Bioethanol production from defatted biomass of *Nannochloropsis oculata* microalgae grown under mixotrophic conditions

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Abstract

Microalgal biomass is one of the most promising third-generation feedstocks for bioethanol production because it contains significantly reduced sugar amounts which, by separate hydrolysis and fermentation, can be used as a source for ethanol production. In this study, the defatted microalgal biomass of *Nannochloropsis oculata* (NNO-1 UTEX Culture LB 2164) was subjected to bioethanol production through acid digestion and enzymatic treatment before being fermented by *Saccharomyces cerevisiae* (NRRLY-2034). For acid hydrolysis (AH), the highest carbohydrate yield 252.84 mg/g DW was obtained with 5.0% (v/v) H_2SO_4 at 121°C for 15 min for defatted biomass cultivated mixotrophically on SBAE with respect to 207.41 mg/g DW for defatted biomass cultivated autotrophically (control treatment), Whereas, the highest levels of reducing sugars was obtained With 4.0%(v/v) H_2SO_4 157.47 ± 1.60 mg/g DW for defatted biomass cultivated mixotrophically in compared with 135.30 mg/g DW for the defatted control treatment. The combination of acid hydrolysis 2.0% (v/v) H_2SO_4 followed by enzymatic treatment (AEH) increased the carbohydrate yields to 268.53 mg/g DW for defatted biomass cultivated mixotrophically on SBAE with respect to 177.73 mg/g DW for the defatted control treatment. However, the highest levels of reducing sugars were obtained with 3.0% (v/v) H_2SO_4 followed by enzyme treatment gave 232.39 ± 1.77 for defatted biomass cultivated mixotrophically on SBAE and 150.75 mg/g DW for the defatted control treatment. The sugar composition of the polysaccharides showed that glucose was the principal polysaccharide sugar (60.7%-62.49%) of *N. oculata* defatted biomass. Fermentation of the hydrolysates by *Saccharomyces cerevisiae* for the acid pretreated defatted biomass samples gave ethanol yield of 0.86 g/l (0.062 g/g sugar consumed) for control and 1.17 g/l (0.069 g/g sugar consumed) for SBAE mixotrophic. Whereas, the maximum ethanol yield of 6.17 ± 0.47 g/l (0.26 ± 0.11 g/g sugar consumed) was obtained with samples from defatted biomass grown mixotrophically (SBAE mixotrophic) pretreated with acid coupled enzyme hydrolysis.

Introduction

Bioenergy has gained more attention in recent decades due to the depletion of fossil fuel forms and increasing requirements for eco-friendly energy to alleviate global warming. Thus, biofuels are now a viable alternative for fossil fuels (Koyande et al. 2019). Sustainable processing of biomass to produce biofuels, biochemicals, and bioenergy is referred to as biorefining (Taylor et al. 2011; Knoshaug et al. 2018; Osman et al. 2020; Demiray et al. 2019; El-Sheekh et al. 2019; Aristizábal-Marulanda et al. 2020; Dong et al. 2016; Yao et al. 2020; Malhotra et al. 2021; Rodríguez-Valderrama et al. 2020; Attia et al. 2021; Abdelsalam et al. 2019; Hijazi et al. 2020a,b). Biorefineries can be considered as environment-friendly analogous of oil refineries. Biofuels have the advantage of being renewable and emitting less environmental contaminants, resulting in a reduction of global warming. Because of its high oxygen content, ethanol is an excellent example of a biofuel that could be combined with gasoline to increase octane number, preventing cylinder knocking in engines and lowering greenhouse gas emissions (Wyman 1996; Sanchez and Cardona 2008; Walker 2011). Bioethanol is a liquid biofuel derived from biomass by fermentation technique. Unlike gasoline, ethanol is a cleaner fuel that can be fully burned. It can be used

either as an independent fuel or fuel enhancer (Sánchez and Cardona 2008). As a transportation fuel, it can be either used in blended form along with gasoline (gasohol) or as pure ethanol. One of the most well-established and mature bioprocesses is ethanol fermentation (Swana et al. 2011). The development of biofuel from microalgae biomass is regarded as one of the most promising third-generation technologies due to the effective photosynthetic process combined with a higher growth rate results in a greater ability to sequester carbon dioxide than other biomasses (Matsumoto et al. 1997). However, there are still significant complications to commercial microalgae development, such as economic barriers, a lack of technical readiness, and a shortage of common products developed to make overall energy production from algae biomass desirable. Microalgae in autotrophic cultivation depend on light energy to produce carbohydrates, while in heterotrophic cultivation organic carbon sources are directly used instead of light (Ceron-Garcia et al. 2005). Microalgae may use both inorganic carbons fixed by photosynthesis and organic carbons such as glucose, acetate, and sucrose (Wen et al. 2019; Cheah et al. 2018; Dragone et al. 2010). Mixotrophic cultivations provide a viable method for producing algal biomass and valuable metabolites through both autotrophic and heterotrophic pathways. Furthermore, using organic carbon sources lowers the expense of continuous light energy usage. (Ceron -Garcia et al. 2005; Sipauba-Tavares et al. 2020). The use of additional carbon sources in the culture medium will increase algal biomass and fat accumulation. Lowering the cost of microalgae production by using low-cost carbon sources is more cost-effective than using refined glucose. As a result, sugars extracted from sugarcane residues have been established as a promising energy source for mixotrophic microalgae cultivation (Nguyen et al. 2018). Many microalgae species can be transit from the photoautotrophic to the mixotrophic pathway. Furthermore, it can only depend on autotrophic or heterotrophic metabolic pathways for growth (Choi and Lee 2015). However, in the presence of organic carbon sources, photosynthetic activity in certain microalgae may be hindered (Cecchin et al. 2018). Defatted microalgal biomass can be hydrolyzed to produce a considerable amount of reducing sugars which can be fermented to ethanol (Sivasankar et al. 2017). In order to overcome economic problems combined with microalga production (cultivation and processing), the biomass remaining after extracting oil and other valuable compounds should be used in the production of ethanol after proper pretreatment to liberate fermentable sugars (Sivasankar et al. 2017; Nobre et al. 2013; Chaudhary et al. 2014). Before being used as a raw material for ethanol processing, microalgae biomass can be mechanically, chemically, or enzymatically pretreated (Maurya et al. 2016; El-Sayed et al. 2017 and 2015).

