

Code	Village	Region & Province	Storage	Storage duration	pH	a_w
S0603-05	Awatip	Ambunti, ESP	plastic bag	4 days	7.04	1.000
S0603-06	Malu	Ambunti, ESP	freshly made	NA	4.63	0.999
S0603-07	Waiawas	Ambunti, ESP	plastic bag	2 weeks	3.98	0.995
S0603-08	Appan	Ambunti, ESP	unwrapped	4 days	5.91	0.998
S0603-10	Miko	Wosera, ESP	open plastic bag	1 day	5.41	0.998
S0602-12	Marienberg station	Angoram, ESP	freshly made	NA	4.53	0.996
S0603-13	Imbando	Angoram, ESP	basket	8 days	3.90	0.992
S0603-14	Angoram	Angoram, ESP	plastic bag	1 day	4.03	0.995
S0603-16	Biwat (Yaul)	Angoram, ESP	plastic bag	4 weeks	4.68	0.998
S0603-18	Arepan	Angoram, ESP	large plastic bag	2 weeks	4.25	0.997
S0603-20	Wariman	Wewak, ESP	sago leaves	1 week	4.39	0.998
W0404-01	Kubut	Lake Murray, WP	woven bag	3 weeks	3.75	0.998
W0404-03	Kubut	Lake Murray, WP	woven bag	3 weeks	2.73	0.992
W0404-05	Kubut	Lake Murray, WP	unknown	1 day	6.14	0.975
W0404-06	Kuem	Middle Fly, WP	woven bag	1 month	3.81	0.999
W0404-07	Kuem	Middle Fly, WP	woven bag	unknown	6.44	0.93
W0404-08	Mipan	Middle Fly, WP	woven bag	2 months	3.74	0.93
W0404-09	Mipan	Middle Fly, WP	woven bag	2-3 months	4.67	0.999
W0404-10	Mipan	Middle Fly, WP	woven bag	2 months	3.57	0.943
W0404-13	Manda	Middle Fly, WP	woven bag	3 months	4.19	0.930
W0404-14	Riti	Suki, WP	woven bag	1 week	7.30	0.998
W0404-15	Riti	Suki, WP	woven bag	1 month	4.49	0.998
W0404-18	Balimo	Balimo, WP	freshly made	NA	7.17	0.999
W0404-21	Madirr	Mabane, WP	sago leaves	3 weeks	7.34	0.997
W0404-22	Lenada	Mabane, WP	sago leaves	2 months	3.85	0.999
W0405-01	Aewa	Suki, WP	woven bag	1 month	4.18	NT
W0405-02	Aewa	Suki, WP	sago leaves	1 week	4.98	NT
W0405-03	Awaba	Balimo, WP	sago leaves	1 week	4.94	NT
W0405-04	Awaba	Balimo, WP	sago leaves	1 week	4.29	NT
W0405-05	Awaba	Balimo, WP	sago leaves	2 weeks	5.15	NT
W0405-06	Kuta 2	Balimo, WP	sago leaves	1 day	6.16	NT
W0405-07	Kuta 2	Balimo, WP	sago leaves	1 day	6.62	NT
W0405-08	Kuta 2	Balimo, WP	woven basket	1 day	6.40	NT
W0405-09	Ali	Balimo, WP	woven bag	1 month	4.63	NT
W0405-10	Ali	Balimo, WP	woven bag	1 day	4.60	NT
W0405-11	Ali	Balimo, WP	woven bag	2 weeks	6.81	NT
W0405-12	Ali	Balimo, WP	woven bag	2 weeks	4.18	NT
W0605-01	Aewe	Suki, WP	woven bag	4-5 weeks	4.22	NT

Legend: ESP: East Sepik Province
MBP: Milne Bay Province
WP: Western Province
NA: Not applicable
NT: Not tested

Appendix 3.2 Enumeration of *Bacillus cereus*

Samples S1102-01 to S1102-10 and M1102-01 were enumerated using the spread plate technique, resulting in a lower limit of detection of 1.0×10^2 cfu/g. All other samples were enumerated using the MPN technique, and consequently have a lower limit of detection of 3 cfu/g. *Bacillus cereus* was present in samples S1102-04, S1102-08 and S1102-10, although exact numbers were not able to be determined.

Table A3.02: Number of *B. cereus* per gram of sago starch enumerated using either spread plate technique (S1102 and M1102) or MPN (all other samples).

Sago sample	organisms/g sago	Sago sample	organisms/g sago
S1102-01	1.3×10^3	W0303-16	$< 3.0 \times 10^0$
S1102-02	$< 1.0 \times 10^2$	W0303-17	4.6×10^2
S1102-03	4.5×10^2	W0303-18	1.5×10^2
S1102-04	$>1.0 \times 10^4$	W0303-19	1.4×10^1
S1102-05	$< 1.0 \times 10^2$	W0303-20	$< 3.0 \times 10^0$
S1102-06	$< 1.0 \times 10^2$	S0603-01	7.0×10^0
S1102-07	$< 1.0 \times 10^2$	S0603-02	9.3×10^1
S1102-08	$> 1.0 \times 10^2$	S0603-03	9.0×10^0
S1102-09	8.0×10^3	S0603-04	9.0×10^0
S1102-10	$>2.5 \times 10^2$	S0603-05	$< 3.0 \times 10^0$
M1102-01	$< 1.0 \times 10^2$	S0603-06	$< 3.0 \times 10^0$
W0303-01	4.6×10^2	S0603-07	1.5×10^2
W0303-02	4.3×10^1	S0603-08	$< 3.0 \times 10^0$
W0303-03	1.5×10^2	S0603-10	3.0×10^0
W0303-06	$>1.1 \times 10^3$	S0603-12	9.3×10^1
W0303-07	4.6×10^2	S0603-13	2.3×10^1
W0303-09	4.6×10^2	S0603-14	2.1×10^1
W0303-11	2.4×10^2	S0603-16	4.0×10^0
W0303-12	4.0×10^0	S0603-18	1.1×10^3
W0303-13	1.5×10^2	S0603-20	1.1×10^3
W0303-14	7.0×10^0	W0605-01	$<1.0 \times 10^1$
W0303-15	4.0×10^0		

Appendix 3.3 Enumeration of *Clostridium perfringens* and Saccharolytic Clostridia

Table A3.03: Number of *C. perfringens* and saccharolytic clostridia per gram isolated from sago starch.

Sago sample	*C. perfringens* organisms/g sago	saccharolytic clostridia (cfu/g)
W0404-01	$< 3.0 \times 10^0$	$< 1.0 \times 10^2$
W0404-03	$< 3.0 \times 10^0$	$< 1.0 \times 10^2$
W0404-06	$< 3.0 \times 10^0$	$< 1.0 \times 10^2$
W0404-07	1.5×10^1	$< 1.0 \times 10^2$
W0404-08	$< 3.0 \times 10^0$	$< 1.0 \times 10^2$
W0404-09	$< 3.0 \times 10^0$	$< 1.0 \times 10^2$
W0404-10	$< 3.0 \times 10^0$	$< 1.0 \times 10^2$
W0404-13	$< 3.0 \times 10^0$	$< 1.0 \times 10^2$
W0404-14	$< 3.0 \times 10^0$	$< 1.0 \times 10^2$
W0404-15	$< 3.0 \times 10^0$	$< 1.0 \times 10^2$
W0404-18	2.3×10^1	$< 1.0 \times 10^2$
W0404-19	$< 3.0 \times 10^0$	$< 1.0 \times 10^2$
W0404-21	$< 3.0 \times 10^0$	$< 1.0 \times 10^2$
W0404-22	$< 3.0 \times 10^0$	$< 1.0 \times 10^2$
W0404-05	$< 3.0 \times 10^0$	$< 1.0 \times 10^2$
W0605-01	9.0×10^0	1.0×10^2

Appendix 3.4 Isolation and Enumeration of *Enterobacteriaceae*

Table A3.04: Detection of *Enterobacteriaceae* in sago starch. Results for total coliforms, faecal coliforms and *E. coli* are organisms/g sago starch.

