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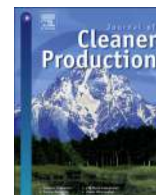

Fungal pretreatment as a sustainable and low cost option for bioethanol production from marine algae

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ABSTRACT

Marine algae are promising alternative feedstocks for bioethanol production since they are fast growing and do not compete with food crops for land. However, the commercial viability of bioethanol production from marine algae is limited by the high cost of the pretreatment required to break down the cell wall and the high cost of nitrogen supplements used during fermentation. Development of alternative pretreatment and nutritional supplementation strategies is therefore crucial to the development of economically viable methods for producing these third generation biofuels. This research develops a novel method for fungal pretreatment of marine macroalgae. The method is both an effective pretreatment capable of breaking down the complex algae cell wall, and allows for fungal biomass to be recovered from the pretreatment stage and used as a nutrient supplement during fermentation. The study demonstrates that fungal pretreatment prior to enzyme hydrolysis increased sugar yields 2.3 fold compared to untreated algae, and nutrient supplementation using the recovered fungal biomass increased ethanol yields by up to 38.23%. This data suggests that the use of fungi for pretreatment and nutrient supplementation could greatly increase the economic viability of production of these third-generation biofuels.

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1. Introduction

The global population is predicted to reach 10 billion by 2050 (Goujon, 2019), increasing demand for fuel (Rempel et al., 2019). It is estimated that fossil fuel supplies will be exhausted by the middle of this century (Shokrkar et al., 2017), necessitating alternative energy sources (Wood and Roelich, 2019). Bioethanol is a potential alternative fuel source due to its renewable nature and low carbon emissions (Chamnipa et al., 2017). However, first and second generation bioethanol feedstocks compete with other crops for land, limiting their viability (Nanda et al., 2015). Fast-growing, renewable biomass sources such as marine algae, have attracted attention in the search for alternatives (Kamyab et al., 2016). Marine algae is a promising third-generation feedstock for bioethanol production due to its rapid and sustainable growth (Sulfahri et al., 2017), and the absence of competition for land (Ashokkumar et al., 2019).

Red seaweeds in particular are fast growing and low-value species. Just three species of red seaweed account for over 43% of global seaweed production, 68% of which is produced in Indonesia (FAO, 2020). This study develops a pretreatment method for bioethanol production using the marine algae *Kappaphycus alvarezii* and *Gelidium amansii* as raw materials to produce fermentable sugars for conversion to bioethanol. *K. alvarezii* (elkhorn sea moss) is an algae of global economic importance. Its primary use is in the production of carrageenan, a gelling agent widely used in food processing. Indonesia and the Philippines produce nearly all of the global supply of this species (FAO, 2020). *K. alvarezii* is produced almost exclusively by smallholder farmers in these countries, due to the low cost barriers to entry and the need for a highly flexible

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workforce that can work according to tidal and moon cycles. The seaweeds act as a carbon sink (Duarte et al. 2017) and provide income to rural households (Mariño et al., 2019). *G. amansii* is another red seaweed widely available in Indonesia, which although currently limited in commercial cultivation, has been targeted as a potential algae variety for commercial development. Given the high economic importance of these seaweeds and future government supported plans to encourage rapid increases in production, these algae are potentially important feedstocks for bioethanol production, being low cost, carbon negative feedstocks which do not compete for arable land and provide a range of social benefits to low-income smallholder farmers.

These algae are potentially suitable for bioethanol production given their fast growth rates and high carbohydrate contents of 88.6% for *K. alvarezii* (Ra et al., 2016) and 60.0% for *G. amansii* (Jeong et al., 2011). These carbohydrates are converted to monosaccharides during the pretreatment and fermentation processes. However, bioethanol production from marine macroalgae poses a unique challenge due to the presence of hydrocolloid polymers in the cell walls (Guedes et al., 2019), which make their cell walls are stronger than terrestrial plants (Keris-Sen and Gurol, 2017). Pretreatment is necessary to breakdown the complex cell wall structure and facilitate the conversion of cellular carbohydrates to glucose prior to ethanol fermentation. This stage of the process is expensive, typically representing up to 20% of the costs of production (Travaini et al., 2016). A range of pretreatment methods have been tested, including acid, enzymatic, ultrasonication, microwave, and ozone pretreatments (Sulfahri et al., 2020). Despite high yields, most of these approaches are high cost, require extreme reaction conditions, and generate by-products that inhibit the subsequent fermentation process (Farkas et al., 2019). The development of low-cost pretreatments which do not form fermentation inhibiting by-products is therefore a crucial step in the development of low-cost bioethanol production processes from marine macroalgae.

