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Fungal pretreatment of marine algae for enhanced enzymatic hydrolysis followed by bioethanol production

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Abstract

Algae have been considered as renewable biomass for bioethanol production. But pretreatment is required to breakdown the complex structure of algae and render it accessible by enzymes hydrolysis and bioethanol fermentation. This research aims to develop an economic pretreatment process through the use of fungal for algal cell disruption to improve enzymatic hydrolysis for bioethanol production. The effectiveness of this process was evaluated both for algae *Kappaphycus alvarezii* and *Gelidium amansii*. The new fungal pretreatment method utilised *Trichoderma harzianum* followed by enzymatic hydrolysis, to produce sugar. Lastly, the residual biomass of *T.harzianum* obtained from pretreatment then used as a supplemented nutrient for bioethanol production. The results showed that fungal pretreatment, followed by enzymatic hydrolysis, increased sugar yield by 2.3-fold than enzymatic hydrolysis without fungal pretreatment. Fungal pretreatment also produces residual biomass that able to increased ethanol yield up to 38.2%.

Keywords: marine algae; sugar; pretreatment; fermentation; ethanol

1. Introduction

Fuel demands are increasing, along with the addition of the human population (Jankowska *et al*., 2017; Rempel *et al.,* 2019). In 2050, the human population is predicted to reach 10 billion, which requires fuel every day (Goujon, 2019). Fossil fuels which are the primary energy source for the world, are non-renewable and estimated to be used up by the middle of the century (Shokrkar *et al.*, 2017; Ortigueira *et al.,* 2015). The high dependence on fossil fuels has led to uncertainty of petroleum resources, which mandates for search of an alternative and eco-friendly energy source (Wood & Roelich, 2019). One of the renewable fuels that have the potential to replace fossil fuel is bioethanol (Ko *et al.,* 2016; Chamnipa *et al.,* 2017). Bioethanol is one of the renewable, eco-friendly energy, and produces low carbon gas compared to fossil fuel such as gasoline (Kaewkrajay *et al.,* 2014; Quresh *et al.,* 2015). New, renewable energy sources, such as algae has attracted attention and become an important issue related to the generation of alternative energy (Sulfahri *et al.,* 2016).

Algal biomass is promising third-generation feedstock for bioethanol production due to its rapid and sustainable growth (Jambo et al., 2019; Sulfahri *et al.,* 2017). Besides, algal biomass doeas not compete with other food crops. Therefore, in this study, we used marine algae *Kappaphycus alvarezii* and *Gellidium amansii* as a raw material to produce fermentable sugars and convert them to bioethanol. These algal are high carbohydrate content, high growth rate, and easy to cultivate. The carbohydrate content of *K. alvarezii* and *G. amansii* was 88.6% and 60.0%, respectively (Ra *et al.,* 2016: Jeong *et al.,* 2011). The carbohydrate content of algal biomass converted to mon sugars during the

pretreatment process. This is followed by ethanol fermentation as these sugars will be converted into bioethanol.

In order to convert the algal biomass to bioethanol, it needs to disrupt the cell wall and converted to fermentable sugar by pretreatment methods. Unlike lignocellulosic biomasses, the conversion of the algal biomass into fermentable sugars is easier, because algal biomass have a lignin-free biomass composition. However, algae have stronger cell walls compared to those of terrestrial plant cells (Keris-Sen *et al.,* 2017). The structural cell walls of algae usually consist of polysaccharides, which are dominated by cellulose fibrils (Abd-rahim *et al.,* 2014). Thus, before the ethanol fermentation step, pretreatment is required to breakdown the complex cell wall structure of algae to facilitate the conversion of cellular carbohydrates to glucose. Various pretreatment methods, including acid, enzymatic, ultrasonication, microwave, and ozone pretreatment, have been tested. Although these pretreatments can obtain high glucose yields, most of the methods are having limitations such as high cost, extreme reaction conditions, and produce inhibitors that interfere with the fermentation process. Therefore, a low-cost pretreatment for cell disruption without inhibitor formation is needed.

