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Utilization of Algal Consortium to Produce Biofuels and Byproducts For Reducing Pollution load

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ABSTRACT: Algal biorefinery process utilizes every component of algal biomass to produce multiple useful fuel products. In this technique, acid pretreatment of algal biomass hydrolyzes microalgal carbohydrates into fermentable sugars, makes lipids more extractable and a protein part accessible for additional products. In the present study, *Chlorella sorkiniana* produced higher quantity of biodiesel than *Botryococcus braunii* and biomass in *Botryococcus braunii* was higher than the *Chlorella sorkiniana. Botryococcus braunii* produces 11% more lipid content than *Chlorella sorkiniana* which was consistent with biomass content. The total sugar (oligomeric and monomeric) yield attained by Combined Algal Processing (CAP) was 89.9%. 29 g/L ethanol was produced during the fermentation in the Pretreated Algal Slurry. The recovery of lipids from CAP was reported as 84–89% after fermentation and ethanol removal. CAP preserves the PUFA (Poly-Unsaturated Fatty Acids) and utilizes these high-value PUFAs to further reduce the cost of biofuel production and replace petroleum products.

Keywords: Algal biofuel, Biorefinery, Pretreatment, Fermentation, Extraction.

INTRODUCTION

Fast depleting stocks of fossil fuels, increasing oil prices along with the increasing levels of greenhouse gas emissions poses unavoidable dangers to global environment. This has demanded the exploration of cost effective sustainable energy sources (Wijffels & Barbosa, 2010; Demirbas, 2009). Currently among alternative fuels, biofuels such as biodiesel are the promising sustainable resources with tremendous future prospects (Singh et al. 2016). The various sources of biofuels are sunflower seeds, soybean, canola, peanuts, jatropha, coconut and palm oil for biodiesel, and wheat, sugar beet, sweet sorghum for bioethanol whereas algae can offer numerous and diverse types of alternative biofuels from single source. Methane is produced by anaerobic digestion of algal biomass (Sialve et al,

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2009), biodiesel is also derived from algal oil; bioethanol is derived from the fermentation of intracellular starch of some algae (Schenk et al, 2008) and bio-hydrogen is photo-biologically produced from algae (Angenent et al, 2004; Das, 2009; Cantrell, 2008). The concept of using algae as a source of fuel is not novel, but it is now being taken seriously. Current research developments have established that microalgae biomass can serve as one of the favorable source of renewable biofuel which is capable of fulfilling the global demand for transport fuels with higher efficiency to overcome global warming (Chisti, 2008). It can be attributed to several facts such as their growth rate is higher; they have higher photosynthetic efficiency; they require lesser cultivation area and have higher biomass production (Khan & Singh, 2008). On comparing the oil yield potential of various food crops with algae, it seems that algae have the potential to produce up to ten times more oil per hectare land than other traditional biofuel crops like oil palm, jatropha, soybeans, rapeseed etc.(Emily, 2009; Ramachandra et al, 2009) Therefore the biofuel (biodiesel) obtained from microalgae will also not affect production of food, fodder and other products derived from crops and it has the potential to completely displace fossil fuel (Singh et al, 2016).

Using microalgal biomass for extracting one product might not be commercially feasible, thus the concept of bio-refinery has appeared. The integration of different algal biomass conversion processes to produce energy and value added chemicals into a single facility is called an algal biorefinery. The conversion of biomass to different products is sustainable because the processes produce minimal wastes to the environment (Li et al, 2008). This concept is similar to the crude oil refinery where various products are produced at different stages of petroleum refining. The key components of microalgal biomass are carbohydrates, lipids and proteins. Carbohydrates are present in the form of α-(1-4)-glucans, β-(1-3)-glucans, fructans, and they can serve as the excellent source of ethanol. Two types of lipids viz. storage lipids and membrane lipids are present in algae. Triacylglycerides (TAGs) are present as storage lipids which constitute 20- 50% of dry cell weight; which can be extracted from the wet biomass with the help of organic solvent. These can further be used as biodiesel after transesterification. Polyunsaturated fatty acids (PUFAs) constitute about 40% of membrane lipids. Some examples of these acids are as α-linolenic acid, eicosapentaenoic acid and docosaesaenoic acid. The other valuable components from the algal biorefinery are pigments, antioxidants, essential fatty acids, vitamins, sterols, anti-fungal microbial, anti-viral toxins etc[11].

One of the huge challenges associated with algal biofuels production in a biorefinery-type concept is increasing the yields of the main products in the process and also providing economically viable co-product concepts (Laurens et al, 2015; Khan & Singh, 2008). Since most of the reports deal with processes that focus on a lipid-only pathway, improvements in process energetic yields by taking advantage of additional fuel options, such as those derived from carbohydrates have the potential to significantly improve the overall algae process environmental footprint. In the present investigation, the efficiency of integrated approach based on moderate temperatures and low pH to convert the carbohydrates in algal biomass slurry to soluble sugars is explained which is further fermented to bioethanol whereas lipids are made more accessible for downstream extraction and leaving a protein-enriched fraction behind.

Lipid, carbohydrate and protein content of algal biomass can be fractionated into three major streams which can be converted into respective (co-)products with added-

value. Here we demonstrate an acid-based fractionation process for algal biomass in a Parallel Algal Processing (PAP) and Combined Algal Processing (CAP). Under PAP, dilute acid pretreatment hydrolyze storage polysaccharides to release monomeric sugars (mainly glucose and mannose) into an aqueous stream. Sugars were separated from solid residue that was rich in lipids and protein by solid/liquid separation (SLS). The sugars collected in the liquor phase were then fermented to bioethanol whereas lipids could be recovered from the solid fraction using hexane extraction leaving a residue stream enriched in protein.

It has been observed that liquid present in the solid mass after solid liquid separation (SLS) contains significant amount of sugars which cannot be fermented to yield ethanol and thereby leading to considerable loss in the yield of fuel. CAP is the cost effective approach to recover the hydrolyzed sugar for fermentation. In this process the entire algal slurry obtained after acid pretreatment was first subjected to fermentation from which ethanol is obtained by subjecting the post-fermented broth to distillation. From the same broth (after distillation) which still contains lipids and non-volatile residues, lipids can be recovered by extracting with n-hexane.

