

Article

Bio-Refining of Carbohydrate-Rich Food Waste for Biofuels

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Abstract: The global dependence on finite fossil fuel-derived energy is of serious concern given the predicted population increase. Over the past decades, bio-refining of woody biomass has received much attention, but data on food waste refining are sorely lacking, despite annual and global deposition of 1.3 billion tons in landfills. In addition to negative environmental impacts, this represents a squandering of valuable energy, water and nutrient resources. The potential of carbohydrate-rich food waste (CRFW) for biofuel (by *Rhodotorulla glutinis* fermentation) and biogas production (by calculating theoretical methane yield) was therefore investigated using a novel integrated bio-refinery approach. In this approach, hydrolyzed CRFW from three different conditions was used for *Rhodotorulla glutinis* cultivation to produce biolipids, whilst residual solids after hydrolysis were characterized for methane recovery potential via anaerobic digestion. Initially, CRFW was hydrolysed using thermal- (Th), chemical- (Ch) and Th-Ch combined hydrolysis (TCh), with the CRFW-leachate serving as a control (Pcon). Excessive foaming led to the loss of TCh cultures, while day-7 biomass yields were similar (3.4–3.6 g dry weight (DW) L⁻¹) for the remaining treatments. Total fatty acid methyl ester (FAME) content of *R. glutinis* cultivated on CRFW hydrolysates were relatively low (~6.5%) but quality parameters (*i.e.*, cetane number, density, viscosity and higher heating values) of

biomass extracted biodiesel complied with ASTM standards. Despite low theoretical RS-derived methane potential, further research under optimised and scaled conditions will reveal the potential of this approach for the bio-refining of CRFW for energy recovery and value-added co-product production.

Keywords: anaerobic digestion; biodiesel; fatty acid methyl ester (FAME); fermentation; *Rhodotorula glutinis*; yeast

1. Introduction

Bio-refining is an alternative to fossil fuel-based refining yielding high-value, low-volume, marketable products, *i.e.*, polymers, pigments, nutraceuticals and biofuels as co-products [1,2]. It is defined as a more sustainable, efficient and flexible process for complete conversion of biomass into value-added products through integrated approaches [3]. Conventionally a variety of processes such as fractionation, liquefaction, hydrolysis, fermentation, pyrolysis, hydrothermal carbonization and bio-gasification are included to recycle waste biomass into value-adding products [2]. Employed refining processes are grouped into four main categories based on energy, economic and by-product developments, *i.e.*, gasification-, pyrolysis-, hydrothermal- and fermentation-based bio-refineries [1,2,4]. A wide range of feedstocks can be bio-refined, but the selection of appropriate processes and their integration is important, yet little information is available at this stage for effective strategies for the bio-refining of food wastes, *i.e.*, more than 80% biomass feedstock used in bio-refineries are wood and shrubs [2,5]. Technically feasible separation operation of the biomass, which would enable separate use or subsequent processing of whole biomass as feedstock through integrated biorefinery approaches are still in initial stages of investigation [6].

Globally, ~1.3 billion metric tons of food waste is generated annually which is estimated to increase in parallel with population growth [7,8]. Within the Australian context, the disposal of 2.29 million tons of food annually to landfills and the resulting biogenic degradation leads to emission of 11 million tons of greenhouse gas emissions (as CO₂ (g) equivalents; [9]). The current technological capability offers few options for the transformation of food waste into bio-energy, but the net energy yields often exceed total energy input (e.g., pyrolysis-based bio-refining exceeded by 20%; [10]). Fermentation-based bio-refining (*i.e.*, anaerobic digestion (AD), composting and direct fermentation processes) are widely accepted methods for food waste treatment yielding favourable end products such as biogas, bio-fertilizers and industrially important acids and alcohols (*i.e.*, lactic acid, succinic acid, acetic acid, ethanol, methanol, *etc.*) requiring low energy inputs [11–13]. The main drawbacks with these conventional technologies are time efficiency (21 and 60 days for AD and composting, respectively), foot print area requirements, GHG emissions (*i.e.*, CO₂ and ammonia during composting), and process-associated health risks due to pathogen spread/odours (*i.e.*, composting). Fermentation-based approaches are widely used either on their own or in combination with other treatment technologies for bio-refining of food waste, mainly to: (i) maximize recycling of nutrients/energy; and (ii) reduce treatment cost, time requirements and environmental burdens. However, there is no appropriate technology integration for mixed food waste treatment coupling energy and nutrient recovery potentials.