In this study, a new pretreatment method which uses fungal *Trichoderma harzianum* was developed. This new fungal pretreatment has many superior properties, such as not generate any inhibitors and is environmentally friendly with low chemical input. The microbe *T. harzianum* is a fungal producing enzymes cellulase, β-glucosidase, and xylanase (Delabona *et al.,* 2012). Theses enzyme can disrupt the cell wall of the algae and converted to mono sugars. Besides, theses enzyme and another protein in fungal *T. harzianum* consist of amino acid that rich in nitrogen content that potential to be applied

as a supplemented nutrient in next ethanol fermentation stages. Ethanol fermentative microorganisms require nitrogen as a supplemented nutrient for better performance. Some studies reported that nitrogen as supplemented nutrient increased ethanol production (Lainez *et al*., 2019; Aleman-Ramirez *et al*., 2019). Therefore, we further identified the optimal reaction conditions to maximize the ethanol production from fungal pretreated of *K. alvarezii* and *G. amansii* by using supplemented nutrient from residual biomass of *T. harzianum*.

Until recently, only a limited number of studies reported the use of fungal for algal cell disruption to release their polysaccharides content. Therefore, in this study, the effect of fungal on sugar yield was analyzed for different process variables, including algal concentrations, inoculum size of fungal, supplemented nutrient, and pretreatment time. The polysaccharides which have been obtained through the pretreatment then converted into monosaccharides through enzyme hydrolysis. The monosaccharides then converted into bioethanol through fermentation using *Saccharomyces cerevisiae* with different process variables, including fermentation time and fermentation nutrient.

2. Materials and Methods

2.1. Raw Materials

Algae *K. alvarezii* and *G. amansii* were purchased from Seaweed Research Unit of Hasanuddin University, Indonesia. Algal biomass 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. The screened algal powders were used for cell disruption studies.

T. harzianum and *S. cerevisiae* used in this study obtained from Indonesian Culture Collection (InaCC). *T. harzianum* was cultured on PDA (Potato Dextrose Agar) medium, whereas *S. cerevisiae* was cultured on PDA (Sabouraud Dextrose Agar) medium. Both *T. harzianum* and *S. cerevisiae* culture at 30°C for 48 hours.

2.2. Fungal Pretreatment

T. harzianum was employed in this fungal pretreatment process. Algal concentrations, inoculum size of fungal, supplemented nutrient and reaction time were evaluated in this fungal pretreatment. Algae biomass pretreated with heat treatments at 100 °C for 2 h was used for the fungal pretreatment experiments. The fungal pretreatment experiments was performed in 100 mL tubes with 50 mL of working volume. Algal concentrations were varied from 1 to 5% w/w, dry algae. The inoculum size of fungal was varied from 5 to 20% (v/v). In this study, we used yeast extract as supplemented nutrient. Supplemented nutrient was varied from 0.5 to 3% w/w, dry algae. The pH in the reactors was adjusted to a pH of 5.0 using Na-citrate buffer. The reaction time was varied from 0 to 96 h. After hydrolysis, the algal solution was pasteurized to stop the reaction and separated from the liquid by centrifugation at 9,000 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.

2.3. Enzymatic Hydrolysis

The polysaccharides which have been obtained through the best pretreatment process converted into monosaccharides through enzyme hydrolysis. Algae biomass from

pretreatment process was used for the enzyme pretreatment experiments. Enzymatic hydrolysis of pretreated algal biomass was carried out using commercial enzymes (Cellic CTec2; 150 KNU; Novozymes, Denmark). The enzymatic hydrolysis was performed in 100 mL tubes with 50 mL of working volume. The amount of enzyme was 100 KNU with constant algal biomass of 5 g for enzymatic hydrolysis. The reaction time of the enzymatic hydrolysis process was 48 h at 30°C. The algal concentration was 2% (w/w) based on experimental results, which determined the optimum algal concentration. After hydrolysis, the algal solution was separated from the liquid by centrifugation at 9,000 rpm for10 min at 4 °C followed by filtration. The liquid fraction was then collected to determine the total sugar and reducing sugar yields.

2.4. Ethanol Fermentation

S. cerevisiae was employed in this ethanol fermentation process. Fermentation condition and fermentation time and were evaluated in this ethanol fermentation experiment. The algae hydrolysates obtained from the best pretreatment followed by a hydrolysis process were used as a fermentation medium for bioethanol 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, 48, and 60 h) and supplemented nutrient using fungal biomass in previous step (supplemented and no supplemented nutrient). The fermentation process was performed under anaerobic conditions at 30 °C with the agitation of 150 rpm. 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.

2.5. Analytical

2.5.1 Cell Biomass

The measurement of cell biomass of *T. harzianum* was performed using the cell dry weight method. The algal solution from microbial pretreatment using *T. harzianum* was centrifuged at 9,000 rpm for 10 min at 4 °C. Pellets were then resuspended in distilled water. They aspirated using a pipette, before being transferred to a paper filter, which had previously been put into the oven until its weight was constant (W_1). The pellets on the filter paper were then dried in an oven at 80 °C until its weight was constant (W_2). The dry cell weight was calculated based on the difference between the weight of the final filter paper and the weight of the initial filter paper ($W_2 - W_1$) and is expressed in $g.L^{-1}$.