The objective of the present study is to demonstrate that CAP designing approach will lead to higher energy yield and provide better option for the low cost algal biofuel production.

MATERIALS AND METHODS

Batch Photo-Bioreactors were inoculated with selected green algae, *Chlorella sorokiniana* (UTEX 1230), and *Botryococcus braunii* (UTEX 2441), brought from their mother cultures. 1mL of mother culture was inoculated with a Distilled water concentration of approximately 140 mg/L. As a form of pure culture, those microalgae species were obtained from the Biological Science department of MDU University, Ajmer. Selected microalgal consortium were grown in photobiorectors using nitrate deplete cultivation media. The dried *Chlorella sorkiniana* and *Botryococcus braunii* biomass was centrifuged and filtered through 1mm sieve and the material was frozen until needed. To reach the targets of 40% each of carbohydrate and lipid content, 7 to 10 days were required for lipid accumulation under nutrient deplete conditions (Laurens et al, 2014). Experimental set up has been described in Fig 1.

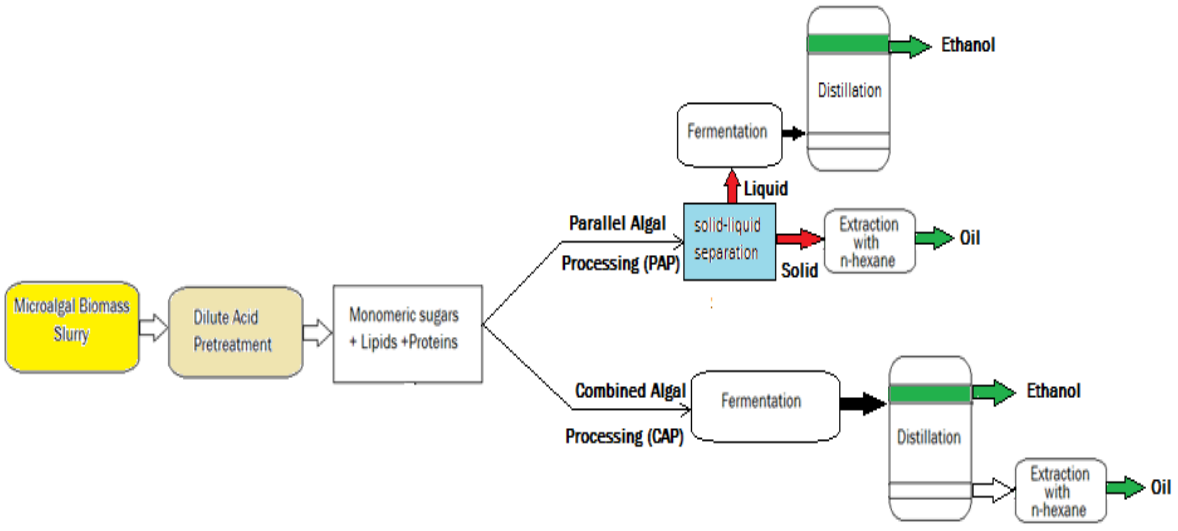

Fig. 1. Experimental Setup

Biomass Pretreatment: Algal biomass pretreatment was performed in a 4-L (2-L working volume) batch reactor. The reactor is well equipped with an electrical heating blanket set at reaction temperature to lessen steam condensation due to heat losses through the reactor wall. Steam was directly injected into the bottom of the reactor through ports in a rotary-plow type agitator and constant temperature was achieved by controlling the steam pressure in the reactor during pretreatment. The contents within the reactor were reached reaction temperature within 5 to 10 s of starting the steam flow. The steam pressure was slowly released through a condenser over a period of 25 to 40 s to eliminate boil-over while still allowing for steam escape to reduce slurry dilution by condensate at the end of pretreatment (Weiss, 2009; Ciesielski, 2014).

After determining total solid content of starting biomass paste at 105°C, 400 g wet algal paste, H_2SO_4 and water were added to the sample canister sequentially achieving a final solids loading of 30% (*w*/*w*) and an acid concentration of 3% (*w*/*w*). All samples were pretreated at 150 °C for 20 min (Laurens et al., 2014). The sample canister was removed from the reactor and cooled in ice water at the end of the reaction. Sufficient substrate for the fermentation experiments was provided by conducting a set of 12 identical pretreatment reactions. The pretreated algal slurry was combined and refrigerated until required.

Glucose and mannose yields were calculated by subtracting the weight of fraction insoluble solids from the slurry weight and mass of the remaining liquor was converted into volume using a density conversion. Fermentable monosaccharides in the hydrolysate liquor were determined by HPLC. Mass of sugar was determined by multiplying concentration by liquor volume, and normalized against the initial mass (Sluiter, 2008).

The pretreated algal slurry (PAS) was split into two fractions. The first fraction was centrifuged for 20 minutes to separate hydrolysate liquor from pelleted solid fraction. The liquor collected after centrifugation was neutralized to pH 5.2 using NaOH prior to fermentation via parallel algal processing. The second fraction of pretreated algal slurry was neutralized to pH 5.2 using NaOH prior to fermentation without solid/ liquid separation and parallel algal processing.

Fermentation: Fermentation by *Chlorella sorkiniana* and *Botryococcus braunii* biomass by *Saccharomyces cerevisiae*: An overnight seed culture of *Saccharomyces cerevisiae* was grown in 200 mL Yeast, Peptone and Dextrose (YPD) by adding 1% yeast extract, 2% peptone, 20 g/L glucose in a 500 mL baffled flask at 37 °C, a 1:10 dilution, 225 rpm from a fresh culture grown overnight the previous night (Bailey,1982). 10% of *Saccharomyces cerevisiae* was added to the fermenters for production of bioethanol by fermentation. The process was carried out for a period of another six days at 37°C. Samples were taken for the estimation of alcohol (bioethanol) during every 24 hours of the process.