Mixed food wastes are rich in sugars, proteins, lipids, vitamins and minerals which are easily assimilated as raw materials for the production of high-value chemicals, bio-oil and polymers by microorganisms such as bacteria, yeast, fungi or algae [8,14]. Yeast is considered superior over other microorganisms due to high biomass growth rates, lipid accumulation capacity, fatty acid profile, and carotenoid, biopolymer and nutraceutical potential [15,16]. Although there are 600 identified yeast species, fewer than 30 species can be categorised as oleaginous with the lipidome accounting for >20% biomass and only very few produce carotenoids and biopolymers [17,18]. In addition, data on bio-refining of carbohydrate-rich food waste using yeast are limited, *i.e.*, largely confined to reported yeast biomass production on other media. For instance, *Yarrowia lipolytica* (phylum Ascomycota) has been used for bio-refining of lipid-rich restaurant-food waste, which proved to be an excellent cultivation medium when coupled with hydrolysis of the complex sugars [19]. In contrast, the red yeast *Rhodotorula glutinis* (phylum Basidiomycota) has been cultivated on different wastewater media [16], food waste and municipal wastewater mixed media [20], glycerol- [15] and starch-containing wastewaters [21]. Another yeast, *Cryptococcus curvatus* showed higher productivity than *Y. lipolytica* when cultured on glucose-based media, but lipid content was 10% lower than *R. glutinis* cultured on food waste hydrolysates [20]. More than 70% increase in lipidome content per gram dry biomass has been achieved in *R. glutinis* under nutrient-limited (nitrogen and phosphate) culture conditions [22,23]. Of the total lipidome accumulated and synthesized, $\geq 90\%$ is comprised solely of triacylglycerides (TAG), free fatty acids (<1%) (FFA) and steryl esters (SE) [24]. Furthermore, *R. glutinis* accumulate the high value bio-product β -carotene (1 kg \sim 1,600 USD) used commercially as a food colorant, nutritional supplement, cosmetic colorant, antioxidant and anticancer agent in pharmaceuticals [16].

These industrially relevant characteristics of *R. glutinis* make it a potential candidate for developing a fermentative bio-refinery concept for food waste treatment. As such, this study aimed to investigate an integrated bio-refinery process consisting of hydrolysis of carbohydrate-rich food waste (CRFW) and yeast fermentation of the resulting hydrolysate using *R. glutinis* for lipid accumulation. The residual solid from the hydrolysis pre-treatment were characterized for bioenergy potential to maximize energy recycling in the proposed bio-refinery approach (Figure 1). Effects of three different hydrolytic pre-treatments (thermal, chemical and thermo-chemical) on fermentative biolipid production and bioenergy recovery potential during the fermentative bio-refinery process of CRFW were investigated and results are discussed.

2. Materials and Methods

2.1. *R. glutinis* Culturing and Acclimatization

A pure culture of *R. glutinis* FRR-4522, an isolate from dairy produce was supplied by the Commonwealth Scientific and Industrial Research Organisation (CSIRO), Australia. *R. glutinis* was subcultured and maintained on 2% agar plates prepared with Yeast Malt (YM) medium (3 g L⁻¹ yeast extract; 3 g L⁻¹ malt extract; 5 g L⁻¹ casein peptone; 10 g L⁻¹ dextrose). Cultivation of *R. glutinis* for CRFW conversion consisted of 350 mL YM broth and 100 mL hydrolysed CRFW (hydrolysis procedures are detailed in section 2.2) at a pH of 4 ± 0.01 in 1 L Erlenmeyer flasks. Seed cultures were maintained in YM liquid medium on a rotary shaker at 95 rpm for a minimum of 5 days (to a maximum of 7 days) at 28 ± 2 °C for biomass enrichment ($\geq 5 \times 10^8$ cells mL⁻¹) prior to inoculations.

The axenic state of seed cultures was confirmed by light microscopy (at 400× magnification on an Olympus CX21LED, Philippines) at every stage of the cultivation process.

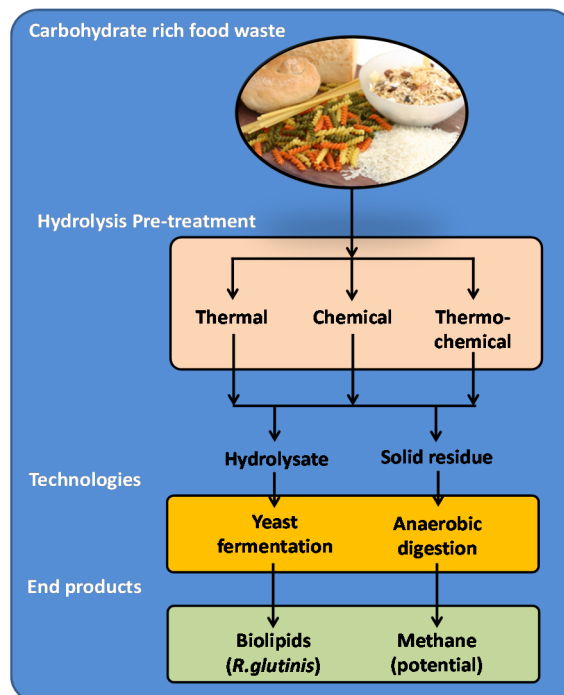


Figure 1. Proposed integrated bio-refining approach for carbohydrate-rich food waste recycling for biofuels

2.2. Bio-Refining of Carbohydrate-Rich Food Waste

CRFW was a mixture of bread (25.9%), oats (29.8%), cooked pasta (27.7%) and boiled rice (16.6%) which was homogenised (<1 cm³ particle size) by grinding (PB7600s MultiBlender™ Pro-Sunbeam). The homogenized CFRW was characterized for various parameters before and after pre-treatment as detailed in Section 2.3.