In this study, a new pretreatment method using the fungus *Trichoderma harzianum* was developed. This pretreatment method avoids generating fermentation inhibiting compounds and is environmentally friendly with low chemical inputs. *T. harzianum* produces the enzymes cellulase, xylanase, and β-glucosidase (Delabona et al., 2012). Theses enzymes disrupt the algae cell wall, converting polysaccharides to monosaccharides. In addition, biomass produced during this pretreatment process contains enzymes and proteins rich in nitrogen that can be used as a supplemental nutrient in the subsequent ethanol fermentation stage, where there is a need for additional nitrogen inputs (Aleman-Ramirez et al., 2019), and this has the potential to substantially reduce input costs. This study optimizes reaction conditions using the residual biomass of *T. harzianum* as a nitrogen supplement.

A limited number of studies have used fungal pretreatment for algal cell disruption, and these studies have not optimized pretreatment and fermentation conditions across the entire bioethanol production process (Kumar et al., 2015). This study addresses this gap by analysing the effect of fungal pretreatment on bioethanol production by altering multiple variables, including algal concentration, fungal inoculum size, nutrient supplementation and pretreatment time. This paper therefore provides a wholistic analysis of bioethanol production process, capable of optimising the effects of fungal pretreatment across the process. The study represents a crucial step in development of economically viable bioethanol production from marine algae.

2. Materials and Methods

The bioethanol production process involves an initial fungal pretreatment stage using *T. harzianum*. This is followed by enzyme hydrolysis, in which polysaccharides are converted to monosaccharides. These monosaccharides are then fermented using *Saccharomyces cerevisiae* to produce ethanol.

2.1. Raw Materials

Samples of *K. alvarezii* and *G. amansii* were purchased from the Seaweed Research Unit of Hasanuddin University, Indonesia, in unprocessed form. The 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 was ground to an average size of 100 mesh using a laboratory mill.

T. harzianum cultured on Potato Dextrose Agar (PDA) medium and *S. cerevisiae* cultured on Sabouraud Dextrose Agar (SDA) medium were obtained from the Indonesian Culture Collection (InaCC) and maintained at 30 °C for 48 h.

Yeast extract (Merck, Germany) was used as a supplemental nutrient in fungal pretreatment, and commercial enzymes (Cellic CTec2; 150 KNU; Novozymes, Denmark) were used in enzymatic pretreatment.

2.2. Fungal Pretreatment

Fungal pretreatment was undertaken, varying algal concentrations, fungus inoculum size, nutrient supplementation and reaction time. This process involves initially heating the algae solution at 100 °C for 2 h. This was performed in 100 mL tubes with 50 mL working volume. Algal concentrations varied from 1 to 5% w/w, dry algae. The inoculum size of fungal was varied from 5 to 20% (v/v). Yeast extract was used as a supplemental nutrient and was varied from 0 to 3% w/w dry algae. The pH in the reactors was adjusted to a pH of 5.0 using Na-citrate buffer. The pretreatment reaction time was varied from 0 to 120 h at 30 °C. Following the process of hydrolysis, the algal solution was pasteurized to stop the reaction. The algae biomass was separated from the liquid through centrifugation at 9,000 rpm for 10 min at 4 °C. The liquid fraction was measured to determine the total sugar and reducing sugar yields.

2.3. Enzymatic Hydrolysis

The polysaccharides obtained through the optimized pretreatment process were converted to monosaccharides through enzyme hydrolysis. Commercial enzymes (Cellic CTec2; 150 KNU; Novozymes, Denmark) were used. Enzymatic hydrolysis was performed in 100 mL tubes with a 50 mL working volume. 100 KNU of enzyme was added to 5 g algae biomass dissolved in solution at 2% (w/w) and reacted for 48 h at 30 °C. These parameters are based on previously determined conditions for enzyme hydrolysis of these algae (Abd-rahim et al., 2014). After hydrolysis, algal biomass was removed from solution by centrifugation at 9,000 rpm at 4 °C for 10 min. The liquid fraction was collected and used to determine the total and reducing sugar yields.

2.4. Ethanol Fermentation

The algae hydrolysates selected from the optimized enzyme hydrolysis stage were used as inputs to the fermentation process. Ethanol fermentation was performed in 100 mL tubes with a 50 mL operating volume. Fermentation time (0, 12, 24, 36, 48, and 60 h) and presence of nutritional supplementation (with and without supplementation) was varied. This fermentation process was performed anaerobically at 30 °C with 150 rpm agitation. The pH in the reactor was adjusted to 5.0 using Na-citrate buffer. The final sugar content, ethanol content and cell biomass were measured.

2.5. Analytical

The cell biomass, sugar levels and ethanol levels were measured using established equipment and techniques as described below.

2.5.1. Cell Biomass

The measurement of *T. harzianum* cell biomass was performed using the dry cell weight method, after centrifuging the algal solution obtained from microbial pretreatment at 9,000 rpm and 4 °C for 10 min. The pellets were resuspended in distilled water, and subsequently aspirated using a pipette, before being transferred to filter paper prepared by oven drying to ensure a constant weight (W_1). The pellets on the filter paper were dried in an oven at 80 °C until constant weight (W_2) was attained. The dry cell weight was calculated based on the difference between the final and initial filter weight ($W_2 - W_1$) and expressed in $g.L^{-1}$.