For triplicate shake flask experiments, 50 mL of neutralized algal pretreated liquor or PAS was either supplemented with 0.30 g/L yeast extract and 0.5 g/L peptone (YP) termed as YP +, or with 2 of the same volume of water (YP −) in baffled flask of 125 mL .After that, flasks were inoculated with cells and water bringing the final volume to 60 mL, capped with a water trap, and incubated at 37 °C and 150 rpm. Samples were taken periodically for HPLC to tracking consumption of sugar and ethanol formation.

For triplicate fermenter experiments, 400 mL of neutralized pretreated algal slurry supplemented with YP (YP +) or water(YP −), YPDM (1% yeast extract, 2% peptone, 40 g/L glucose, and 20 g/L mannose), and a pure sugar control media.

Fermenters were controlled pH 5 and at 37 °C. Samples were further taken for HPLC analysis for sugar consumption and ethanol formation during the fermentation. The fermentation broth was recovered and ethanol was removed for both flask and fermenter experiments, as described below.

Ethanol removal: Using operating conditions established for the biorefinery distillation column, ethanol was removed by heating the fermentation broth. The fermentation broth having residual solids was heated in laboratory water bath to 80°C and held for 5 min. The samples were cooled to room temperature and the weights were taken to ensure ethanol removal which termed as a stillage.

Lipid extraction: The total lipids from the harvested cell mass were extracted three times by mixing chloroform-methanol (1:1 v/v) with a proportion of 1:1. The mixtures were transferred into a separatory funnel and shaken for 5 min. The lipid fraction was then separated from the separatory funnel and the solvent was evaporated using a rotary evaporator. The weight of the crude lipid was obtained from each sample was measured. The FAME (Fatty Acids Methyl Esters) content of the extracted lipid stream and extracted stillage was measured as described below. The FAME recovery was determined based on the baseline-measured FAME content of the starting material.

Moisture and ash analysis: Samples containing crucibles were preconditioned in the 570°C muffle furnace overnight to remove any combustible contaminants. Once the crucibles came to room temperature, their weights were calculated. In each crucible 50 ± 2.5 mg of dried algae was added and the weight of each sample was recorded. Biomass samples were then kept in a 40°C vacuum oven overnight to remove moisture and once the samples had come to room temperature, the oven dry weight of the sample was recorded the next day. Finally the samples were kept in the 570°C oven overnight. The weight of the ash content was calculated from a final weight (Wychen & Laurens, 2013).

Carbohydrate analysis: Lyophilized biomass (25 mg) and 250 µL of sulphuric acid (72% *w*/*w*) were added into a 10 mL glass tube. The first step hydrolysis was performed in 30°C water bath for 1 h. After that, 7 mL of highly purified water was added to the tube. The tube was sealed and autoclaved for 62 minutes at 121°C. The tube was allowed to cool down to room temperature and an aliquot of sample was neutralized to pH 6–8 by using calcium carbonate. For HPLC analysis, the neutralized sample was filtered through a 0.2 µm nylon membrane filter. The sugar analysis was performed on high performance liquid chromatography (HPLC) and sample was kept at 85°C and the detector was set at 55°C. Flow rate was 0.6 mL/min with water as mobile phase. Injection volume was 60 µL and the run time was 45min. The carbohydrate composition was tested with a calibration range of 0.05 g/L to 6 g/L for cellobiose, glucose, xylose, galactose, arabinose, and mannose (Wychen & Laurens, 2013).

Samples were filtered through a 0.2 µm nylon filter, diluted as necessary, and run by high performance anion exchange (HPAE) to quantify glucose and mannose at the start and end of fermentation. A high performance anion exchange (HPAE containing PA20 column) was run at 0.5 mL/min and 37 °C for both column and detector compartments using the quadruple waveform for carbohydrate detection. Samples were injected at 15 µl and an eluent of 27.5 mM sodium hydroxide was used to separate the monosaccharides followed by a gradient from 2–19% of 1 M sodium acetate and 100 mM sodium hydroxide.

Ethanol analysis: Each samples aliquot was filtered through a 0.2 µm nylon filter and run by high performance liquid chromatography (HPLC) to calculate

ethanol produced throughout fermentation and a refractive index detector was placed with a 0.01 N sulphuric acid at mobile phase at 0.6 mL/min.

FAME analysis: After a whole biomass *in situ* transesterification procedure, optimized for algae, lipid content in biomass was measured as total fatty acid methyl ester (FAME) content. A total of 7 to 10 mg of lyophilized biomass was transesterified with the presence of 0.2 mL of 2:1, *v*/*v*chloroform/methanol and 0.3 mL of 5%, *v*/*v*HCl/methanol for 1 h at 85°C with a known amount of tridecanoic acid (C13) methyl ester as an internal standard. FAMEs were extracted with 1 mL hexane at room temperature for 1 h and analyzed by gas chromatography–flame ionization detection. Quantification of the FAMEs was based on integration of individual fatty acid peaks in the chromatograms and quantified using a 5-point calibration curve. The individual FAME concentrations were normalized against the internal standard tridecanoic acid methyl ester (Laurens, 2012).

Protein analysis: Nitrogen content in dry biomass was determined by thermo Analyzer using the classical Dumas method, with thermal conductivity detection (TCD). Weighed samples were combusted in oxygen at 1000 °C. The combustion products were swept by a helium carrier gas through combustion catalysts, scrubbers and through a tube filled with reduced copper. The copper removes excess oxygen and reduces the NO_x to N_2. The N_2 was then separated from other gases on a chromatography column and measured with a TCD. A nitrogen-to-protein conversion factor of 4.78 was used to estimate the protein content in algal biomass (Lourenço, 2004).

Calculation of theoretical conversion yields: Theoretical yields were calculated by converting all fermentable sugars with a 51% theoretical ethanol fermentation yield (i.e. metabolic yield) from glucose and conversion of total fatty acid content of the biomass to hydrocarbon-based renewable diesel at a 78 wt.% renewable diesel yield from total fatty acids (Balat & Balat, 2009; Davis, 2014).

Results and Discussion

Comparison of Algal species in Extraction of oil and biomass production : Comparison of algal species shows that *Chlorella sorkiniana* produce higher quantity of biodiesel than *Botryococcus braunii* (Table 1, Fig.2) and biomass in *Botryococcus braunii* was higher than the *Chlorella sorkiniana* (Fig.2).