2.2.1. Hydrolysis of CRFW

Feed slurry was prepared by adding 250 g CRFW to 1 L⁻¹ of deionised (DI) water and refrigerated at 4 °C for 24 h to allow passive leaching of nutrients, whilst minimising microbial growth. The resultant mixtures represented the physical control (PCon) throughout the study (*i.e.*, 4 °C 24 h-leachate). The slurry was hydrolyzed using three different approaches:

- (i) **Chemical hydrolysis (Ch)**—acidic hydrolysis of the CRFW slurry at a pH of 3 ± 0.01 adjusted with 2 M HCl (Sigma Aldrich, Australia) for 24 h at room temperature (25 °C);
- (ii) **Thermal hydrolysis (Th)**—autoclaving of the CRFW slurry using a standard moisture-heat procedure of 121 °C at 1013.25 hPa for 30 min (Tomy, VWR International, Murarrie, QLD 4172, Australia);
- (iii) **Thermochemical hydrolysis (TCh)**—a combined double hydrolysing procedure, where the chemical hydrolysis of the slurry preceded the thermal hydrolysis.

Following hydrolysis, hydrolysates were centrifuged at $15,900 \times g$ for 20 min with 10 min deceleration at $4\text{ }^{\circ}\text{C}$ (Avanti®J-26 XPI, Beckman Coulter, USA). The supernatants (hydrolysates) were decanted into sterilised 2 L Simax bottles for *R. glutinis* enrichment (as detailed in Section 2.2.2). Prior to inoculation with *R. glutinis*, total carbohydrate was determined for each hydrolysate (as detailed in Section 2.3) and pH was adjusted to 4 with addition of either 1 M HCl or NaOH (WP-81, Thermofisher Scientific, Australia). pH probes were cleaned and sterilized with an ethanol wash before each measurement. Residual solids (RS) were characterized for biogas and corresponding bioenergy (methane) potential was estimated using Buswell equation (as detailed in Section 2.3.3).

2.2.2. Cultivation of *R. Glutinis* in Hydrolysates for Bio-Product Development

500 mL of undiluted hydrolysates of each treatment were inoculated with *R. glutinis* $\sim 1.0 \times 10^9$ cells in sterile 1 L Simax reagent bottles with a modified polypropylene cap for aeration and ventilation. The culture bottles were maintained with a continuous airflow of $130 \pm 0.7\text{ L h}^{-1}$ at $28 \pm 2\text{ }^{\circ}\text{C}$ for 7 days. Filtered air 0.45 μm syringe filter (Advantec, VWR International) was supplied via a 2 mL glass pipette connected to a Precision Air Pump 7500 (Aqua One, Local Aquarium, Townsville, Australia) and venting occurred via pipette tips filled with cotton wool. Although photo periods and light intensities are not limiting factors for lipid accumulation in saprophytic microorganisms, *R. glutinis* experiments were conducted under a 12:12 light:dark cycle as cultivation was carried out in the algal culture room at the North Queensland Algal Culture Identification Facilities (NQAIF, James Cook University, Australia). Growth of *R. glutinis* was monitored daily by cell count (Neubauer improved bright-line haemocytometer) and measuring dry weight (DW) gravimetrically [25] at days 0, 1, 3, 5 and 7. Total carbohydrates were measured using the UV-sulphuric acid method [26] at days 1, 4 and 7 and the system pH (portable pH meter-Oaklon®, Singapore) was measured at days 0 and 7. Experiments were performed in triplicates and all sampling occurred in a sterile laminar flow cabinet (AES Environmental Pty LTD fitted with HEPA filter, Australia) to minimise contamination. *Rhodotorula glutinis* biomass was harvested from hydrolysates by centrifugation, was freeze-dried and extracted for transesterification into fatty acid methyl esters (FAME) [27]. In brief, $\sim 30 \pm 4\text{ mg}$ lyophilised *R. glutinis* biomass was measured into 8 mL Teflon capped glass vials (Supelco, Sigma-Aldrich). An equal volume (50 mg) of 0.5 mm zirconium oxide beads (Next Advance) was added to the biomass, serving as abrasive particles for mechanical cell wall disruption. 2 mL of freshly prepared methylation mixture, HPLC-grade methanol and acetyl chloride (95:5 v/v), was added and supplemented with 300 μL internal standard solution (Nonadecanoic acid-C19:0, 0.2 mg mL⁻¹ HPLC-methanol). The methylated-biomass mixture was vortexed at 2,200 rpm for 30 s at 30 s intervals (Schneiter and Daum, 2006). Once homogenised, vials were placed into a block heater (Ratek DBH30, Australia) at $100\text{ }^{\circ}\text{C}$ for 60 min to facilitate transesterification of fatty acids to methyl esters. Heated samples were allowed to cool to room temperature, before adding 1 mL non-polar organic solvent (0.01% BHT w/w HPLC-Hexane) and mixing by inversion. Sample vials were replaced into the warm block heater for 60 s, enabling the formation of a miscible mixture. Once cooled, addition of 1 mL UltraPure water (MilliQ, Life Technologies) separated the two phases. The upper FAME-hexane mixture was collected and filtered through a 0.2 μm PTFE filter (Agilent) prior to its injection into GC vials. Gas chromatography determination of FAME profiles were carried out on an Agilent 7890 GC with flame

