

Full Length Article

A combined pretreatment, fermentation and ethanol-assisted liquefaction process for production of biofuel from *Chlorella* sp.Quazi Mahzabin Rahman^a, Bo Zhang^b, Lijun Wang^{b,*}, Abolghasem Shahbazi^b^a Energy and Environmental Systems, North Carolina A&T State University, Greensboro, NC, USA^b Department of Natural Resources and Environmental Design, North Carolina A&T State University, Greensboro, NC, USA

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ABSTRACT

A combined pretreatment, fermentation and ethanol-assisted liquefaction approach was investigated to produce biofuel and biochemicals from freshwater microalga *Chlorella* sp. Microalgal biomass was pretreated with dilute sulfuric acid in different acid concentration (1–3 wt.%), biomass concentration (40–150 g/L) and pretreatment time (15–60 mins) to observe the effects on carbohydrate yield. The glucose concentration of 10.88 g/L was obtained with a glucose yield of 90.7% from biomass loading of 80 g/L pretreated with 3% (wt%) dilute sulfuric acid at 121 °C for 60 min. The usability of the pretreated hydrolysate as fermentation medium was determined using two yeast species—*Saccharomyces cerevisiae* and *Pichia stipitis*. Similar ethanol yields were obtained from both yeasts at lower biomass loading of 40 g/L. However, at increased biomass loading of 100 g/L, *P. stipitis* obtained higher ethanol yield of 74.73% with higher glucose and xylose utilization compared to ethanol yield of 68.59% from *S. cerevisiae*. Following fermentation, liquefaction assisted with 15% (v/v) ethanol at 265 °C converted fermented microalgae to crude biodiesel, aqueous products and solid residue. This newly developed process could increase the crude biodiesel yield by 40.7%, compared to liquefaction of original microalgae. The main advantage of this combined approach is the utilization of fermented algae in essential ethanol production within the process to enhance the crude biodiesel yield by ethanol-assisted liquefaction.

1. Introduction

Biodiesel is mono alkyl esters of long chain fatty acids that can be produced from acyl-glycerol (usually triglyceride) in vegetable oils or animal fats [1–3]. Biodiesel offers many advantages as it is nontoxic and biodegradable and can be used in most diesel engines with no or only minor modification [1,2,4]. Among many different forms of feedstocks used to produce biodiesel, microalgae have been considered as one of the most promising feedstocks due to their higher growth rate and high lipid/oil content [5–7]. Depending on the species and growth condition, microalgae possess a significant amount of carbohydrate, lipid and protein [8–10]. For this reason, microalgae can be used for the production of bioethanol, biodiesel, biohydrogen and biogas [11–13]. Different methods have been explored to produce biodiesel from microalgae. The general approach involves extraction of algal oil from dried algae followed by transesterification of the oil to biodiesel using an alcohol in presence of a catalyst [10,14]. Non-catalytic lipid hydrolysis and esterification were also achieved under supercritical or subcritical condition with or without using organic solvents [15–17]. Organic solvents can assist the thermal treatment of microalgae for

multiple purposes, such as extraction of algal lipids, in situ transesterification of lipids as well as assisting liquefaction yield [18]. Increased bio-oil quality and biodiesel yield have been reported in the literature for organic solvent (examples-ethanol and methanol) assisted thermal treatment of wet microalgae [14,19–21]. Because methanol is readily available at a low price, it has been widely used in supercritical transesterification [22,23]. However, the toxic properties of methanol and its production from petroleum-based resources restrict the development of byproducts from residual biomass such as livestock feed for cattle and aqua culture [20,24]. The use of ethanol is advantageous as ethanol can be produced exclusively from carbohydrate rich renewable sources which can make the process more sustainable and renewable [20,25,26].

In fact, some microalgal species have higher carbohydrate contents with the absence of lignin which makes them excellent substrates for bioethanol production [27–29]. *Saccharomyces cerevisiae* is the primary species that has been used for ethanol production [30,31]. But the widely used fermenting yeast *S. cerevisiae* cannot metabolize pentose sugars, such as xylose and L-arabinose, which are also available in microalgae biomass [32]. A complete and efficient conversion of the

* Corresponding author.

E-mail address: lwang@ncat.edu (L. Wang).<https://doi.org/10.1016/j.fuel.2019.116026>

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lignocellulosic hydrolysate containing hexose and pentose sugars to ethanol has been explored in the literature using xylose fermenting yeast *Pichia stipitis* in co-culture with glucose fermenting yeast [33,34]. However, *Pichia stipitis* has not been used for fermenting microalgae hydrolysate as per our knowledge which could enhance the ethanol yield from total carbohydrates available in algae. Making full use of lipids and carbohydrates in microalgae biomass for joint production of biodiesel and bioethanol has been described as an economic method for biofuel production from microalgae [11,35]. Wang et al. [35] reported joint production of biodiesel and bioethanol via acid hydrolysis of *Tribonema* sp. utilizing both lipid and carbohydrate. Biorefinery concept with wet microalgae *Chlorella* and *Scenedesmus* for integrated lipid and carbohydrate-based biofuels production was demonstrated by Lauren et al. [36]. In our previous study, a combined fermentation and ethanol-assisted liquefaction approach was explored with high lipid content saltwater species *Nannochloropsis* sp. [37]. The direct use of in-situ ethanol in the fermented hydrolysate through combined fermentation and liquefaction for freshwater species *Chlorella* sp. has not been explored so far.