Table 1. Composition and theoretical fuel potential of *algal* biomass (all data shown as % DW) used for pretreatment and fermentation experiments. (Composition and calculated theoretical fuel equivalent in *algal* biomass)

Composition of Algal biomass	*Chlorella sorkiniana*	*Botryococcus braunii*
Total carbohydrates (% DW)	21	28
Glucose (% DW)	14	15
Mannose (% DW)	3	5
Galactose (% DW)	0.5	1
Ethanol (% DW)[a]	8	10
Ethanol (gallon/ton)	29	39
Gasoline equivalent (gallon/ton)[b]	28	37
Btu equivalent ($\times 10^3$)	2520	3210
Fatty acids (FAME) (% DW)	23	34
Hydrocarbon (% DW)[c]	17	26
Diesel equivalent (gallon/ton)	67	78
Btu equivalent ($\times 10^3$)	7,180	8,180
Total fuel energy ($\times 10^3$ Btu)	6,700	8,400
Total gasoline gallon equivalent (GGE/short ton)	89	98
Protein (% DW)	3	5
Ash (% DW)	2	3

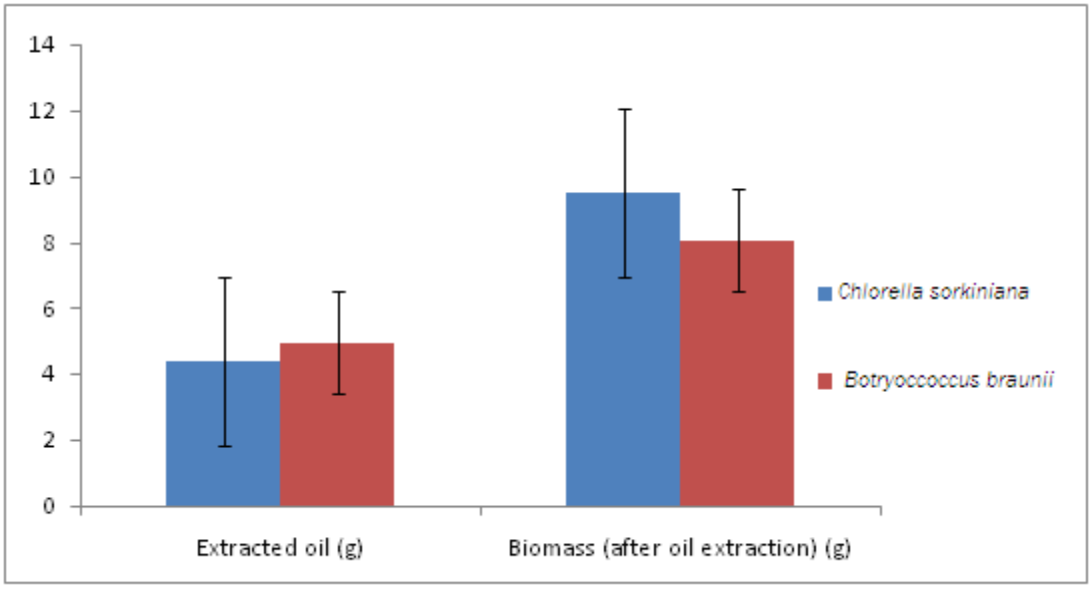

Fig. 2. Biodiesel and biomass production from *Chlorella sorkiniana* and *Botryococcus braunii* . Error bars are one sample standard deviation and all experiments were performed in triplicate

Table 2. Glucose and Mannose yield after acid pretreatment (Total = monomeric + oligomeric)

Sugar format	Glucose		Mannose	
	Yield %	Conc. g/g biomass	Yield %	Conc. g/g biomass
Monomeric	79.7 ± 0.7	0.28	83..8 ± 2.1	0.09
Total	88.2 ± 1.7	0.30	89.9 ± 2.3	0.10

Analysis of the biomass composition: Composition of the algal biomass used in these experiments is shown in Table 2. The typical compositional profile after an extended cultivation period under nitrogen-depleted conditions showed accumulation of both algal lipids and carbohydrates. The lipid content measured as total FAME was over 23% and 34 % with *Chlorella sorkiniana* and *Botryococcus braunii* respectively consistent with biomass of high lipid-based biofuel potential. In addition, the fermentable sugars, glucose and mannose made up over 38.5% of dry cell weight of the both algal biomasses. The low protein content in this batch of biomass is observed in both algal biomasses which were cultivated under the nitrogen-deplete condition.

Based on the composition data, theoretical conversion of carbohydrates and fatty acid to fuels was calculated (Laurens et al, 2014). The fuel yields are shown on a BTU energy basis and then converted to gallon gasoline equivalent (GGE) per ton dry biomass. The theoretical ethanol and hydrocarbon yields (Table 1) were calculated 86 wt. % (glucose-to-ethanol metabolic limit) and 34 wt. % (FAME-to-hydrocarbon), respectively. Both of the algal biomass was determined to have the potential to support 187 GGE per dry ton based on both sugar-derived ethanol and lipid-derived renewable diesel (Table 1). Total fuel energy ($\times 10^3$ Btu) (both of the algal biomass) was calculated 15,100. Notably, the fuel potential derived from fermentable sugar is 38% of that calculated from lipid, indicating a considerable amount of fuel energy can be recovered by utilizing the carbohydrate stream. Thus, a processing approach in which the total amount of sugar is utilized during fermentation is needed (Davis et al, 2014; Davis et al, 2012).

Sugar yield after dilute acid pretreatment: Results from the pretreatment of algal biomass are shown in Table 2. Samples were analyzed from 8 to 10 runs used to produce biomass for fermentation and lipid recovery. The average variation between samples was 0.7% and 1.7%, for monomeric and total (oligomeric + monomeric) sugars respectively. The concentration of degradation products and potential fermentation inhibitors and furfural was determined as ranging from 1.15 to 1.24 g/L and 0 to 0.26 g/L, respectively. The concentration of total solids in the pretreated slurry was 21.1%, and the soluble solid content in the pretreated liquors was 13.6%.