ionisation detector (FID) and Electron Ionisation (EI) Turbo Mass Spectrometer (MS) (Agilent 5975C, Agilent Technologies Australia Pty Ltd). A DB-23 column with cyanopropyl stationary phase (60 m × 0.55 mm id × 0.15 μm) with He₂ (g) injection (33 cm s⁻¹ at 50 °C) at 230 kPa was used for sample separation. Constant inlet temperatures for injector and FID were maintained at 150 °C and 250 °C with split injection of 1/50, respectively. Oven and column temperature settings were based on instrumental protocols by the manufacturer. Unknown FAME profiles were determined via comparison of peaks and retention times of pure external standards (C8-C24, Sigma-Aldrich), whilst the recovery potential was corrected using a factor derived from the known concentrations of nonadecanoic acid (C19:0) used as internal standards. Review of the literature and supporting data from procedure blanks confirmed that C19:0 is not produced by *R. glutinis* [23,28,29].

2.3. Analytical Procedure

2.3.1. Reagents and Standard Calibration Gases

All chemicals and reagents were obtained from Sigma-Aldrich, Australia. The calibration CH₄ gases (*i.e.*, 10%–50%), helium and compressed air (N₂-78.08% and O₂-20.94%) for GC-TCD-FID were supplied by BOC a member of the Linde group, Townsville, Australia. All gases were ISO certified for purity.

2.3.2. Characterization of CRFW

25 ± 2 g CRFW and RS were freeze-dried over 48 h (Virtis benchtop 2K, VWR International, Australia). The subsequent lyophilised products were then homogenised in pre-dried (105 ± 2 °C for 4 h) porcelain mortars into a fine powder, which were passed through a 1 mm² stainless steel mesh to exclude large fragments for CHNS-O analysis. The sample analysis was outsourced to Organic Elemental Analysis Laboratories (OEA Laboratory Ltd., Cornwall, UK). Total and volatile solids (TS and VS, [25]) for CRFW and RS were measured and moisture contents were back calculated (moisture% = 100–TS).

2.3.3. Calculating Bio-diesel and Bio-energy Potential of Bio-refined CRFW

The potential physicochemical properties of biodiesel were calculated based on levels of (un)saturation and carbon length of the individual FAMEs using established models [30,31]. For this study, the values of cetane (CN), kinematic viscosity (ν), density (ρ), and higher heating values (HHV_B) of biodiesel were calculated using Equations 1–4, respectively.

$$CN = \sum_i (-7.8 + 0.302 \times M_i - 20 \times N) \quad (1)$$

$$\ln(\nu_i) = -12.503 + 2.496 \times \ln(M_i) - 0.178 \times N \quad (2)$$

$$\rho_i = 0.8463 + \frac{4.9}{M_i} + 0.0118 \times N \quad (3)$$

$$HHV_{B(i)} = 46.19 + \frac{1794}{M_i} - 0.21 \times N \quad (4)$$

where N_i , M_i , and D_i represent the percentage, molecular weight and number of double bonds in the respective i^{th} FAME.

Based on the elemental analysis, theoretical biogas (B_{th}) yields of RS were calculated using Equations 5 and 6 [32] and compared with actual CH_4 potential as reported in literature [33]:

$$B_{th} \left[\frac{m^3}{kg_{VS}} \right] = \frac{a * 22.415}{12a + b + 16c + 14d} \quad (5)$$

$$M_{th} \left[\frac{m^3}{kg_{VS}} \right] = \frac{\left(\frac{4a + b - 2c - 3d}{8} \right) * 22.415}{12a + b + 16c + 14d} \quad (6)$$

where, a: carbon-C%; b: hydrogen-H%; c: oxygen-O% d: nitrogen-N%.