	Total coliforms	**Faecal coliforms**	***E. coli***	***Salmonella***
S1102-01	$< 3.0 \times 10^0$	$< 3.0 \times 10^0$	$< 3.0 \times 10^0$	negative
S1102-02	$> 1.1 \times 10^3$	$> 1.1 \times 10^3$	$> 1.1 \times 10^3$	positive
S1102-03	$< 3.0 \times 10^0$	$< 3.0 \times 10^0$	$< 3.0 \times 10^0$	negative
S1102-04	$> 1.1 \times 10^3$	$> 1.1 \times 10^3$	$> 1.1 \times 10^3$	negative
S1102-05	$> 1.1 \times 10^3$	$> 1.1 \times 10^3$	$> 1.1 \times 10^3$	negative
S1102-06	$> 1.1 \times 10^3$	$> 1.1 \times 10^3$	$> 1.1 \times 10^3$	negative
S1102-07	$> 1.1 \times 10^3$	$> 1.1 \times 10^3$	$> 1.1 \times 10^3$	positive
S1102-08	$> 1.1 \times 10^3$	$> 1.1 \times 10^3$	4.6×10^2	negative
S1102-09	$> 1.1 \times 10^3$	$> 1.1 \times 10^3$	$> 1.1 \times 10^3$	positive
S1102-10	$< 3.0 \times 10^0$	$< 3.0 \times 10^0$	$< 3.0 \times 10^0$	negative
M1102-01	$> 1.1 \times 10^3$	$> 1.1 \times 10^3$	4.6×10^2	negative
W0303-01	$< 3.0 \times 10^0$	$< 3.0 \times 10^0$	$< 3.0 \times 10^0$	negative
W0303-02	$> 1.1 \times 10^3$	$> 1.1 \times 10^3$	$> 1.1 \times 10^3$	negative
W0303-03	$< 3.0 \times 10^0$	$< 3.0 \times 10^0$	$< 3.0 \times 10^0$	negative
W0303-06	$< 3.0 \times 10^0$	$< 3.0 \times 10^0$	$< 3.0 \times 10^0$	negative
W0303-07	1.1×10^3	1.1×10^3	9.0×10^0	negative
W0303-09	$< 3.0 \times 10^0$	$< 3.0 \times 10^0$	$< 3.0 \times 10^0$	negative
W0303-11	$> 1.1 \times 10^3$	$> 1.1 \times 10^3$	$> 1.1 \times 10^3$	negative
W0303-12	$> 1.1 \times 10^3$	$> 1.1 \times 10^3$	$> 1.1 \times 10^3$	negative
W0303-13	$> 1.1 \times 10^3$	$> 1.1 \times 10^3$	$> 1.1 \times 10^3$	negative
W0303-14	$> 1.1 \times 10^3$	$> 1.1 \times 10^3$	$> 1.1 \times 10^3$	negative
W0303-15	$> 1.1 \times 10^3$	1.5×10^2	$< 3.0 \times 10^0$	negative
W0303-16	$> 1.1 \times 10^3$	$> 1.1 \times 10^3$	4.3×10^1	negative
W0303-17	$> 1.1 \times 10^3$	$> 1.1 \times 10^3$	$> 1.1 \times 10^3$	negative
W0303-18	$> 1.1 \times 10^3$	$> 1.1 \times 10^3$	$> 1.1 \times 10^3$	negative
W0303-19	$> 1.1 \times 10^3$	$> 1.1 \times 10^3$	$> 1.1 \times 10^3$	negative
W0303-20	$> 1.1 \times 10^3$	$> 1.1 \times 10^3$	$> 1.1 \times 10^3$	negative
S0603-01	$> 1.1 \times 10^3$	$> 1.1 \times 10^3$	$> 1.1 \times 10^3$	negative
S0603-02	$> 1.1 \times 10^3$	2.4×10^2	4.3×10^1	negative
S0603-03	$> 1.1 \times 10^3$	$> 1.1 \times 10^3$	$> 1.1 \times 10^3$	negative
S0603-04	$> 1.1 \times 10^3$	4.3×10^1	4.0×10^0	negative
S0603-05	$> 1.1 \times 10^3$	$> 1.1 \times 10^3$	$> 1.1 \times 10^3$	negative
S0603-06	$> 1.1 \times 10^3$	$> 1.1 \times 10^3$	$> 1.1 \times 10^3$	negative
S0603-07	$> 1.1 \times 10^3$	$< 3.0 \times 10^0$	$< 3.0 \times 10^0$	negative
S0603-08	$> 1.1 \times 10^3$	$> 1.1 \times 10^3$	$> 1.1 \times 10^3$	negative
S0603-10	$> 1.1 \times 10^3$	$> 1.1 \times 10^3$	$> 1.1 \times 10^3$	negative
S0603-12	$> 1.1 \times 10^3$	1.1×10^3	4.6×10^2	negative
S0603-13	$< 3.0 \times 10^0$	$< 3.0 \times 10^0$	$< 3.0 \times 10^0$	negative
S0603-14	$> 1.1 \times 10^3$	1.5×10^2	4.0×10^0	negative
S0603-16	$> 1.1 \times 10^3$	$> 1.1 \times 10^3$	$> 1.1 \times 10^3$	negative

	Total coliforms	**Faecal coliforms**	***E. coli***	***Salmonella***
S0603-18	$> 1.1 \times 10^{3}$	4.6×10^{2}	2.4×10^{2}	negative
S0603-20	$< 3.0 \times 10^{0}$	$< 3.0 \times 10^{0}$	$< 3.0 \times 10^{0}$	negative
W0404-01	$> 1.1 \times 10^{4}$	2.4×10^{3}	$< 3.0 \times 10^{1}$	negative
W0404-03	$< 3.0 \times 10^{1}$	$< 3.0 \times 10^{0}$	$< 3.0 \times 10^{0}$	negative
W0404-06	$> 1.1 \times 10^{4}$	$> 1.1 \times 10^{4}$	4.3×10^{2}	negative
W0404-07	2.3×10^{2}	2.3×10^{2}	2.3×10^{2}	negative
W0404-08	$> 1.1 \times 10^{4}$	$> 1.1 \times 10^{4}$	$< 3.0 \times 10^{1}$	negative
W0404-09	$> 1.1 \times 10^{4}$	$> 1.1 \times 10^{4}$	4.0×10^{1}	negative
W0404-10	$> 1.1 \times 10^{4}$	2.4×10^{3}	$< 3.0 \times 10^{1}$	negative
W0404-13	$> 1.1 \times 10^{4}$	$> 1.1 \times 10^{4}$	9.0×10^{1}	negative
W0404-14	$> 1.1 \times 10^{4}$	$> 1.1 \times 10^{4}$	$> 1.1 \times 10^{4}$	negative
W0404-15	$> 1.1 \times 10^{4}$	$> 1.1 \times 10^{4}$	$> 1.1 \times 10^{4}$	negative
W0404-18	$> 1.1 \times 10^{4}$	$> 1.1 \times 10^{4}$	$> 1.1 \times 10^{4}$	positive
W0404-19	2.3×10^{2}	$< 3.0 \times 10^{1}$	$< 3.0 \times 10^{1}$	negative
W0404-21	$> 1.1 \times 10^{4}$	$> 1.1 \times 10^{4}$	1.1×10^{4}	positive
W0404-22	$< 3.0 \times 10^{1}$	$< 3.0 \times 10^{1}$	$< 3.0 \times 10^{1}$	negative
W0404-05	$> 1.1 \times 10^{3}$	1.1×10^{3}	1.1×10^{3}	negative
W0405-01	NT	$> 1.1 \times 10^{5}$	$> 1.1 \times 10^{5}$	negative
W0405-02	NT	$> 1.1 \times 10^{5}$	$> 1.1 \times 10^{5}$	negative
W0405-03	NT	$< 3.0 \times 10^{1}$	$< 3.0 \times 10^{1}$	negative
W0405-04	NT	$< 3.0 \times 10^{1}$	$< 3.0 \times 10^{1}$	negative
W0405-05	NT	$> 1.1 \times 10^{5}$	4.3×10^{3}	negative
W0405-06	NT	$> 1.1 \times 10^{5}$	$> 1.1 \times 10^{5}$	negative
W0405-07	NT	$> 1.1 \times 10^{5}$	$> 1.1 \times 10^{5}$	negative
W0405-08	NT	$> 1.1 \times 10^{5}$	$> 1.1 \times 10^{5}$	negative
W0405-09	NT	$< 3.0 \times 10^{1}$	$< 3.0 \times 10^{1}$	negative
W0405-10	NT	$> 1.1 \times 10^{5}$	$> 1.1 \times 10^{5}$	negative
W0405-11	NT	$> 1.1 \times 10^{5}$	$> 1.1 \times 10^{5}$	negative
W0405-12	NT	$< 3.0 \times 10^{1}$	$< 3.0 \times 10^{1}$	negative

Legend: NT: Not tested

Appendix 3.5 Enumeration of Coagulase Positive Staphylococci

Table 3.05: Enumeration of coagulase positive staphylococci (cfu/g) in sago starch.

Sago sample	Coagulase positive staphylococci (cfu/g)	Sago sample	Coagulase positive staphylococci (cfu/g)
S1102-01	$< 1.0 \times 10^2$	S0603-03	$< 1.0 \times 10^2$
S1102-02	$< 1.0 \times 10^2$	S0603-04	$< 1.0 \times 10^2$
S1102-03	$< 1.0 \times 10^2$	S0603-05	5.0×10^4
S1102-04	$< 1.0 \times 10^2$	S0603-06	$< 1.0 \times 10^2$
S1102-05	$< 1.0 \times 10^2$	S0603-07	$< 1.0 \times 10^2$
S1102-06	$< 1.0 \times 10^2$	S0603-08	6.6×10^3
S1102-07	$< 1.0 \times 10^2$	S0603-10	3.9×10^4
S1102-08	$< 1.0 \times 10^2$	S0603-12	$< 1.0 \times 10^2$
S1102-09	$< 1.0 \times 10^2$	S0603-13	$< 1.0 \times 10^2$
S1102-10	$< 1.0 \times 10^2$	S0603-14	$< 1.0 \times 10^2$
M1102-01	$< 1.0 \times 10^2$	S0603-16	$< 1.0 \times 10^2$
W0303-01	$< 1.0 \times 10^2$	S0603-18	$< 1.0 \times 10^2$
W0303-02	$< 1.0 \times 10^2$	S0603-20	$< 1.0 \times 10^2$
W0303-03	$< 1.0 \times 10^2$	W0404-01	$< 1.0 \times 10^2$
W0303-06	$< 1.0 \times 10^2$	W0404-03	$< 1.0 \times 10^2$
W0303-07	$< 1.0 \times 10^2$	W0404-06	$< 1.0 \times 10^2$
W0303-09	$< 1.0 \times 10^2$	W0404-07	$< 1.0 \times 10^2$
W0303-11	4.5×10^5	W0404-08	$< 1.0 \times 10^2$
W0303-12	4.4×10^5	W0404-09	$< 1.0 \times 10^2$
W0303-13	4.5×10^4	W0404-10	$< 1.0 \times 10^2$
W0303-14	9.4×10^2	W0404-13	$< 1.0 \times 10^2$
W0303-15	$< 1.0 \times 10^2$	W0404-14	$< 1.0 \times 10^2$
W0303-16	9.0×10^2	W0404-15	$< 1.0 \times 10^2$
W0303-17	7.8×10^2	W0404-18	$< 1.0 \times 10^2$
W0303-18	1.8×10^2	W0404-19	$< 1.0 \times 10^2$
W0303-19	1.2×10^3	W0404-21	$< 1.0 \times 10^2$
W0303-20	5.4×10^3	W0404-22	$< 1.0 \times 10^2$
S0603-01	$< 1.0 \times 10^2$	W0605-01	$< 1.0 \times 10^2$
S0603-02	$< 1.0 \times 10^2$		

Appendix 3.6 Enumeration of Total Culturable Aerobic Bacteria

Table A3.06: Enumeration of total culturable aerobic bacteria (cfu/g) in sago starch.