This study explores a biorefinery approach combining pretreatment, fermentation and ethanol-assisted liquefaction to produce biofuels from microalga *Chlorella* sp. In addition, xylose fermenting yeast *P. stipitis* fermentation was also explored to maximize carbohydrate utilization. The main concept of this study is to develop a sustainable biorefinery approach from microalgae utilizing all three major components of the algal biomass.

2. Method

2.1. Biomass preparation

Microalga *Chlorella* sp. in dry powder form was purchased from food store Nuts.com (Cranford, New Jersey, US) and stored at room temperature. According to the description from this company, this alga was cultivated in a closed sterilized indoor facility in Gunsan, South Korea. It was grown in heterotrophic tanks and was processed into dry powder by subsequent heating and cooling treatment. This dry alga was referred as original dry biomass during experiments.

2.2. Microalgae characterization

Microalgal biomass were analyzed for the ash content, the solid content and carbohydrates using the laboratory analytical procedures developed by the National Renewable Energy Laboratory (NREL) [38,39]. The lipid content of microalgal biomass was determined according to NREL's protocol [40], in which the total lipids are expressed as fatty acid methyl esters (FAME). This procedure involves a whole biomass transesterification of lipids to FAME, which eliminates the need for extraction and therefore is able to access all fatty acids in the biomass and represent an accurate reflection of the biofuels potential [40].

Elemental analyses for carbon (C), hydrogen (H), and nitrogen (N) contents were determined using a Perkin–Elmer 2400 CHN/S analyzer (Waltham, MA). The contents of C, H, N and S were calculated on a dry basis. All experiments and analyses were performed in duplicate.

2.3. Bioethanol production

2.3.1. Microalgae pretreatment

Dilute sulfuric acid was chosen for pretreatment in this study. To observe the carbohydrate yield, a range of pretreatment temperature, time (15–60 min), biomass concentration (40–150 g/L) and acid concentration (1–3% w/w) parameters were set at different levels. For initial pretreatment experiments, algae biomass was loaded in a 30 mL heavy walled pressure tube. Required weight of microalgae and acid solution were added and the tube was sealed with rubber stopper and

aluminum crimp cap. After adding the acid to biomass, it was shaken in a vortex mixture. An autoclave was used to pretreat the biomass at required temperature and time.

After the pretreatment, the hydrolysate was cooled down to room temperature and 5 mL of hydrolysate was adjusted to pH 5 using 4 N NH_4OH . From the neutralized hydrolysate, 1 mL sample was collected for high-performance liquid chromatography (HPLC) analysis.

2.3.2. Yeast preparation

Two microorganism *Saccharomyces cerevisiae* (ATCC 24858) and *Pichia stipitis* (ATCC 58785) were cultured using yeast mold (YM) broth medium (Dickinson and Company, Sparks, Maryland, USA). The initial culture was prepared in the test tube with a volume ratio of 1:4 for seed inoculation to culture volume. The yeast cultures were scaled up by 10 times on a volumetric basis by transferring 0.5 mL of previously cultured yeast to 5 mL YM broth solution and grown at 30 °C for 24 h. After cultivation, the yeast cells were harvested by centrifuging and washed twice with peptone water. The centrifuged and washed yeast cells were used for fermentation.

2.3.3. Combined pretreatment and fermentation

Both pretreatment and fermentation were carried out in a 250 mL shake flask with 50 mL working volume containing 8% (w/v) algal biomass and 3% (w/w) sulfuric acid. Weight of algae used was 4 g dry weight in total 50 mL volume. An autoclave was used to maintain the pretreatment temperature at 121 °C for 60 min. Loss of solvent during autoclaving was measured and compensated during pH adjustment step. The acid pretreated algae slurry was adjusted to pH 5 by adding 4 N ammonium hydroxide and was directly used as the fermentation medium for bioethanol production. Centrifuged and washed yeast cells prepared as section 2.3.2 (5 mg dry basis) were added to hydrolysate and anaerobically fermented for 48 h in the shaker at 30 °C and 150 rpm. The liquid samples were obtained and analyzed in a Waters HPLC (Milford, MA, USA) for residual sugar and ethanol concentrations. A small amount of fermented sample was dried for the determination of lipid content and characterization. Yeast was also grown in YM medium separately as a control.

Fermentation of the algae hydrolysate with a nutrient supplement of yeast extract and inorganic salt was conducted to observe the change in the product yield. Yeast extract and inorganic salt supplement solution was prepared according to the method reported by Fu et al. [33] with modification. The fermentation medium was supplemented with nutrients to final concentrations of 1 g/L ammonium sulfate, 0.5 g/L potassium phosphate, 0.25 g/L magnesium sulfate, 2 g/L yeast extract, 1 g/L peptone and 80 g/L microalgae concentration as carbon source.

