BioEnergy Research (2020) 13:1269–1279
https://doi.org/10.1007/s12155-020-10131-w

Ozonolysis as an Effective Pretreatment Strategy for Bioethanol Production from Marine Algae

Sulfahri[1] · Siti Mushlihah[2] · Alexandra Langford[3] · Asmi Citra Malina A. R. Tassakka[4]

Published online: 22 April 2020

Abstract

Marine algae are promising third-generation feedstocks for bioethanol production as they are fast growing, require minimal inputs, and do not compete for land. However, marine algae have complex cell walls which necessitate pretreatment prior to fermentation, and this represents a major component of the cost of bioethanol production. Standard pretreatment processes using acids are costly and generate hazardous waste streams. This study aims to develop an economic and environmentally friendly pretreatment process using ozonolysis for the marine algae *Kappaphycus alvarezii* and *Gelidium amansii.* Acid and ozone pretreatments were compared across the pretreatment, enzyme hydrolysis, and fermentation stages of bioethanol production. Acid pretreatment outperformed ozonolysis over the pretreatment and enzyme hydrolysis stages. However, it also generated as by-products the compounds 5-hydroxymethyl furfural (5-HMF) and levulinic acid (LA), which inhibited ethanol fermentation and reduced the efficiency of the process overall. Ozone pretreatment did not produce these inhibitory compounds, and as such outperformed acid pretreatment across the process as a whole. These results indicate the potential of ozonolysis as an economic and environmentally friendly pretreatment for the production of bioethanol from marine algae.

Keywords Acid · Biofuel · Fermentation · Ozone · Seaweed

Introduction

Bioethanol is a promising alternative to fossil fuels due to its capacity for sustainable and environmentally friendly production [1, 2]. Seaweed (macroalgae) is a promising third-generation feedstock for bioethanol production, because unlike first- and second-generation feedstocks, it does not compete for land or freshwater resources [3]. Many seaweeds have high carbohydrate contents and rapid growth rates, making them a potentially sustainable bioethanol feedstock [3, 4]. Macroalgae are grouped into three types: Rhodophyceae (red seaweed), Phaeophyceae (brown seaweed), and Chlorophyceae (green seaweed) [5]. Of these, red seaweeds are the most widely commercially cultivated in the world because of their high productivity and rapid growth [6, 7]. In 2017, red seaweeds accounted for 46% of the $11 billion global seaweed market [8]. Indonesia produces 56% of the global supply of red seaweed by weight [8], with 70,000 households depending on small-scale seaweed production for their livelihoods [9, 10]. This seaweed is primarily used to produce carrageenan and agar, both inputs for food manufacturing [9]. Production of these red algae offers substantial socio-economic benefits to small farmers [11], who in Indonesia typically earn around $5000 per year from this activity [11, 12]. However, farmers are vulnerable to price fluctuations and this can have severe implications for local livelihoods [13]. Diversifying options for processing red seaweed domestically, such as through bioethanol production, therefore promises benefits for poverty reduction in Indonesia, as well as being a potentially sustainable method of bioethanol

✉ Sulfahri
sulfahri@unhas.ac.id

Siti Mushlihah
siti.mushlihah@rmit.edu.au; siti.mushlihah@indonegri.id

Alexandra Langford
zannie.langford@uq.edu.au

Asmi Citra Malina A. R. Tassakka
Citra@unhas.ac.id

1 Faculty of Mathematics and Natural Sciences, Hasanuddin University, Makassar, Indonesia

2 School of Engineering, RMIT University, Melbourne, Australia

3 School of Agriculture and Food Sciences, University of Queensland, Brisbane, Australia

4 Faculty of Marine Science and Fisheries, Hasanuddin University, Makassar, Indonesia

production. Two species of red seaweed are of particular interest in this context. *Kappaphycus alvarezii* represents 15% of the global red seaweed market value [8] and is one of the main carrageenophyte species produced in Indonesia [13]. *Gelidium amansii* grows wild in Indonesian waters and is commonly used for agar production [14], and may be a promising new species for commercial cultivation.

Production of bioethanol from algae biomass is undertaken in three stages: pretreatment of algal biomass, hydrolysis of sugar polymers, and fermentation of the derived sugars into ethanol [15]. Pretreatment of algae is required to break the polysaccharides down to monosaccharides [16]. The carbohydrate polymer of *K. alvarezii* is D-galactose, while *G. amansii* contains both D-galactose and L-galactose [17, 18]. Thus both D-type and L-type carbohydrates are used as a carbon source of the yeast for ethanol production. Pretreatment breaks down the complex cell wall structure of algae to facilitate the conversion of cellular carbohydrates to glucose [19] and is a major cost of production, typically representing up to 20% of the total cost of bioethanol production [20]. Therefore, development of effective low-cost pretreatment options is an important priority for improving the cost effectiveness of these processes.

A variety of pretreatment processes are available, including alkali, acid, enzyme, microwave, and hydrothermal pretreatments [3, 21]. Of these, acid pretreatment is most commonly used to improve accessibility to cell wall degrading enzymes [14, 22]. This method involves the use of acids as catalysts for chemical pretreatment and the use of enzymes for enzymatic hydrolysis [23]. However, acid pretreatment has several disadvantages, including high temperature reaction conditions and the production of inhibitors, such as 5-hydroxymethyl furfural (5-HMF) and levulinic acid (LA), that interfere with the fermentation process [24, 25]. Removal of 5-HMF and LA requires additional treatment, generating additional costs [26–28]; a pretreatment method that avoids producing these components is therefore desirable.

Ozone pretreatment involves the use of ozone to oxidize, solubilize, and degrade the cell wall. It generates pretreated algae with excellent characteristics for hydrolysis, such as a lack of inhibitory compounds, the generation mainly of weak carboxylic acids, limited effects on sugar polymers, and selective degradation of lignins [20, 29]. Ozone pretreatment may also be undertaken at a lower operational cost as it is undertaken at ambient temperature and pressure [20]. Ozone is widely used for disinfection purposes due to its destructive effect on cell walls [20]. However, its application as pretreatment strategy for bioethanol production has been limited to only a few studies [30] and although ozone pretreatment has been used on algae in other processes [31], to date no studies have explored the effectiveness of ozonolysis as a pretreatment strategy in bioethanol production. This study addresses this gap by investigating the feasibility of ozonolysis as a low-cost pretreatment strategy for the red algae *K. alvarezii* and *G. amansii*, through comparison with a commonly used acid pretreatment method. The study integrates analysis of the full fermentation process, including pretreatment, enzymatic hydrolysis, and fermentation, in order to produce a more holistic comparison of acid and ozone pretreatments than is afforded by a focus on pretreatment alone. The algae is first pretreated with algae or ozonolysis, and the production of the common fermentation inhibitors 5-HMF and LA evaluated, as these compounds negatively affect cell growth and ethanol fermentation [32, 33]. The obtained sugar hydrolysates were then fermented using commercial yeast, *Saccharomyces cerevisiae*. Each pretreatment strategy was optimized at the pretreatment, enzyme hydrolysis, and fermentation stages. The performance of these two pretreatment strategies across the optimized bioethanol production processes was evaluated.