3. Results and Discussion

3.1. Characteristics of Hydrolysates from Pre-treated CRFW

Hydrolysis pre-treatment of CRFW converts complex organic structures into simpler molecules (mainly sugars) making them readily available for microbial conversion [34]. Hydrolysed CRFW had a pH range of 3.77–5.79 based on the different pre-treatments. Therefore, HCl or NaOH were used to reduce/increase the pH of hydrolysates for cultivation of *R. glutinis*. Buffering capacities of the hydrolysates differed, requiring variable volumes and concentrations of acids/bases. Compared to Pcon leachate total carbohydrate content (~ 45 mg eq-Gluc.g⁻¹), carbohydrate release was higher ($\sim 25\%$; *i.e.*, ~ 65 mg eq-Gluc.g⁻¹) in the thermal hydrolysis leading to a change in leachate pH from 4.01 to 5.79, whereas, the Ch or TCh pre-treatments did not improve total carbohydrate release from CRFW. These results are in contrast to acid-hydrolysed fruit and vegetable waste which released more carbohydrates than when subjected to alkali or Th hydrolysis [35]. However, another study showed, that, whilst acid directly hydrolysed/solubilised starch and hemicellulose from CRFW, thermal hydrolysis was more efficient [36]. Furthermore, Th hydrolysis also simultaneously reduces pathogen levels and viscosity of the medium [37], which resulted in easier handling of RS and *R. glutinis* cultivation.

3.2. Growth of *R. glutinis* in CRFW Hydrolysates

Whilst final cell concentrations were similar for Th-, Ch- and Pcon-treated CRFW, growth responses of *R. glutinis* in the early stages of cultivation varied. Foam formation occurred in the culture based on TCh-hydrolysate causing $>80\%$ loss of *R. glutinis* cells and leading to termination on day 4 (Figure 2). Foaming could be due to high aeration and inhibited fermentation processes led to protein degradation in the systems. Culture growth was accompanied by a shift in system pH from 4 to 7.62 ± 0.17 and 6.36 ± 0.02 in Pcon and Th cultures, respectively. Growth of *R. glutinis* was comparable between Th and Ch hydrolysates, peaking within 2 days. However, Ch hydrolysates better supported the growth of *R. glutinis* and achieved maximum cell counts within 24 h. On the other hand, Th hydrolysates contained more carbohydrates, which were expected to provide higher *R. glutinis* biomass.

All systems reached *R. glutinis* densities of $2.60\text{--}2.67 \times 10^8$ cells within 4 days are remained stable during stationary phase until day 7, which is consistent with another report [15]. In terms of

biomass yield, $\sim 3.42\text{--}3.61$ g DW_{biomass} L⁻¹ was measured from Pcon (3.61 ± 0.25 g DW_{biomass} L⁻¹), Ch (3.42 ± 0.41 g DW_{biomass} L⁻¹) and Th (3.53 ± 0.94 g DW_{biomass} L⁻¹) hydrolysates and no significant differences were observed between the cultivation media. Our results were also comparable to biomass yields of $4.3\text{--}6.9$ gDW_{biomass}.L⁻¹ achieved when cultivating *R. glutinis* on carbon sources such as glucose, xylose, arabinose at a pH 5.8 and temperature 28 °C [38]. Carbohydrate analysis at harvest indicated that secondary or continuous cultivation of *R. glutinis* over a longer period may be possible, as $\sim 29\%$ (from Pcon and TCh hydrolysates) and $\sim 24\%$ (from Ch hydrolysate) of carbohydrates were assimilated within 7 days. As nitrogen was likely limiting biomass yield and also carbon utilization [23], nitrogen supplementation could enhance these process performance criteria.

In the context of biofuel potential, for which green microalgae have been identified as a potential biomass source [30] total lipid contents of 14.05% (*Chlorella sp.*) of dryweight biomass have been reported [39]. In contrast, achieved CRFW hydrolysate-cultivated *R. glutinis* biomass yields were 3.5× higher (~ 3.5 g DW L⁻¹ with a total lipid content of 40%) compared to typical yields of green microalgae cultivated in open system suspension cultures [40].