The ethanol yield was expressed as the percentage of the theoretical yield (Eq. (1)) following the formula used in literature for lignocellulosic biomass [41] with some modification, which is given by:

$$\% \text{Yield Ethanol} = \frac{[\text{Ethanol}]_f - [\text{Ethanol}]_o}{0.568 \times f \times [\text{Biomass}]} \times 100\% \quad (1)$$

where, $[\text{Ethanol}]_f$ – Ethanol concentration at the end of the fermentation (g/L); $[\text{Ethanol}]_o$ – Ethanol concentration at the beginning of the fermentation (g/L); $[\text{Biomass}]$ – Dry biomass concentration at the beginning of the fermentation (g/L); f – Combined Cellulose and hemicellulose fraction of dry biomass (g/g); 0.568 – Conversion factor from cellulose and hemicellulose to ethanol.

2.4. Crude biodiesel production from fermented algae

Fermented algal broth obtained with initial dry algae weight of 4 g and biomass concentration of 8% (w/v) was thermally treated via ethanol-assisted liquefaction to produce crude biodiesel. The experiments were performed in a Parr 75 mL stainless steel bench top reactor accompanied by a controller unit (Moline, Illinois, USA). Additional

amount of ethanol (pure 200 proof) was added to obtain a final ethanol concentration of 15% (by volume) in the reactor. For liquefaction of non-fermented original *Chlorella* sp., ethanol and deionized water were added to obtain the same ethanol concentration of 15% (by volume). The ethanol concentration of 15% was chosen since the commercial starch-to-bioethanol fermentation process could yield a 10–15% ethanol concentration.

The reactor was heated to 265 °C at a heating rate of 15 °C/min and held at the final temperature for 30 min. The temperature was measured by a thermocouple inserted into the slurry, and the reactor pressure was monitored by a pressure gauge connected to the reactor. The liquefaction temperature and time used in this study were chosen based on the findings from the literature [20] and our previous study [18]. After 30 min, the reactor was cooled down to the room temperature quickly by using an electric fan. Gaseous products were then released through a control valve, and the content in the reactor was collected. The solid product was separated from the liquid by vacuum filtration and dried at 105 °C overnight for elemental analysis. The liquid fraction was further extracted with 50 mL hexane in a separatory funnel, and the hexane phase (upper layer) and the aqueous products (bottom layer) were separated. Hexane was evaporated under nitrogen and the weight remained was recorded to calculate the crude biodiesel yield.

The product yield was expressed in wt.% and calculated by following equations-

$$\text{Solid residue yield \%} = \frac{\text{Weight of solid residue}}{\text{Initial weight of microalgae}} \times 100 \quad (2)$$

$$\text{Crude Biodiesel yield \%} = \frac{\text{Weight of crude biodiesel}}{\text{Initial weight of microalgae}} \times 100 \quad (3)$$

$$\text{Aqueous product yield \%} = 100 - \text{Solid residue yield\%} - \text{Crude Biodiesel yield\%} \quad (4)$$

The gas yields were calculated from the final pressure after reactions, the volume of free space in the reactor and the gas composition using the ideal gas law, and was considered negligible (< 2%) [24].

Both the crude biodiesel and the aqueous products were analyzed by using an Agilent 7890 GC/5975 MS equipped with a DB-1 nonpolar capillary column (Santa Clara, CA, USA). The injection temperature was set at 250 °C. The oven temperature was set at 40 °C and held for 2 min, followed by a ramp at 10 °C/min to 250 °C and then held for 10 min. The components in the samples were identified by comparing to the mass spectra library of the National Institute of Standards and Technology (NIST, USA).

Fatty acid ethyl esters (FAEE) content in the crude biodiesel was quantified using the FAEE standard of Supelco #49454-U (College Park, GA, USA). FAEE yield was calculated as the weight percentage (wt%) of total lipid content. The elemental composition of crude biodiesel and solid residue was analyzed by using a Perkin Elmer 2400 series II CHNS/O Analyzer (Waltham, MA, USA).

3. Results and discussion

3.1. Characterization of *Chlorella* sp.

The total solid content and the ash content of the dry *Chlorella* sp. were measured as 99.1% and 4.54% respectively (Table 1). Total carbohydrates in algal biomass were determined as glucose, xylose and arabinose present after inorganic acid hydrolysis and total carbohydrate content obtained was 23.9% of dry ash-free weight. Lipid content was determined as 8.2%. Both carbohydrate and lipid contents were found at close range to the reported value from the seller.

Fatty acid profile of *Chlorella* sp. contains saturated and unsaturated carbon chain lengths from C14 to C24 (Table 2). The major components of lipids were saturated palmitic acid (C16:0, 22%) and unsaturated

Table 1

Proximate and ultimate analysis of algal biomass.