2.5.2 Sugar Levels

Sugar levels were measured using a digital Brix refractometer (Milwaukee MA871) and 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 x 7.8 mm). The column was maintained at 65 °C with a flow of 0.6 mL/min of 5 mM H_2SO_4 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 measured based on the percent theoretical yield was calculated by the percent ratio of the

average ethanol production to the ethanol theoretically produced (0.511 g ethanol.g sugar^{-1}) in the biochemical conversion of the sugars consumed.

2.6. Statistical Analysis

All of the experiments were conducted in triplicate, and data is presented as mean ± standard deviation (SD). The experimental data were statistically analyzed by one-way analysis of variance (ANOVA) using Tukey–Kramer multiple comparison test using GraphPad Prism version 8.2.1 for Mac (GraphPad Software, Inc., USA). Values were considered significant when P value was <0.05.

3. Results and Discussion

3.1. Effect of Substrate Concentration on Sugar Yield

In this study, we optimize sugar yield production by fungal cell disruption followed by enzymatic hydrolysis using substrate concentrations of 1-5% and reaction time of 0-96 h. Figure 1 presents the formation of sugar yields during reaction time. Sugar yields increased in a similar pattern according to the reaction time. The highest sugar yields of *K.alvarezii* and *G.amansii* were obtained with 96 h of reaction time, with sugar yields of 0.43 g.g^{-1} (g sugar.g dry algae^{-1}) and 0.40 g.g^{-1}, respectively. The sugar yield of *K.alvarezii* and *G.amansii* was increased significantly from 38 to 80 % every 24 h when substrate concentration was 1-3%. In contrast, the sugar yield not increased significantly from 8 to 33% every 24 h when substrate concentration was 4-5%.

The effect of substrate concentration was more profound when substrate concentration was increased from 1 to 3% compared to a change from 4 to 5%. Sugar yields of *K.alvarezii* and *G.amansii* were increased by 14.28% and 5.00 % when substrate

concentration was increased from 1 to 2%, respectively. In contrast, when substrate concentration was 3%, sugar yields of *K.alvarezii* and *G.amansii* were decreased by 2.5% and 2.3%, respectively. Similarly, sugar yiled of *K.alvarezii* and *G.amansii* were decreased by 66.67% and 71.79% when substrate concentration was increased to 4-5%, respectively. The decreased of sugar yields with increasing substrate concentration may be because the high viscosity of the solid content of both *K.alvarezii* and *G.amansii* in 4-5% makes it hard to utilize by fungal *T. harzianum*. As explained by Martínez-patiño *et el.,* (2018) and Shirkavand *et al.,* (2016), substrate concentration was reported as the main factor controlling sugar yield of fungal pretreatment. The highest sugar yield was achieved at 2% of substrate concentration for both algae *K. alvarezii* and *G.amansii*; accordingly, this substrate concentration was applied for further experiments in this study.

Figure 2 presents the comparison of enzymatic hydrolysis and fungal pretreatment followed by enzymatic hydrolysis on sugar yield. Enzymatic hydrolysis of *K.alvarezii* and *G.amansii* without fungal treatment produce the highest sugar yield of 0.18 $g.g^{-1}$ and 0.15 $g.g^{-1}$, respectively. Under fungal pretreatment followed by enzymatic hydrolysis of *K.alvarezii* and *G.amansii* produce the highest sugar yield of 0.43 $g.g^{-1}$ and 0.40 $g.g^{-1}$, respectively. The fungal pretreatment of *K.alvarezii* and *G.amansii* followed by enzymatic hydrolysis resulting up to a 2.3 fold increase of sugar yield than enzymatic hydrolysis without fungal pretreatment. These findings indicate fungal pretreatment using *T.harzianum* can be a promising candidate for algae pretreatment via a fermentation process.