Sugarcane bagasse (SBA) is one of the lignocellulosic materials that are rich in cellulose, which can be hydrolyzed into glucose. However, this polysaccharide is associated with lignin and hemicellulose, materials that limit its conversion. SBA contains about 22.4 % lignin (Siqueira et al. 2013), therefore delignification process must be carried out before hydrolysis. Moreover, during delignification process, toxic compounds are formed (Mesa et al. 2011). Therefore, conversion of lignocellulosic residues to cellulosic ethanol consists of three steps: (i) raw material pretreatment to minimize lignin content and increase polysaccharide exposure; (ii) hydrolysis to transform the polysaccharides into glucose and xylose monomers; and (iii) sugar fermentation to ethanol. Thus, ethanol production from lignocellulosic material is non -economic. On the other hand, the microalgae biomass is one of the most promising third

generation feedstocks for biofuel production because their rapid growth rate, rich oil content, strong ability to sequester carbon dioxide and rapid conversion of carbon dioxide into a reusable gas such as methane or hydrogen. According to the above-mentioned points conversion of sugarcane bagasse into ethanol is more expensive, non-economic and less effective than microalgal biomass. The present study has used non-toxic, copious agricultural waste, sugarcane bagasse aqueous extract (soluble extract) for cultivating *Nannochloropsis oculata* microalga as the potential source of traditional nutritional substitutes to minimize the costs of cultivation. In addition, the zero-value defatted N. oculata microalga was used as a feedstock source for bioethanol production after hydrolysis under variable acid concentration and/or constant enzyme dosage.

Materials And Methods

Algae and growth conditions

Marine microalga *Nannochloropsis oculata* (NNO-1 UTEX Culture LB 2164) belonging to **Chrysophyta** were obtained from Algal Biotechnology Unit, National Research Centre. Cultures were grown under conditions of F2 (Guillard 1975). According to the previously described structure (El- Sayed et al. 2020), a zigzag-shaped photobioreactor with a capacity of 1000 L was used to cultivate *N. oculata* grown on mixotrophic nutrition way by integrating both autotrophic and heterotrophic-machineries by absorbing provided organic substrates carbon source. Prior to cultivation, the unit was sterilized by hypochlorite solution before inoculation overnight, then washed by water till cleaning and then filled by 1000 L tap water containing 25% F2 artificial growth medium, 10% sugarcane bagasse aqueous extract (SBAE) filtrate, and enriched by commercial fertilizer compounds as described by El-Sayed et al. (2020). Night illumination was provided by the upper surface fluorescent lamp (6 lamps × 40 watts) along with the growth surface. When the alga reached its maximum growth dry weight (g/l), stress was induced with 10 Kg NaCl (1%), and samples were collected continuously from the bioreactor till the end of the experiments. Control treatment of N. *oculata* was grown by the autotrophic way of nutrition on the F2 medium.

Lipid-extracted alga biomass (LEAB)

Aeration of the culture was switched off at the end of the incubation cycle to allow algal cells to settle. The transparent supernatant was removed and concentrated the algal slurry by centrifugation using Thermo laboratory centrifuge (4,000 rpm/1800g). The lipids were extracted by using a hexane/isopropanol mixture (3:2) after drying the biomass. The extracted biomass was removed using the Soxhlet method, and the residual hexane was evaporated by bubbling with either nitrogen or carbon dioxide gas in a downflow mode. The procedure was repeated until no odor of hexane was detected. The LEAB was collected as dry biomass and held at 4°C until it was used.

Acid hydrolysis and preparation of defatted biomass hydrolysates

Single-stage acid hydrolysis was accomplished in 250 mL conical flasks with (1%, 2%, 3%, 4%, and 5%) of 98 percent H_2SO_4 for 15 minutes at 121°C. The solid/liquid ratio of defatted biomass content was set at 10% (w/v). As a control, no H_2SO_4 was used in the previous conditions, only distilled water (Miranda et al. 2012). The liquid fraction was isolated after hydrolysis by centrifugation at 14000 rpm (26342 RCF with radius of rotor (120mm)) for 15 minutes. The supernatant was neutralized to pH 7 with 5.0 M NaOH. The neutralized hydrolysates were then used to determine total carbohydrates and reducing sugars before being stored at 5–7^{o}C until used to produce ethanol. All experiments were three times replicated.

Enzyme hydrolysis and preparation of defatted biomass hydrolysates

Under different conditions, the liquefaction process with diluted sulfuric acid was used for pretreatment of *Nannochloropsis oculata* biomass. In 250 mL flasks, 10g of defatted dry powder biomass were mixed in 1:10 (w/v) ratios with 0.0, 1.0, 2.0, and 3.0 percent H_2SO_4 solutions. The flasks were autoclaved for 15 minutes at 121°C.The autoclaved solutions were cooled, neutralized with 5 M NaOH, and subjected to commercially available hydrolytic enzymes α-amylase 1000U at 95^{o}C with a pH of 6.0 for 6 hours, while the saccharification process was carried out with a mixture of commercially available enzyme mixtures from *Trichoderma reesei* ATCC 26921 and *Trichoderma longibrachiatum* C 9748 containing multiple enzyme activities mainly ; exoglucanase (1.6 FPU/mL), endoglucanase (33.3 U/mL), cellobiohydrolase (30.02 U/mL), xylanase (21.0 U/mL), and β-glucosidase content (12.4 U/mL).The hydrolysis was carried out by incubating the mixture in a water bath at 60 ^{0}C, pH 5.5, for 24 hours (pH was maintained using 0.05M citrate buffer). The residual materials were separated by centrifugation at 14,000 rpm for 15min. For complete free sugar analysis, the resulting hydrolysate has been used and then cooled up to 5-7^{o}C until used for ethanol production (Mirsiaghi and Reardon 2015). All tests have been conducted three times. The commercial enzymes used in this study were procured from Sigma-Aldrich.

Ethanol production

Inoculum of *Saccharomyces cerevisiae* (NRRLY-2034) was obtained from department of Microbiology, Soils, Water and Environment Research Institute, Agriculture research center-Giza, Egypt. It was cultured on yeast extract peptone dextrose agar (YPD) and incubated at 30^{o}C for 48 hours. After 24 hours of incubation at 30 °C and 150 rpm, a single colony was transferred into 50 mL YPD broth (20.0 g/L dextrose, 10.0 g/L yeast extract, and 20.0 g/L peptone). The cell growth in the inoculum was 5 X 10^{7} cells/mL. It was used to inoculate the fermentation medium. The medium used for ethanol fermentation was composed of (%) 0.25 Yeast extract, 0.25 $(NH_4)_2SO_4$, 0.1 KH_2PO_4, and 0.05 $MgSO_4.7H_2O$, which were added to 100 mL of the filtrate from saccharified biomasses and sterilized at 121°C for 15 min. The medium was cooled at room temperature after sterilization and then *Saccharomyces cerevisiae* was inoculated at 2.0 % V/V. For the first four days of fermentation, it was incubated under static conditions at 30°C. The produced ethanol was determined and the ethanol yield was calculated using the formula stated in Yoswathana et al. (2010). At the start and end of the 12-hour fermentation cycle, samples were

taken for glucose and ethanol analysis. All the measurements were duplicated and the data reported are average of two replications. The following equation was used to calculate ethanol yield.:

Ethanol Yield = Measured Ethanol in the sample (g) / Theoretical Ethanol (g)

where theoretical ethanol (g) = Amount of initial sugar content (g) in fermentation solution X 0.5