Proximate Analysis of Microalgae, wt% (dry basis)	
Water content	0.9
Dry algae content	99.1
Ash content	4.54
Biochemical components:	
Carbohydrate	23.9
Protein	55.2
Lipid	8.2
Elemental Analysis, wt%	
Carbon	54.35
Hydrogen	8.53
Nitrogen	8.83
Sulfur	1.54
Oxygen ^a	26.76
algae HHV ^b (MJ/Kg)	26.61

^a Calculated by difference as O = 100-(C + H + N + S)

^b Calculated by using Boie's formula HHV = 0.3516C + 1.16225H + 0.0628N - 0.1109O

Table 2

Fatty acid profile (as FAME) of *Chlorella* sp.

FAME components	Area (% of total methyl esters)
7,10-Hexadecadienoic acid, methyl ester (C16:2)	18.6
9-Hexadecenoic acid, methyl ester (C16:1)	1.3
Hexadecanoic acid, methyl ester (C16:0)	22.0
9,12-Octadecadienoic acid, methyl ester (C18:2)	53.8
9-Octadecenoic acid, methyl ester (C18:1)	4.0
Methyl stearate (C18:0)	0.3

linoleic acid (C18:2, 53.8%).

No more than two double bonds polyunsaturated fatty acids were found, which is good for biodiesel properties. It has been reported that polyunsaturated acid with four or more double bonds are susceptible to oxidation during storage and this reduces the acceptability as biodiesel [42].

3.2. Pretreatment of *Chlorella* sp.

3.2.1. Effect of acid concentration on pretreatment

Effect of acid concentration on pretreatment was observed for 1–3% (wt.%) sulfuric acid concentration. Pretreatment temperature was set to 121 °C and 60-minute pretreatment time and 80 g/L biomass concentration was used. Deionized water was used as the control. Glucose, xylose and arabinose concentration after pretreatment was measured by using HPLC. It was observed that carbohydrate concentration increased for all three components with increasing acid concentration (Fig. 1). The highest 10.88 g/L concentration was obtained for glucose, and the second most abundant monosaccharide observed was xylose and then arabinose. Similar trend in result was reported in the literature [35,43].

The highest glucose yield of 90.7% was obtained for 3% acid concentration (Fig. 2). Ho et al. [44] reported that, total carbohydrate yields of nearly 100% was obtained when the acid concentration was 2.5% or higher, but the biomass loading (solid-to-liquid ratio, w/v) used for that study was 1%, quite lower than 8% used in our study.

However, the increase of the concentration of inhibitors were also reported in higher acid concentration pretreatment in literature [43]. Considering the need to minimize the dosage of acid, sulfuric acid concentration of 3.0% (wt.%) seems to be preferable in the acid hydrolysis, which was also recommended and used in the literature [35,45].

3.2.2. Effect of biomass concentration on pretreatment

Effect of biomass concentration on pretreatment was observed by

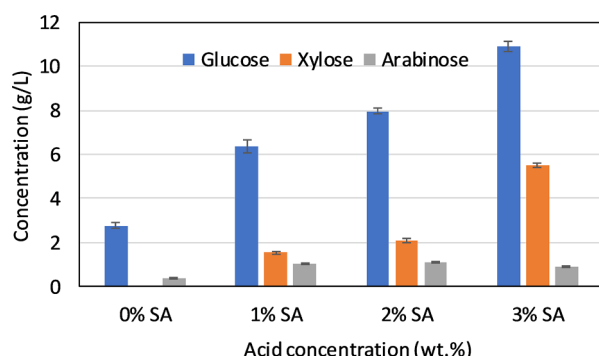


Fig. 1. Effect of sulfuric acid (SA) concentration on carbohydrate concentration.

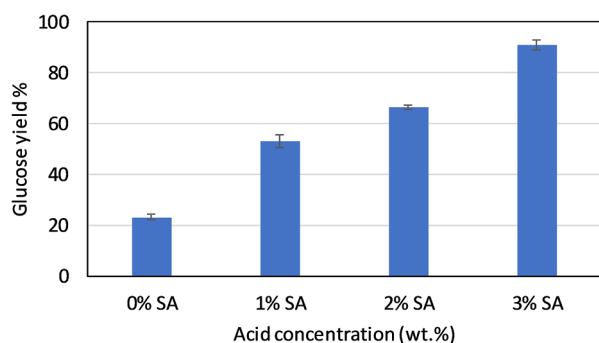


Fig. 2. Effect of acid concentration on glucose yield.

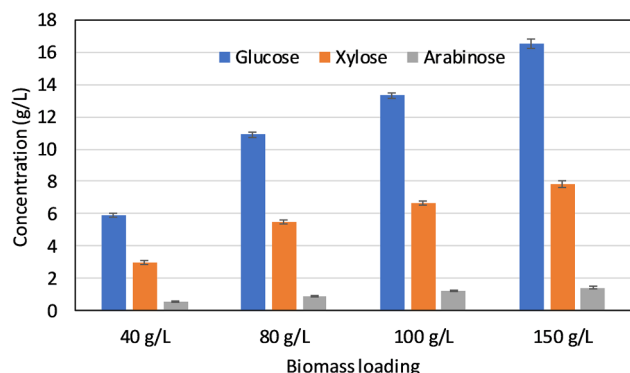


Fig. 3. Effect of biomass concentration on carbohydrate concentration.

varying biomass concentration from 40 to 150 g/L. Pretreatment temperature was set to 121 °C and 60-minute pretreatment time and 3% acid concentration was used. It was observed that glucose and xylose concentration increased with the increasing biomass concentration (Fig. 3). The highest glucose concentration of 16.53 g/L was obtained for 150 g/L biomass concentration.