Materials and Methods

Raw Materials

Samples of the marine algae *K. alvarezii* and *G. amansii* were purchased from Seaweed Research Unit of Hasanuddin University, Indonesia. Algal biomass was washed in water several times to remove salts. The clean algae were then dried for 48 h at 60 °C. Dry algae were ground to an average size of 100 mesh using a laboratory mill. The carbohydrate content of the algal biomass was characterized using phenol sulfuric acid [17]. The total carbohydrate of *K. alvarezii* and *G. amansii* was 67.08% and 59.13%, respectively.

S. cerevisiae used in this study was *S. cerevisiae* InaCC Y614 obtained from Indonesian Culture Collection (InaCC). *S. cerevisiae* was cultured on SDA (Sabouraud Dextrose Agar) medium at 30 °C for 48 h.

Pretreatment

The algal cell wall structure was disrupted using two pretreatment methods (ozonolysis and acid pretreatment) prior to enzymatic hydrolysis. The polysaccharides which were obtained through pretreatment were then converted into monosaccharides through enzyme hydrolysis.

Ozone Pretreatment

Ozone pretreatment was performed in a glass column reactor with working volume 300 mL. The reaction was performed at 30 °C and 150 rpm, at a range of algae concentrations (1%, 2%, 3%, 4%, and 5%) diluted with distilled water at a ratio of 1:10 (w/v), and stirred throughout the treatment using a magnetic stirrer. The column was then exposed to the ozone gas stream that was produced by ozone generator (Sander 301).

The ozone generator was fed with industrial grade oxygen. The ozone concentration in the gas current was kept at 400 mg O_3 L^{-1} and gas flow rate was maintained constant at 0.5 L min^{-1} with varied reaction time (0, 30, 60, 90, and 120 min).

Acid Pretreatment

Acid hydrolysis process was performed at 100 °C using sulfuric acid (H_2SO_4). The reaction was performed in an autoclave with 100-mL tubes (working volume 50 mL). The parameters selected for optimization in the acid hydrolysis experiments were (1) reaction time (0, 30, 60, 90, and 120 min), (2) substrate concentrations (1%, 2%, 3%, 4%, and 5%), and (3) acid concentrations (0.00 M, 0.05 M, 0.10 M, 0.15 M, 0.20 M, and 0.25 M). After the reaction, NaOH was added to neutralize the material. After the neutralization process, the algal solution was separated from the liquid by centrifugation at 9000 rpm for 10 min at 4 °C, before filtration was undertaken. The liquid fraction was then collected to determine the total sugar and reducing sugar yields.

Enzymatic Hydrolysis

Pretreated algal biomass was used for enzyme hydrolysis to convert the polysaccharides produced in pretreatment into monosaccharides. Enzymatic hydrolysis of pretreated algal biomass was carried out using commercial enzymes (Cellic CTec2; Novozymes, Denmark). The enzyme activity used in this study was 100 FPU/g (measured in our laboratory). The enzymatic hydrolysis was performed in 100 mL tubes with 50 mL of working volume. The pH in the reactors was adjusted to a pH of 5.0 using Na-citrate buffer. The amount of enzyme was 100 KNU with a constant algal biomass of 5 g for enzymatic hydrolysis. The reaction time of the enzymatic hydrolysis process was 48 h at 30 °C. After hydrolysis, the algal solution was separated from the liquid by centrifugation at 9000 rpm for 10 min at 4 °C followed by filtration. The liquid fraction was then collected to determine the total sugar and reducing sugar yields.

Ethanol Fermentation

S. cerevisiae was employed in this ethanol fermentation process. The algae hydrolysates obtained from ozone and acid pretreatment followed by hydrolysis were used as fermentation mediums for ethanol production. The ethanol fermentation experiments were performed in 100 mL tubes with 50 mL of working volume. The parameters selected for optimization in ethanol fermentation experiments were fermentation time (0, 12, 24, 36, and 48 h). The fermentation process was performed under anaerobic conditions at 30 °C with agitation of 150 rpm. Anaerobic conditions were obtained through gas stripping, undertaken by streaming hydrogen into the fermenter. The fermenter was closed with a rubber stopper cover with a gas valve and then was purged with gas hydrogen for 2 min. The pH in the reactors was adjusted to a pH of 5.0 using Na-citrate buffer. Cell biomass, sugar, and ethanol were measured at the end of the fermentation treatment time. Sacrificial tests were set up to measure the samples at each time point.

Analytical

Sugar, 5-HMF, and LA Levels

In this study, sugars refer to the sum of glucose and galactose contents of hydrolysates. Sugar, 5-HMF, and LA levels were measured using the Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA, USA). The HPLC was fitted with a refractive index detector, equipped with a Bio-Rad Aminex HPX-87H column (300 × 7.8 mm). The column was maintained at 65 °C with a flow of 0.6 mL/min of 5 mM H2SO4 mobile phase. The standards consisted of solutions of glucose and galactose in a concentration range between 0.01 and 20 g/L, whereas standard solutions of 5-HMF and LA in a concentration range between 0.01 and 10 g/L.

Ethanol Levels

Ethanol levels were measured using specific gravity and gas chromatography methods using a Techcomp GC7900 fitted with a thermal conductivity detector, TM-5 column, injector at 250 °C oven at 80 °C and FID of 250 °C. The ethanol yield (Yp/s) was calculated based on the actual ethanol production and expressed as grams of ethanol per grams of sugar utilized (g/g). The fermentation efficiency was calculated by the percent ratio of the average ethanol production to the ethanol theoretically produced (0.511 g ethanol/g sugar) in the biochemical conversion of the sugars consumed.

Statistical Analysis

Ozone pretreatment, acid pretreatment, enzymatic hydrolysis, and ethanol fermentation were conducted in triplicate. The experimental data were statistically analyzed by one-way analysis of variance (ANOVA) and Tukey analysis using GraphPad Software (GraphPad Prism version 8.2.1). Values were considered significant when p value was < 0.05.

Results and Discussion

Ozone Pretreatment

Effect of Substrate Concentration on Ozone Pretreatment

This study optimizes sugar yield production by ozone pretreatment, followed by enzymatic hydrolysis using substrate concentrations of 1–5%. Figure 1 presents the formation of sugar yields at 60 min of reaction time. Ozone pretreatment of *K. alvarezii* resulted in up to a 3.2-fold increase in sugar yield compared to that without ozone pretreatment. For *G. amansii*, a 6.8-fold increase was obtained. The high sugar yield of *K. alvarezii* compared to *G. amansii* may be because of the high carbohydrate content of *K. alvarezii* (67%) than *G. amansii* (59%) [7, 17]. As Kim et al. [14] explain, the carbohydrate content of substrates is the main factor controlling sugar yield in biomass pretreatment.