Analytical procedures

The measurement of the total carbohydrate was performed by the method described by Dubois et al. (1956). Estimation for the amount of reducing sugars was carried out by the dinitro salicylic acid (DNS) method using glucose as standard (Miller 1959). The monosaccharides and hydroxymethylfurfural (HMF) in the hydrolysates were analyzed using high performance liquid chromatography (HPLC, Agilent, USA). The chromatographic conditions were waters amino chromatographic column with refractive index detector, elution influent. The mobile phase was deionized water that had been degassed in an ultrasonic bath under vacuum. At a temperature of 40^{o}C and an injection volume of 10L, the flow rate was 0.7 mL/min. The repeatability relative standard deviation of this method is below 5% and the recovery is over 97%. A satisfactory result is obtained under these optimum conditions, and the whole separation can be finished within 25 min. The quantification was achieved by comparison with analytical curves using glucose, fructose, sucrose, fucose, ribose, arabinose, xylose, and mannose standards. HMF was determined using a diode-array detector and an ODS analytical column with detection at 280 nm. A filtered and degassed mobile phase was prepared at a flow rate of 1 mL/min with a sodium acetate buffer (0.08 M) and adjusted to pH 3.6 with glacial acetic acid. Gas Chromatography was used to determine the concentration of bioethanol. Purification by distillation is very low when the retention time of impurities is considered (below the boiling point of ethanol). The fermentation efficiency (FE %) was determined by dividing the average generated ethanol by the theoretically produced ethanol in the biochemical conversion of the sugars consumed.

Statistical analysis

Statistical analysis was done by ANOVA test using the Microsoft Excel program. The difference in values was indicated in the form of probability ($p < 0.05$) values.

Results

To increase the economic viability and environmental sustainability of microalgal bioethanol production, sugarcane bagasse aqueous extract (SBAE) as a nontoxic and abundant agricultural waste was used for cultivating *Nannochloropsis oculata* microalga as potential sources of substitutes for conventional nutrition to reduce cultivation costs. The primary goal of this study was to produce ethanol from the hydrolysate of defatted biomass of *N. oculata* grown mixotrophically on SBAE and CO_2 as carbon sources. The content of carbohydrates in microalgae is mainly present within the cell wall, its disruption and collapses are required to release monosaccharides and make them accessible to fermentative

microorganisms as carbon sources. The efficiency of each pre-treatment method was determined by measuring reducing sugars in hydrolysates. The sugar composition of the polysaccharides and hydroxymethylfurfural (HMF) in hydrolysates was determined by HPLC after each pre-treatment. Effective pre-treatment processes take into consideration the cost of the production process, degradation capacity of fermentable sugars, energy efficiency, and ease of application (Sritrakul et al. 2017; Phwan et al. 2019).

Effect of acid hydrolysis on total carbohydrates and total reduced sugar content

Giang et al. (2019) showed that the acid concentration is a limiting factor that can impair microalgal biomass saccharification in the autoclave. The total carbohydrate yields were increased with increasing acid concentration during the hydrolysis processes with H_2SO_4 pre-treatment in the autoclave at 121 °C for 15 min as shown in figure 1. The highest carbohydrate yields due to acid hydrolysis were 252.84 mg/g DW for defatted biomass cultivated mixotrophically on SBAE that obtained with 5.0%(v/v) H_2SO_4 concentration with respect to 207.41 mg/g DW for defatted biomass cultivated autotrophically (defatted control treatment). Regarding the reducing sugar, results are shown in figure (1) revealed that increasing acid concentration from 1.0 % (v/v) to 4.0 % (v/v) increased the concentration of H^+ to a level that was optimum for hydrolyzing the biomass. The highest level of reducing sugars due to acid hydrolysis (4.0% (v/v) H_2SO_4) was 157.47 mg/g DW for defatted biomass cultivated mixotrophically in comparison to 135.30 mg/g DW for defatted control treatment.

<Figure 1>

Effect of enzymatic hydrolysis on total carbohydrates and total reduced sugar content

The synergistic effect of enzymatic hydrolysis coupled with acid pretreatment was investigated and shown in Fig. (2). The highest carbohydrate yield was obtained after a 15-minute pretreatment with 2.0% sulfuric acid at 121^{o}C. It was recorded as 268. 53 and 177.73 mg/g DW for defatted biomass cultivated mixotrophically and for defatted biomass cultivated autotrophically treatment, respectively. However, the highest levels of reducing sugars were 232.39 mg/g obtained with 3.0% (v/v) H_2SO_4 concentration for defatted biomass cultivated mixotrophically and 150.75 mg/g for the defatted control treatment (Fig. 2). By the same respect, 8.56 and 4.25 mg/g were obtained with 0.0% H_2SO_4 digestion comparing with 40.39 and 32.17 mg/g of 0.5 %(v/v) H_2SO_4 digestion or with 1.0% H_2SO_4 digestion that resulted in 66.25 and 45.09 mg/g. Whereas, pretreatment with 2.0%(v/v) H_2SO_4 digestion gave 152.39 and 87.68 mg/g for the defatted biomass cultivated mixotrophically on SBAE and control treatment, respectively. Therefore, combination with acidic pre-treatment increased the enzymatic scarification performance of algal biomass.

<Figure 2>

Sugar composition of the polysaccharides

Enzymatic hydrolysis after pretreatment with 3.0% (v/v) H_2SO_4 yielded the highest saccharification rates of 92.2 % and 90.6% for defatted biomass cultivated mixotrophically on SBAE and control treatment, respectively. At high acid hydrolysis concentration 4.0% (v/v), both defatted biomasses were more resistant than enzymatic hydrolysis with released sugar yields of 76.29% and 75.17% for defatted biomass cultivated mixotrophically on SBAE and control treatment, respectively. Thus, both treatments were subjected to HPLC analysis of hydrolase products before bioethanol production. Results in Table (1) and Fig (SI 1 A and B) showed the sugar composition of the defatted biomass of *N. oculata* pretreated with 4.0% (v/v) H_2SO_4, unexpectedly, acid hydrolysis of the algal biomass grown mixotrophically or autotrophically(control) gave low glucose yield (22.5% and 18.2%, respectively).

However, the enzymatic hydrolysis coupled with acid pretreatment showed higher saccharification ratio (total reducing sugar yield to total carbohydrates) for the defatted biomass of *N. oculata* grown mixotrophically or autotrophically (control treatment). Both the defatted biomasses showed the same suite of sugars. Glucose was the principal polysaccharide sugar (62.49% and 60.7%) followed by galactose, fucose, rhamnose, xylose, arabinose, and mannose (Table 1, Fig. SI 1C and D). Figure SI 2 showed the HPLC chromatograms of hydroxymethylfurfural (HMF): (A) standard and (B) sample of *N. Oculata* extracts from acid enzyme hydrolysis.