Although carbohydrate concentration increased with the increasing biomass load which is expected, the glucose yield decreased by 25% with 150 g/L biomass concentration (Fig. 4). The highest glucose yield was obtained from the lowest biomass concentration of 40 g/L. Similar result was reported in the literature for dilute sulfuric acid pretreatment of *Chlorella* sp. Giang et al. [43] reported, biomass concentration beyond 40 g/L did not improve the sugar production.

The result suggested that the low dispersion of sugar due to the inadequate space in the reaction vessel might reduce the hydrolysis rate at a higher biomass load which is reported in literature [35]. Higher biomass load of 150 g/L decreased the accessibility of acid to biomass by forming thick slurry. It has also been reported that higher biomass concentrations can decrease the bioethanol production levels due to

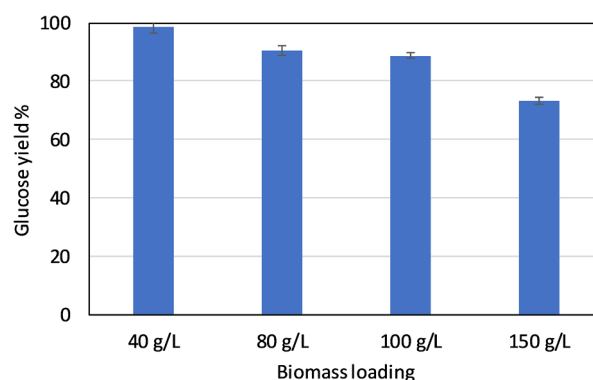


Fig. 4. Effect of biomass concentration on glucose yield.

overburdened metabolism and the toxicity of high ethanol concentrations towards yeast cells [46,47]. Hence, the 40–80 g/L biomass concentration range was used in this study.

3.2.3. Effect of acid pretreatment time

Effect of pretreatment time of 15–60 min was observed. Pretreatment temperature was set to 121 °C with 80 g/L biomass loading and 3% dilute sulfuric acid was used for all experiments. Glucose, xylose and arabinose concentrations after pretreatment were measured by HPLC. It was observed that carbohydrate concentration increased for all three components with increasing pretreatment time (Fig. 5).

Similar to carbohydrate concentration, glucose yield also increased with pretreatment time (Fig. 6). The glucose yield of 70% was obtained from pretreatment time of 30 min whereas the 90% glucose yield was obtained by 60 min pretreatment.

In the literature, similar results were reported for dilute sulfuric acid pretreatment of microalgae biomass. Giang et al. [43] reported an increased production of reducing sugars with the increase of pretreatment time up to 180 min. Wang et al. [35] reported linear increase of sugar concentration upto 45 min pretreatment time. Hence, pretreatment time of 60 min was chosen and used for other pretreatment experiments in this study.

3.3. Fermentation

3.3.1. Effect of salt supplement on fermentation

Inorganic salt supplement along with yeast extract was added to acid-hydrolyzed microalgae, and the ethanol yields of different conditions were compared. Fig. 7 reveals that both *S. cerevisiae* and *P. stipitis* preferred glucose as the utilization of glucose was close to 100%. The result indicated that *P. stipitis* can utilize a higher amount of xylose than *S. cerevisiae*, although similar ethanol yields were observed for both yeast species. Higher ethanol yield was observed in the hydrolysate

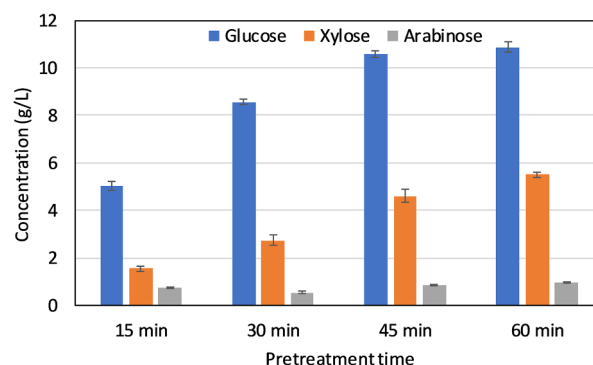


Fig. 5. Effect of pretreatment time on carbohydrate concentration.

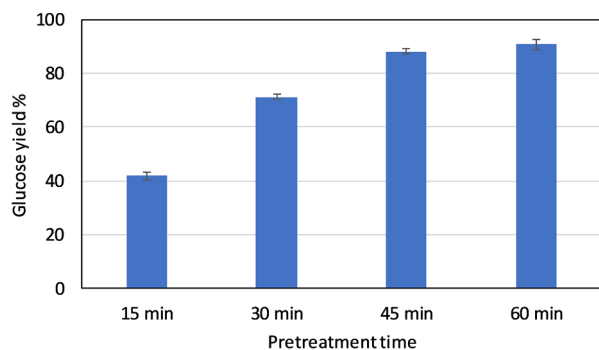


Fig. 6. Effect of pretreatment time on glucose yield.