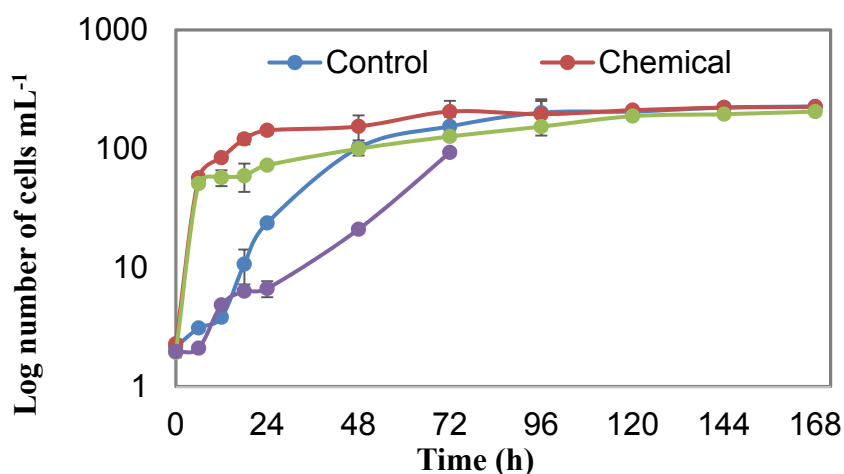


Figure 2. Growth of *R. glutinis* in carbohydrate-rich food waste (CRFW) hydrolysates.

3.2.1. FAME Profile of *R. glutinis* Cultivated in CRFW Hydrolysates

The total fatty acid (TotFA) contents and FAME profiles of *R. glutinis* enriched in CRFW hydrolysates are shown in Table 1. TotFA contents were 41.79 ± 12.6 , 38.05 ± 8.2 and 65.56 ± 30.9 mg_{TotFA}.g⁻¹DW_{biomass} for Pcon-, Ch- and Th-cultivated *R. glutinis*, respectively. TotFA content achieved for Th-cultivated *R. glutinis* is comparable to reported values for its biomass derived from food waste and wastewater ($62\text{--}63$ mg_{TotFA}.g⁻¹DW_{biomass} [20]). A positive correlation between medium carbohydrate content and TotFA content of the derived biomass was also demonstrated in Braunwald, Schwemlein [23]. Compared to Pcon, biomass TotFA of Ch-cultivated *R. glutinis* was lower, suggesting that stored fatty acids may be re-utilized (*i.e.*, lipid turnover) during prolonged cultivation [41]. This is consistent with the literature, reporting a 63% reduction of TotFA content (*i.e.*, from 190 (3rd day) to 120 (5th day) mg_{Lipids}.g⁻¹DW_{biomass}) for *R. glutinis* with prolonged cultivation when cultivated in wastewater [42].

The FAME profiles of CRFW hydrolysate-cultivated *R. glutinis* are shown in Figure 3. Mono- and poly-unsaturated fatty acids (MUFA and PUFA, respectively) accounted for 61%–67%, while

saturated fatty acids (SFA) were only 32%–39% of the biomass. While general fatty acid profiles were similar, the percentage distributions of fatty acids differed. Highest MUFA and PUFA contents were achieved in *R. glutinis* cultivated in Th and PCon hydrolysates, respectively. Irrespective of cultivation medium, fatty acid profiles and elongation and desaturation patterns were typical for *R. glutinis* and *de novo* lipid synthesis [43]. In line with other reports, oleic acid (C_{18:1}) and linoleic acid (C_{18:2}) were the main MUFA and PUFA of *R. glutinis* cultivated in CRFW hydrolysates [16,23,44]. Oleic acid (C_{18:1}) accounted for ~42% in Th-cultivated *R. glutinis* biomass, whilst the levels were 35 and 44% lower in Ch- and Pcon cultivates, respectively. On the other hand, palmitic acid (C_{16:0}) was elevated by 43% in Ch-cultures, while contents were similar for Th- and Pcon cultivated *R. glutinis* biomass. Highest levels of C_{18:2} (26%) were achieved in Pcon-cultivated *R. glutinis* declining by 37.5 and 65% with hydrolysis treatment (Ch- and Th-cultures, respectively) with an opposite trend observed for stearic acid (C_{18:0}). A-linolenic acid (C_{18:3}) was highest in Ch-cultures, slightly lower in Pcon and lowest in Th-cultivated biomass, with an opposite trend observed for palmitoleic acid (C_{16:1}). While margaric acid (C_{17:0}) was not detectable in Th-cultivated *R. glutinis*, heptadecenoic acid (C_{17:1}), C_{16:1} and myristic acid (C_{14:0}) was observed in small amounts under all cultivation conditions. Overall, the FAME profiles obtained from all *R. glutinis* cultures are considered to be ideal precursors for the biofuel industry [44–46].