<Table 1>

Ethanol productivity

The algae hydrolysates were then used as a substrate for the producer microorganisms to convert to bioethanol. After every 12 hours, samples were taken from the fermentation media and were analyzed for alcohol contents through the GC method. Also, the reduction in sugar content was simultaneously measured. Based on chromatogram analysis by comparing ethanol standard and sample, ethanol production from hydrolysates of defatted *N. oculata* biomass treated with acid hydrolysis and acid coupled enzyme hydrolysis is shown in Fig. (3). Among the samples, the ethanol yield of the acid pretreated defatted biomass samples was only 0.86 g/l (0.062 g/g sugar consumed) for defatted biomass grown autotrophically (control) and 1.17 g/l (0.069 g/g sugar consumed) for SBAE mixotrophic. However, the highest ethanol yield of 6.17±0.47 g/l (0.26±0.11 g/g sugar consumed) was obtained at the samples from defatted biomass grown mixotrophically (SBAE mixotrophic) pretreated with acid coupled enzyme hydrolysis (AEH). Whereas the initial reducing sugar concentration was 24.33±1.41 g/l at 48 hours corresponding to 64.50% of theoretical fermentation yield. The reducing sugar in *N. oculata* hydrolysate was not fully consumed after 72 hours and it was 2.47±1.63 g/l.

<Figure 3>

Discussion

The results clearly indicate that, when 5.0 percent (v/v) H_2SO_4 was added, the sugar release values were decreased. Increasing the acid concentration to 5.0 percent (v/v) H_2SO_4 led to lower pre-treatment

efficiency with excessive H^+ degrading the sugar progressively into inhibitors. These results correspond to the findings of Miranda et al. (2012), which found that a decrease in reducing sugars is observed when a concentration higher than 2 N (5.4 percent v/v) H_2SO_4 was treated for *Scenedesmus Oblique*. Results in Fig. 2 confirmed that the enzymatic saccharification efficiency of algal biomass was enhanced by coupling with 3.0% H_2SO_4 pretreatments. The higher saccharification rate is obtained due to the greater exposure of algal biomass to enzymes degradation. A decrease in reducing sugars in samples treated with 4.0% H_2SO_4 may be attributed to the degradation of monosaccharides into hydroxymethylfurfural in agreement with that obtained by Ben-Hafsa et al. (2017). Our results showed highest levels of reducing sugars with 4% H_2SO_4 in accordance with that reported by Hernández et al. (2015) in which pre-treatments for *Chlorella sorokiniana*, *Nannochloropsis gaditana*, and *Scenedesmus almeriensis* were carried out using H_2SO_4 at different concentrations (4%, 7%, and 10% (v/v)) at 121°C for 30 min. Hernández et al. reported that the highest reducing sugar was obtained with 7% H_2SO_4 concentration and values of 84, 93, and 55 mg/g DW for *C. sorokiniana*, *N. gaditana* and *Scenedesmus almeriensis*, respectively with small differences between 4% and 7%. *Nannochloropsis* cells have a high cell rupture resistance with a structurally complex and mechanically rigid cell wall consisting of an outer layer of algaenan and a cellulose layer in the inner layer (~75 wt% of the cell wall) (Ajjawi et al. 2017). Therefore, cell wall conferred microalgal cells with structural rigidity as well as resistance to cell rupture and chemical digestion (Gerken et al. 2013). Algaenan is an aliphatic hydrocarbon capable of being resistant to harsh oxidative treatment and acid/alkaline hydrolysis (Scholz et al. 2014; Angles et al. 2017; Baudelet et al. 2017). Sharma et al. (2015) described the liquefaction of starch that occurs during the treatment of *Chlamydomonas reinhardtii* (UTEX90). The commercially available hydrolytic enzymes α-amylase and amyloglucosidase were employed to perform liquefaction and saccharification, respectively. Under heterotrophic and mixotrophic conditions, energy storage molecules such as lipids and carbohydrates (starch and glycogen) are strongly accumulated. Consequently, the biomass content of these compounds is higher than that under photo-autotrophic conditions (Choix et al. 2012). Cellulases are enzymes that break down β-bonds in order to catalyze the hydrolysis of cellulose and hemicellulose, but they may also break down α-bonds from carbohydrates such as starch (Lee et al. 2011). On the other hand, amylases are enzymes that catalyze the hydrolysis of starch breaking down mixotrophically α-bonds, but they are unable to break down β -bonds (Möllers et al. 2014).

The results of the polysaccharides composition showed that acid hydrolysis was not sufficient to hydrolyze high amounts of cellulosic materials. The existence of galactose may be attributed to the high concentrations of galactolipids (e.g., MGDG, DGDG) that compose the photosynthetic membranes of actively growing cells (Harwood 1998) or to the 1–6 linked galactans that decorate the cell wall glycoproteins (Noda et al. 1996). Murano et al. (1997) evaluated the effect of H_2SO_4 concentration and reaction time on hydrolysis efficiency of the biomass from *Solieria filiformis* seaweed and they obtained similar effects of acid on galactose. An increase in acid concentration up to 0.2 M resulted in a sharp increase in galactose. Most galactose presences in hydrolysates are confirmed by the high contents of the pure iota-carrageenan of the *Solieria filiformis* (Harwood 1998). *Nannochloropsis* has complex cell

walls that comprise an outer algaenan layer and an inner layer of cellulose (Ajjawi et al. 2017). *Nannochloropsis* algaenans appeared to be much the same biopolymer as the cutan found in drought-resistant land plants such as Agave and Clivia (Blokker et al. 1998; Gupta et al. 2006). Moreover, the presence of a high amount of HMF (Table 1 and Fig. 3) is due to the degradation of glucose to HMF. Whereas, HMF was presented in low concentrations in defatted biomass pretreated with 3.0% H_2SO_4 followed by enzymatic hydrolysis biomass grown mixotrophically and autotrophically. Appropriate pretreatment methods are required to overcome the recalcitrance of lignocellulosic materials such as dilute 0.5–2.5% H_2SO_4 performed at temperatures from 100 to 200ºC (Sun et al. 2016).

The results of monosaccharide analysis after AEH were in relative agreement with that obtained by Volkman et al. (1993); which also showed glucose (45.2–66.2% of total sugars) to be the most dominant monosaccharide in *Nannochloropsis* biomass. Other sugars included fucose, galactose, mannose, rhamnose, ribose, and xylose (2.0– 14.0). The most prominent monosaccharide in *Nannochloropsis sp.* is glucose, fucose, galactose, mannose, ribose, and xylose (Templeton et al. 2012). Bioethanol yields of *Nannochloropsis gaditana* grown in various municipal wastewaters (0, 30, 60, and 100 %) ranged between 70.3 ± 2.4 mg/g biomass and 94.3 ± 5.5 mg/g biomass (Onay 2018). The maximum bioethanol production and yield by the microalgal hydrolysate were found to be 4.84 g/l and 0.37 g/g, respectively (Lee et al. 2019) when *Brettanomyces custersii* H1-603 was used to convert *Nannochloropsis gaditana* hydrolysate.