The highest sugar yield that was achieved at a substrate concentration was 1%. Based on Tukey's multiple comparison test, the sugar yield of *G. amansii* at 1% of substrate concentration was not significantly different ($p > 0.05$) compared to 2% of substrate concentration. In contrast, the sugar yield of *K. alvarezii* decreased significantly when substrate concentration was increased to 2%. This difference is likely due to a higher viscosity of the solid contents of *K. alvarezii* compared to *G. amansii*. The higher viscosity reduces the rate of penetration of ozone and of enzymatic hydrolysis. The

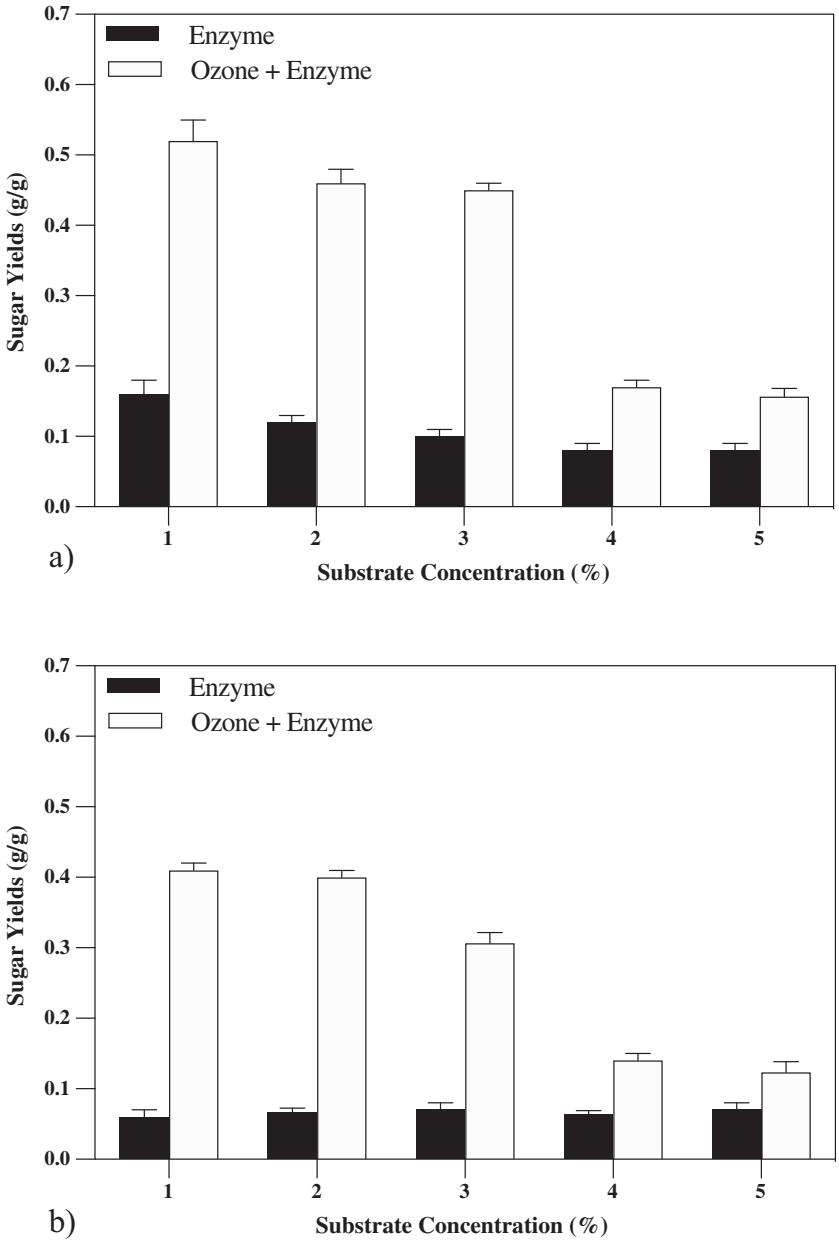

Fig. 1 Effect of substrate concentration on ozone pretreatment. **a** *K. alvarezii*. **b** *G. amansii*

carbohydrate polymer of *K. alvarezii* consists mainly of carrageenan, while *G. amansii* consists mainly of agar [7, 17]. The major repeating unit of carrageenan is carrabiose, which is composed of β-D-galactose-4-sulfate-β-1,4–3,6-anhydro-D-galactose-2-sulfate, while the major repeating unit of agar is agarobiose, which is a disaccharide composed of 1,3-linked-D-galactose and 1,4-linked 3,6-anhydrous-L-galactose [18, 34]. The structure of carrageenan gel is harder than agar, so it requires higher dilution and harder to disrupt by ozonolysis at higher substrate concentrations. This may be the reason why the sugar yield of *K. alvarezii* was decreased significantly when substrate concentration was increased to 2%.

Sugar yields of *K. alvarezii* decreased slightly by 2.2% when substrate concentration was increased from 2 to 3%, whereas sugar yields of *G. amansii* sharply decreased by 25%. When substrate concentration was increased from 3 to 4%, sugar yields of *K. alvarezii* and *G. amansii* were sharply decreased by 53% and 62%, respectively. In contrast, sugar yields of *K. alvarezii* and *G. amansii* did not decline significantly when substrate concentration was increased from 4 to 5%. This might be due to the high viscosity of the solid content of both *K. alvarezii* and *G. amansii* when substrate concentration was 4–5%. The high viscosity is attributed to residual biomass, which is predominantly in the form of complex hydrocolloids [35]. The highest sugar yield was achieved at 1% of substrate concentration *for K. alvarezii* and *G. amansii*; accordingly, this substrate concentration was applied for further experiments in this study.

Effect of Reaction Time on Ozone Pretreatment

The effect of reaction time on ozone pretreatment from *K. alvarezii* and *G. amansii* biomass at a substrate concentration of 1% is shown in Fig. 2. Sugar yields increased with increasing reaction time. The sugar yield of *K. alvarezii* and *G. amansii* sharply increased up to a 60- and 30-min reaction time, respectively. As such a reaction time of 60 min was selected to ensure both species reached equilibrium, with *K. alvarezii* and *G. amansii* producing 0.52 g g^{-1} (g sugar g dry $algae^{-1}$) and 0.41 g g^{-1}, respectively.