Sago sample	Standard plate count (cfu/g)	Sago sample	Standard plate count (cfu/g)
S1102-01	5.4×10^5	S0603-03	3.0×10^8
S1102-02	4.4×10^7	S0603-04	4.2×10^8
S1102-03	2.5×10^6	S0603-05	9.6×10^7
S1102-04	3.1×10^7	S0603-06	3.4×10^7
S1102-05	8.7×10^7	S0603-07	7.4×10^6
S1102-06	6.7×10^7	S0603-08	2.0×10^8
S1102-07	4.3×10^7	S0603-10	5.9×10^9
S1102-08	1.8×10^8	S0603-12	7.2×10^7
S1102-09	3.1×10^7	S0603-13	1.8×10^7
S1102-10	9.4×10^6	S0603-14	3.9×10^7
M1102-01	1.5×10^8	S0603-16	5.5×10^8
W0303-01	1.3×10^7	S0603-18	4.0×10^7
W0303-02	6.1×10^7	S0603-20	1.2×10^8
W0303-03	4.8×10^7	W0404-01	8.6×10^7
W0303-06	$< 1.0 \times 10^4$	W0404-03	1.2×10^7
W0303-07	1.8×10^8	W0404-06	1.2×10^8
W0303-09	1.8×10^7	W0404-07	1.5×10^7
W0303-11	1.1×10^8	W0404-08	1.1×10^8
W0303-12	1.2×10^8	W0404-09	2.7×10^7
W0303-13	1.2×10^8	W0404-10	1.2×10^7
W0303-14	9.9×10^7	W0404-13	1.5×10^8
W0303-15	1.4×10^8	W0404-14	1.6×10^9
W0303-16	1.5×10^8	W0404-15	2.2×10^9
W0303-17	3.2×10^7	W0404-18	$< 1.0 \times 10^2$
W0303-18	1.5×10^8	W0404-19	$< 1.0 \times 10^2$
W0303-19	4.9×10^7	W0404-21	$< 1.0 \times 10^2$
W0303-20	3.6×10^7	W0404-22	$< 1.0 \times 10^2$
S0603-01	3.4×10^8	W0605-01	1.3×10^8
S0603-02	6.2×10^8		

APPENDIX 4: ENUMERATION, IDENTIFICATION AND HAEMOLYTIC ACTIVITY OF FILAMENTOUS FUNGI FROM SAGO STARCH

Appendix 4.1 Enumeration of Yeasts and Moulds in Sago Starch

Table A4.01: Enumeration of yeasts and moulds (cfu/g) in sago starch cultured on DRBC agar.

Sample	Yeasts (cfu/g)	Moulds (cfu/g)	Sample	Yeasts (cfu/g)	Moulds (cfu/g)
S1102-01	1.2×10^3	1.5×10^4	S0603-10	1.8×10^6	1.4×10^3
S1102-02	4.8×10^4	5.0×10^3	S0603-12	1.4×10^7	8.5×10^2
S1102-03	1.2×10^4	6.5×10^2	S0603-13	4.8×10^5	$< 1.0 \times 10^2$
S1102-04	1.5×10^5	1.4×10^5	S0603-14	3.8×10^6	$< 1.0 \times 10^2$
S1102-05	1.4×10^5	1.0×10^4	S0603-16	9.4×10^5	1.6×10^3
S1102-06	4.6×10^5	$< 1.0 \times 10^2$	S0603-18	4.8×10^5	7.1×10^3
S1102-07	1.2×10^5	4.0×10^4	S0603-20	1.4×10^7	5.0×10^4
S1102-08	6.9×10^5	1.0×10^4	W0404-01	3.2×10^6	3.5×10^4
S1102-09	5.2×10^5	2.5×10^2	W0404-03	5.3×10^6	1.8×10^5
S1102-10	1.8×10^4	2.5×10^3	W0404-05	5.7×10^6	8.5×10^4
M1102-01	1.4×10^5	2.5×10^3	W0404-06	2.5×10^6	4.8×10^5
W0303-01	3.0×10^6	3.8×10^5	W0404-07	3.8×10^5	2.0×10^5
W0303-02	3.4×10^7	6.5×10^2	W0404-08	1.7×10^6	2.4×10^5
W0303-03	3.5×10^5	2.8×10^5	W0404-09	4.5×10^6	8.4×10^5
W0303-06	$< 1.0 \times 10^2$	$<1.0 \times 10^2$	W0404-10	1.6×10^6	3.0×10^6
W0303-07	3.8×10^6	4.5×10^4	W0404-13	1.7×10^6	3.0×10^5
W0303-09	1.4×10^7	4.4×10^5	W0404-14	1.0×10^7	2.8×10^5
W0303-11	5.2×10^6	1.6×10^5	W0404-15	6.4×10^6	1.4×10^5
W0303-12	3.2×10^6	4.5×10^4	W0404-18	4.0×10^5	1.2×10^4
W0303-13	6.5×10^6	3.2×10^4	W0404-19	6.1×10^5	4.0×10^2
W0303-14	1.2×10^6	9.0×10^4	W0404-21	3.2×10^6	3.0×10^4
W0303-15	1.1×10^6	4.0×10^3	W0404-22	1.3×10^4	1.0×10^2
W0303-16	8.9×10^5	8.5×10^3	W0404-25	1.0×10^4	4.4×10^4
W0303-17	4.7×10^6	3.8×10^5	W0405-01	1.0×10^7	9.0×10^4
W0303-18	5.5×10^6	1.4×10^4	W0405-02	1.3×10^7	3.0×10^3
W0303-19	1.7×10^6	1.0×10^3	W0405-03	2.0×10^7	1.0×10^2
W0303-20	2.2×10^6	7.5×10^3	W0405-04	6.8×10^6	1.0×10^2
S0603-01	5.5×10^6	3.5×10^3	W0405-05	3.4×10^7	3.0×10^3
S0603-02	6.4×10^5	7.0×10^2	W0405-06	2.7×10^6	2.3×10^3
S0603-03	2.6×10^6	1.8×10^5	W0405-07	$< 1.0 \times 10^2$	$< 1.0 \times 10^2$
S0603-04	4.0×10^6	1.6×10^5	W0405-08	1.3×10^7	2.0×10^3
S0603-05	5.8×10^5	3.3×10^3	W0405-09	1.4×10^7	4.0×10^2
S0603-06	8.8×10^5	4.2×10^3	W0405-10	6.1×10^6	6.0×10^3
S0603-07	7.4×10^5	1.0×10^3	W0405-11	5.6×10^6	1.1×10^5
S0603-08	3.0×10^6	9.0×10^2	W0405-12	8.3×10^7	2.0×10^2

Appendix 4.2 Enumeration of *Geotrichum* species

Table A4.02: Enumeration of *Geotrichum* spp. (cfu/g) cultured on *Geotrichum* selective agar isolated from sago starch.

Sample	Geotrichum cfu/g	Sample	Geotrichum cfu/g	Sample	Geotrichum
S1102-01	$< 1.0 \times 10^2$	W0303-14	$< 1.0 \times 10^2$	S0603-16	$< 1.0 \times 10^2$
S1102-02	$< 1.0 \times 10^2$	W0303-15	$< 1.0 \times 10^2$	S0603-18	$< 1.0 \times 10^2$
S1102-03	$< 1.0 \times 10^2$	W0303-16	$< 1.0 \times 10^2$	S0603-20	$< 1.0 \times 10^2$
S1102-04	$< 1.0 \times 10^2$	W0303-17	$< 1.0 \times 10^2$	W0404-01	$< 1.0 \times 10^2$
S1102-05	$< 1.0 \times 10^2$	W0303-18	$< 1.0 \times 10^2$	W0404-03	$< 1.0 \times 10^2$
S1102-06	$< 1.0 \times 10^2$	W0303-19	$< 1.0 \times 10^2$	W0404-05	$< 1.0 \times 10^2$
S1102-07	$< 1.0 \times 10^2$	W0303-20	$< 1.0 \times 10^2$	W0404-06	$< 1.0 \times 10^2$
S1102-08	$< 1.0 \times 10^2$	S0603-01	5.5×10^2	W0404-07	$< 1.0 \times 10^2$
S1102-09	$< 1.0 \times 10^2$	S0603-02	$< 1.0 \times 10^2$	W0404-08	4.5×10^2
S1102-10	$< 1.0 \times 10^2$	S0603-03	5.1×10^4	W0404-09	$< 1.0 \times 10^2$
M1102-01	$< 1.0 \times 10^2$	S0603-04	$< 1.0 \times 10^2$	W0404-10	$< 1.0 \times 10^2$
W0303-01	$< 1.0 \times 10^2$	S0603-05	$< 1.0 \times 10^2$	W0404-13	1.00×10^2
W0303-02	$< 1.0 \times 10^2$	S0603-06	$< 1.0 \times 10^2$	W0404-14	5.00×10^2
W0303-03	$< 1.0 \times 10^2$	S0603-07	1.0×10^2	W0404-15	7.4×10^4
W0303-06	$< 1.0 \times 10^2$	S0603-08	1.0×10^2	W0404-18	$< 1.0 \times 10^2$
W0303-07	$< 1.0 \times 10^2$	S0603-10	$< 1.0 \times 10^2$	W0404-19	$< 1.0 \times 10^2$
W0303-09	$< 1.0 \times 10^2$	S0603-12	$< 1.0 \times 10^2$	W0404-21	1.0×10^3
W0303-11	$< 1.0 \times 10^2$	S0603-13	$< 1.0 \times 10^2$	W0404-22	$< 1.0 \times 10^2$
W0303-12	$< 1.0 \times 10^2$	S0603-14	$< 1.0 \times 10^2$	W0404-25	$< 1.0 \times 10^2$
W0303-13	$< 1.0 \times 10^2$				

Appendix 4.3 Ergosterol Analysis of Sago Starch

Table A4.03: Ergosterol level (µg/g) of sago samples.