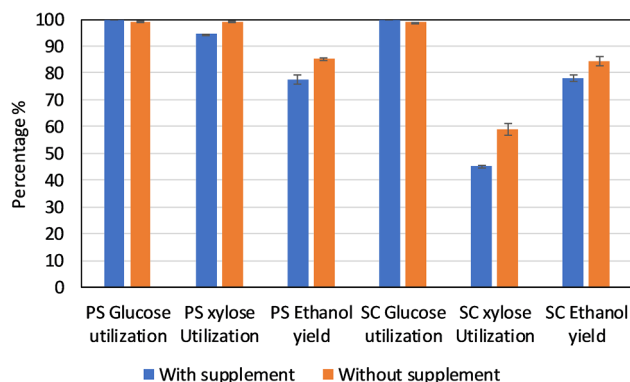


Fig. 7. Effect of salt supplement on ethanol fermentation.

without adding salt medium which indicates the direct usability of hydrolysates for fermentation. High conversion of xylose by *P. stipitis* has been reported in the literature [33,48].

As adding yeast extract and salt supplement does not affect ethanol yield, acid hydrolysate was directly used after pH adjustment for the other fermentation experiments in this study.

3.3.2. Effect of biomass concentration on fermentation

Effect of biomass concentration was observed on glucose, xylose utilization and ethanol yield during fermentation for 40 g/L and 100 g/L biomass concentration. Fig. 8 shows the glucose, xylose and ethanol profiles during fermentation at 100 g/L biomass concentration. From the concentration profile, it is clear that both yeast preferred glucose at the beginning as substrate. For *S. cerevisiae*, glucose was utilized faster and ethanol concentration did not increase after 18–24 h. *P. stipitis* also preferred glucose first and started to use xylose until glucose in medium was depleted to half. Similar trend is reported in literature. Agbogbo et al. [49] reported *P. stipitis* requires glucose concentration in the medium to be below 2% (w/v) before significant xylose utilization is initiated in a mixed substrate fermentation.

The maximum ethanol concentration achieved from *S. cerevisiae* was 9.57 g/L, whereas *P. stipitis* fermentation obtained a slightly higher ethanol concentration of 10.31 g/L from 100 g/L biomass loading. It was noticeable from the concentration profile (Fig. 8) that *S. cerevisiae* produced ethanol faster almost within 18-h whereas *P. stipitis* produced a similar amount of ethanol after 24-h fermentation.

Fig. 9 shows the effects of biomass concentration on sugar utilization and ethanol yield for both yeasts for 48-h fermentation. Glucose was utilized completely for both 40 g/L and 100 g/L biomass concentration, suggesting no obvious effect of biomass concentration within this range on yeast growth. Xylose utilization was higher for *P. stipitis* fermentation as observed earlier, but a higher biomass concentration dropped the utilization rate of xylose from 99.10% to 64.42% (Fig. 9). As a higher biomass concentration provided a higher amount of glucose, it was consumed before xylose fermentation. This is

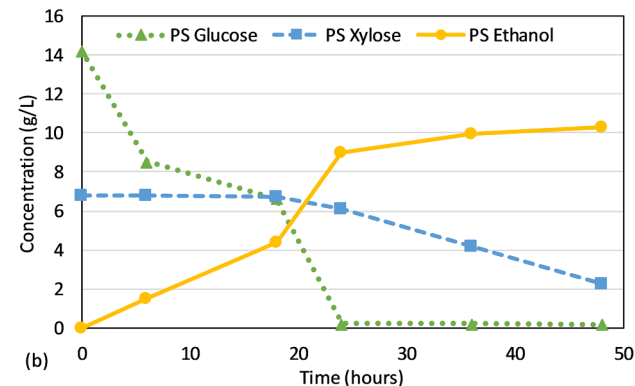
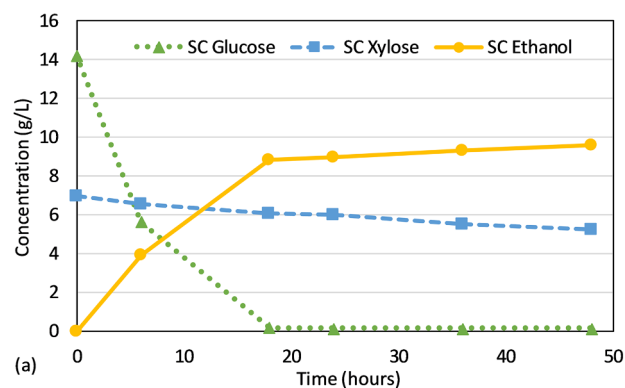
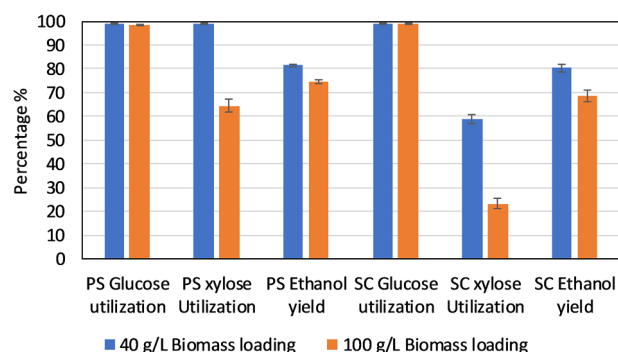
Fig. 8. Glucose, xylose and ethanol profile of a) *S. cerevisiae* and b) *P. stipitis* fermentation with 40 g/L biomass concentration.