2.5.2. Sugar Levels

Sugar levels were evaluated using a digital Brix refractometer (Milwaukee MA871) and High Performance Liquid Chromatography (HPLC) (Agilent 1100 HPLC system, Agilent Technologies, Santa Clara, CA, USA). The HPLC was fitted with a refractive index detector and equipped with a Bio-Rad Aminex HPX-87H column (300 × 7.8 mm). The column was maintained at 65 °C with a flow rate of 0.6 mL/min 5 mM H_2SO_4 was used as the mobile phase.

2.5.3. 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 fermentation efficiency was evaluated based on the percent theoretical yield, calculated as the ratio of the average ethanol production to the maximum possible theoretical production (0.511 g ethanol.g sugar^{-1}) (Li et al., 2016).

2.6. Statistical Analysis

All experiments were conducted in triplicate with mean values and standard deviation reported. One-way analysis of variance (ANOVA) and Tukey Multiple Comparison Test were undertaken using GraphPad Prism version 8.2.1 for Mac (GraphPad Software, Inc., USA). Values were considered significant when P was <0.05.

3. Results and Discussion

The bioethanol production process was optimized according to multiple variables. The dried and ground algae were first pretreated using *T. harzianum*. This was followed by enzyme hydrolysis and fermentation to produce ethanol.

3.1. Effect of Substrate Concentration on Sugar Yield

T. harzianum is a fungus capable of producing the enzyme cellulase over short fermentation times (Li et al., 2019). The cell wall of marine algae consists of polysaccharides dominated by cellulose fibrils (Abd-rahim et al., 2014). *T.harzianum* produces the enzyme cellulase, which breaks down the structure of cell wall of the marine algae. In addition, *T. harzianum* produces the enzymes β-glucosidase and xylanase which also convert polysaccharides to monosaccharides. The yield of sugar produced by fungal cell disruption and enzymatic hydrolysis was optimized varying substrate concentration (1–5%) and reaction time (0–120 h). Fig. 1 shows increasing sugar yields with reaction time. Yields are substantially lower at concentrations above 3% likely due to the increased viscosity inhibiting fungal activity. The sugar yield was highest at 2% of substrate concentration. Similarly, Kim et al. (2015) reported that the highest sugar production of marine algae was achieved at 2% of substrate concentration via acid pretreatment for the marine algae *Gracilaria verucosa*. At substrate concentrations over 3%, the high viscosity of the solution inhibited fungal growth. This reduced the production of enzymes necessary for breaking down cell wall polysaccharides into monosaccharides, leading to reduced sugar production from the process. This is consistent with other reports that substrate concentration is a critical determinant of sugar yield in fungal pretreatment (Martínez-patiño et al., 2018).

Sugar yields increased with reaction time, with the rate of yield increase declining after 96 h for concentrations of 1–3%. Tukey's Multiple Comparison Test revealed significant differences in the reaction extent of both *G.amansii* and *K.alvarezii* at 0, 24, 48, 72, and 96 h ($p < 0.05$). Reaction extents were not significantly different at 96 and 120 h ($p > 0.05$). This indicates that fungal pretreatment reaction time is optimized at 96 h. Wang et al. (2020) reported comparable findings, noting that *T. harzianum* required 96–144 h to produce cellulase enzyme with activity up to 14.79 IU/mL. Zheng et al. (2020) also obtained similar results using a *Trichoderma* sp. for pretreatment of wheat straw, reporting an optimum reaction time of 96 h.

Fig. 2 presents the results of enzymatic hydrolysis of algae with and without fungal pretreatment. Fungal pretreatment of *K.alvarezii* and *G.amansii* prior to enzymatic hydrolysis resulted in an up to 2.3 fold increase in sugar. The mechanism by which this occurred is as follows. The cell wall of marine algae consists of polysaccharides dominated by cellulose (Abd-rahim et al., 2014). The cellulose is