In the present study, the ethanol production rate and fermentation efficiency of the acid hydrolysate (AH) were low, due to the presence of galactose as the main reducing sugar in the dilute acid hydrolysate, and the fact that ethanol yield from galactose is lower than glucose (Hong et al. 2011; Hessami et al. 2019). D- Galactose undergoes conversion via the Leloir pathway; basically, in this pathway, a five-step enzymatic pathway converts D-Galactose to glucose-6-phosphate (Timson 2007). Therefore, lower fermentation yield from galactose compared with glucose is due to higher energy consumption in galactose metabolism.

Conclusion

In general, this is the first study describing bioethanol and carbohydrate productions from defatted biomass of *Nannochloropsis Oculata* grown mixotrophically on SBAE. The comparison between different chemical pretreatment methods has shown that acid treatment combined with subsequent enzyme treatment is a suitable method for the conversion of cellulose to sugar. Up to 92 % of sugar yield could be observed after this method. Acid hydrolysis was not sufficient to hydrolyze large quantities of celluloses. The maximum ethanol production was obtained from defatted biomass hydrolyzed by acid coupled with enzyme hydrolysis of defatted biomass. The acid coupled enzyme hydrolysis introduces a leap in bioethanol production from microalgae such as *N. Oculata.* However, this method recommended efficient improvement in fermentation technology to make it an industrially feasible feedstock for bioethanol production in terms of economic viability.

Declarations

Ethics approval and consent to participate: Not applicable

Consent for publication: Not applicable

Data Availability Statement: All data generated or analyzed during this study are included in this published article.

Competing interests: The authors declare that they have no conflict of interest.

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Authors' Contributions: MWS, ABE, and YAA conceived, designed research, analyzed data, wrote the manuscript, reading and approving the manuscript. FMI and NAHF conducted experiments and contributed with reagents and test methods and analytical tools. All authors read and approved the manuscript.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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References

- Abdelsalam E, Samer M, Attia Y, Abdel-Hadi MA, Hassan HE, Badr Y (2019) Effects of Laser Irradiation and Ni Nanoparticles on Biogas Production from Anaerobic Digestion of Slurry. Waste and Biomass Valorization 10(11):3251–3262.
- Ajjawi I, Verruto J, Aqui M, Soriaga L, Coppersmith J, Kwok K, Peach L, Orchard E, Kalb R, Xu W, Carlson TJ, Francis K, Konigsfeld K, Bartalis J, Schultz A, Lambert W, Schwartz AS, Brown R, Moellering ER (2017) Lipid production in *Nannochloropsis gaditana* is doubled by decreasing expression of a single transcriptional regulator. Nat Biotechnol 35(7):647–652.
- Angles E, Jaouen P, Pruvost J, Marchal L (2017) Wet lipid extraction from the microalga Nannochloropsis sp.: Disruption, physiological effects and solvent screening. Algal Research 21:27-34.
- Aristizábal-Marulanda V, Solarte-Toro JC, Cardona Alzate CA (2020) Study of biorefineries based on experimental data: production of bioethanol, biogas, syngas, and electricity using coffee-cut stems as raw material. Environ Sci Pollut Res. https://doi.org/10.1007/s11356-020-09804-y

- Attia YA, Samer M, Moselhy MA, Arisha AH, Abdelqader AA, Abdelsalam E (2021) Influence of laser photoactivated graphitic carbon nitride nanosheets and nickel nanoparticles on purple non-sulfur bacteria for biohydrogen production from biomass. Journal of Cleaner Production, 299:126898.
- Baudelet PH, Ricochon G, Linder M, Muniglia L (2017) Grand Challenges in Algae Biotechnology. Algal Research 25:333-371.
- Ben -Hafsa M, Ben Ismail M, Garrab M, Aly R, Gagnon J, Naghmouchi K (2017) Antimicrobial, antioxidant, cytotoxic and anticholinesterase activities of water-soluble polysaccharides extracted from microalgae *Isochrysis galbana* and *Nannochloropsis oculata*. J Serbian Chem Soc 82(5):509-522.
- Blokker P, Schouten S, Vanen EH, De Leeuwm JW, Hatcher PG, Damste JSS (1998) Chemical structure of algaenans from the fresh water algae *Tetraedron minimum*, *Scenedesmus communis* and *Pediastrum boryanum*. Org Geochem 29:1453-1468.
- Cecchin M, Benfatto S, Griggo F, Mori A, Cazzaniga S, Vitulo N, Delledonne MM (2018) Molecular basis of autotrophic vs mixotrophic growth in *Chlorella sorokiniana*. Sci Rep 8(1):6465.
- Ceron-Garcia MC, Sanchez MA, Fernandez SJM, Molina GE, Garcia CF (2005) Mixotrophic growth of the microalga *Phaeodactylum tricornutum*: influence of different nitrogen and organic carbon sources on productivity and biomass composition. Process Biochem 40(1):297-305.
- Chaudhary L, Priya P, Soni N, Singh P, Tiwari A (2014) Algae as a Feedstock for Bioethanol Production: New Entrance in Biofuel World. Int J Chemtech Res 6(2):1381-1389.
- Cheah WY, Show PL, Juan JC, Chang JS, Ling TC (2018) Enhancing biomass and lipid productions of microalgae in palm oil mill effluent using carbon and nutrient supplementation. Energy Convers Manag 164:188-197.
- Choi HJ, Lee SM (2015) Biomass and oil content of microalgae under mixotrophic conditions. Environ Eng Res 20(1):25-32.
- Choix FJ, De-Bashan LE, Bashan Y (2012) Enhanced accumulation of starch and total carbohydrates in alginate-immobilized Chlorella spp. induced by *Azospirillum brasilense*: II. Heterotrophic conditions. Enzyme Microb Technol 51(5):300–309.
- Demiray E, Karatay SE, Dönmez G (2019) Improvement of bioethanol production from pomegranate peels via acidic pretreatment and enzymatic hydrolysis. Environ Sci Pollut Res 26:29366–29378.
- Dong T, Knoshaug EP, Davis R, Laurens LML, Van Wychen S, Pienkos PT, Nagle N (2016) Combined algal processing: A novel integrated biorefinery process to produce algal biofuels and bioproducts. Algal Research 19:316-323.
- Dragone G, Fernandes B, Vicente A, Teixeira JA (2010) Third generation biofuels from microalgae, current research, technology and education. Appl Microbiol Biotechnol 2:1355–1368.
- Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Calorimetric methods for determination of sugars and related substances. Anal Chem 28:350- 356.
- El-Sayed AB, Battah MG, El-Sayed EW (2015) Utilization efficiency of artificial carbon dioxide and corn steam liquor by *Chlorella vulgaris*. Biolife 3(2):391-403.