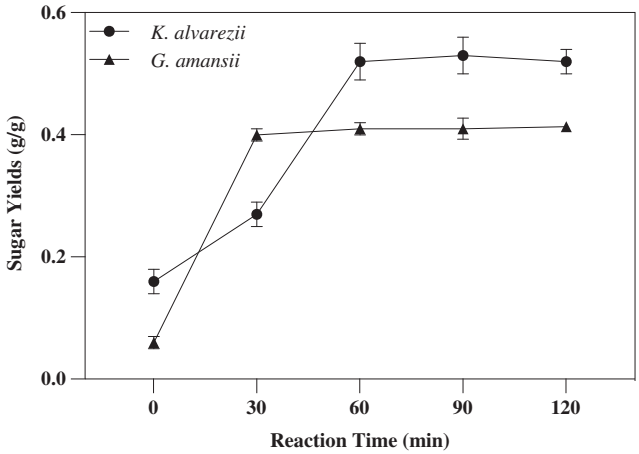

Fig. 2 Effect of reaction time on ozone pretreatment

Based on the Tukey's multiple comparison test, the reaction extent of *G. amansii* at 30, 60, 90, and 120 min was not significantly different ($p > 0.05$). Similarly, the reaction extent of *K. alvarezii* at 60, 90, and 120 min was not significantly different ($p > 0.05$). This indicates that ozone pretreatment produces maximum sugars from *G. amansii* and *K. alvarezii* at 30 and 60 min, respectively. Travaini et al. [20] reported similar findings, noting that ozone only requires short reaction times to react with olefinic, aromatic, and phenolic compounds due to their electron density. Similarly, Bule et al. [36] reported that ozone required only 30–60 h to generate reactive hydroxyl radicals through the formation of a superoxide (the primarily formed radical), which reacted with carbohydrates resulting in the random cleavage of glycosidic bonds, and hence led to enhanced hydrolysis of wheat straw, resulting in sugar production increase of more than 50%.

Acid Pretreatment

Effect of Substrate Concentration on Acid Pretreatment

Acid pretreatment using H_2SO_4 followed by enzymatic hydrolysis was optimized using substrate concentrations of 1–5%, reaction times of 0–120 min, and acid concentrations of 0.00–0.25 M. Figure 3 presents sugar yields at different substrate concentrations. Based on the Tukey's multiple comparison test, sugar yield of *K. alvarezii* and *G. amansii* at 4–5% of substrate concentration was significantly different at 1–3% of substrate concentration. The results indicate that sugar yields decrease at higher algal concentrations, likely because the high viscosity reduces the rate at which acid permeates the material. The decreased sugar yield in acid pretreatment at concentrations above 3% indicates that the acid could not properly hydrolyze the substrate at a high substrate concentration. The efficiency of acid hydrolysis decreased because the H^+ ion concentration in the acid was not sufficient to hydrolyze high amounts of cellulosic materials. This is consistent with the report of Abd-Rahim et al. [18], which states that H_2SO_4 more efficiently hydrolyzes cellulose materials in seaweed. This makes it more suitable to hydrolyze carrageenan in *K. alvarezii*.

Effect of Reaction Time on Acid Pretreatment

Figure 4 presents the effect of reaction time on acid pretreatment at a substrate concentration of 1%. Based on Tukey's multiple comparison test, the sugar yield of *K. alvarezii* and *G. amansii* at 30–120 min of substrate concentration was not significantly different ($p > 0.05$). Equilibrium was reached for

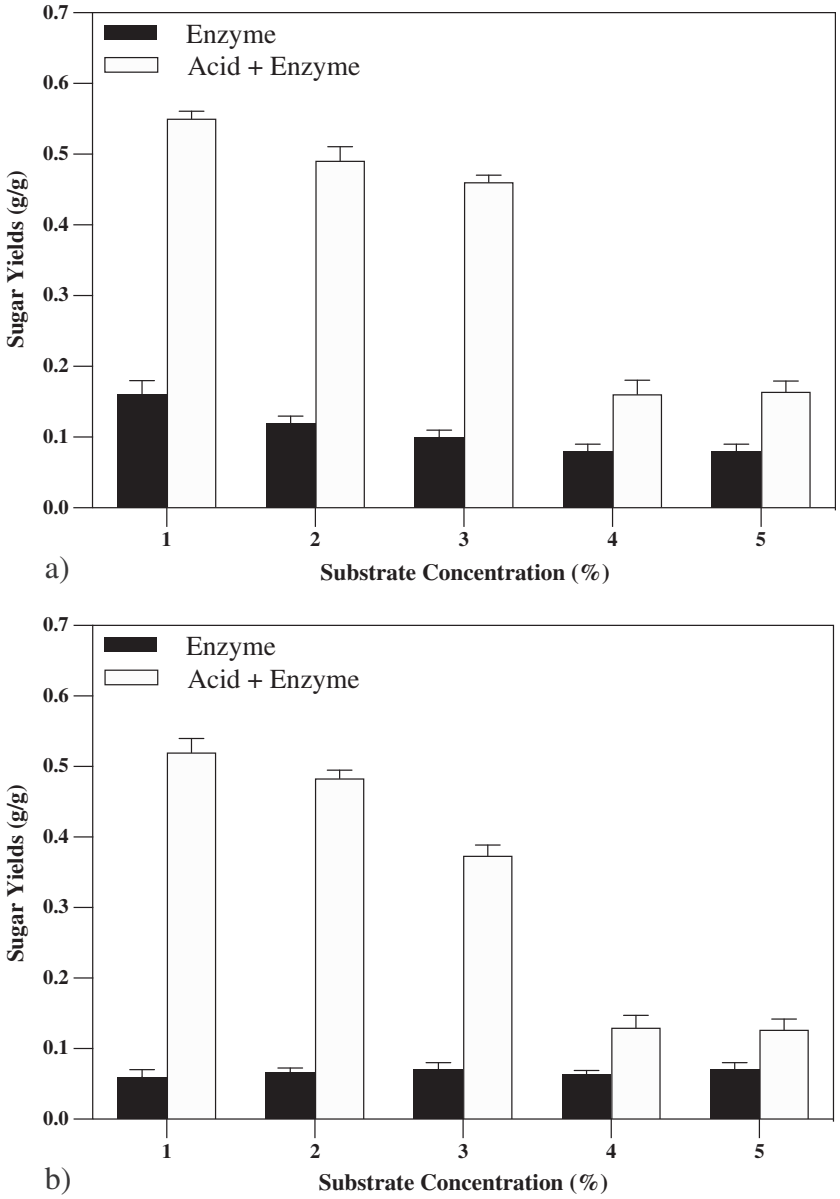

Fig. 3 Effect of substrate concentration on acid pretreatment. **a** *K. alvarezii*. **b** *G. amansii*

both *K. alvarezii* and *G. amansii* at 30 min of reaction time, with sugar yields of 0.56 g g^{-1} (g sugar g dry $algae^{-1}$) and 0.51 g g^{-1}, respectively. These results are consistent with reports by Kim et al. [14] that longer reaction time were ineffective in pretreatment as they led to the acceleration of sugar degradation and increased energy and cost.

Effect of Acid Concentration on Acid Pretreatment

Figure 4 presents the effect of reaction time and acid concentration on acid pretreatment at 1% of substrate concentration. The highest sugar yields of *K. alvarezii* and *G. amansii* were obtained with 30 min of reaction time and 0.15 M of acid concentration, with sugar yields of 0.56 g g^{-1} (g sugar g dry $algae^{-1}$) and 0.51 g g^{-1}, respectively. After 30 min, ethanol yields increased with reaction time. This is consistent with reports from Kim et al. [14] and Guarnieri et al. [37] that long reaction times during acid pretreatment reduces the formation of reducing sugars and produces more by-products such as furfural or levulinic acid.