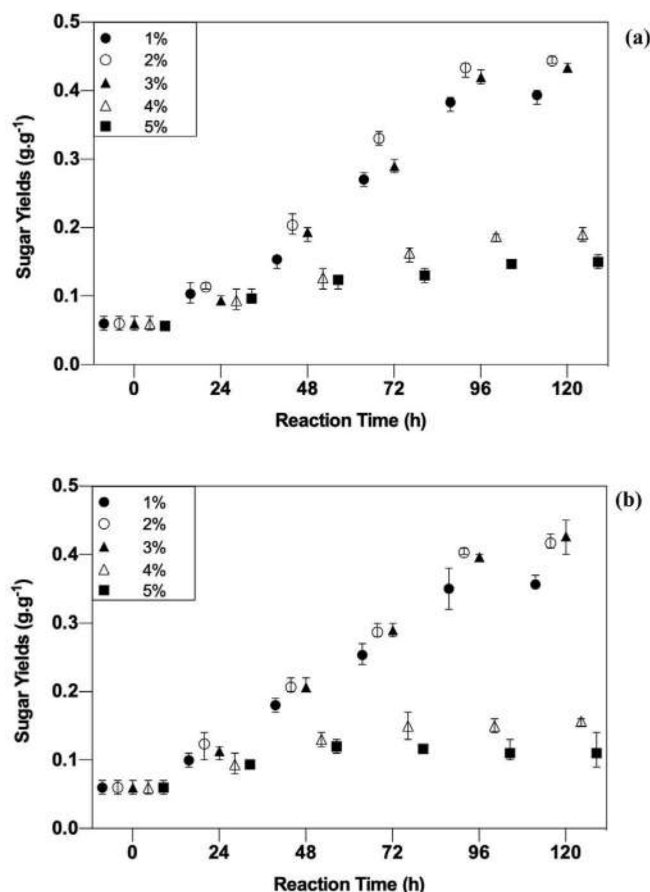

Fig. 1. Effect of substrate concentration and reaction time on sugar yield of (a). *K. alvarezii*, (b) *G. amansii*.

broken down into monosaccharides by the enzyme cellulase, which is produced by *T. harzianum.* In addition, *T. harzianum* produces the enzymes β-glucosidase and xylanase which also convert polysaccharides to monosaccharides (Wang et al., 2020). These degraded cell walls are more easily hydrolysed than untreated algae, increasing sugar production from the process. As reported by Rouches et al. (2016), fungal pretreatment can transform structural carbohydrates into non-structural soluble carbohydrates using cellulolytic enzymes. In addition, fungal pretreatment can increase pore sizes and overall surface area, increasing accessibility to enzymatic attacks (Yahmed et al., 2017). These combines processes are responsible for the efficacy of fungal pretreatment of *K.alvarezii* and *G.amansii* in cell wall degradation for improved enzyme hydrolysis and increased sugar yield compared unpretreated algae.

3.2. Effect of Inoculum Size on Sugar Yield

The effect of inoculum size and reaction time on the formation of sugars from *K.alvarezii* and *G.amansii* biomass using 2% of substrate concentration is shown in Fig. 3. The maximum sugars yields were obtained at 20% of inoculum size and at 96–120 h reaction time. Based on Tukey's Multiple Comparison Test, the inoculum size of 10–20% showed similar sugar production from *K.alvarezii* and *G.amansii*, in the range 0.43–0.48 g g^{-1} and 0.41–0.42 g g^{-1} respectively ($p > 0.05$). The lower inoculum rate of 5% showed lower sugar production at 96–120 h from *K.alvarezii* and *G.amansii* in the range of 0.14–0.18 g g^{-1} and 0.11–0.15 g g^{-1} respectively ($p < 0.05$). Other studies have shown that increasing inoculum rate is ineffective beyond required amounts (Shirkavand et al., 2016). An inoculum size of 10% was determined to be optimal in this experiment as higher inoculum sizes did not significantly increase sugar yields according to Tukey's Multiple Comparison Test.

3.3. Effect of Supplemented Nutrient on Sugar Yield

Commercial yeast extract was used to supplement nutrients during fungal pretreatment. The effect of nutrient supplementation and reaction time on the formation of sugars from *K.alvarezii* and *G.amansii* biomass was tested using a substrate concentration of 2% and an inoculum size of 10%, as shown in Fig. 4. The highest sugar yields both in *K.alvarezii* and *G.amansii* were achieved at 96–120 h of reaction time. The rate of sugar production remained significant up to 96–120 h based on Tukey's Multiple Comparison Test.

In unsupplemented treatments, the highest sugar yields of *K.alvarezii* and *G.amansii* was 0.43 g g^{-1} and 0.39 g g^{-1}, respectively. In nutrient supplementation, the highest sugar yields of *K.alvarezii* and *G.amansii* was 0.55 g g^{-1} and 0.53 g g^{-1}, respectively (Fig. 5). Nutrient supplementation increased sugar yield by 16.3–27.9% (Fig. 6). The highest sugar yield was achieved with 3.0 g.L^{-1} of supplemented nutrient in both *K.alvarezii* and *G.amansii*. However, based on Tukey's Multiple Comparison Test, supplemented nutrient of 2 g.L^{-1} was not significantly different from rates of 2.5–3.0 g.L^{-1}. As such, the optimal supplementation rate was determined to be 2 g.L^{-1} for both *K.alvarezii* and *G.amansii*.