- El-Sayed AB, Nashwa AHF, Ibrahim FM, Fayed SA, Sadik MW (2020) Application of bagasse extract in economic *Nannochloropsis oculata* mass production. Egypt J Chem 63(12):5183-5192.
- El-Sayed AB, Nashwa AHF, Magda K (2017) Bioethanol Production from Egyptian alga *Enteromorpha sp.* Middle East J Appl Sci 7(1):216-225.
- El-Sheekh MM, Gheda SF, El-Sayed AEKB, Abo Shady AM, El-Sheikh ME, Schagerl M (2019) Outdoor cultivation of the green microalga Chlorella vulgaris under stress conditions as a feedstock for biofuel. Environ Sci Pollut Res 26:18520–18532.
- Gerken HG, Donohoe B, Knoshaug EP (2013) Enzymatic cell wall degradation of *Chlorella vulgaris* and other microalgae for biofuels production. Planta 237(1):239–253.
- Giang TT, Lunprom S, Liao Q, Reungsang A, Salakkam A (2019) Improvement of hydrogen production from *Chlorella sp.* biomass by acid-thermal pretreatment. PeerJ 7:6637.
- Guillard RRL (1975) Culture of phytoplankton for feeding marine invertebrates. In: Smith, W. L. and Charley, M. M. (eds.). Culture of marine invertebrates' animals. New York, Plenum, 29-60.
- Gupta NS, Collinson ME, Briggs DEG, Evershed RP, Pancost RD (2006) Reinvestigation of the occurrence of cutan in plants: implications for the leaf fossil record. Paleobiology 32:432–449.
- Harwood JL, Siegenthaler PA, Murata N (Eds.) (1998) Lipids in Photosynthesis: Structure, Function and Genetics. Springer, Netherlands, pp 53.
- Hernández D, Riaño B, Coca M, García-González MC (2015) Saccharification of carbohydrates in microalgal biomass by physical, chemical and enzymatic pre-treatments as a previous step for bioethanol production. Chem Eng J 262:939-945.
- Hessami MJ, Cheng SF, Ambati RR, Yin YH, Phang SM (2019) Bioethanol production from agarophyte red seaweed, *Gelidium elegans*, using a novel sample preparation method for analyzing bioethanol content by gas chromatography. J Biotechnol (3):9-25.
- Hijazi O, Abdelsalam E, Samer M, Amer BMA, Yacoub IH, Moselhy MA, Attia YA, Bernhardt H (2020a) Environmental impacts concerning addition of the trace metals in the process of biogas production from anaerobic digestion of slurry. Journal of Cleaner Production 243:118593.
- Hijazi O, Abdelsalam E, Samer M, Attia YA, Amer BMA, Amer MA, Badr M, Bernhardt H (2020b) Life cycle assessment of the use of nanomaterials in biogas production from anaerobic digestion of manure. Renewable Energy 148:417-424.
- Hong KK, Vongsangnk W, Vemuri GN, Nielsen J (2011) Unravelling evolutionary strategies of yeast for improving galactose utilization through integrated systems level analysis. PNAS 108(29):12179–12184.
- Knoshaug EP, Dong T, Spiller R, Nagle N, Pienkos PT (2018) Pretreatment and fermentation of salt-water grown algal biomass as a feedstock for biofuels and high-value biochemicals. Algal Research 36:239-248.
- Koyande AK, Show P, Guo R, Tang B, Ogino O, Chang J (2019) Bio- processing of algal bio-refinery: a review on current advances and future perspectives. Bioengineered 10(1):574-592.

- Lee JH, Lee HU, Lee JH, Lee SK, Yoo HY, Park C, Kim SW (2019) Continuous production of bioethanol using microalgal sugars extracted from *Nannochlorpsis gaditana*. Korean J Chem Eng 36:71-76.
- Lee S, Oh Y, Kim D, Kwon D, Lee C, Lee J (2011) Converting carbohydrates extracted from marine algae into ethanol using various ethanolic *Escherichia coli* strains. Appl Biochem Biotechnol 164:878–888.
- Malhotra M, Suman SK (2021) Laccase-mediated delignification and detoxification of lignocellulosic biomass: removing obstacles in energy generation. Environ Sci Pollut Res. https://doi.org/10.1007/s11356-021-13283-0
- Matsumoto H, Hamasaki A, Sioji N, Ikuta Y (1997) Influence of CO_2, SO_2 and NO in flue gas on microalgae productivity. JCEJ 30:620–624.
- Maurya R, Paliwal C, Ghosh T, Pancha I, Chokshi K, Mitra M, Ghosh A, Mishra S (2016) Applications of de-oiled microalgal biomass towards development of sustainable biorefinery. Bioresour Technol 214:787-796.
- Mesa L, González E, Cara C, González M, Castro E, Mussatto SI (2011) The effect of organosolv pretreatment variables on enzymatic hydrolysis of sugarcane bagasse. Chem Eng J 168:1157-1162.
- Miller GL (1959) Use of dinitrosalicylic acid for determination of reducing sugar. Anal Chem 31:426-428.
- Miranda JR, Passarinho PC, Gouveia L (2012) Pre-treatment optimization of *Scenedesmus obliquus* microalga for bioethanol production. Bioresour Technol 104:342-348.
- Mirsiaghi M, Reardon KF (2015) Conversion of lipid-extracted *Nannochloropsis salina* biomass into fermentable sugars. Algal Res 8:145-152.
- Möllers KB, Canella D, Jorgensen H, Frigaard NU (2014) Converting Carbohydrates Extracted from Marine Algae into Ethanol Using Various Ethanolic *Escherichia coli* Strains. Biotechnol Biofuels 7:64.
- Murano E, Toffanin R, Cecere E, Rizzo R, Knutsen SH (1997) Investigation of the carrageenans extracted from *Solieria filiformis* and *Agardhiella subulata* from Mar Piccolo, Taranto. Mar Chem 58(3-4):319-325.
- Nguyen HC, Su CH, Yu YK, Huong DTM (2018) Sugarcane bagasse as a novel carbon source for heterotrophic cultivation of oleaginous microalgae *Schizochytrium sp.* Ind Crops Prod 121:99-105.
- Nobre BP, Villalobos F, Barragan BE, Oliveira AC, Batista AP, Marques PASS, Mendes RL, Sovova L, Palavra AF, Gouveia L (2013) A biorefinery from *Nannochloropsis sp*. microalga - Extraction of oils and pigments. Production of biohydrogen from the leftover biomass. Bioresour Technol 135:128-36.
- Noda K, Ohno N, Tanaka K, Kamiya N, Okuda M, Yadomae T, Nomoto K, Shoyama Y (1996) A water-soluble antitumor glycoprotein from *Chlorella vulgaris*. Planta Med 62(5):423–426.
- Onay M (2018) Bioethanol production from *Nannochloropsis gaditana* in municipal wastewater. Energy Procedia 153:253-257.
- Osman MEH, Abo-Shady AM, Elshobary ME, Abd El-Ghafar MO, Abomohra A (2020) Screening of seaweeds for sustainable biofuel recovery through sequential biodiesel and bioethanol production.