Sample	Ergosterol /g	Sample	Ergosterol µg /g	Sample	Ergosterol µg /g
S1102-01	0.52	W0303-06	0.11	S0603-09	0.85
S1102-02	1.45	W0303-07	7.04	S0603-10	0.73
S1102-03	2.13	W0303-09	23.24	S0603-11	< 0.1
S1102-04	0.83	W0303-12	7.80	S0603-12	1.83
S1102-05	1.50	W0303-14	7.54	S0603-13	0.07
S1102-06	3.69	W0303-16	3.89	S0603-14	< 0.1
S1102-07	1.14	W0303-17	32.98	S0603-15	1.02
S1102-08	5.09	W0303-18	6.72	S0603-16	0.85
S1102-09	4.99	S0603-01	1.98	S0603-17	1.23
S1102-10	0.38	S0603-02	1.34	S0603-18	0.67
M1102-01	0.32	S0603-03	0.79	S0603-19	0.41
W0303-01	20.29	S0603-04	2.46	W0405-09	0.23
W0303-02	29.43	S0603-05	1.81	W0405-13	4.70
W0303-03	5.54	S0603-06	0.81	W0605-01	41.48
W0303-04	3.53	S0603-07	1.90	**Mean**	**5.23**
W0303-05	3.29	S0603-08	1.81	**Median**	**1.81**

Appendix 4.4 Identification of Filamentous Fungi in Sago Starch

All filamentous fungi (moulds) isolated were identified, typically to genus level. Some fungi were unable to be identified due to lack of sporing structures. Fungal enumeration was carried out using DRBC agar. Colonies were differentiated using a stereomicroscope. Representative colonies were subsequently grown in pure culture for identification purposes, and on blood agar for haemolytic screening. In some cases only low numbers of an individual colony type were present on a high dilution DRBC plate. As such, it is difficult to accurately enumerate some isolates. The numbers provided below (Table A4.04) are a maximum estimate.

Table A4.04: Identification, enumeration and haemolytic activity on blood agar of filamentous fungi isolated from sago starch

CODE	IDENTIFICATION	cfu/g (≤)	HAEMOLYSIS	
			SBA	HBA
	Sago sample: S1102-01			
S1-0103	*Aspergillus carbonarius*	1.0×10^2	-	NT
	Sago sample: S1102-02			
S1- 0201	*Penicillium brevicompactum*	6.0×10^2	++	NT
S1-0202	*Fusarium merismoides*	1.0×10^3	+	NT
S1- 0203	*Penicillium brevicompactum*	2.0×10^3	++	NT
S1- 0204	*Scytalidium* sp.	2.0×10^4	-	NT
	Sago sample: S1102-03			
S1-0301	*Geotrichum*-like	1.0×10^2	+	NT
S1-0302	*Aspergillus niveus* affinities	1.0×10^3	+	NT
	Sago sample: S1102-04			
S1-0401	*Scytalidium* sp.	2.0×10^4	-	NT
S1-0402	*Aspergillus versicolor*	1.0×10^4	α	NT
S1-0404	*Fusarium* sp.	2.0×10^4	α	NT
S1-0406	*Fusarium semitectum*	1.0×10^5	++	NT
S1-0407	*Fusarium* sp. & dematiaceous hyphomycete	1.0×10^3	α	NT
S1-0408	*Acremonium* sp.	9.0×10^4	-	NT
S1-0410	*Penicillium brevicompactum*	1.0×10^4	++	NT
S1-0411	*Aspergillus* sp.	1.0×10^4	-	NT
	Sago sample: S1102-05			
S1-0501	*Trichoderma viride* aggregate	1.0×10^2	α	NT
S1-0502	*Trichoderma atroviride*	1.0×10^2	-	NT
S1-0503	*Acremonium* sp.	1.0×10^3	NG	NT
S1-0504	*Zythia* sp.	1.0×10^3	α	NT
S1-0505	*Fusarium* sp.	1.0×10^3	-	NT
S1-0506	Unknown sterile, septate, dematiaceous hyphae	3.0×10^3	NG	NT
S1-0507	*Cladosporium cladosporioides*	1.0×10^3	-	NT
	Sago sample: S1102-06			
	No filamentous fungi isolated.			
	Sago sample S1102-07			
S1-0701	*Scytalidium* sp.	5.1×10^3	-	NT
S1-0702	*Penicillium* sp.	1.0×10^2	NG	NT
S1-0703	Unknown sterile, septate, dematiaceous hyphae	1.0×10^2	-	NT

CODE	IDENTIFICATION	cfu/g	HAEMOLYSIS	
		(≤)	SBA	HBA
S1-0704	*Pestalotia* sp.	1.0×10^2	-	NT
S1-0705	Unknown sterile, septate, dematiaceous hyphae	2.0×10^2	α	NT
S1-0706	*Penicillium* sp. & sterile, septate, dematiaceous hyphae	3.0×10^2	-	NT
S1-0707	*Aureobasidium* sp.	4.5×10^4	-	NT
S1-0711	Unknown sterile, septate, hyline hyphae	6.0×10^3	-	NT
S1-0712	*Curvularia* sp.	1.0×10^3	NG	NT
	Sago sample: S1102-08			
S1-0801	*Penicillium brevicompactum*	1.8×10^3	++	NT
S1-0802	*Aspergillus*sp.	4.0×10^2	α	NT
S1-0803	*Penicillium variabile* affinities 1	1.0×10^3	+	NT
S1-0804	*Penicillium variabile* affinities 2	1.0×10^4	+	NT
	Sago sample: S1102-09			
S1-0901	*Aureobasidium* sp.	2.0×10^4	-	NT
S1-0902	*Acremonium* sp.	3.0×10^3	α	NT
S1-0903	*Gliomastix* sp.	2.0×10^3	-	NT
S1-0907	Unknown sterile, septate, hyline hyphae	1.0×10^3	++	NT
S1-0908	*Penicillium brevicompactum*	7.0×10^2	++	NT
S1-0909	*Aspergillus candidus* affinities	1.0×10^2	α	NT
S1-0910	*Aspergillus flavipes*	1.0×10^2	+++	NT
S1-0911	*Penicillium mirabile* affinities	1.0×10^2	α	NT
S1-0913	*Penicillium verruculosum* affinities	1.0×10^2	+	NT
	Sago sample: S1102-10			
S1-1001	*Cladosporium cladosporioides*	1.0×10^4	α	NT
S1-1002	*Penicillium brevicompactum*	1.0×10^3	+++	NT
S1-1003	*Chrysosporium* sp.	2.0×10^3	α	NT
S1-1004	*Penicillium variabile* affinities 1	6.0×10^2	α	NT
S1-1005	Unknown sterile, septate, hyaline hyphae	1.0×10^2	α	NT
S1-1006	*Penicillium variabile* affinities 2	2.0×10^2	+	NT
S1-1007	*Scytalidium* sp.	1.0×10^2	-	NT
S1-1008	*Aspergillus fumigatus*	1.0×10^2	++	NT
S1-1010	*Penicillium mirabile* affinities	8.0×10^2	+	NT
S1-1011	*Moniliella* sp.	5.0×10^2	α	NT
S1-1012	*Aspergillus* sp.	1.0×10^2	+	NT
	Sago sample: M1102-01			
M1-0101	*Aspergillus* sp.	1.0×10^4	+	NT
M1-0102	Unknown dematiaceous, septate hyphae	1.0×10^4	-	NT
M1-0103	*Cladosporium cladosporiodes*	1.0×10^4	NG	NT
M1-0104	Unknown sterile, dematiaceous septate hyphae	1.0×10^4	NG	NT
M1-0105	Unknown hyaline, septate hyphae	1.0×10^4	-	NT
M1-0106	*Aspergillus giganteus*	2.0×10^3	α	NT
M1-0107	*Aspergillus clavatus*	1.0×10^3	+	NT
M1-0108	*Scytalidium* sp.	1.0×10^3	NG	NT
M1-0109	*Asperillus giganteus*	1.0×10^2	+	NT
M1-0110	*Aspergillus* sp.	1.0×10^2	NG	NT
M1-0111	*Fusarium moniliforme* affinities	1.0×10^2	NG	NT
	Sago sample: W0303-01			
W1-0101	*Penicillium* sp.	3.2×10^5	+	NT
W1-0102	*Scytalidium* sp.	5.5×10^4	-	NT

CODE	IDENTIFICATION	cfu/g (≤)	HAEMOLYSIS	
			SBA	HBA
W1-0103	*Trichoderma* sp.	1.0×10^4	-	NT
W1-0104	*Paecilomyces* sp.	1.0×10^4	++	NT
	Sago sample: W0303-02			
W1-0201	Unidentified #1	1.5×10^2	+	NT
W1-0202	Unidentified #2	2.0×10^2	-	NT
W1-0203	*Trichoderma* sp.	1.5×10^2	-	NT
W1-0204	*Cylindrocarpon* sp.	3.0×10^2	-	NT
	Sago sample: W0303-03			
W1-0301	*Scytalidium* sp.	2.7×10^5	-	NT
W1-0302	*Geotrichum* sp.	1.5×10^4	+	NT
W1-0304	*Stachybotrys*-like	1.0×10^4	-	NT
	Sago sample: W0303-06			
	No filamentous fungi isolated.			
	Sago sample: W0303-07			
W1-0701	*Paecilomyces* sp.	4.5×10^4	+	NT
	Sago sample: W0303-09			
W1-0901	*Phialophora* sp.	4.1×10^5	-	NT
W1-0902	*Cladosporum* sp.	1.0×10^4	-	NT
W1-0903	*Paecilomyces* sp.	3.0×10^4	-	NT
W1-0904	*Aspergillus* sp.	1.0×10^4	+	NT
	Sago sample: W0303-11			
W1-1101	*Penicillium steckii*	2.0×10^4	++	NT
W1-1102	Mixed: *Penicillium sp.* & *Acremonium* sp.	1.0×10^4	-	NT
W1-1103	*Cladosporium* sp.	1.0×10^4	-	NT
W1-1104	*Gliomastix* sp.	4.0×10^4	-	NT
W1-1105	*Penicillium steckii*	2.0×10^4	+++	NT
W1-1106	*Aspergillus flavipes*	2.5×10^4	+++	NT
W1-1107	Unidentified isolate (no growth on subculture)	2.0×10^4	NG	NT
W1-1108	Unidentified isolate (no growth on subculture)	1.0×10^4	NG	NT
W1-1109	*Penicillium steckii*	3.0×10^4	+	NT
W1-1110	*Aspergillus sydowii*	1.0×10^4	++	NT
	Sago sample: W0303-12			
W1-1201	*Scytalidium* sp.	3.5×10^4	+	NT
W1-1202	Mixed: *Penicillium, Cylindrocarpon* & *Fusarium* sp.	1.5×10^4	++ , +, -	NT
	Sago sample: W0303-13			
W1-1301	*Penicillium steckii*	1.6×10^4	+++	NT
W1-1302	*Scytalidium* sp.	1.5×10^4	-	NT
W1-1303	*Helicostylum spiriforme*	1.0×10^3	-	NT
W1-1304	Unidentified isolate (no growth on subculture)	1.0×10^3	NG	NT
	Sago sample: W0303-14			
W1-1401	*Scytalidium* sp.	7.5×10^4	-	NT
W1-1403	*Fusarium* sp. & *Acremonium* sp.	1.0×10^4	+	NT
W1-1404	Mixed: *Scytalidium* & *Cylindrocarpon*	1.0×10^4	-	NT
	Sago sample: W0303-15			
W1-1501	*Penicillium steckii*	2.0×10^3	+++	NT
W1-1502	*Geotrichum* sp.	2.5×10^3	+	NT
	Sago sample: W0303-16			
W1-1601	Unidentified isolate (no growth on subculture)	6.5×10^3	NG	NT