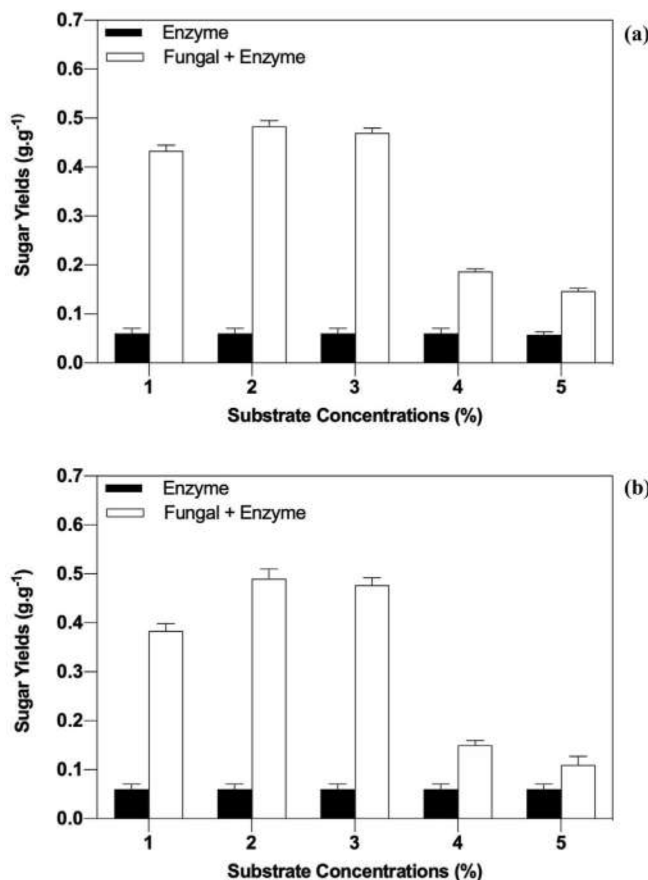

Fig. 2. Comparison of enzymatic hydrolysis and fungal pretreatment followed by enzymatic hydrolysis on sugar yield of (a) *K. alvarezii*, (b) *G. amansii*.

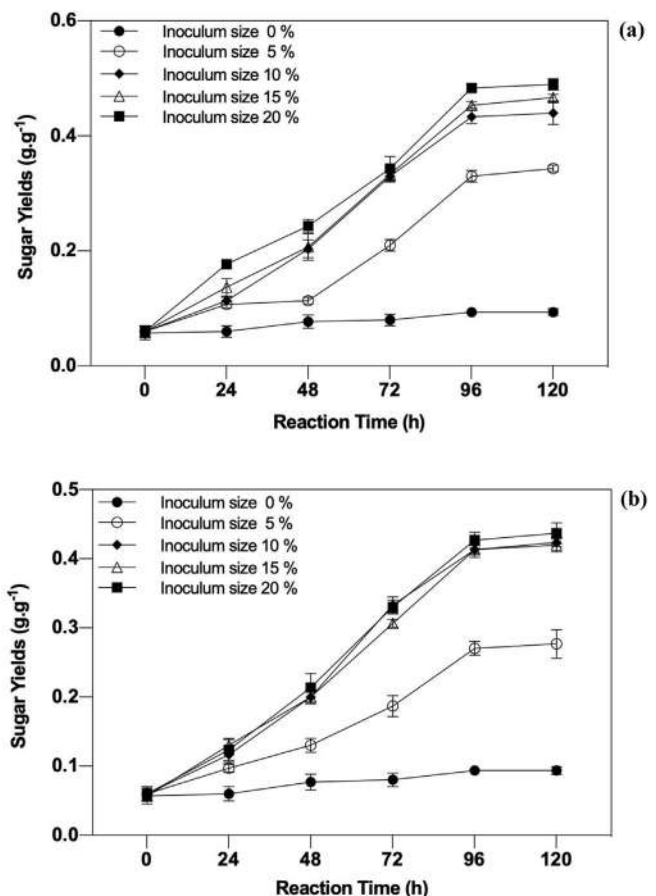

Fig. 3. Effect of inoculum size and reaction time on sugar yield of (a). *K. alvarezii*, (b) *G. amansii*.

3.4. Fungal Biomass During Pretreatment

Fungal pretreatment may be a more effective option than commonly employed acid pretreatment processes, as acid pretreatment generates compounds that inhibit ethanol fermentation, such as hydroxymethylfurfural, furfural, and levulinic acid (Ahmed et al., 2017). In contrast, the *T. harzianum* biomass recovered from the fungal pretreatment stage of this process does not contain any such inhibitors. In addition, *T. harzianum* beneficially produces the enzymes cellulase, β-glucosidase, and xylanase (Delabona et al., 2012). These enzymes contain amino acids that are rich in nitrogen, and adequate nitrogen levels during ethanol fermentation protect yeast from osmotic stress (Yue et al., 2012). These features suggest that fungal pretreatment of marine algae using *T. harzianum* may function as both an effective pretreatment which does not generate fermentation inhibiting by-products, and as a source of supplemental nutrients.