Sugar yields of *K. alvarezii* and *G. amansii* pretreated at 0.05 M acid concentration were 50% higher than non-pretreated algae. Similarly, sugar yields of *K. alvarezii* and *G. amansii* increased by 72% and 59% when acid concentration was increased from 0.05 to 0.10 M, respectively. In contrast, based on Tukey's multiple comparison test, sugar yields did not increase in a similar pattern from 0.10 to 0.25 M ($p > 0.05$). Therefore, the most effective acid concentration

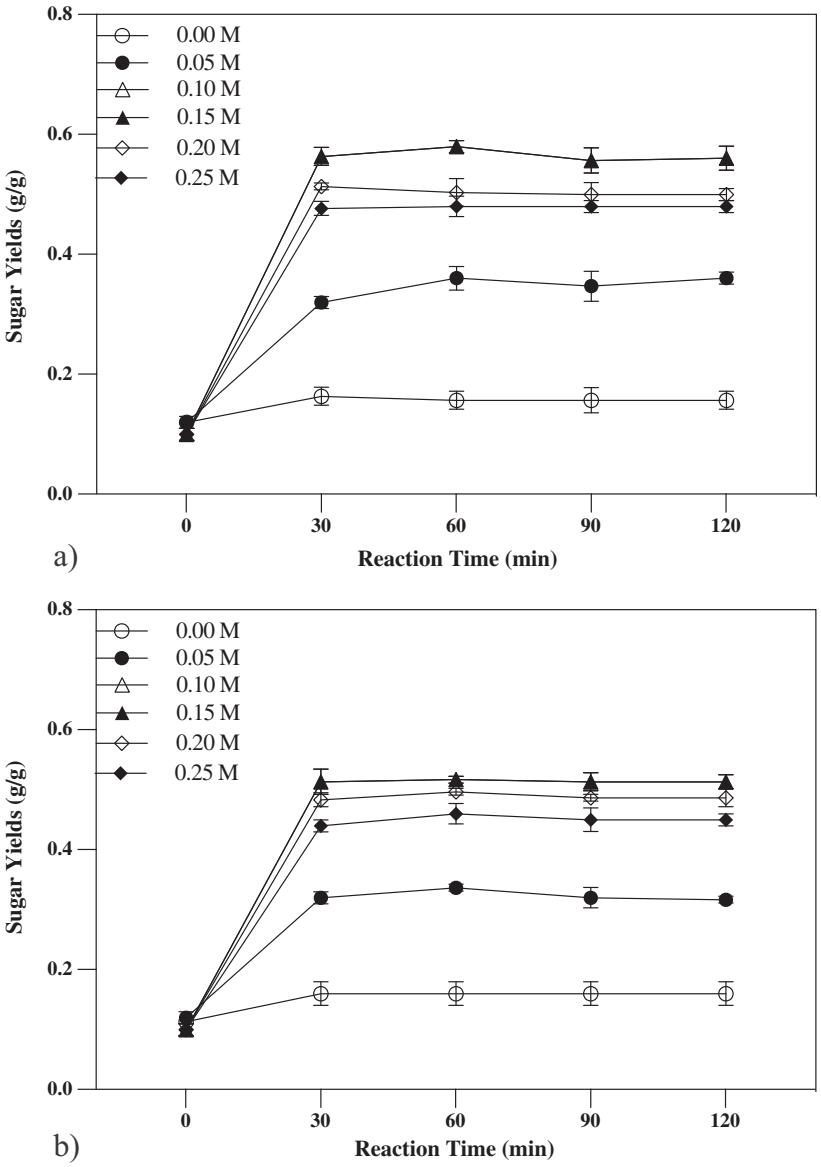

Fig. 4 Effect of acid concentration on acid pretreatment. **a** *K. alvarezii*. **b** *G. amansii*

was 0.10 M both in *K. alvarezii* and *G. amansii*. These results are consistent with the study by Meineta et al. [17], which reports that the increase in the concentration of acid affects the formation of LA and 5-HMF, which is converted from mono sugars.

According to Ge et al. [38], lower acid concentration is desirable due to lower costs and reduced environmental impacts. In addition, a higher acid concentration requires greater amounts of the neutralizing agent, thereby increasing pretreatment costs. It is therefore desirable to perform acid hydrolysis at a lower concentration. The optimal acid concentration as determined in this study (0.1 M) half the concentration used in other studies (0.2 M) [14, 39]. It therefore represents a potential method for acid pretreatment of both *K. alvarezii* and *G. amansii* because it is able to produce a high sugar yield at a lower acid concentration.

Comparison of Ozone and Acid Pretreatment

The comparison of sugar production using different pretreatment processes is summarized in Table 1. The highest sugar production was achieved by ozone pretreatment followed by enzymatic hydrolysis at 60 min of reaction time at 1% of substrate concentration. The highest sugar production was achieved by acid pretreatment at 30 min of reaction time, 1% of substrate concentration, and 0.10 M of acid

Table 1 Comparison of sugar production using different pretreatment processes

Pretreatment	Algae	Time (min)	Substrate concentration (%)	Sugar yield (g/g)	5-HMF (g/g)	LA (g/g)
Ozone	*K. alvarezii*	60	1	0.52	0	0
Ozone	*G. amansii*	60	1	0.41	0	0
Acid	*K. alvarezii*	30	1	0.56	0.0488	0.0102
Acid	*G. amansii*	30	1	0.51	0.0511	0.0037

concentration. These results showed that acid pretreatment of *K. alvarezii* outperformed ozone pretreatment and led to a 7.69% increase in sugar yield. Similarly, acid pretreatment of *G. amansii* also outperformed ozone pretreatment and led to a 24.39% increase in sugar yield. Acid pretreatment yielded more sugar and needed a shorter reaction time (30 min) than ozone pretreatment (60 min). Although ozone pretreatment required more time than acid pretreatment, ozone pretreatment is performed at a lower temperature (30 °C) than acid pretreatment (100 °C), potentially reducing operational costs.

5-HMF and LA production was evaluated at optimized pretreatment parameters for both ozone and acid pretreatments. 5-HMF and LA have been reported as the most common inhibitors referenced for pretreatment processes [14]. Acid pretreatment of *K. alvarezii* and *G. amansii* produced 5-HMF at 0.0488 and 0.0511 g/g, respectively, and LA at 0.0102 and 0.0037 g/g, respectively. In contrast, ozone pretreatment did not produce 5-HMF and LA (Table 1). These results are consistent with a previous report from Venegas et al. [40] that acid pretreatment may lead to the decomposition of sugars into inhibitors of the fermentation process such as 5-HMF and LA [40, 41]. As has been reported by Travaini et al. [20], ozone pretreatment does not produce these inhibitors using sugarcane as raw materials. Removal of 5-HMF and LA requires additional treatments, thereby generating additional costs, adding complexity to the process and generating additional waste products [14, 37, 41]. As such, ozone pretreatment may be economically viable despite its lower sugar yields because it does not produce inhibitors, does not require pH neutralization, and does not require cooling from high temperatures. The opportunities for ozone pretreatment to be developed as a lower-cost alternative should therefore be investigated in more detail to compare these processes at an industrial scale.