CODE	IDENTIFICATION	cfu/g (≤)	HAEMOLYSIS	
			SBA	HBA
W1-1602	*Penicillium brevicompactum*	1.0×10^3	++	NT
W1-1603	*Geotrichum* sp.	1.0×10^3	+	NT
W1-1604	*Fusarium solani*	1.0×10^3	+	NT
W1-1605	*Penicillium* sp.	1.0×10^3	+	NT
	Sago sample: W0303-17			
W1-1701	*Scytalidium* sp.	2.8×10^5	-	NT
W1-1702	*Talaromyces emersonii*	4.5×10^4	++	NT
W1-1704	*Penicillium steckii*	1.5×10^4	+	NT
W1-1705	Unidentified isolate (no growth on subculture)	2.5×10^4	NG	NT
W1-1706	*Penicillium* sp.	2.0×10^4	++	NT
W1-1707	*Cylindrocarpon* sp.	1.5×10^4	NG	NT
W1-1708	*Aspergillus* sp.	1.0×10^4	-	
	Sago sample: W0303-18			
W1-1801	*Penicillium steckii*	7.0×10^3	+++	NT
W1-1802	*Cylindrocarpon* sp.	3.0×10^3	+	NT
W1-1803	Unknown dematiaceous, septate hyphae, sterile	2.5×10^3	+	NT
W1-1804	*Geotrichum* affinities	2.0×10^3	+	NT
	Sago sample: W0303-19			
W1-1901	*Penicillium steckii*	1.5×10^2	+++	NT
W1-1902	*Cladosporium* sp.	9.0×10^2	-	NT
	Sago sample: W0303-20			
W1-2001	*Penicillium steckii*	7.5×10^3	+	NT
W1-2002	*Penicillium steckii*	1.0×10^3	+++	NT
	Sago sample: S0603-01			
S2-0101	*Acremonium* sp. 1	2.1×10^3	+	NT
S2-0102	*Geotrichum* sp.	1.1×10^4	-	NT
S2-0103	*Scytalidium* sp.	1.0×10^3	-	NT
S2-0105	*Acremonium* sp. 2	1.0×10^3	-	NT
S2-0108	*Cladosporium* sp.	1.1×10^5	-	NT
	Sago sample: S0603-02			
S2-0201	*Geotrichum* sp.	5.5×10^2	+	NT
S2-0202	Unknown	2.0×10^2	-	NT
S2-0203	*Scytalidium* sp.	1.0×10^2	-	NT
S2-0207	*Aspergillus flavipes*	1.0×10^5	+++	NT
S2-0208	*Cladosporium* sp.	1.0×10^5	-	NT
	Sago sample: S0603-03			
S2-0301	*Geotrichum* sp.	1.8×10^5	-	NT
	Sago sample: S0603-04			
S2-0401	*Scytalidium* sp.	1.6×10^5	-	NT
	Sago sample: S0603-05			
S2-0501	*Scytalidium* sp.	2.2×10^3	-	NT
S2-0502	*Aureobasidium* sp.	8.5×10^2	-	NT
S2-0503	*Penicillium steckii*	2.5×10^2	++	NT
S2-0505	*Acremonium* sp.	1.0×10^2	+	NT
S2-0506	Unknown	1.0×10^2	-	NT
	Sago sample: S0603-06			
S2-0601	*Scytalidium* sp.	2.9×10^3	+	NT
S2-0602	Unknown	1.0×10^2	-	NT

CODE	IDENTIFICATION	cfu/g (≤)	HAEMOLYSIS	
			SBA	HBA
S2-0603	*Penicillium steckii*	1.1×10^3	++	NT
S2-0604	*Geotrichum* sp.	2.0×10^2	+	NT
S2-0605	Unknown dermataceous septate non sporing	1.0×10^2	-	NT
S2-0608	Unknown hyaline septate blastospore former	1.0×10^2	++	NT
	Sago sample: S0603-07			
S2-0701	*Aspergillus clavatus*	1.0×10^2	+	NT
S2-0702	*Trichoderma aureoviride* affinities	1.0×10^2	+++	NT
S2-0703	*Geotrichum* sp.	1.0×10^2	+	NT
S2-0704	*Scytalidium* sp.	1.0×10^2	-	NT
S2-0705	*Aspergillus flavipes*	1.0×10^2	++	NT
	Sago sample: S0603-08			
S2-0801	*Geotrichum* sp.	3.5×10^2	+	NT
S2-0802	*Scytalidium* sp.	5.0×10^2	-	NT
S2-0804	*Aureobasidium* sp.	1.5×10^2	-	NT
	Sago sample: S0603-10			
S2-1001	*Geotrichum*-like	9.0×10^2	-	NT
S2-1003	*Acremonium* sp.	1.0×10^2	-	NT
S2-1004	*Penicillium steckii*	1.0×10^2	++	NT
S2-1005	*Curvularia* sp.	1.0×10^2	+	NT
	Sago sample: S0603-12			
S2-1201	*Aureobasidium* sp.	5.0×10^2	++	NT
S2-1202	Unidentified isolate (no growth on subculture)	1.0×10^2	NG	NT
S2-1203	*Trichoderma longibrach* or *T. reesei*	1.5×10^2	++	NT
S2-1204	*Aureobasidium* sp.	2.0×10^2	-	NT
	Sago sample: S0603-13			
S2-1301	*Humicola* affinities	1.5×10^2	-	NT
S2-1302	*Cladosporium* sp.	1.0×10^2	-	NT
S2-1303	*Penicillium* sp.	1.0×10^4	+++	NT
S2-1304	Unknown, hyline hyphae, arthrospore producer	1.0×10^5	-	NT
S2-1305	*Penicillium steckii*	3.0×10^5	+++	NT
	Sago sample: S0603-14			
	No filamentous fungi isolated.			
	Sago sample: S0603-16			
S2-1601	*Acremonium* sp.	4.5×10^3	-	NT
S2-1602	*Penicillium* sp.	1.0×10^3	++	NT
S2-1604	*Geotrichum* sp.	1.0×10^3	+	NT
S2-1605	Unknown hyline hyphae, blastospores on denticles, very large chlamydospores	1.5×10^2	-	NT
S2-1606	*Penicillium steckii*	1.0×10^2	-	NT
	Sago sample: S0603-18			
S2-1801	*Aureobasidium* sp.	6.8×10^3	-	NT
S2-1803	Unknown, sterile, hyline, septate hyphae	1.0×10^2	-	NT
S2-1804	*Penicillium steckii*	3.5×10^2	+++	NT
S2-1806	*Penicillium steckii*	1.0×10^4	++	NT
S2-1807	*Cladosporium* sp.	1.0×10^5	++	NT
	Sago sample: S0603-20			
S2-2001	*Paecilomyces* sp.	1.0×10^2	-	NT
S2-2002	*Penicillium* sp.	1.0×10^3	+	NT

CODE	IDENTIFICATION	cfu/g (≤)	HAEMOLYSIS	
			SBA	HBA
	Sago sample: W0404-01			
W2-0101	*Penicillium* sp.	2.0×10^4	-	NT
W2-0102	Rhizomorph producing sterile fungi	2.0×10^4	+	NT
W2-0103	*Acremonium* sp.	2.0×10^3	-	NT
W2-0104	*Trichoderma atroviride*	3.3×10^3	-	NT
W2-0105	*Trichoderma atroviride*	1.0×10^3	+++	NT
	Sago sample: W0404-03			
W2-0301	*Penicillium* sp.	1.2×10^5	+++	NT
W2-0302	*Penicillium* sp.	3.5×10^4	-	NT
W2-0303	*Penicillium brevicompactum*	1.0×10^4	+++	NT
W2-0304	*Aureobasidium* sp.	1.0×10^4	++	NT
W2-0305	*Fusarium* sp.	2.5×10^4	-	NT
W2-0306	*Trichoderma sp.*	1.5×10^3	++	NT
	Sago sample: W0404-05			
W2-0501	*Penicillium brevicompactum*	5.5×10^4	+	NT
W2-0502	*Penicillium sp.*	1.0×10^4	-	NT
W2-0503	*Aureobasidium* sp.	1.0×10^4	++	NT
W2-0504	*Aspergillus* sp.	1.0×10^3	-	NT
	Sago sample: W0404-06			
W2-0601	*Aureobasidium* sp.	4.4×10^5	++	NT
W2-0602	*Fusarium* sp.	2.5×10^4	++	NT
W2-0603	*Penicillium sp.*	1.0×10^4	++	NT
W2-0604	*Penicillium brevicompactum*	1.0×10^4	++	NT
W2-0607	*Penicillium brevicompactum*	1.1×10^4	++	NT
W2-0608	*Aphanocladium*-like	2.5×10^3	+++	NT
	Sago sample: W0404-07			
W2-0701	*Aureobasidium* sp.	1.9×10^5	-	NT
W2-0702	*Scopulariopsis* sp.	2.0×10^3	+	NT
W2-0703	*Penicillium brevicompactum*	3.5×10^3	-	NT
W2-0704	Unknown non sporing hyline septate	1.0×10^3	-	NT
W2-0705	*Penicillium sp.*	7.5×10^3	-	NT
W2-0706	*Acremonium* sp.	1.0×10^3	-	NT
W2-0707	*Penicillium brevicompactum*	2.0×10^3	+	NT
	Sago sample: W0404-08			
W2-0801	*Aureobasidium* sp.	2.0×10^5	++	NT
W2-0802	Unknown	2.0×10^4	-	NT
W2-0803	*Absidia* sp.	1.0×10^4	-	NT
W2-0804	*Cephalosporiopsis* sp.	1.5×10^4	-	NT
	Sago sample: W0404-09			
W2-0901	*Acremonium* sp.	6.2×10^5	-	NT
W2-0902	*Aureobasidium* sp.	2.1×10^5	-	NT
W2-0903	*Aphanocladium* sp.	2.0×10^4	++	NT
W2-0906	Unknown, non-sporing, hyline, septate	1.0×10^4	+	NT
	Sago sample: W0404-10			
W2-1001	*Aureobasidium* sp.	2.9×10^6	-	NT