In order to investigate the potential for nutrient supplementation using fungal biomass during ethanol fermentation, the rate of *T.harzianum* biomass production was monitored during pretreatment. Fig. 7 shows the formation of *T.harzianum* biomass during pretreatment for *K.alvarezii* and *G.amansii.* The highest biomass of *T.harzianum* was achived in 96–120 h of reaction time both in *K.alvarezii* and *G.amansii* substrate, at 8.09 g.L^{-1} and 7.42 g.L^{-1} respectively. This rate of fungal biomass production was sufficient for use in nutrient supplementation in the ethanol production stage. Ardalan et al. (2018) reported that nutrient supplementation using yeast extracts of 5 g.L^{-1} were sufficient to improve ethanol fermentation performance using the yeast *S. cerevisiae.* Similarly, Li et al. (2016) reported that nutrient supplementation improved fermentation above 2.5 g.L^{-1}. This indicates the potential for fungal biomass to be recovered from the pretreatment stage and used as a nutrient supplement during fermentation.

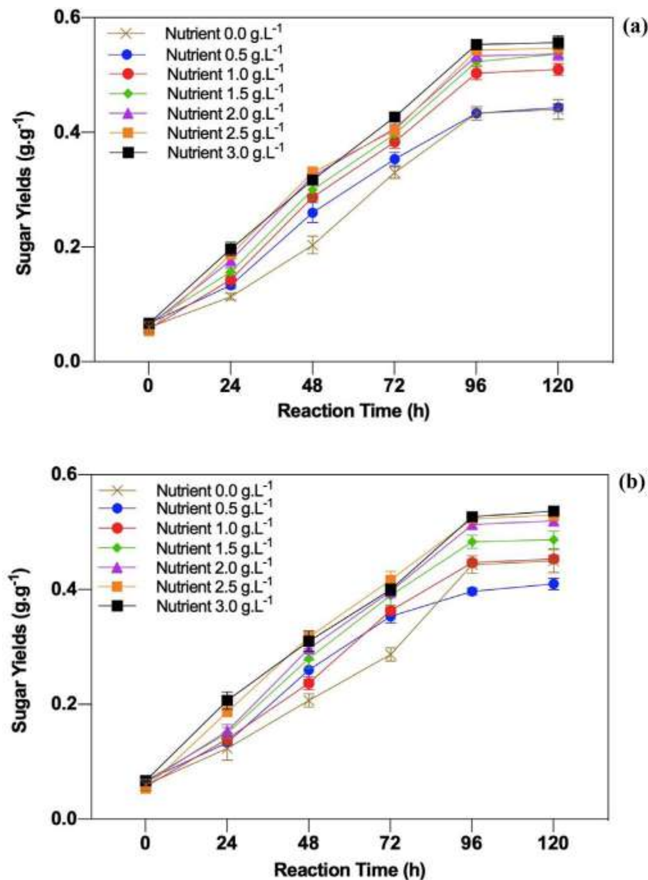

Fig. 4. Effect of supplemented nutrient and reaction time on sugar yield of (a). *K. alvarezii*, (b) *G. amansii*.

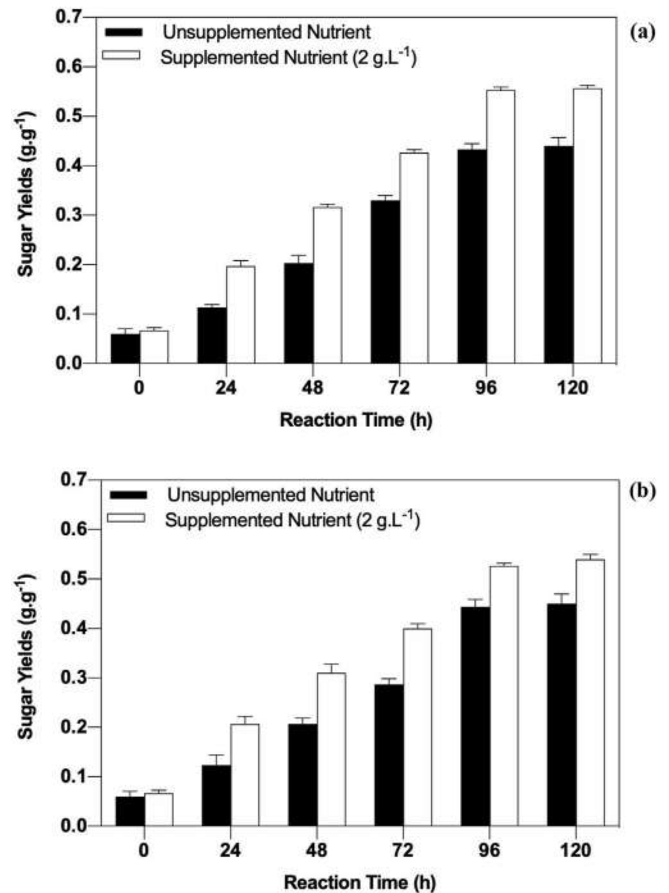

Fig. 5. Comparison of supplemented nutrient (2 g.L^{-1}) and unsupplemented nutrients on sugar yield during fungal pretreatment of (a). *K. alvarezii*, (b) *G. amansii*.