Environ Sci Pollut Res 27:32481–32493.

- Phwan CK, Chew KW, Sebayang AH, Ong HC, Ling TC, Marlinda AM, Ho Y, Show PL (2019) Effects of acids pre-treatment on the microbial fermentation process for bioethanol production from microalgae. Biotechnol Biofuels 12:191.
- Rodríguez-Valderrama S, Escamilla-Alvarado C, Rivas-García P, Magnin J-P, Alcalá-Rodríguez M, García-Reyes RB (2020) Biorefinery concept comprising acid hydrolysis, dark fermentation, and anaerobic digestion for co-processing of fruit and vegetable wastes and corn stover. Environ Sci Pollut Res 27:28585–28596.
- Saddler JN, Mabee WE, Simms R, Taylor M (2011) The Biorefining Story: Progress in the Commercialization of Biomass-to-Ethanol. Forests in Development: A Vital Balance. 39–51, Springer Netherlands.
- Sanchez OJ, Cardona CA (2008) Trends in biotechnological production of fuel ethanol from different feedstocks. Bioresour Technol 99(13):5270–95.
- Scholz MJ, Weiss TL, Jinkerson RE, Jing J, Roth RU, Goodenough MCP, Gerken HG (2014) Ultrastructure and composition of the *Nannochloropsis gaditana* cell wall. Eukaryot Cell 13:450-1464.
- Sharma P, Khetmalas MB, Tanon GD (2015) Evaluation of *Chlorella ellipsodea* for biodiesel and bioethanol production. Asian J Microbiol Biotechnol Environ Sci 17(4):207-211.
- Sipauba TLH, Scardoeli TB, Fenerick DC, Tedesque MG (2020) Comparison of photoautotrophic and mixotrophic cultivation of microalgae *Messastrum gracile* (Chlorophyceae) in alternative culture media. Braz J Biol Sci 7-23.
- Siqueira G, Várnai A, Ferraz A, Milagres AMF (2013) Enhancement of cellulose hydrolysis in sugarcane bagasse by the selective removal of lignin with sodium chlorite. Applied Energy 102:399–40.
- Sivasankar P, Dhandayuthapani K, Shanthi K (2017) Production of ethanol using hydrolysates derived from acid pretreated defatted biomass *Nannochloropsis limnetic*. Int J Recent Sci 8(8):19392-19395.
- Sritrakul N, Nitisinprasert S, Keawsompong S (2017) Evaluation of dilute acid pretreatment for bioethanol fermentation from sugarcane bagasse pith. Agric. Nat Resour 51(6):512–9.
- Sun S, Sun S, Cao X, Sun R (2016) The role of pretreatment in improving the enzymatic hydrolysis of lignocellulosic materials. Bioresour Technol 199(1):49-58.
- Swana J, Yang Y, Behnam M, Thompson R (2011) An Analysis of Net Energy Production and Feedstock Availability for Biobutanol and Bioethanol. Bioresource Technology 102(2):2112–17.
- Timson DJ (2007) Galactose metabolism in *Saccharomyces cerevisiae*. Dyn Biochem Process Biotechnol Mol Biol 1(1):63–73.
- Volkman JK, Brown MR, Dunstan GA, Jeffrey S W (1993) The biochemcial composition of marine microalgae from the class Eustigmatophyceae. J Phycol 29:69-78.

- Walker GM (2011) Fuel alcohol: current production and future challenges. J Inst Brew 117(1):3–22.
- Wen X, Tao H, Peng X, Wang Z, Ding Y, Xu Y, Liang L, Du K, Zhang A, Liu C, Geng Y, Li Y (2019) Sequential phototrophic-mixotrophic cultivation of oleaginous microalgae *Grasiella sp.* WBG-1 in a 1000 m^2 open raceway pond. Biotechnol. Biofuels 12(1):27.
- Wyman C (1996) Handbook on Bioethanol. NewYork Routledge, https://doi.org/10.1201/9780203752456.
- Yao P, You S, Qi W, Su R, He Z (2020) Investigation of fermentation conditions of biodiesel by-products for high production of β-farnesene by an engineered Escherichia coli. Environ Sci Pollut Res 27:22758–22769.
- Yoswathana N, Phuriphipat P, Treyawutthiwat P, Eshtiaghi MN (2010) Bioethanol Production from Rice Straw. Energy Res J 1(10):26-31.

Tables

Table 1. Sugar composition of hydrolysates of *N. oculata* microalgae biomass cultivated autotrophically and mixotrophically on SBAE after optimum pre-treatments.

nts	AH*				AEH**			
	Autotrophic		Mixotrophic		Autotrophic		Mixotrophic	
	g/L	wt %	g/L	wt%	g/L	wt %	g/L	wt %
se	6.12	44.014	8.281	49.030	1.16	10.312	2.29	9.256
e	3.13	22.514	3.62	18.203	9.50	60.736	15.47	62.488
e	3.65	26.210	4.90	29.016	2.65	16.965	4.31	17.396
se	0.338	2.432	0.933	3.5233	0.865	5.527	1.70	6.880
	0.105	0.755	0.079	0.465	0.080	0.512	0.119	0.484
se	0.061	0.435	0.0262	0.155	0.139	0.893	0.126	0.509
e	0.505	3.630	0.444	2.630	0.773	4.945	0.704	2.845
	0.019	19.0µg/mL	0.081	81.0 µg/mL	0.007	7.0 µg/mL	0.011	11.0 µg/mL

AH*: acid hydrolysis with 4% (v/v) H_2SO_4

AEH**: acid enzyme hydrolysis with 3% (v/v) H_2SO_4 followed by enzymatic treatments