Fig. 9. Effect of biomass concentration on glucose, xylose utilization and ethanol yield.

due to repression of xylose uptake by glucose [50]. In the literature [49], required fermentation time of 120 h has been reported for complete utilization of xylose at higher biomass concentration whereas 48-h fermentation time was used in this study. Longer fermentation time could provide more information towards total carbohydrate utilization.

The ethanol yields were similar for both species- 81.42% and 80.33% for *P. stipitis* and *S. cerevisiae* respectively at biomass loading of 40 g/L. When biomass loading was increased to 100 g/L, *P. stipitis* obtained higher ethanol yield of 74.73% compared to ethanol yield of 68.59% from *S. cerevisiae*. Although *P. stipitis* performed better in higher biomass concentration compared to *S. cerevisiae*, overall a slight decrease of the ethanol yield was observed for higher biomass concentration from both yeast. The overall decrease in ethanol yield at higher biomass concentration is expected due to overburdened metabolism and the toxicity of high ethanol concentrations towards yeast cells [46,47]. Similar result from *P. stipitis* fermentation at higher biomass concentration was also reported in literature. Agbogbo et al. [49] reported *P. stipitis* has a slightly higher ethanol concentration and yield

Table 3
Fatty acid profile (as FAME) of non-fermented and fermented *Chlorella* sp.

Fatty acid components	Area (% of total methyl esters)	
	Original biomass	Fermented biomass
7,10-Hexadecadienoic acid, methyl ester (C16:2)	18.6	18.9
9-Hexadecenoic acid, methyl ester (C16:1)	1.3	1.7
Hexadecanoic acid, methyl ester (C16:0)	22	21
9,12-Octadecadienoic acid, methyl ester (C18:2)	53.8	53
9-Octadecenoic acid, methyl ester (C18:1)	4	4.7
Methyl stearate (C18:0)	0.3	0.6

when growing on high xylose fractions because the cells used the xylose for ethanol production rather than for cell growth after initial glucose utilization. This result indicates *P. stipitis* can tolerate higher ethanol concentration and higher biomass loading compared to *S. cerevisiae* which could be beneficial for fermentation involved in higher biomass loading.

3.3.3. Effect of pretreatment and fermentation on fatty acid profile

The total lipid contents of feedstocks were measured before and after pretreatment and fermentation. It was observed that total lipid content increased from 8.2% to 12% of dry weight after pretreatment and fermentation. Table 3 shows the changes in the fatty acid profile of original and fermented biomass. Similar fatty acid profiles were observed before and after fermentation, which matches the findings obtained in our previous study for *Nannochloropsis* sp. [37]. This result makes the fermented broth a potential feedstock for biodiesel production via hydrothermal treatment or liquefaction.

3.4. Ethanol-assisted liquefaction of fermented algae

3.4.1. Effect of acid hydrolysis and pretreatment on product yield

Product yields (wt% of microalgae) after ethanol-assisted liquefaction of fermented and original algal biomass are shown in Fig. 10. Compared to original microalgae, liquefaction of fermented microalgae resulted in a lower solid residue yield. Application of pretreatment and fermentation prior to liquefaction of microalgae increased crude biodiesel production by 40.7% from 11.26 wt% to 15.84 wt%. This result is in accordance with our previous finding that pretreatment and fermentation enhances the crude biodiesel yield [37]. The increase of total lipid in fermented algae may have attributed to this increase in the crude biodiesel yields.

FAEE yields (wt% of total lipid) were quantified as 33.7% and 13.6% for ethanol-assisted liquefaction of fermented and non-fermented microalgae respectively. Hence, the FAEE yield of fermented dry *Chlorella* sp. increased by 2.5 times of original biomass liquefaction with a 2:1 ethanol-to-algae ratio, which indicated that pretreatment and fermentation can improve the FAEE yield at lower ethanol concentration in reaction. The biodiesel yields obtained in this study are

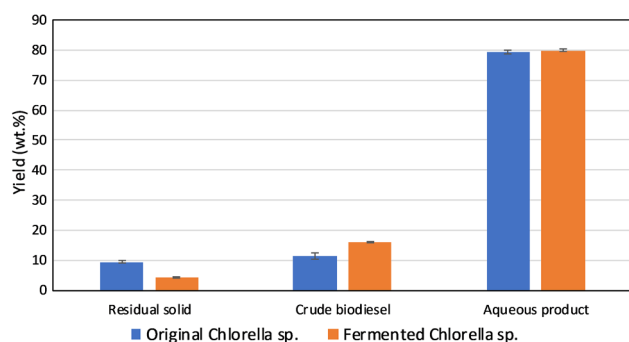


Fig. 10. Ethanol-assisted liquefaction product yield comparison from original and fermented *Chlorella* sp.

relatively low, which can be due to the low ethanol-to-algae ratio (2:1) and low lipid content in the reaction mixture. Necessity of higher ethanol-to-algae ratios is mentioned to drive the ethyl ester production reaction at a faster rate and shift the equilibrium towards the product side in non-catalytic supercritical alcohol processes [20,21]. But higher ethanol concentration can lead to higher energy requirement for downstream processing as using excess alcohol makes the separation of final product energy intensive [20].