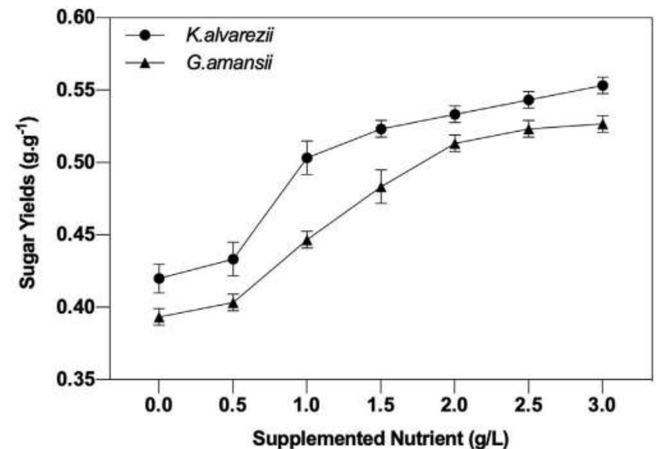

Fig. 6. Effect of supplemented nutrient in 96 h of reaction time on sugar yield.

3.5. Ethanol Production

Ethanol fermentation was undertaken on algae pretreated under the optimized conditions of 2% of substrate concentration, 96 h

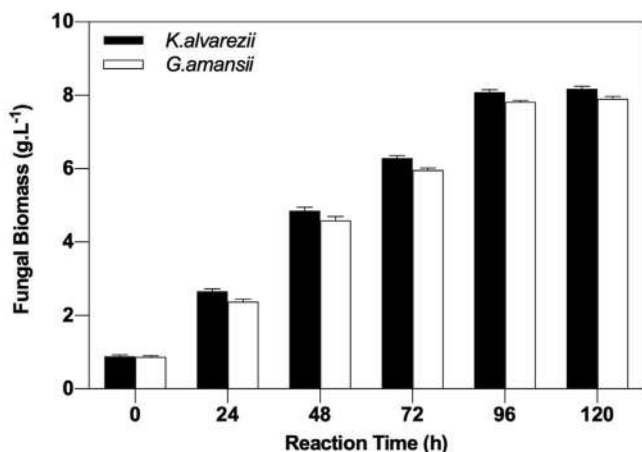

Fig. 7. Biomass formation of *T.harzianum* during fungal pretreatment.

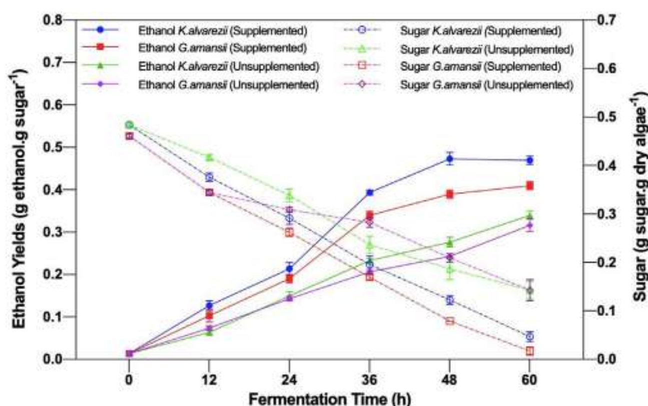

Fig. 8. Effect of supplemented nutrient and fermentation time on ethanol yield and sugar consumption.

reaction time, an inoculum size of 10%, and with 2 g.L^{-1} nutrient supplementation. The algae slurry of *K.alvarezii* and *G.amansii* produced by fungal pretreatment and enzyme hydrolysate contained sugars at 0.56 g g^{-1} and 0.53 g g^{-1} respectively. Ethanol fermentation was performed using nutrition supplements obtained from the residual biomass of *T.harzianum* recovered from the pretreatment stage and was compared to unsupplemented fermentation. Bioethanol production and sugar concentration during fermentation are presented in Fig. 8.

In unsupplemented treatments, the highest ethanol yields of *K.alvarezii* and *G.amansii* was 0.34 g g^{-1} and 0.32 g g^{-1}, respectively. With nutrient supplementation, the highest ethanol yields of *K.alvarezii* and *G.amansii* were 0.47 g g^{-1} and 0.41 g g^{-1} respectively (Fig. 8). Nutrient supplementation therefore significantly increased ethanol yields, by 28.12% and 38.23% for *K.alvarezii* and *G.amansii* respectively. The highest ethanol yields both in *K.alvarezii* and *G.amansii* were achieved at 60 h of reaction time under supplemented nutrient treatment. Fermentation rates decreased by up to 65.2% after 48 h. Based on Tukey's Multiple Comparison Test, a 60 h fermentation time did not significantly increase ethanol yields compared to 48 h of fermentation time. As such, a 48 h fermentation time is optimal for both *K.alvarezii* and *G.amansii*.