Bioethanol Production

This study evaluated ethanol production using both ozone and acid pretreatments. Figure 5 shows the formation of ethanol using different pretreatment strategies. The results indicate that ozone pretreatment substantially outperformed acid pretreatment, leading to a 1.7- and 1.4-fold increase in ethanol yield for *K. alvarezii* and *G. amansii*, respectively. The increased ethanol yield can be attributed to the lack of 5-HMF and LA. These results indicate the advantage of ozone pretreatment over acid pretreatment for efficient conversion of *K. alvarezii* and *G. amansii* to ethanol.

The fermentation efficiency was calculated as the ratio of the actual and theoretically possible ethanol production (0.511 g ethanol g sugar^{-1}). A comparison of ethanol production using different pretreatment processes (Table 2) shows that the fermentation efficiency of ozone pretreatment is higher than for acid pretreatment. This indicates that the ozone pretreatment method was more effective than acid pretreatment in ethanol production.

Ozone pretreatment followed by enzymatic hydrolysis produced 0.52 and 0.41 g sugar g dry algae^{-1} dry algae for *K. alvarezii* and *G. amansii*, respectively. The resulting sugar was converted into ethanol using *S. cerevisiae* with a fermentation efficiency of 92% and 76%, respectively. The fermentation efficiencies achieved in this study were higher than those reported in other studies (Table 3).

The fermentation efficiency achieved in this study (92% for *K. alvarezii*) was higher than previous studies for both acid and enzyme pretreatment (maximum 65.36%). Rahman et al. [13] reported a fermentation efficiency of 57.32% using *Nannochloropsis* sp. as a raw material using acid hydrolysis (H_2SO_4 0.2 M) and with 48 h of

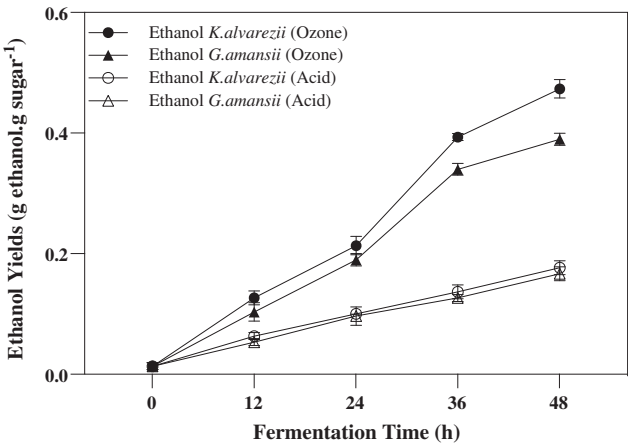

Fig. 5 Effect of different pretreatment strategies on ethanol yields

Table 2 Comparison of ethanol production using different pretreatment processes

Pretreatment	Algae	Fermentation time (h)	Ethanol yield (g/g)	Yeast biomass (g/L)	Fermentation efficiency (%)
Ozone	*K. alvarezii*	48	0.47	8.09	92
Ozone	*G. amansii*	48	0.39	7.82	76
Acid	*K. alvarezii*	48	0.17	3.54	33
Acid	*G. amansii*	48	0.16	3.28	31

fermentation time. Xia et al. [39] reported a fermentation efficiency of 47.74% using *Laminaria digitata* as a raw material and using acid hydrolysis (H_2SO_4 0.2 M) with 72 h of fermentation time. Jang et al. [43] reported a fermentation efficiency of 65.36% using *Saccharina japonica* as a raw material using acid hydrolysis (H_2SO_4 0.4 M) with 60 h of fermentation time. These higher fermentation efficiencies reported in the present study could result from the use of different algae species and application of different pretreatment conditions.

The higher fermentation efficiency under ozone pretreatment that was achieved in this study confirmed that ozone pretreatment has a potential application in bioethanol production. Ozone generates reactive hydroxyl radicals through the formation of superoxide, which reacts with carbohydrate resulting in random cleavage of glycosidic bonds [36]. Ozone pretreatment did not produce inhibitory compounds such as HMF and LA, make it the resulting sugar more suitable for ethanol fermentation. 5-HMF and LA are formed from the dehydration of hexoses during sugar degradation

Table 3 A comparative study of various pretreatment and fermentation processes for bioethanol production using algae biomass

Algae species	Pretreatment		Fermentation			References
	Pretreatment method	Sugar yield (g sugar/g biomass)	Microorganism	Condition	Fermentation efficiency (%)	
K. alvarezii	Ozone (400 mg O_3 L^{-1}) + enzyme	0.52	*Saccharomyces cerevisiae*	48 h, 30 °C, 150 rpm	92.00	This study
K. alvarezii	Acid (H_2SO_4 0.1 M) + enzyme	0.56	*Saccharomyces cerevisiae*	48 h, 30 °C, 150 rpm	33.00	This study
G. amansii	Ozone (400 mg O_3 L^{-1}) + enzyme	0.41	*Saccharomyces cerevisiae*	48 h, 30 °C, 150 rpm	76.00	This study
G. amansii	Acid (H_2SO_4 0.1 M) + enzyme	0.51	*Saccharomyces cerevisiae*	48 h, 30 °C, 150 rpm	31.00	This study
Nannochloropsis sp.	Acid (H_2SO_4 0.2 M)	0.13	*Saccharomyces cerevisiae* (ATCC 245858)	48 h, 30 °C, 150 rpm	57.32	Rahman et al. [42]
Laminaria digitata	Acid (H_2SO_4 2.5%, 0.2 M)	0.23	*Saccharomyces cerevisiae*	72 h, 37 °C, 30 rpm	47.74	Xia, et al. [39]
Saccharina japonica	Acid (H_2SO_4, 0.4 M)	0.21	Mixed culture of *Pichia angophorae, Saccharomyces cerevisiae*, *Phacysolen thannophylus*	60 h, 30 °C, 200 rpm	65.36	Jang et al. [43]
Ulva fasciata	Enzymatic (cellulase)	0.21	*Saccharomyces cerevisiae* MTCC180	48 h, 28 °C, 120 rpm	88.20	Trivedi et al. [44]
Chlamydomonas mexicana	Enzymatic (cellulase)	0.44	*Saccharomyces cerevisiae* YPH499	30 °C, pH 5, 120 rpm, 72 h,	80.21	El-Dalatony et al. [45]
Gelidium amansii	Enzymatic (cellulase)	0.50	*Saccharomyces cerevisiae KCTC 7906*	24 h, 37 °C	84.90	Kim et al. [14]

[17, 37]. These compounds inhibit yeast growth, thereby decreasing ethanol production [17]. These inhibitors work by breaking down the yeast's DNA and inhibiting protein synthesis [14, 41]. The absence of these by-products ozone pretreatment means there is no inhibition of the DNA synthesis in the yeast, leading to greater ethanol production during fermentation than that observed for algae pretreated using the acid method. This is supported by cell biomass data, which shows that yeast cell biomass in acid pretreatment is up to 56% lower compared to cell biomass in ozone pretreatment (Table 2). This study therefore provides a new approach to third-generation ethanol production through the combination of ozone pretreatment followed by enzymatic hydrolysis and ethanol fermentation.