3.4.2. Effect of acid hydrolysis and pretreatment on crude biodiesel

Major components found in crude biodiesel obtained from fermented *Chlorella* sp. were fatty acids, fatty acid ethyl esters (FAEE) and nitrogenated compounds (Table 4). The major FAEE components (up to 33 wt% of crude biodiesel) found in crude biodiesel were palmitic acid ethyl ester (C16:0), oleic acid ethyl ester (C18:1) and linoleic acid ethyl ester (C18:2). Higher amounts of fatty acids were observed for both original and fermented crude biodiesel product. Higher percentage of fatty acids in the crude biodiesel indicates incomplete esterification reaction in the process [15]. The lower liquefaction temperature and lower ethanol concentration used can attribute to incomplete esterification during liquefaction. Fatty acids recovered from the crude biodiesel could be recycled back to the supercritical reactor, generating additional FAEE [15].

Lower percentage of nitrogenated components were observed from fermented algae compared to original which is good in terms of fuel properties. Protein degradation during pretreatment and fermentation might contribute to this lower nitrogen percentage.

Percentage of FAEE and hydrocarbons slightly increased for fermented *chlorella*. The decrease of nitrogenated compounds and the increase of FAEE and hydrocarbons indicated a higher potential of the use of fermented algae for thermal treatment.

3.4.3. Solid residue and aqueous product analysis

Solid residue obtained from ethanol-assisted liquefaction of fermented biomass was also analyzed (Table 5). Carbon, hydrogen and nitrogen content decreased from original biomass in solid residue after liquefaction process due to thermal decomposition of biochemical components. The sulfur content increased due to the use of sulfuric acid pretreatment. A significant amount of nitrogen was left (4.4%) which makes it a potential candidate for soil application as organic fertilizer.

Aqueous product was analyzed in HPLC to determine unreacted ethanol concentration. It was observed that about 77% (v/v) of added ethanol remains unreacted in the aqueous product which can be separated and recycled back to the liquefaction step to increase the sustainability of the whole process. Based on this preliminary fermentation study, the pretreatment and fermentation of the microalgae (80 g/L biomass concentration) could provide ~18% (v/v) of ethanol required for ethanol-assisted liquefaction reactions. This result is advantageous as it can save additional solvent for the liquefaction process from algae.

4. Conclusion

The feasibility of an integrated process combining pretreatment,

Table 4
Effect of acid pretreatment and fermentation on crude biodiesel component

Chemical Components available in crude biodiesel	From original <i>Chlorella</i> sp. (area% of total)	From fermented <i>Chlorella</i> sp. (area% of total)
Hydrocarbons	3.37%	10.75%
Fatty acids	53.20%	38.90%
Saturated fatty acid ethyl esters	7.80%	5.23%
Unsaturated fatty acid ethyl esters	17.70%	28.00%
Nitrogenated compounds	17.90%	9.62%
Other (acetate, esters)	0.00%	7.40%

Table 5
Solid residue ultimate analysis

Liquefaction solid residue ultimate analysis, wt%	
Carbon	36.62
Hydrogen	4.56
Nitrogen	4.4
Sulfur	8.04
Oxygen ^a	46.39
HHV ^b	13.47

^a Calculated by difference as $O = 100 - (C + H + N + S)$

^b Calculated by Boie's formula $HHV = 0.3516C + 1.16225H + 0.0628N - 0.1109O$

fermentation and liquefaction for production of biofuel from *Chlorella* sp. was studied and confirmed. Glucose yield increased with the increase of acid concentration and pretreatment time. Higher biomass loading at 150 g/L resulted in lower glucose yield. The highest glucose yield of 98.6% was obtained at 3% acid concentration, 120 °C, 60-minute pretreatment time and 40 g/L biomass concentration. *S. cerevisiae* fermented microalgae hydrolysate with a 100% utilization of glucose whereas *P. stipitis* fermentation obtained 100% utilization of both glucose and xylose at 40 g/L biomass concentration. At higher biomass loading of 100 g/L, higher ethanol yield of 74.73% was obtained by *P. stipitis* compared to 68.59% from *S. cerevisiae*. *P. stipitis* could be a better alternative for microalgae fermentation with high biomass concentration. Fermented hydrolysate was converted to crude biodiesel via an ethanol-assisted liquefaction process. This approach increased the crude biodiesel yield by 40.7%, comparing to that of liquefaction of original biomass. Residual solid obtained from liquefaction contained more than 4% nitrogen which can be used as organic fertilizer.

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