The total sugar (oligomeric and monomeric) yield of both mannose and glucose reached 89.9%. Over 6.4% and 7.4% of the total glucose and mannose was in the oligomeric form which contributed significantly to the overall sugar stream. Results showed that the pretreatment severity (a combination of time and temperature) was not sufficient for complete hydrolysis of the algal sugars into a monomeric form. On the other hand, increasing the severity may also increase the concentration of degradation products, which are currently minimal. Additional hydrolysis would require for oligomeric sugars using either enzymatic or thermochemical means to release monomeric sugars however the increased yield would come at additional cost (Shekiro, 2012).

Fermentation: After proceeding shake flask fermentations of either the liquor or slurry rapidly, it was observed that and ethanol production (Fig. 3) had reached a maximum and glucose was not detected in the fermentation broth after 18 h (mannose was not followed). The results obtained with fermentations run without yeast extract and peptone supported that algal hydrolysate contains sufficient nutrients to support ethanol production.

Fermentation of neutralized PAS in fermenters proceeded rapidly and 97.5% and 98.9% of the glucose was gone in 12 h for the YP + and YP − slurry respectively, confirmed that the algal hydrolysate can provide enough nutrients for effective fermentation (Fig. 4 and Fig 5). Complete utilization of glucose was observed within 8 h, in the clean sugar control, YPDM. The other major sugar present in the PAS, mannose, was also completely utilized (Fig. 4 and Fig 5). Up to 29 g/L ethanol was produced during the fermentation in the PAS (Fig.4). Overall, fermentation of the pre treated slurry is a rapid and robust process that does not require any additional nutrients for *S. cerevisiae* as it performed nearly as rapid with nearly the same ethanol production even in fermentations without added yeast extract and peptone.

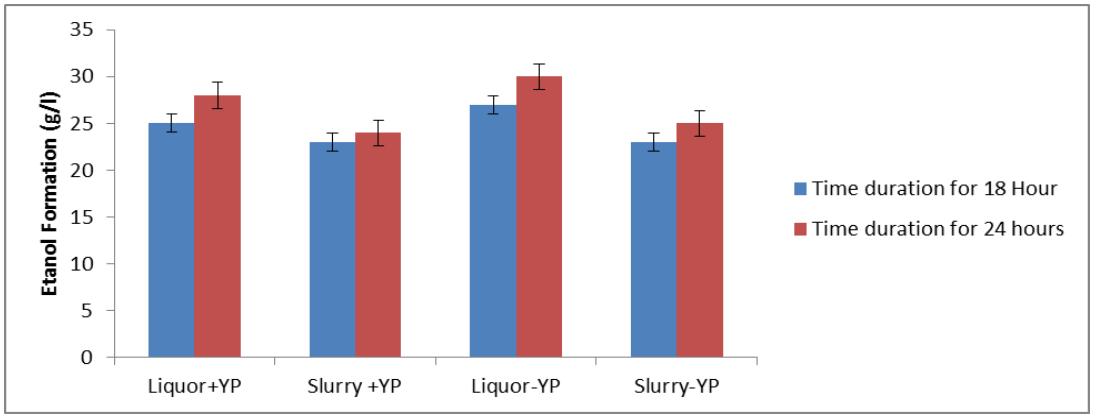

Fig. 3. Ethanol formation after 18 h and 24 h in shake flasks by *S. cerevisiae* fermenting pretreated algal liquor or slurry with or without YP supplementation. Error bars are one sample standard deviation and all experiments were performed in triplicate.

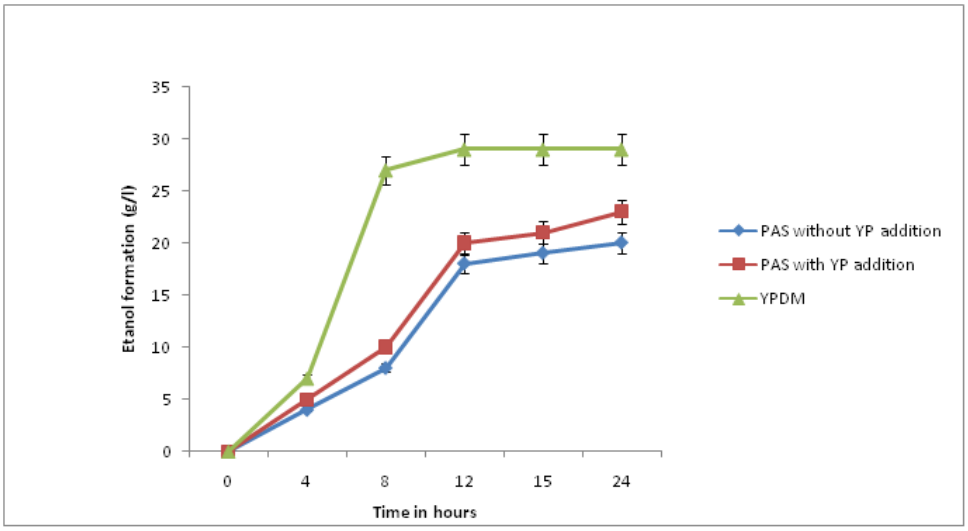

Fig. 4. Ethanol production and sugar utilization on PAS and YPDM (control) in fermenters. Error bars are one sample standard deviation and all experiments were performed in triplicate.