CODE	IDENTIFICATION	cfu/g (≤)	HAEMOLYSIS	
			SBA	HBA
W2-1003	*Penicillium* sp.	3.5×10^4	-	NT
W2-1004	Unknown, sterile	2.0×10^4	-	NT
W2-1005	*Acremonium* sp.	2.0×10^4	-	NT
	Sago sample: W0404-13			
W2-1301	*Trichoderma* sp.	3.0×10^4	-	NT
W2-1302	*Penicillium brevicompactum*	2.0×10^5	+	NT
W2-1303	Unidentified isolate (no growth on subculture)	6.5×10^4	NA	NT
	Sago sample: W0404-14			
W2-1401	*Penicillium brevicompactum*	2.8×10^4	++	NT
W2-1402	Unknown	1.0×10^2	-	NT
	Sago sample: W0404-15			
W2-1501	*Penicillium brevicompactum*	1.5×10^4	+	NT
W2-1502	*Fusarium* sp.	3.5×10^2	-	NT
W2-1503	Unknown sterile	1.5×10^5	NG	NT
	Sago sample: W0404-18			
W2-1801	Unknown, hyline, non-sporing septate hyphae	3.5×10^3	++	NT
W2-1802	*Penicillium brevicompactum*	2.0×10^3	++	NT
W2-1803	*Penicillium* sp.	1.5×10^3	++	NT
W2-1804	*Penicillium brevicompactum*	1.0×10^3	++	NT
W2-1806	*Fusarium* sp.	1.0×10^3	++	NT
W2-1807	*Auriobasidium* sp.	1.0×10^3	-	NT
W2-1808	*Aspergillus* sp.	1.0×10^3	++	NT
W2-1809	Unknown, sterile	1.0×10^2	-	NT
W2-1810	Unknown, sterile, septate hyphae	1.0×10^2	-	NT
	Sago sample: W0404-19			
W2-1901	*Aureobasidium* sp.	3.0×10^2	-	NT
W2-1902	*Trichoderma citroviride*	1.0×10^2	+++	NT
W2-1903	*Geotrichum* sp.	1.0×10^2	-	NT
	Sago sample: W0404-21			
W2-2101	*Neurospora* sp.	2.0×10^4	-	NT
W2-2102	Unknown	1.0×10^4	NG	NT
	Sago sample: W0404-22			
W2-2201	Unidentified (no growth on sub-culture)	1.0×10^2	NG	NT
W2-2202	Unidentified (no growth on sub-culture)	1.0×10^4	NG	NT
	Sago sample: W0404-25			
W2-2501	*Penicillium* sp.	5.0×10^3	-	NT
W2-2502	*Penicillium brevicompactum*	4.0×10^3	+	NT
W2-2503	Non-sporing dematiaceous septate unknown 1	2.0×10^3	-	NT
W2-2504	Non-sporing dematiaceous septate unknown 2	2.9×10^4	-	NT
W2-2505	*Paecilomyces* sp.	2.0×10^2	-	NT
W2-2506	*Aspergillus* sp.	1.0×10^2	-	NT

CODE	IDENTIFICATION	cfu/g (≤)	HAEMOLYSIS	
			SBA	HBA
	Sago sample: W0405-01			
W3-0101	*Scytalidium* sp.	1.0×10^5	-	NT
W3-0102	*Acremonium* sp.	2.0×10^4	-	NT
W3-0103	*Cladosporium* sp.	1.5×10^4	-	NT
W3-0105	*Phialophora* sp.	4.5×10^4	+	NT
	Sago sample: W0405-02			
W3-0201	*Penicillium brevicompactum*	2.5×10^3	++	NT
W3-0202	*Fusarium* sp.	1.0×10^3	-	NT
	Sago sample: W0405-03			
W3-0301	*Penicillium funiculosum*	1.0×10^2	++	NT
W3-0302	Unknown (aleuriospores borne on synnemata)	1.0×10^2	+	NT
	Sago sample: W0405-04			
W3-0401	*Cladosporium* sp.	1.0×10^3	+	NT
W3-0402	*Curvularia* sp.	1.0×10^2	-	NT
	Sago sample: W0405-05			
W3-0501	*Scytalidium* sp.	1.0×10^4	+	NT
W3 0502	*Scytalidium* sp.	2.8×10^3	-	NT
W3-0503	*Penicillium brevicompactum*	1.0×10^3	++	
	Sago sample: W0405-06			
W3-0601	*Penicillium brevicompactum*	2.0×10^3	++	NT
W3-0602	*Penicillium* sp.	1.0×10^3	-	NT
W3-0603	*Fusarium* sp.	1.0×10^3	-	NT
W3-0605	*Geotrichum* like	3.0×10^2	++	NT
	Sago sample: W0405-07			
	No filamentous fungi isolated.			
	Sago sample: W0405-08			
W3-0801	*Cylindrocarpon* sp.	1.5×10^3	-	NT
W3-0802	*Penicillium brevicompactum*	1.0×10^3	+++	NT
	Sago sample: W0405-09			
W3-0901	*Aspergillus* sp.	1.0×10^4	++	NT
W3-0902	*Penicillium brevicompactum*	1.0×10^3	++	NT
	Sago sample: W0405-10			
W3-1002	*Penicillium brevicompactum*	1.0×10^3	+++	NT
W3-1003	*Cylindrocarpon* sp.	1.0×10^2	NA	NT
W3-1004	*Trichoderma* sp.	1.0×10^2	-	NT
W3-1005	*Aspergillus* sp.	1.0×10^2	-	NT
	Sago sample: W0405-11			
W3-1101	*Scytalidium* sp.	5.5×10^4	+	NT
W3-1102	*Aphanocladium* sp.	5.5×10^4	NA	NT
W3-1104	*Penicillium brevicompactum*	1.1×10^3	+++	NT
	Sago sample: W0405-12			
W3-1201	*Penicillium brevicompactum*	2.0×10^2	+++	NT
	Sago sample: W0605-01 implicated sago			
W4-0101	*Trichoderma viride* aggregates	9.5×10^6	-	+++
W4-0102	*Penicillium brevicompactum*	2.0×10^6	-	+++
W4-0103	*Penicillium pinophilum*	1.0×10^6	++	++

CODE	IDENTIFICATION	cfu/g (≤)	HAEMOLYSIS	
			SBA	HBA
W4-0115	*Penicillium oxalicum*	1.0×10^6	α	-
W4-0120	*Penicillium funiculosum*	1.0×10^7	+	++
W4-0124	*Aureobasidium* sp.	1.1×10^5	NG	NG
W4-0131	*Aphanocladium*-like	1.0×10^6	+	+
W4-0132	*Penicillium aculeatum*	Not enum.	+	+
W4-0133	*Fusarium*-like	Not enum.	NG	NG
W4-0134	*Cladosporium* sp.	Not enum.	NG	NG
W4-0135	*Cylindrocarpon* affinities	Not enum.	++	+
	Sago sample: W1105-01 implicated sago			
W5-0101	*Penicillium steckii*	2.3×10^4	+++	+
W5-0102	*Fusarium* sp. 1	4.5×10^4	-	+
W5-0105	*Fusarium* sp. 2	5.0×10^3	-	-
W5-0106	*Aureobasidium* sp.	7.0×10^4	-	-
W5-0107	*Penicillium* sp.	2.0×10^4	+++	-
W5-0113	*Scytalidium*-like	1.0×10^3	NG	NG
W5-0115	*Penicillium coffeae*	4.0×10^3	NG	NG
W5-0118	*Fusarium*-like	2.5×10^3	-	-
W5-0119	*Penicillium citrinum*	4.2×10^3	+++	+
W5-0127	*Acremonium* sp.	5.0×10^4	-	-
W5-0128	*Acremonium*-like	8.0×10^3	-	-
W5-0129	*Scytalidium* sp.	5.0×10^2	-	-
W5-0130	Unknown, hyline, septate hyphae	2.0×10^3	NG	NG

Legend: -: no haemolysis
α: alpha (partial) haemolysis
NG: no growth on blood agar plate, so haemolysis could not be determined
NT: not tested: not enum: Fungi not enumerated, as isolation technique did not allow enumeration

Appendix 4.5 Haemolytic Activity of Filamentous Fungal Isolates From Implicated Sago

All fungi isolated from the two samples of implicated sago (W0605-01 and W1105-01) that could be subcultured were grown on SBA and HBA to decrease the likelihood of haemolytic isolates being overlooked.

Table A4.05: Haemolytic activity of filamentous fungal isolates from the implicated sago sample W0605-01.

CODE	IDENTIFICATION	HAEMOLYSIS	
		SBA	HBA
W0605-0101	*Trichoderma viride* aggregates	-	+++
W0605-0102a	*Penicillium pinophilum*	α	++
W0605-0102b	*Penicillium brevicompactum*	+	+++
W0605-0102c	*Penicillium brevicompactum*	+	++
W0605-0102d	*Penicillium oxalicum*	α	-
W0605-0103a	*Penicillium pinophilum*	++	++
W0605-0103b	*Penicillium brevicompactum*	+	++
W0605-0111	*Trichoderma viride* aggregates	-	-
W0605-0112	*Trichoderma viride* aggregates	++	+++
W0605-0113	*Trichoderma viride* aggregates	-	-
W0605-0114	*Penicillium pinophilum*	α	-
W0605-0115	*Penicillium oxalicum*	α	-
W0605-0116	*Penicillium pinophilum*	α	-
W0605-0119	*Trichoderma viride* aggregates	-	++
W0605-0120	*Penicillium funiculosum*	++	+++
W0605-0121	*Trichoderma* sp.	NT	+++
W0605-0123	*Penicillium pinophilum*	α	-
W0605-0128	*Penicillium brevicompactum*	++	++
W0605-0131	*Aphanocladium*-like	++	++
W0605-0132	*Penicillium aculeatum*	α	α
W0605-0135	*Cylindrocarpon* affinities	++	+

Legend: -: no haemolysis
α: alpha (partial) haemolysis
NT: not tested

Table A4.06: Haemolytic activity of filamentous fungal isolates from the implicated sago sample W1105-01.