Ethanol production was accompanied by declining sugar content. At the beginning of fermentation (0 h) sugar content ranged from 0.53 to 0.56 g g^{-1} (g sugar.g dry algae^{-1}). By the end of fermentation (96 h) the sugar level had decreased to 0.05–0.02 g g^{-1}. Nutrient supplementation increased sugar consumption both of *K. alvarezii* and *G. amansii* by 25.2%.

The fermentation efficiency was calculated as the ratio of the average ethanol production (0.511 g g^{-1}) to the theoretical yield possible from biochemical conversion of the consumed sugar. The results showed that the fermentation efficiency of *K.alvarezii* and *G.amansii* in unsupplemented treatments was 66.7% and 62.7%, respectively. Under supplemented nutrient treatment, the fermentation efficiency of *K.alvarezii* and *G.amansii* was 92.2% and 80.4% respectively. The fermentation efficiency achieved in this study was higher than other studies using *S. cerevisiae*. Others have reported a 61.7% fermentation efficiency (Li et al., 2016), a 78.4% fermentation efficiency (Saravanan et al., 2018) and a 70.3% fermentation efficiency (Kim et al. (2017) (Table 1). The higher fermentation efficiency achieved in this study was obtained as a result of the novel step of using residual *T.harzianum* biomass for nutrient supplementation in the ethanol fermentation stage. The dry *T.harzianum* biomass contains nitrogen levels up to 28.3% higher than commercial nutrient supplements (Ahmed et al., 2017). Nitrogen protects *S. cerevisiae* cells from osmotic stress during ethanol production (Appiah-Nkansah et al., 2018*)*, significantly improving ethanol production.

Walker and Walker (2018) report that fermentation media represents over 50% of ethanol production cost. Yeast extracts show promise for increasing ethanol production as supplemented nutrient, but they are usually expensive (Salakkam *et al.*, 2017). As such, development of an inexpensive alternative nitrogen source is crucial for the economic production of bioethanol from macroalgae. Residual biomass of *T.harzianum* was readily available from the previous fungal pretreatment stages. This suggests that fungal pretreatment of marine algae has the added benefit of producing residual biomass that can be utilized as a nutrient supplement during fermentation, increasing fermentation yields at a low cost.

4. Conclusion

The study indicates that fungal pretreatment by *T.harzianum* followed by enzymatic hydrolysis of the marine macroalgae

Table 1
Comparison of ethanol fermentation efficiency using different supplemented nutrient

Supplemented Nutrient	Raw Materials	Microbe	Fermentation Efficiency (%)	References
Fungal residual biomass	*K.alvarezii*	*S.cerevisiae*	92.2	This study
Fungal residual biomass	*G.amansii*	*S.cerevisiae*	80.4	This study
$(NH_4)2SO_4$ + KH_2PO_4	*Ulva prolifera*	*S.cerevisiae*	61.7	Li et al. (2016)
Yeast extract + $(NH_4)2SO_4$	*Gracilaria* sp.	*S.cerevisiae*	78.4	Saravanan et al. (2018)
Yeast extract and peptone	*Porphyridium cruentum*	*S.cerevisiae*	70.3	Kim et al. (2017)
Yeast extract and peptone	*Gelidium amansii*	*S.cerevisiae*	84.9	Kim et al. (2015)
Peptone and yeast extract	*Ulva fasciata*	*S.cerevisiae*	88.2	Trivedi et al. (2013)
Yeast extract	*Sargassum angustifolium*	*S.cerevisiae*	73.0	Ardalan et al. (2018)

K. alvarezii and *G. amansii* produced sugar contents of 0.55 g g^{-1} and 0.53 g g^{-1}, respectively. Fungal pretreatment prior to enzymatic hydrolysis resulted in up to a 2.3 fold increase in sugar yield compared to enzymatic hydrolysis without fungal pretreatment. Produced sugars were converted to ethanol using *S. cerevisiae* under nutrient supplemented conditions. The fermentation efficiency under these optimized conditions reached 92.2%. This is substantially higher than previously reported studies. This high efficiency was achieved using nutrient supplementation with fungal biomass recovered from the pretreatment stage, increasing ethanol production by 38.2%. These results illustrate the potential of fungal pretreatment to substantially increase the efficiency of bioethanol production from marine algae. This pretreatment generates high sugar yields, is low cost, does not produce fermentation inhibiting compounds, and generates biomass with a high nitrogen content that is suitable for nutrient supplementation during fermentation. These results indicate the potential of fungal pretreatment with biomass recovery for the cost-effective production of third-generation bioethanol from marine algae. Future research could usefully consider a full economic optimisation of this bioethanol production process at industrial scale.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Sulfahri: Conceptualization, Formal analysis, Methodology, Software. **Siti Mushlihah:** Data curation, Writing - original draft, Writing - review & editing. **Dirayah R. Husain:** Supervision, Investigation, Validation. **Alexandra Langford:** Conceptualization, Writing - original draft, Writing - review & editing. **Asmi Citra Malina A.R. Tassakka:** Software, Funding acquisition.

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Appendix A. Supplementary data

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