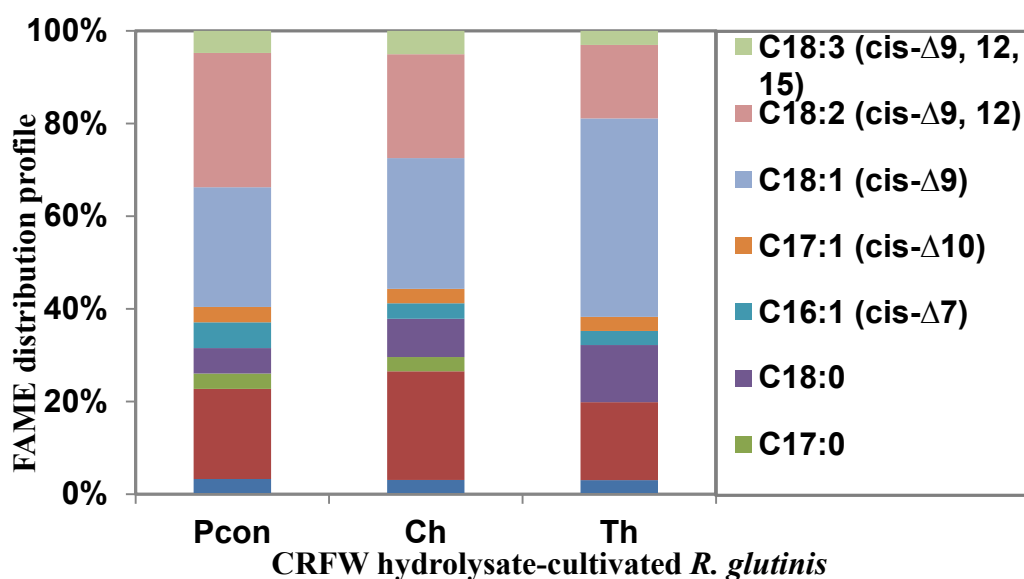


Figure 3. Fatty acid methyl ester (FAME) profiles (%) of *R. glutinis* grown in CRFW hydrolysates.

Table 1. Total fatty acid contents and distribution of fatty acid categories in *R. glutinis* cultivated in CRFW hydrolysates.

Particulars	Pre-treatment of CRFW		
	PCon	Ch	Th
Fatty Acids (mg g ⁻¹ DW)	41.79 ± 12.67	38.05 ± 8.23	65.56 ± 30.91
Branching Fatty Acid (%)	9.88 ± 6.02	3.79 ± 2.18	<3
Saturated Fatty Acid (%)	37.81 ± 7.41	39.5 ± 2.78	32.53 ± 11.50
Monounsaturated Fatty Acid (%)	28.64 ± 12.90	31.82 ± 12.77	51.65 ± 34.85
Polyunsaturated Fatty Acid (%)	33.55 ± 20.81	28.67 ± 12.82	15.82 ± 0.98

3.2.2. Biodiesel Potential of *R. glutinis* Enriched from CRFW Hydrolysates

Biodiesel properties calculated based on FAME profiles of *R. glutinis* biomass cultivated using CRFW-hydrolysates yielded a density of $\rho \sim 0.79$ to 0.85 g cm^{-3} and mean HHVs of $35.79\text{--}39.36 \text{ MJ.kg}^{-1}$. The HHVs were 14%–25% lower than the fossil fuel-derived diesel [47]. Calculated CNs were comparable with the biodiesel standard (ASTM D6751-02), *i.e.*, $\text{CN} > 51$, but higher than demanded standards for fossil fuel-derived diesel (ASTM D975; $\text{CN} \sim 40\text{--}50$). In addition, the calculated viscosity (ν) for the biomass was within the standard limits ($\nu \sim 1.3\text{--}4.1 \text{ mm}^2\text{S}^{-1}$) for fossil fuel-derived diesel (Table 2). In general, higher CN, ρ and HHV are correlated with MUFA content of TotFA of *R. glutinis* harvested from Th hydrolysates. If required, these properties can be tailored to meet the required standards in downstream processes or the biofuel can be used as a diesel blend [48]. While higher SFA contents correlate with higher CN, contributing to shorter ignition delay times and improved oxidative stability of final products [41], cold use properties decline, as the biofuels become more viscous with decreasing temperatures [30]. It should be recognized that at present only B5 (5% biodiesel content) fuels are being used, but the calculated fatty acid-derived properties of CRFW hydrolysate cultivated *R. glutinis* indicates that higher mixing ratios are possible without adverse effects on key fuel properties [30]. In the context of biodiesel production, the fatty acid profiles and contents of the red yeast *R. glutinis* cultivated on CRFW hydrolysates are comparable to those in green algae [40], but biomass yields ($3.5\times$ higher) and total lipid contents ($3\text{--}4.5\times$ higher) were superior than green algal biomass cultivated under phototrophic conditions in open systems. Together with the potential secondary or continuous cultivation option for *R. glutinis* cultivation in CRFW hydrolysates, this would translate into a potentially much smaller production area foot print when compared to green algal biodiesel. However, required energy inputs for the cultivation of the respective biomass will need detailed investigation.

Table 2. Standard biodiesel properties as set by ASTM D6751-02 and EN 14214 and FAME-based calculated biodiesel properties of *R. glutinis* cultivated on CRFW hydrolysate.