Conclusion

K. alvarezii and *G. amansii* may be suitable materials for third-generation bioethanol production, due to their fermentable sugar contents and high fermentation efficiency. Results indicate that acid pretreatment, followed by enzymatic hydrolysis, produced a high amount of sugars compared to ozone pretreatment. However, acid pretreatment also produced the inhibitory compounds HMF and LA, which reduced the fermentation efficiency of this process. In contrast, ozone pretreatment did not produce these inhibitory compounds, and as a result generated higher ethanol yields after fermentation than occurred after acid pretreatment. These results indicate the potential of ozone pretreatment for low-cost, environmentally sustainable bioethanol production from macroalgae.

References

1. Trevorah RM, Huynh T, Vancov T, Othman MZ (2018) Bioethanol potential of *Eucalyptus obliqua* sawdust using gamma-valerolactone fractionation. Bioresour Technol 250:673–682. https://doi.org/10.1016/j.biortech.2017.11.084
2. Tan SJ, Phapugrangkul P, Keong C, Lai Z, Hafizi M, Bakar A, Murugan P (2019) Biocatalysis and agricultural biotechnology banana frond juice as novel fermentation substrate for bioethanol production by *Saccharomyces cerevisiae*. Biocatal Agric Biotechnol 21. https://doi.org/10.1016/j.bcab.2019.101293
3. Dave N, Selvaraj R, Varadavenkatesan T, Vinayagam R (2019) A critical review on production of bioethanol from macroalgal biomass. Algal Res 42. https://doi.org/10.1016/j.algal.2019.101606
4. Sulfahri S, Amin M, Sumitro SB, Saptasari M (2017) Comparison of biomass production from algae *Spirogyra hyalina* and *Spirogyra peipingensis*. Biofuels 8(3):359–366. https://doi.org/10.1080/17597269.2016.1231954
5. Ramachandra TV, Hebbale D (2020) Bioethanol from macroalgae: prospects and challenges. Renew Sust Energ Rev 117. https://doi.org/10.1016/j.rser.2019.109479
6. Greetham D, Zaky A, Makanjuola O, Du C (2018) A brief review on bioethanol production using marine biomass, marine microorganism and seawater. Curr Opin Green Sustain Chem 14: 53–59. https://doi.org/10.1016/j.cogsc.2018.06.008
7. Lee S, Kim S, Hong Y, Jeong G (2016) Optimization of the production of platform chemicals and sugars from the red macroalga, *Kappaphycus alvarezii*. Algal Res 13:303–310. https://doi.org/10.1016/j.algal.2015.12.013
8. Food and Agriculture Organization of the United Nations Fisheries and Aquaculture Department (2020) Global aquaculture production 1950–2017. Retrieved from http://www.fao.org/fishery/topic/16140/en
9. Neish I, Salling P, Klose J (2017) Carrageenan and agar: Indonesia, beyond the land of cottonii and gracilaria. Ministry of Marine Affairs and Fisheries, Swiss Import Promotion Programme
10. Deloitte (2018) Lifting 40,000 Indonesian seaweed farmers out of poverty. Retrieved from https://www2.deloitte.com/au/en/pages/purpose/articles/lifting-40000-indonesian-seaweed-farmers-out-poverty.html
11. Valderrama D, Cai J, Hishamunda N, Ridler N (2013) Social and economic dimensions of carrageenan seaweed farming. FAO Fisheries and Aquaculture Technical Paper 580
12. Neish IC (2013) Social and economic dimensions of carrageenan seaweed farming in Indonesia. Social and economic dimensions of carrageenan seaweed farming, Fisheries and Aquaculture Technical Paper, 580, 61–89
13. Mariño M, Breckwoldt A, Teichberg M, Kase A, Reuter H (2019) Livelihood aspects of seaweed farming in Rote Island, Indonesia. Mar Policy 107:103600. https://doi.org/10.1016/j.marpol.2019.103600
14. Kim MH, Gon S, Jung S, Song Y, Bae H (2015) Efficient approach for bioethanol production from red seaweed *Gelidium amansii*. Bioresour Technol 175:128–134. https://doi.org/10.1016/j.biortech.2014.10.050
15. Sivarathnakumar S, Jayamuthunagai J, Bharathiraja B, Praveenkumar R, Selvakumari IAE, Arokiyaraj S (2019) Bioethanol production from woody stem *Prosopis juliflora* using thermo tolerant yeast *Kluyveromyces marxianus* and its kinetics studies. Bioresour Technol 239. https://doi.org/10.1016/j.biortech.2019.122060
16. Anto S, Mukherjee SS, Muthappa R, Mathimani T, Deviram G, Kumar SS, Verma TN (2019)Algae as green energy reserve: technological outlook on biofuel production. Chemosphere 242. https://doi.org/10.1016/j.chemosphere.2019.125079
17. Meinita MDN, Marhaeni B, Winanto T, Jeong G, Khan MNA, Hong Y (2013) Comparison of agarophytes (Gelidium, Gracilaria, and Gracilariopsis) as potential resources for bioethanol production. J Appl Phycol 25(6):1957–1961. https://doi.org/10.1007/s10811-013-0041-4
18. Abd-Rahim F, Wasoh H, Zakaria MR, Ariff A, Kapri R, Ramli N, Siew-Ling L (2014) Production of high yield sugars from *Kappaphycus alvarezii* using combined methods of chemical and enzymatic hydrolysis. Food Hydrocoll 42(2):309–315. https://doi.org/10.1016/j.foodhyd.2014.05.017
19. Phwan CK, Chyuan H, Chen W, Chuan T, Poh E (2018) Overview: comparison of pretreatment technologies and fermentation processes of bioethanol from microalgae. Energy Convers Manag 173:81–94. https://doi.org/10.1016/j.enconman.2018.07.054
20. Travaini R, Barrado E, Bolado-Rodríguez S (2016) Effect of ozonolysis pretreatment parameters on the sugar release, ozone consumption and ethanol production from sugarcane bagasse. Bioresour Technol 214:150–158. https://doi.org/10.1016/j.biortech.2016.04.102
21. Rastogi M, Shrivastava S (2017) Recent advances in second generation bioethanol production: an insight to pretreatment, saccharification and fermentation processes. Renew Sust Energ Rev 80: 330–340. https://doi.org/10.1016/j.rser.2017.05.225