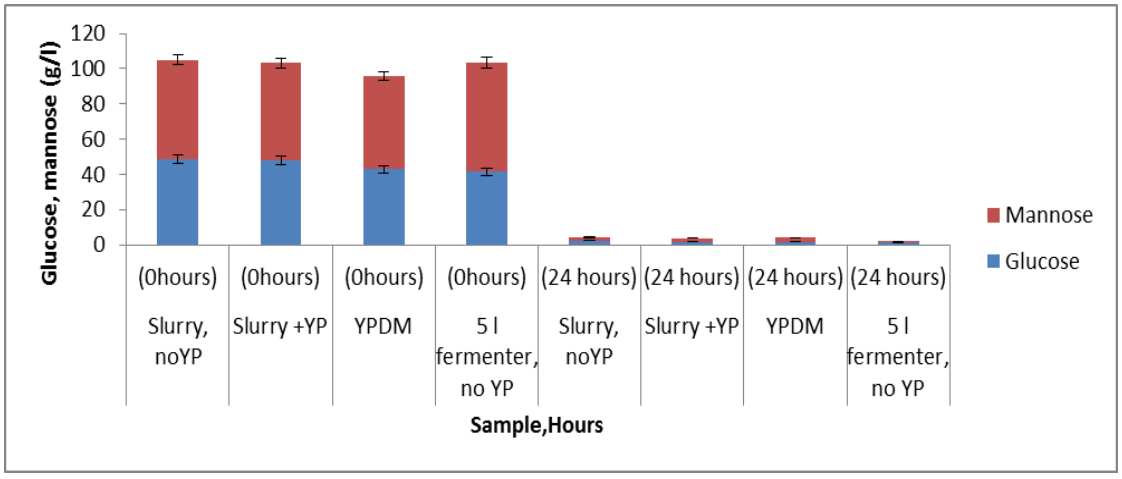

Fig. 5. Sugar utilization at 0 h and after 24 h of fermentation: Error bars are one sample standard deviation and all experiments were performed in triplicate.

Lipid yield after fermentation and ethanol removal: The lipid content in both algal biomasses was 47 wt. %DW, determined by FAME analysis (Table 1). Table 3 showed the percentage of the initial lipid recovered from pretreated solids from the PAP scheme and from the stillage of the CAP scheme. The recovery of lipids from CAP is in the range of 84–89% after fermentation and ethanol removal which indicated that the initial fermentation of soluble sugars in the pretreated algal slurry does not adversely affect lipid recovery. Laurens et al., 2014 reported lipid extractability of 77–90% for *S. acutus* having various compositional profiles under similar pretreatment conditions. There is also no significant impact of the thermal treatment used for ethanol removal compared to rotovapping with respect to lipid recovery. The FAME concentration of extracted oil (though dark green in color) is very high, and therefore the extracted oil is expected to be an excellent feedstock for catalytic upgrading to produce hydrocarbon fuel or biodiesel as shown in Table 3.

Table 3. FAME purity and recovery after fermentation with ethanol recovery by rotary evaporation or by using thermal treatment.

Process	Fermentation condition	EtOH removal	FAME purity[a] (%)	FAME recovery (%)
Parallel algal processing (PAP)	Lipid extraction prior to fermentation	–	98.9 ± 0.6	89.2 ± 1.8
Combined algal Processing (CAP)	PAS + YP (flask)	Rotovap	100.9 ± 0.8	85.6 ± 2.9
Combined algal Processing (CAP)	PAS + YP (flask)	Thermal treatment	97.3 ± 1.3	85.6 ± 0.9
Combined algal Processing (CAP)	PAS − YP (flask)	Thermal treatment	96.9 ± 0.6	84.8 ± 2.5
Combined algal Processing (CAP)	PAS + YP (fermenter)	Thermal treatment	99.9 ± 0.6	87.4 ± 2.6
Combined algal Processing (CAP)	PAS − YP (fermenter)	Thermal treatment	99.5 ± 1.3	89.0 ± 2.7

(All experiments were performed in triplicate)

a. Theoretical mass of fatty acids recovered in extract divided by measured mass of extract.

The extracted crude oil would require processing through a series of purification steps consisting of degumming, demetallization, and bleaching to remove phospholipids, metals, salts, and other impurities (Murphy & Allen, 2011). The elemental analysis of the crude oil extracted from PAP and CAP approaches was conducted to determine the concentrations of impurities, and the results are summarized in Table 4.

Table 4. Elemental analysis of oil extracted using different approaches. Nitrogen was detected by ASTM D4629. Other elements were detected by ICP.

Elements (µg/g)	TXa	Calcium	Iron	Magnesium	Phosphorus	Potassium	Sodium	Sulfur	Nitrogen
Oil extracted in PAP	19	2	< 1	< 1	2	< 1	10	49	319
Oil extracted in CAP	38	4	2	< 1	6	< 1	3	67	288

a. Total halogens (Cl + Br + I) as equivalent chlorine.

The elements tested in this study are generally believed to be the most challenging for hydrodeoxygenation catalysts. It was found that most of these elements were under the detection limit, indicating that a demetallization process may be preventable. The phospholipid in the extracted oil is also found very low as shown by the undetectably low level of phosphorus. This suggests that a degumming process can likely be avoided. It can be speculated that the acid pretreatment could help the hydrolysis of phospholipids to produce free fatty acids (FFAs) which are considered as a preferred feedstock for hydrodeoxygenation. On the other hand, the acid pretreatment process is capable of removing phospholipids because this process is like acidic degumming process applied in the vegetable oil industry (Chen et al, 2012). The sulfur and nitrogen content are relatively high probably due to carryover of these components from protein, chlorophyll (Dong et al, 2015), and other sulfur and nitrogen containing compounds inhabitant in the biomass or derived from the process. The need for additional cleanup procedures for removing nitrogen and sulfur from the crude extracted oil to facilitate catalytic upgrading is under investigation.

Composition of the extracted stillage: Unutilized carbohydrates and unextracted lipid were identified in the extracted stillage residue (Table 5 and Table 6), The sugar identified in the extracted stillage must be either in an oligomeric form or associated with the solid residue since no monomeric sugars were detectable in the

fermentation beer at the end point of fermentation. As shown in Table 5, about 22% of total glucose and 33% of total mannose were quantified in the post-extracted stillage residue. A total mass balance of 98.7% was obtained for fermentable sugars. This result is consistent with our findings that not all of the sugars were hydrolyzed under current pretreatment conditions. The optimization of pretreatment conditions is under investigation as is an evaluation of the costs and benefits of enzymatic hydrolysis.

Table 5. FAME and sugar distributions in different fractions (FAME or sugar contents were expressed as g FAME or sugar/g original biomass DCW).