	CN	ν	ρ	HHV
ASTM D6751-02	≥ 47	1.9–6.0	0.86 *	NA
EN 14214	≥ 51	3.5–5.0	0.86–0.90	35 **
PCon	51.06 ± 0.12	3.59 ± 0.05	0.79 ± 0.03	35.76 ± 1.23
Ch	56.92 ± 0.36	3.99 ± 0.9	0.84 ± 0.02	38.19 ± 1.11
Th	62.35 ± 0.45	4.46 ± 0.12	0.86 ± 0.06	39.36 ± 1.36

Note: * typical values; ** set by the DIN 51900.

3.3. Characteristics of Residual Solids and Bioenergy Potential

The elemental composition of RS from hydrolysis pre-treatments are given in Table 3. Hydrolysis pre-treatment had no large effect on C, O, N, H, and S contents. Also, high VS ($\sim 96\%\text{--}97\%$) and TS contents suggest that the RS is suitable for bioconversion. The calculated C/N ratios of the RS were between 15–19, similar to those in the organic fraction of municipal solid waste; OF-MSW [49]. For anaerobic digestion, optimal C/N ratios of 27–32 were recommended in order to avoid any build-up of ammonia and associated toxicity effects in reactors that subsequently affect CH_4 yield [49].

Theoretical CH₄ yields could be lower, *i.e.*, 0.16–0.18 m³CH₄.kgVS⁻¹, compared to reported literature values of 0.18–0.24 m³ CH₄.kgVS⁻¹ reported for FW/OF-MSW [32]. This lower CH₄ potential could be due to the removed carbohydrate contents of the waste, supplied as hydrolysates for *R. glutinis* cultivation in the previous experiments, which has been reported in association with the removal of organic material removal following pre-treatment of organic waste [50]. In the context of the proposed bio-refinery concept, the actual CH₄ potential of recycled RS from the hydrolysates of CRFW will require experimental validation.

Table 3. Characteristics of residual solids of CRFW hydrolysates.

Parameters	Pcon	Ch	Th	TCh
Carbon	41.86 ± 0.81	43.02 ± 0.32	42.27 ± 0.28	44.13 ± 0.31
Nitrogen	2.68 ± 0.22	2.83 ± 0.18	2.29 ± 0.26	3.06 ± 0.31
Hydrogen	6.41 ± 0.23	6.4 ± 0.37	6.46 ± 0.18	6.63 ± 0.09
Sulphur	0.12 ± 0.02	0.14 ± 0.01	<0.1	0.19 ± 0.01
Oxygen	48.95 ± 1.11	47.62 ± 0.59	48.99 ± 0.83	46 ± 1.21
C/N ratio	15.62 ± 0.58	15.23 ± 0.67	18.46 ± 0.38	14.42 ± 0.79
Total solids	74.13 ± 2.71	75.92 ± 1.63	74.68 ± 1.42	75.15 ± 2.35
Volatile solids	96.76 ± 0.97	95.75 ± 1.25	95.97 ± 1.54	97.1 ± 1.80

Note: All values are in %.

4. Conclusions

The proposed integrated yeast fermentation and anaerobic digestion process appears to be a promising approach for the bio-refining of CRFW for biolipids and bioenergy production. Biomass yields, total fatty acid content and profile, as well as calculated important diesel characteristics, render *R. glutinis* a suitable alternative to green microalgal biodiesel when cultivated on CRFW hydrolysates under controlled conditions, potentially requiring a fraction of the cultivation foot print. Ch hydrolysates provided better biomass yields, however biodiesel properties and FAME yields were higher with the Th hydrolysate cultivates. In addition, solid residue from the Th pre-treatment was estimated with higher methane potential. Therefore, Th hydrolysis of CRFW followed by *R. glutinis* cultivation under buffered condition should be recommended for further investigation.

Further research is required with regards to outdoor cultivation suitability and competitiveness for CRFW recycling. Residual solids characteristics and theoretical yields show promise for additional energy benefits that can be derived through the bioconversion of CRFW. However, the full potential of this novel integrative bio-refinery concept for CRFW hydrolysates and residual solids requires further optimization of cultivation conditions and field experimentation to validate biomass yields, total lipids yields and actual methane production, as well as a full characterization of the resulting biodiesel characteristics and quantities, which will lay the foundation for a comprehensive techno-economic analysis and energy requirements of bioenergy generation using different feedstocks. In addition, unutilized sugars from *R. glutinis* fermentation could be potentially re-cycled within the fermentation system or anaerobically digested for making this technology more energy efficient and economically viable. Furthermore, heat and power generated from biogas combustion could be potentially re-routed for hydrolysis pre-treatments which would benefit the proposed integrated bio-refining approach.

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Author Contributions

All authors contributed to generating this manuscript. The research data were generated within the Honours research project by Hoang-Tuong Nguyen Hao under the supervision of Obulisamy Parthiba Karthikeyan and Kirsten Heimann.

Conflicts of Interest

The authors declare no conflict of interest.

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