CODE	IDENTIFICATION	HAEMOLYSIS	
		SBA	HBA
W1105-0101	*Penicillium steckii*	-	+
W1105-0102	*Fusarium* sp.1	-	+
W1105-0103	*Fusarium* sp.1	-	-
W1105-0104	*Fusarium* sp.1	-	+
W1105-0105	*Fusarium* sp.2	-	-
W1105-0106	*Aureobasidium* sp.	-	-
W1105-0107	*Penicillium* sp.	+++	-
W1105-0108	*Aureobasidium* sp.	-	
W1105-0109	*Penicillium steckii*	NG	+++
W1105-0110	*Penicillium steckii*	++	+
W1105-0111	*Penicillium steckii*	++	+
W1105-0112	*Penicillium steckii*	+++	++
W1105-0113	*Scytalidium*-like	NG	NG
W1105-0114	*Fusarium* sp.1	-	-
W1105-0115	*Penicillium coffeae*	-	NG
W1105-0116	*Penicillium steckii*	++	++
W1105-0117	*Penicillium steckii*	+++	+++
W1105-0118	*Fusarium*-like	NG	-
W1105-0119	*Penicillium citrinum*	+++	-
W1105-0120	*Penicillium steckii*	+++	+++
W1105-0122	*Penicillium* sp.	+++	+
W1105-0123	*Scytalidium* sp.	-	-
W1105-0124	Fusarium	-	+
W1105-0127	*Acremonium* sp.	-	-
W1105-0128	*Acremonium* like	-	-
W1105-0129	*Scytalidium* sp.	-	-
W1105-0130	Unknown	-	NG
W1105-0131	*Penicillium steckii*	++	++
W1105-0132	*Fusarium* sp.1	-	-
W1105-0133	*Fusarium* sp.1	-	
W1105-0134	*Aureobasidium* sp.	-	-
W1105-0135	*Aureobasidium* sp.	-	-
W1105-0136	*Penicillium steckii*	+++	+

Legend: -: no haemolysis
α: alpha (partial) haemolysis
NG: no growth on blood agar plate, so haemolysis could not be determined.

APPENDIX 5: MYCOTOXIN ANALYSIS

Table A5.01: Village of origin, storage method and storage duration of sago starch samples analysed for mycotoxins.

Code	Village	Region/Province	Storage container	Approximate storage duration
S1102-01	Biwat	Angoram, ESP	banana leaves	2 months
S1102-02	Magendo 4	Angoram, ESP	freshly made	NA
S1102-03	Keram	Angoram, ESP	banana leaves	3 weeks
S1102-04	Ombos	Angoram, ESP	basket	up to 6 weeks
S1102-05	Reugian camp	Angoram, ESP	freshly made	NA
S1102-06	Taway	Angoram, ESP	freshly made	NA
S1102-07	Taway	Angoram, ESP	clay pot	3 weeks
S1102-08	Kaindi	Wewak, ESP	unknown	1 day
S1102-09	Makung	Wewak, ESP	unknown	1 day
S1102-10	Making	Wewak, ESP	smoked	1 month
M1102-01	Misima	Misima Is, MBP	leaves & smoked	2 days
W0303-01	Ali	Balimo, WP	sago leaves	2 months
W0303-02	Ali	Balimo, WP	sago leaves	3 months
W0303-03	Ali	Balimo, WP	sago leaves	3 months
W0303-04	Makapa	Balimo, WP	sago leaves	1 day
W0303-05	Kaniya	Balimo, WP	sago leaves	2 weeks
W0303-06	Kaniya	Balimo, WP	sago leaves	3 weeks
W0303-07	Dimissi	Morehead, WP	sago leaves	3 weeks
W0303-08	Morehead	Morehead, WP	unknown	1 day
W0303-09	Rouku	Morehead, WP	rice bag	1 month
W0303-10	Kuria-1	Bamu, WP	sago leaves	1 week
W0303-11	Kuria	Bamu, WP	woven basket	3 weeks
W0303-12	Alagi	Bamu, WP	rice bag	3 weeks
W0303-13	Gawi	Bamu, WP	sago leaves	1 month
W0303-14	Gawi	Bamu, WP	sago leaves	1 year
W0303-15	Saiwase	Mapodo, WP	rice bag	4 weeks
W0303-16	Kaenewe	Mapado, WP	sago leaves	1 month
W0303-17	Madila	Mapado, WP	sago leaves	2 months
W0303-18	Ugu	Mapado, WP	sago leaves	1 week
W0303-19	Kini	Balimo, WP	sago leaves	1 day
W0303-20	Kini	Balimo, WP	sago leaves	3 days
S0603-01	Yame	Maprik, ESP	banana leaves	6 days
S0603-02	Kukal	Maprik, ESP	large plastic bag	11 days
S0603-03	Hamahup 2	Maprik, ESP	banana leaves & palm frond	7 days
S0603-04	Srankwandu	Wosera, ESP	plastic bag	5 days
S0603-05	Awatip	Ambunti, ESP	plastic bag	4 days
S0603-06	Malu	Ambunti, ESP	freshly made	NA
S0603-07	Waiawas	Ambunti, ESP	plastic bag	2 weeks
S0603-08	Appan	Ambunti, ESP	unwrapped	4 days
S0603-09	Miko	Wosera, ESP	unknown*	1 day
S0603-10	Miko	Wosera, ESP	open plastic bag	1 day
S0603-11	Marienberg	Angoram, ESP	unknown*	1 day
S0602-12	Marienberg	Angoram, ESP	freshly made	NA
S0603-13	Imbando	Angoram, ESP	basket	8 days
S0603-14	Angoram	Angoram, ESP	plastic bag	1 day
S0603-15	Angoram	Angoram, ESP	unknown*	3 days
S0603-16	Biwat	Angoram, ESP	plastic bag	4 weeks

Code	Village	Region/Province	Storage container	Approximate storage duration
S0603-17	Waskurin	Angoram, ESP	unknown*	3 days
S0603-18	Arepan	Angoram, ESP	large plastic bag	2 weeks
S0603-19	Awain	Wewak, ESP	unknown*	2 days
S0603-20	Wariman	Wewak, ESP	sago leaves	1 week
W0605-01	Aewe	Suki, WP	woven bag	4 - 5 weeks
W1105-01	Erecta	North Fly, WP	unknown	unknown

Legend: ESP: East Sepik Province
MBP: Milne Bay Province
WP: Western Province
NA: Not applicable, samples were not stored.
* Storage was not documented at time of collection, but in these areas sago starch is typically stored in pots covered with water.

APPENDIX 6: HAEMOLTYIC ACTIVITY OF BACTERIA ISOLATED FROM SAGO STARCH AND SAGO STARCH *PER SE*

See Appendix 4 for haemolytic activity of fungal isolates.

Appendix 6.1 Enumeration of Haemolytic Bacteria

Table A6.01 (below) lists the predominant species of haemolytic bacteria isolated from sago starch.

Table A6.01: Predominant haemolytic bacteria isolated from sago starch.

Sago sample	cfu/g	**Predominant species**
W0303-01	1.0×10^3	*Bacillus cereus* group
W0303-02	$<1.0 \times 10^2$	No haemolytic bacteria isolated
W0303-03	$<1.0 \times 10^2$	No haemolytic bacteria isolated
W0303-06	6.0×10^2	*Bacillus cereus* group
W0303-07	9.0×10^2	*Bacillus cereus* group
W0303-09	1.5×10^2	*Bacillus cereus* group
W0303-11		Entire plate partially haemolysed, but isolates non-haemolytic in pure culture
W0303-12	2.5×10^4	Isolates non-haemolytic in pure culture
W0303-13	2.3×10^3	Isolates non-haemolytic in pure culture
W0303-14	1.0×10^4	α haemolytic actinomycete-like and *Pseudomonas aeruginosa*
W0303-15	5.0×10^1	*Bacillus cereus* group
W0303-16	1.0×10^2	unidentified α haemolytic bacteria
W0303-17	2.0×10^3	α haemolytic actinomycete-like
W0303-18	2.4×10^3	*Bacillus cereus* group and Bacillus sp.
W0303-19	3.5×10^2	*Bacillus cereus* group
W0303-20	1.4×10^3	*Bacillus cereus* group
S0603-01		Entire plate partially haemolysed, but isolates non-haemolytic in pure culture
S0603-02	9.5×10^4	*Bacillus* spp.
S0603-03	2.5×10^4	Isolates non-haemolytic in pure culture
S0603-04	2.8×10^5	α haemolytic *Streptococcus* sp. predominant
S0603-05	$<1.0 \times 10^2$	Entire plate partially haemolysed, but isolates non-haemolytic in pure culture

Sago sample	cfu/g	**Predominant species**
S0603-06	$<1.0 \times 10^2$	Entire plate partially haemolysed, but isolates non-haemolytic in pure culture
S0603-07	2.5×10^3	*Bacillus cereus* group
S0603-08	2.5×10^2	*Bacillus cereus* group
S0603-10	1.5×10^5	*Pseudomonas aeruginosa* and *Bacillus cereus*
S0603-12	2.5×10^4	*Bacillus cereus* group and *Bacillus* sp.
S0603-13	1.0×10^2	*Bacillus* sp.
S0603-14	1.5×10^2	*Bacillus* sp.
S0603-16	$<1.0 \times 10^2$	No haemolytic bacteria isolated
S0603-18	1.2×10^4	*Bacillus* spp.
S0603-20	1.0×10^3	non-haemolytic in pure culture
W0404-01	$<1.0 \times 10^2$	No haemolytic bacteria isolated
W0404-03	$<1.0 \times 10^2$	No haemolytic bacteria isolated
W0404-05	$<1.0 \times 10^2$	No haemolytic bacteria isolated
W0404-06	7.0×10^3	*Bacillus cereus* group
W0404-07	4.0×10^3	*Bacillus cereus* group
W0404-08	1.5×10^3	*Bacillus cereus* group and *B. pumilus*
W0404-09	5.0×10^3	*Pseudomonas aeruginosa* and *Bacillus cereus* group
W0404-10	2.5×10^2	*Bacillus cereus* group
W0404-13	1.5×10^3	*Bacillus cereus* group
W0404-14	$<1.0 \times 10^2$	No haemolytic bacteria isolated
W0404-15	$<1.0 \times 10^2$	No haemolytic bacteria isolated
W0404-18	2.0×10^3	*Pseudomonas aeruginosa*
W0404-19	1.0×10^2	*Bacillus cereus* group
W0404-21	$<1.0 \times 10^2$	No haemolytic bacteria isoalted
W0404-22	3.5×10^2	*Bacillus amyloliquefaciens* and *B. pumilus*
W0605-01	1.0×10^5	*Bacillus cereus*

Appendix 6.2 Sago Samples Tested for Haemolytic Activity

Sago starch samples used for analysis of haemolytic activity, using blood agar and hexane extraction:

S1102-01	S1102-07
W0303-01	W0303-02
W0303-03	W0303-05
W0303-10	W0303-17
S0603-07	S0603-12
S0603-15	W0605-01
W1105-01	

Appendix 6.3 Preliminary Studies of Substrate Suitability for Growth of Haemolytic Isolates

Table A6.02: Comparison of haemolytic activity (% haemolysis) after 90 min and 240 min for a selection of haemolytic fungi on different growth substrates.