Original biomass	FAME	g/g biomass	0.497
	Sugar a		0.402
Liquor	Sugar b	g/g biomass	0.287
Extracted oil	FAME	g/g biomass	0.401
Extracted stillage	FAME	g/g biomass	0.034
	Sugar c		0.098
Mass balance	FAME	%	93.9
	Sugar		98.7

a. Total glucose and mannose in original biomass.

b. Monomeric glucose and mannose in the liquor after acid pretreatment.

c. Total glucose and mannose in extracted stillage.

About 6.2% of total FAME in the original biomass ended in the extracted stillage, leading to a total lipid mass balance of 93.9% for FAME (Table 5). A number of intercellular oil droplets are exposed and tend to adhere to the reactors and fermenters, resulting in oil loss during the operations after the acid pretreatment. The loss of oil is speculated to be reduced in a continuous operation, which will be a future target of process optimization.

Table 6. Fatty acids profile in biomass, crude extracted oil and extracted stillage.

Fatty acid[a]	C16:0	C16:1n9	C16:2	C16:3	C16:4	C18:0	C18:1n9	C18:1n7	C18:2n6	C18:3n3	C20:1
Biomass	14.7	5.8	3.9	2.9	2.7	4.4	62.8	0.7	7.2	10.3	1
Extracted Crude oil (YP-)	13.6	5.7	3.8	2.9	2.8	3.9	58.9	0.8	7.3	10.6	1
Extracted Crude oil (YP+)	14.1	5.2	3.7	2.8	2.5	3.9	59.2	0.8	7.6	10.7	1
Extracted stillage (YP-	14.8	6.1	4.1	3.4	2.8	3.6	59.5	0.9	7.9	10.8	1
Extracted stillage (YP+	14.9	6	4.1	3.4	2.8	3.6	60.1	0.9	8.1	10.9	1

a. Fatty acids with relative content lower than 0.5% are not shown.

As shown in Table 6, the fatty acid profile of lipids in the original biomass, extracted oil and extracted stillage residue is very similar and comparable. In previous studies, it was speculated that the incomplete extraction of lipids might be ascribed to the polar lipids (Laurens et al, 2014). The current results indicate that this might not be the case due to the similar fatty acid profiles in extracted oil and the oil remaining in the extracted stillage. Incomplete extraction might be due to physical entrapment and chemical interaction of lipids and cell wall residue.

In addition, as shown in Table 6, the polyunsaturated fatty acid (PUFA) content was not changed during the process, suggesting that value-added co-products like omega-3 fatty acids and epoxy products with high oxirane number might be produced via the CAP (Carlson & Chang, 1985. This is particularly important because a number of algae species could accumulate considerable amounts of PUFA along with fuel range fatty acids (Hoffmann et al, 2010; Dong et al, 2013). The CAP preserves the PUFA and utilizes these high-value PUFAs to further reduce the cost of biofuel production and replace petroleum products (Dong et al, 2015, Dong et al, 2015). Use of the biorefinery concept and advances in raceway ponds engineering will further lower the cost of algal fuel production. More and more biorefinery-type facilities can be configured where some combination of chemical, energy and food processing are used to add value to biomass feeds.

CONCLUSION

An algal biorefinery processes (PAP and CAP) are successfully demonstrated. The algal slurry after acid pretreatment is found to be a sufficient medium for cultivating yeast to produce ethanol without any additional nutrients. CAP was capable to utilize almost all the fermentable sugars. The utilization efficiency of carbohydrates is significantly improved. Lipid yield is not adversely affected by fermentation and ethanol removal, reaching 89% of FAME recovery. Removing an additional SLS resulted in a simplified and robust process. It is expected that a number of high-value co-products may also be produced via the CAP concept. High-value co-product opportunities have potential to notably reduce the high cost of algal biofuel production. In present scenario algal biorefinery is a way to link between energy, local environment and climate change and give us energy independence and sustainable future.

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CONFLICT OF INTEREST

The authors declare that there is not any conflict of interests regarding the publication of this manuscript. In addition, the ethical issues, including plagiarism, informed consent, misconduct, data fabrication and/ or falsification, double publication and/or submission, and redundancy has been completely observed by the authors.

LIFE SCIENCE REPORTING

No life science threat was practiced in this research.

REFERENCES

Angenent, L. T., Karim, K., Al-Dahhan, M. H., Wrenn, B. A. and Domíguez-Espinosa, R. (2004). Production of bioenergy and biochemical s from industrial and agricultural wastewater. Trends Biotechnol. 22; 477–485.

Bailey, R. B., Benitez, T. and Woodward, A. (1982). Saccharomyces cerevisiae mutants resistant to catabolite repression: use in cheese whey hydrolysate fermentation. Appl. Environ. Microbiol., 44; 631–639.

Balat, M. and Balat, H. (2009). Recent trends in global production and utilization of bio-ethanol fuel. Appl. Energy, 86; 2273–2282.

Cantrell, K. B., Ducey, T., Ro, K.S., Hunt, P. G. (2008). Livestock waste-to-bioenergy generation opportunities. Biores. Technol. 99; 7941 –7953.

Carlson, K. D. and Chang, S. P. (1985). Chemical epoxidation of natural unsaturated epoxy seed oil

from Vernoniagalamensis and a look at epoxy oil markets. J. Am. Oil Chem. Soc., 62; 934–939.

Chen, L., Liu, T., Zhang, W., Chen, X., Wang, J. (2012). Biodiesel production from algae oil high in free fatty acids by two-step catalytic conversion. Bioresour. Technol., 111; 208–214.

Chisti, Y. (2008). Biodiesel from microalgae beats bio-ethanol. Trends in Biotechnology. 26; 26–31.

Ciesielski, P. N., Wang, W., Chen, X., Vinzant, T. B.and Tucker, M. P. (2014). Effect of mechanical disruption on the effectiveness of three reactors used for dilute acid pretreatment of corn stover Part 2: morphological and structural substrate analysis. Biotechnol. Biofuels, 7(1); 47.

Clarens, A. F., Nassau, H., Resurreccion, E. P., White, M. A. and Colosi, L. M. (2011). Biodiesel from algae. Environ. Sci. Technol, 45; 7554–7560.

Das, D. (2009). Advances in biological hydrogen production processes: An approach towards commercialization. Int. J. Hydrogen Energy. 34; 7349-7357.