Figures

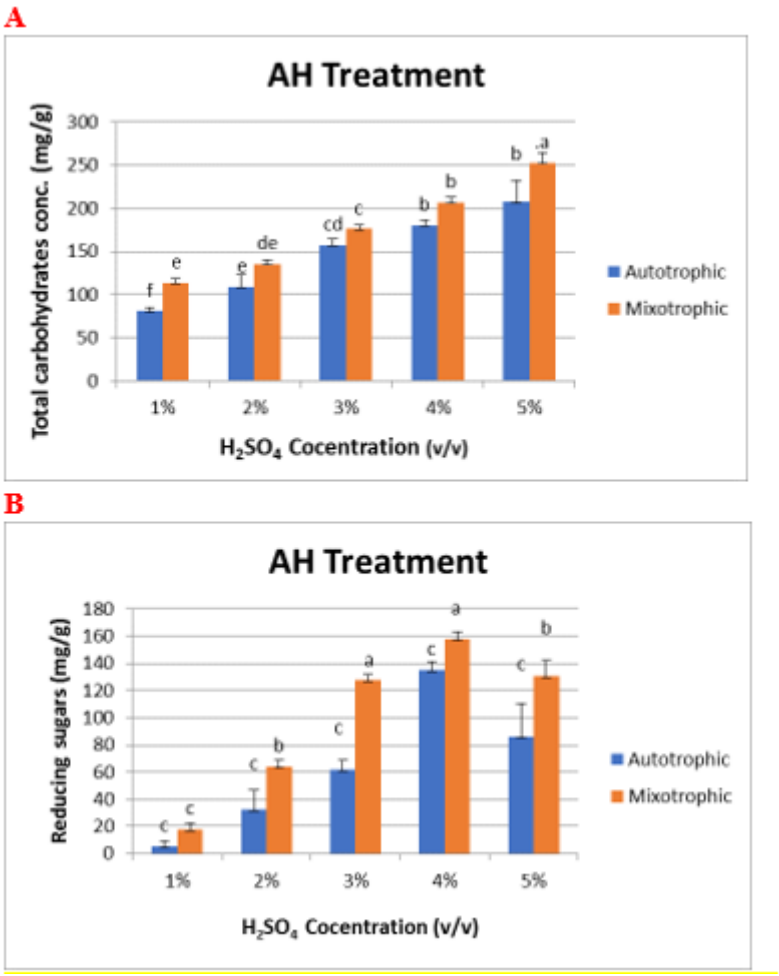

Figure 1

Effect of acid hydrolysis (AH) treatment on total carbohydrates mg/g (A) and reducing sugars mg/g (B) of N. Oculata defatted biomass.

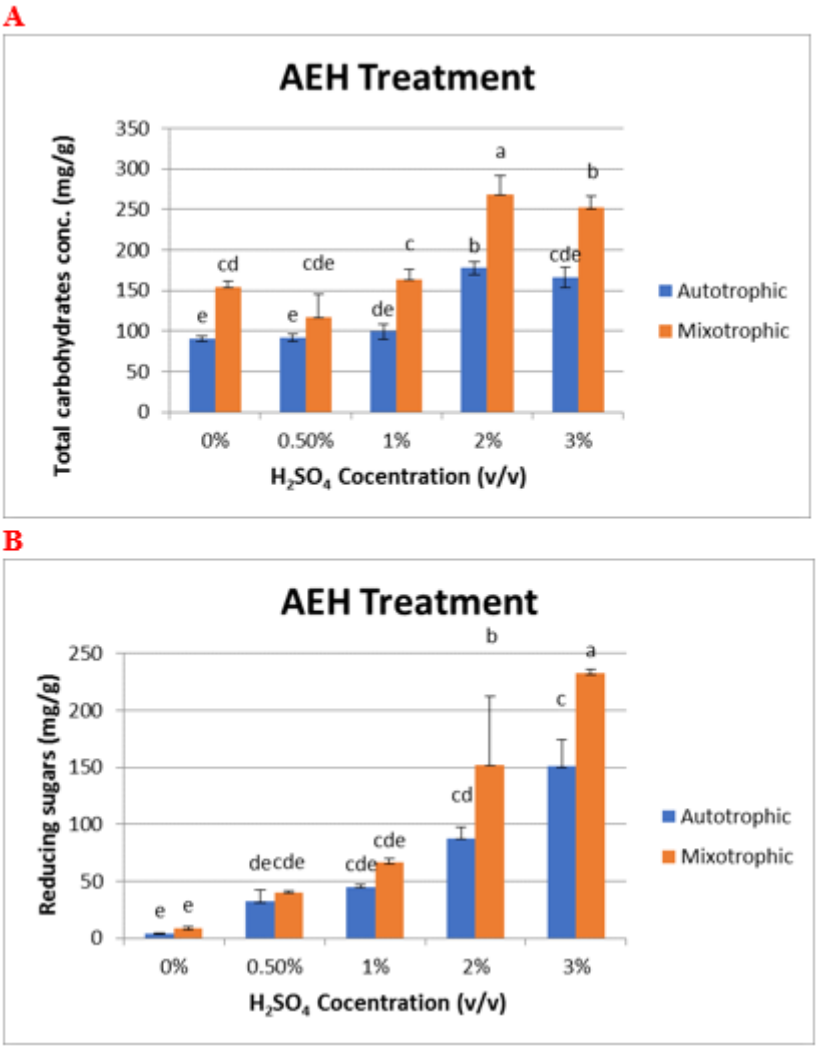

Figure 2

Effect of acid, enzyme hydrolysis (AEH) treatment on total carbohydrates mg/g (A) and reducing sugars mg/g (B) of N. Oculata defatted biomass.

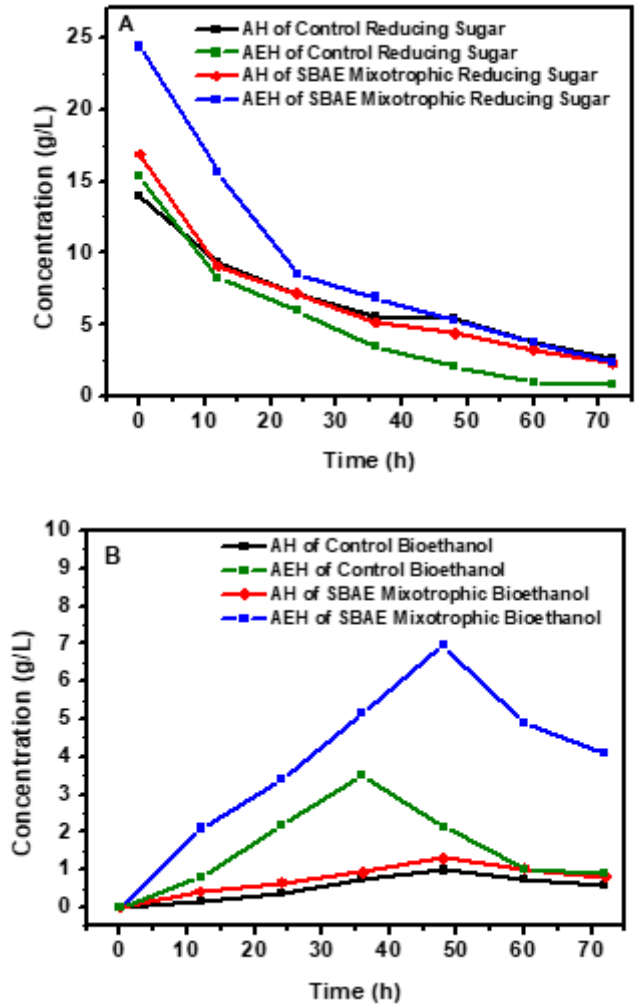

Figure 3

Fermentation with acid hydrolysate and acid enzyme hydrolysate of N. oculata hydrolysate using S. cerevisiae (Reducing Sugar: A and Bioethanol: B)

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