22. Razack A, Surendhiran D (2016) Algae—a quintessential and positive resource of bioethanol production: a comprehensive review. Renew Sust Energ Rev 66:248–267. https://doi.org/10.1016/j.rser.2016.07.024
23. Maurya DP, Singla A, Negi S (2015) An overview of key pretreatment processes for biological conversion of lignocellulosic biomass to bioethanol. 3 Biotech 5(5):597–609. https://doi.org/10.1007/s13205-015-0279-4
24. Harchi ML, Kachkach FZF, Mtili N (2018) Optimization of thermal acid hydrolysis for bioethanol production from *Ulva rigida* with yeast *Pachysolen tannophilus*. S Afr J Bot 115:161–169 https://doi.org/10.1016/j.sajb.2018.01.021
25. Pablo G, Domínguez E, Domínguez VD, Romaní A, Domingues L, Garrote G (2019) Third generation bioethanol from invasive macroalgae *Sargassum muticum* using autohydrolysis pretreatment as first step of a biorefinery. Renew Energy 141:728–735. https://doi.org/10.1016/j.renene.2019.03.083
26. Kostas ET, White DA, Cook DJ (2019) Bioethanol production from UK seaweeds: investigating variable pre-treatment and enzyme hydrolysis parameters. Bioenergy Res:1–15. https://doi.org/10.1007/s12155-019-10054-1
27. Nguyen TH, Sunwoo IY, Jeong GT, Kim SK (2019) Detoxification of hydrolysates of the red seaweed *Gelidium amansii* for improved bioethanol production. Appl Biochem Biotechnol 188(4):977–990. https://doi.org/10.1007/s12010-019-02970-x
28. Kumar G, Sivagurunathan P, Kobayashi T, Xu KQ, Kim SH (2015) Simultaneous removal of 5-hydroxy methyl furfural (5-HMF) and hydrogen production from acid (H_2SO_4) pretreated red-algal hydrolysate via hybrid immobilized cells. Algal Res 11:326–333. https://doi.org/10.1016/j.algal.2015.07.015
29. Keris-Sen UD, Gurol MD (2017) Using ozone for microalgal cell disruption to improve enzymatic saccharification of cellular carbohydrates. Biomass Bioenergy 105:59–65. https://doi.org/10.1016/j.biombioe.2017.06.023
30. Bellido C, González-Benito G, Coca M, Lucas S, García-Cubero MT (2013) Influence of aeration on bioethanol production from ozonized wheat straw hydrolysates using *Pichia Stipitis*. Bioresour Technol 133:51–58. https://doi.org/10.1016/j.biortech.2013.01.104
31. Cardeña R, Moreno G, Bakonyi P, Buitrón G (2017) Enhancement of methane production from various microalgae cultures via novel ozonation pretreatment. Chem Eng 307:948–954. https://doi.org/10.1016/j.cej.2016.09.016
32. Park M, Kim S, Jeong G (2018) Optimization of the levulinic acid production from the red macroalga, *Gracilaria verrucosa* using methanesulfonic acid. Algal Res 31:116–121. https://doi.org/10.1016/j.algal.2018.02.004
33. Jeong G, Kim S, Park D (2015) Bioresource technology application of solid-acid catalyst and marine macro-algae *Gracilaria verrucosa* to production of fermentable sugars. Bioresour Technol 181:1–6. https://doi.org/10.1016/j.biortech.2015.01.038
34. Park JH, Hong JY, Jang HC, Oh SG, Kim SH, Yoon JJ, Kim YJ (2012) Use of *Gelidium amansii* as a promising resource for bioethanol: a practical approach for continuous dilute-acid hydrolysis and fermentation. Bioresour Technol 108:83–88. https://doi.org/10.1016/j.biortech.2011.12.065
35. Li Y, Cui J, Zhang G, Liu Z, Guan H, Hwang H, Wang P (2016) Optimization study on the hydrogen peroxide pretreatment and production of bioethanol from seaweed *Ulva prolifera* biomass. Bioresour Technol 214:144–149. https://doi.org/10.1016/j.biortech.2016.04.090
36. Bule MV, Gao AH, Hiscox B, Chen S (2013) Structural modification of lignin and characterization of pretreated wheat straw by ozonation. J Agric Food Chem 61:3916–3925. https://doi.org/10.1021/jf4001988
37. Guarnieri MT, Franden MA, Johnson CW, Beckham GT (2017) Conversion and assimilation of furfural and 5-(hydroxymethyl) furfural by *Pseudomonas putida* KT2440. Metab Eng Commun 4:22–28. https://doi.org/10.1016/j.meteno.2017.02.001
38. Ge L, Wang P, Mou H (2011) Study on saccharification techniques of seaweed wastes for the transformation of ethanol. Renew Energy 36:84–89. https://doi.org/10.1016/j.renene.2010.06.001
39. Xia A, Jacob A, Tabassum MR, Herrmann C, Murphy JD (2016) Production of hydrogen, ethanol and volatile fatty acids through co-fermentation of macro- and micro-algae. Bioresour Technol 205:118–125. https://doi.org/10.1016/j.biortech.2016.01.025
40. Ra CH, Nguyen TH, Jeong GT, Kim SK (2016) Evaluation of hyper thermal acid hydrolysis of *Kappaphycus alvarezii* for enhanced bioethanol production. Bioresour Technol 209:66–72. https://doi.org/10.1016/j.biortech.2016.02.106
41. Vanegas CH, Hernon A, Bartlett J (2015) Enzymatic and organic acid pretreatment of seaweed: effect on reducing sugars production and biogas inhibition. Int J Ambient Energy 36:2–7. https://doi.org/10.1080/01430750.2013.820143
42. Rahman QM, Zhang B, Wang L, Joseph G, Shahbazi A (2019) A combined fermentation and ethanol-assisted liquefaction process to produce biofuel from *Nannochloropsis* sp. Fuel 238:159–165. https://doi.org/10.1016/j.fuel.2018.10.116
43. Jang JS, Cho YK, Jeong GT, Kim SK (2012) Optimization of saccharification and ethanol production by simultaneous saccharification and fermentation (SSF)from seaweed, *Saccharina japonica*. Bioprocess Biosyst Eng 35:11–18. https://doi.org/10.1007/s00449-011-0611-2
44. Trivedi N, Gupta V, Reddy C, Jha B (2013) Enzymatic hydrolysis and production of bioethanol from common macrophytic green alga *Ulva fasciata* Delile. Bioresour Technol 150:106–112. https://doi.org/10.1016/j.biortech.2013.09.103
45. El-Dalatony MM, Kurade MB, Abou-Shanab RAI, Kim H, Salama ES, Jeon BH (2016) Long-term production of bioethanol in repeated-batch fermentation of microalgal biomass using immobilized *Saccharomyces cerevisiae*. Bioresour Technol 219:98–105. https://doi.org/10.1016/j.biortech.2016.07.113