Davis, R., Fishman, D., Frank, E. D. and Wigmosta, M. S. (2012). Renewable Diesel from Algal Lipids: An Integrated Baseline for Cost, Emissions, and Resource Potential from a Harmonized Model. Golden, CO.

Davis, R., Kinchin, C., Markham, J., Tan, E. C. D., Laurens, L. M. L. (2014). Process design and economics for the conversion of algal biomass to biofuels : algal biomass fractionation to lipid-products, Process Design and Economics for the Conversion of Algal Biomass to Biofuels : Algal Biomass Fractionation to Lipid- and Carbohyd. Analytical Procedure (LAP). Golden, CO.

Demirbas, A. (2009), Progress and recent trends in biodiesel fuels. Energy Conversion and Management, 50; 14–34.

Dong, T., Gao, D., Miao, C., Yu, X., Degan, C. and Garcia-Pérez, M. (2015). Two-step microalgal biodiesel production using acidic catalyst generated from pyrolysis-derived biochar. Energy Convers. Manag., 105; 1389–1396.

Dong, T., Wang, J. M., Zheng, Y. and Chen, S. (2013). Two-step in situ biodiesel production from microalgae with high free fatty acid content. Bioresour. Technol., 136; 8–15.

Dong, T., Yu, L., Gao, D., Yu, X., Miao, C. and Zheng, Y. (2015). Direct quantification of fatty acids in wet microalgal and yeast biomass via a rapid in situ fatty acid methyl ester derivatization approach. Appl. Microbiol. Biot echnol., 36; 1–11.

Dong, T., Yu, X., Miao, C., Rasco, B., Garcia-Perez, M., Sablani, S. S. (2015). Selective esterification to produce microalgal biodiesel and enrich polyunsaturated fatty acid using zeolite as a catalyst, *RSC Adv*. 5; 84894–84900.

Emily, W. (2009). Biotech's green gold. Nature Biotechnol., 27; 15–18.

Hoffmann, M., Marxen, K., Schulz, R. and Vanselow, K. H. (2010). TFA and EPA productivities of Nannochloropsissalina influenced by temperature and nitrate stimuli in turbidostatic controlled experiments. Mar. Drugs, 8; 2526–2545.

Khan, S. A., Singh, R. (2008). Algae: A novel source of renewable energy and carbon sequestration. Ren Energy, 2; 14-18.

Laurens, L. M. L., Nagle, N., Davis, R., Sweeney, N., Wychen, S. V., Lowell, A. and Pienkos, P. T. (2015). Acid-catalyzed algal biomass pretreatment for integrated lipid and carbohydrate-based biofuels production, Green Chem., 17; 1145-1158.

Laurens, L. M. L., Quinn, M., Wychen, S. V., Templeton, D. W. and Wolfrum, E. J. (2012). Accurate and reliable quantification of total microalgal fuel potential as fatty acid methyl esters by in-situ transesterification. Anal. Bioanal. Chem. 403; 167–178.

Laurens, L. M. L., Wychen, S. V., McAllister, J. P., Arrowsmith, S., Dempster, T. A.and McGowen, J. (2014). Strain, biochemistry, and cultivation-dependent measurement variability of algal biomass composition. Anal. Bioc hem., 452;86–95.

Li, Y., Horsman, M., Wu, N., Lan, C. Q. and Dubois-Calero, N. (2008). Biofuels from microalgae. Biotechnol. Prog., 24; 815–820.

Lourenço, S. O., Barbarino, E., Lavín, P. L., Lanfer, M. U. M. and Aidar, E. (2004). Distribution of intracellular nitrogen in marine microalgae: calculation of new nitrogen-to-protein conversion factors. Eur. J. Phycol., 39; 17–32.

Murphy, C. F. and Allen, D. T. (2011). Energy-water nexus for mass cultivation of algae. Environ. Sci. Technol., 45; 5861–5868.

Ramachandra, T. V., Mahapatra, D. M., Karthick, B. and Gordon, R. (2009). Milking diatoms for sustainable energy: biochemical engineering versus gasoline-secreting diatom solar panels. Ind. & Engg. Chem. Res., 48; 87–88.

Schenk, P. M., Skye, R., Thomas, H., Stephens, E., Marx, U. C., Mussgnug, J. H., Posten, C., Kruse, O.

and Hankamer, B. (2008). Second generation biofuels: High-efficiency microalgae for biodiesel production. Bioenergy Res., 1; 20–43.

Shekiro, J., Kuhn, E. M., Selig, M. J., Nagle, N. J., Decker, S. R., Elander, R. T. (2012). Enzymatic conversion of xylan residues from dilute acid-pretreated corn stover. Appl. Biochem. Biotechnol. 168; 421–433.

Sialve, B., Bernet, N. and Bernard, O. (2009). Anaerobic digestion of microalgae as a necessary step to make microalgal biodiesel sustainable. Biotechnology Advances, 27; 409–416.

Singh, P., Bansal, S., Singh, P., Verma, R. and Jagadish, R. S. (2016). Algal Biofuel: A boon for society in future to solve Energy crisis. Int. Res. Ad v., 3; 1-4.

Singh, P., Singh, P., Bansal, S., Srivastava, A., Tripathi, S. and Srivastava, V. (2016), Biodiesel from Jatropha: A step towards Green Alternative, Lenin Media Pvt. Ltd, India, 13-24.

Sluiter, B., Hames, R., Ruiz, C., Scarlata, J. and Sluiter, D. (2008). Determination of Sugars, Byproducts, and Degradation Products in Liquid Fraction Process Samples Laboratory Analytical Procedure (LAP). Golden, CO.

Weiss, N. D., Nagle, N. J., Tucker, M. P., Elander, R. T. (2009). High xylose yields from dilute acid pretreatment of corn stover under process-relevant conditions. Appl. Biochem. Biotechnol., 155; 418–428.

Wijffels, R. H. and Barbosa, M. J. (2010). An outlook on microalgal biofuels. Science, 329; 796–799.

Wychen, S. V. and Laurens, L. M. L. (2013). Determination of Total Solids and Ash in Algal Biomass– Laboratory Analytical Procedure (LAP). Golden, CO.