	90 minutes	**240 minutes**
Corn		
G. candidum	-12 ± 1	8 ± 7
P. steckii (W1-1301)	81 ± 1	80 ± 1
P. steckii (W1-2002)	-3 ± 0	0
P. brevicompactum (S1-0201)	0	1 ± 0
A. flavipes (S2-0207)	69 ± 3	62 ± 4
Rice		
G. candidum	-1 ± 0	0
P. steckii (W1-1301)	0	4 ± 1
P. steckii (W1-2002)	15 ± 1	18 ± 1
P. brevicompactum (S1-0201)	0	0
A. flavipes (S2-0207)	74 ± 0	70 ± 2
Wheat		
G. candidum	-3 ± 0	-2 ± 4
P. steckii (W1-1301)	80 ± 2	76 ± 1
P. steckii (W1-2002)	58 ± 3	55 ± 2
P. brevicompactum (S1-0201)	72 ± 1	74 ± 1
A. flavipes (S2-0207)	55 ± 4	60 ± 14
Sago agar (Sigma)		
G. candidum	5 ± 1	7 ± 1
P. steckii (W1-1301)	1 ± 1	3 ± 0
P. steckii (W1-2002)	2 ± 0	4 ± 1
P. brevicompactum (S1-0201)	3 ± 0	4 ± 0
A. flavipes (S2-0207)	35 ± 3	82 ± 3
Sago agar (Difco)		
G. candidum	5 ± 2	8 ± 3
P. steckii (W1-1301)	Low	Low
P. steckii (W1-2002)	Low	Low
P. brevicompactum (S1-0201)	Low	Low
A. flavipes (S2-0207)	1 ± 1	2 ± 2

Low: Results not documented, but low levels of haemolytic activity observed.
G. = Geotrichum; *P. = Penicillium*; *A. = Aspergillus*

APPENDIX 7: MICROBIAL ECOLOGY OF SAGO STARCH

Appendix 7.1 Nitrogen Levels of Sago Starch

Table A7.01: Percentage nitrogen on a dry weight basis of sago starch. Note that the lower limit of detection using this method is 0.02%. The majority of samples have nitrogen levels below the lower limit of detection.

Sago sample	% Nitrogen	Sago sample	% Nitrogen
S1102-01	0.01	W0303-16	0.04
S1102-02	0.01	W0303-17	0.05
S1102-03	0.01	W0303-18	0.02
S1102-04	0.01	W0303-19	0.01
S1102-05	0.01	W0303-20	0.02
S1102-06	0.01	S0603-01	0.01
S1102-07	0.02	S0603-02	0.02
S1102-08	0.01	S0603-03	0.01
S1102-09	0.02	S0603-04	0.02
S1102-10	0.01	S0603-05	0.02
M1102-01	0.03	S0603-06	0.01
W0303-01	0.08	S0603-07	0.02
W0303-02	0.09	S0603-08	0.01
W0303-03	0.02	S0603-09	0.01
W0303-04	0.02	S0603-10	0.01
W0303-05	0.03	S0603-11	0.01
W0303-06	0.01	S0603-12	0.01
W0303-07	0.02	S0603-13	0.01
W0303-08	0.02	S0603-14	0.08
W0303-09	0.04	S0603-15	0.01
W0303-10	0.03	S0603-16	0.02
W0303-11	0.20	S0603-17	0.02
W0303-12	0.03	S0603-18	0.02
W0303-13	0.03	S0603-19	0.01
W0303-14	0.10	S0603-20	0.02
W0303-15	0.03		

Appendix 7.2 pH of Fermenting Sago Starch

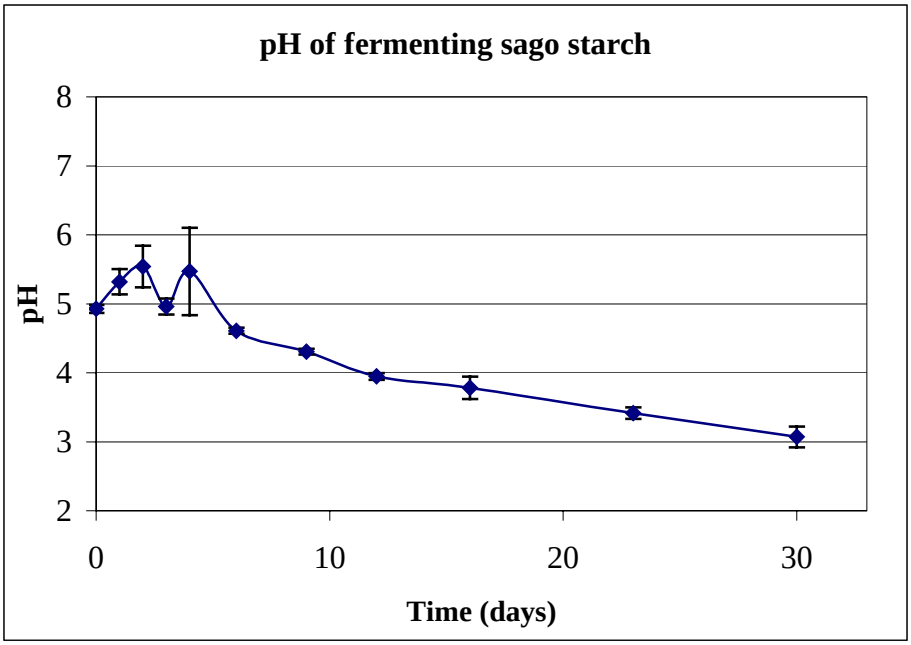

Figure A7.01: pH of laboratory fermented sago starch.

Appendix 7.3 The Role of Lactic Acid bacteria in the Preservation of Sago Starch

Table A7.02: Inhibition of indicator organisms by LAB isolated from sago starch using the inverted ager assay as described in Section 9.2.7.2.

Sample origin	*E. coli*	*E. aerogenes*	*S. salford*	*B. cereus*	*L. monocytogenes*	*S. aureus*
W0405-01 LAB 1	+	+	+	+	+	+
W0405-01 LAB 2	+	+	+	+	+	+
W0405-01 LAB 3	-	+	+	+	+	+
W0405-02 LAB 1	+	+	+	+	+	+
W0405-02 LAB 2	+	+	+	+	+	+
W0405-02 LAB 2	+	+	+	+	+	+
W0405-03 LAB 1	+	+	+	+	+	+
W0405-03 LAB 2	+	+	+	+	+	+
W0405-03 LAB 3	+	+	+	+	+	+
W0405-03 LAB 4	+	+	+	+	+	+
W0405-03 LAB 5	+	+	+	+	+	+
W0405-03 LAB 6	+	+	+	+	+	+
W0405-04 LAB 1	+	+	+	+	+	+
W0405-04 LAB 2	+	+	+	+	+	+
W0405-04 LAB 3	+	+	+	+	+	+
W0405-05 LAB 1	+	+	+	+	+	+
W0405-05 LAB 2	+	+	+	+	+	+
W0405-06 LAB 1	+	+	+	+	+	+
W0405-06 LAB 2	+	+	+	+	+	+

Sample origin	*E. coli*	*E. aerogenes*	*S. salford*	*B. cereus*	*L. monocytogenes*	*S. aureus*
W0405-06 LAB 3	-	+	+	+	+	+
W0405-06 LAB 4	+	+	+	+	+	+
W0405-06 LAB 5	+	+	+	+	+	+
W0405-07 LAB 1	+	+	+	+	+	+
W0405-07 LAB 2	+	+	+	+	+	+
W0405-07 LAB 3	+	+	+	+	+	+
W0405-07 LAB 4	-	-	-	-	-	-
W0405-07 LAB 5	+	+	+	+	+	+
W0405-08 LAB 1	+	+	+	+	+	+
W0405-08 LAB 2	-	+/-	-	+/-	+/-	+/-
W0405-08 LAB 3	+	+	+/-	+/-	+	+/-
W0405-08 LAB 4	+	+	+	+	+	+
W0405-08 LAB 5	+	+	+	+/-	+	+/-
W0405-09 LAB 1	+	+	+	+	+	+
W0405-09 LAB 2	+	+	+	+	+	+
W0405-09 LAB 3	+	+	+	+	+	+
W0405-09 LAB 4	+	+	+	+	+	+
W0405-09 LAB 5	+	+	+	+	+	+
W0405-10 LAB 1	-	+	+	+	+	+
W0405-10 LAB 2	+	+	+	+	+	+
W0405-10 LAB 3	+	+	+	+	+	+
W0405-10 LAB 4	+	+	+	+	+	+
W0405-11 LAB 1	+	+	+	+	+	+
W0405-11 LAB 2	+	+	+	+	+	+
W0405-11 LAB 3	+	+	+	+	+	+
W0405-11 LAB 4	+	+	+	+	+	+
W0405-12 LAB 1	+	+	+	+	+	+
W0405-12 LAB 2	+	+	+	+	+	+
W0405-12 LAB 3	+	+	+	+	+	+
W0405-12 LAB 4	+	+	+	+	+	+
W0405-12 LAB 5	+	+	+	+	+	+

Legend: +: inhibition of indicator organism
-: no inhibition
+/-: reduced inhibition