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 Figure 3. The maximum sugars yields were obtained at 20% of inoculum size and 96 h of reaction time. Based on Tukey's Multiple Comparison Test, the inoculum size of 10-20% showed similar producing sugars performance 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. In contrast, the inoculum size of 5% showed lower producing sugars performance at 96 h from *K.alvarezii* and *G.amansii* in the range 0.14-0.18 $g.g^{-1}$ and 0.11-0.15 $g.g^{-1}$, respectively. A higher amount of inoculum size was ineffective in fungal pretreatment stages, taking into account cost and energy (Shirkavand *et al.,* 2016). Inoculum size of 10% was chosen as the optimal inoculum size for further studies because inoculum size of 10% not significantly different with inoculum size of 15% and 20% based on Tukey's Multiple Comparison Test.

3.3. Effect of Supplemented Nutrient on Sugar Yield

In this study, we used commercial supplemented nutrient, yeast extract as supplemented nutrient for fungal pretreatment. The effect of supplemented nutrient and reaction time on the formation of sugars from *K.alvarezii* and *G.amansii* biomass using 2% of substrate concentration and 10% of inoculum size is shown in Figure 4. The highest sugar yields both in *K.alvarezii* and *G.amansii* were achieved at 96 h of reaction time. Although the sugar production rate of *K.alvarezii* and *G.amansii* was decreased up to 27.5% after 48 h but based on Tukey's Multiple Comparison Test, 96 h was significantly different from 72 h and 48 h. Therefore, the reaction time that produced the most effective sugar was 96 h.

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 (Figure 5). Supplemented nutrient increased sugar yield 16.3-27.9% (Figure 6). The highest sugar yield achieved in 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 $g.L^{-1}$ not significantly different with supplemented nutrient of 2.5-3.0 $g.L^{-1}$. Therefore, the best supplemented nutrient for fungal pretreatment was 2 $g.L^{-1}$, both in *K.alvarezii* and *G.amansii*.

The fungal pretreatment has many superior properties, such as not generate any inhibitors for ethanol production. As explained by Ahmed *et al.* (2017), *T. harzianum* biomass does not contain any inhibitor such as hydroxymethylfurfural, furfural, and levulinic acid. *T. harzianum* is a fungal producing enzymes cellulase, β-glucosidase, and xylanase (Delabona *et al.,* 2012). Whereas theses enzyme consists of amino acid that rich in nitrogen content. Nitrogen protecting the yeast from osmotic stress in ethanol fermentation (Yue *et al*., 2016). This shows that the microbial pretreatment using *T. harzianum* microbes has the potential to be applied to the hydrolysis of complex carbohydrates to mono sugar with algae as raw material. These microbial pretreatment hight yields in producing sugar, low cost, not producing inhibitor, and high nitrogen content that potential to be applied as supplemented nutrients.

3.4. Fungal Biomass During Pretreatment

In order to investigate the possibilities of fungal biomass in the next ethanol fermentation stages, the biomass of fungal *T.harzianum* was monitored during fermentation. Figure 7

presents the formation of *T.harzianum* biomass during pretreatment using both algae substrate of *K.alvarezii* and *G.amansii.* The highest biomass of *T.harzianum* was achived in 96 h of reaction time both in *K.alvarezii* and *G.amansii* substrate. The highest biomass achieved in substrate of *K.alvarezii* and *G.amansii* was 8.09 $g.L^{-1}$ and 7.42 $g.L^{-1}$, respectively. The fungal residual biomass achieved in this study was sufficient for nutrient supplementation for ethanol production. As explained by Li *et al.*, (2016), supplemented nutrient on the media of fermentation is better above 2.5 $g.L^{-1}$. This supported by the results of Ardalan *et al.,* (2018), that supplemented nutrient of yeast extract 5 $g.L^{-1}$ was sufficiently to improve ethanol fermentation performance using yeast *S. cerevisiae.* Therefore, the next fermentation stages using this residual biomass to improve ethanol fermentation.

3.5. Ethanol Production

The optimal pretreatment condition, i.e: 2% of substrate concentration, 96 h of reaction time, 10% of inoculum size, and 2 $g.L^{-1}$ of supplemented nutrient using fungal *T.harzianum* followed by enzymatic hydrolysis was used for ethanol fermentation. Both algae slurry of *K.alvarezii* and *G.amansii* from fungal pretreatment and enzyme hydrolysate obtained was contained sugar of 0.56 $g.g^{-1}$ and 0.53 $g.g^{-1}$, respectively. This fungal pretreatment was performed to determine the potential for this hydrolysate to be utilized for bioethanol production by the commercial yeast, *S.cerevisiae*. Ethanol fermentation was performed by using supplemented nutrition. Supplemented nutrients used in these study obtained from the residual biomass of *T.harzianum* in previous pretreatment stages and compared to unsupplemented nutrients. Bioethanol production and sugar consumption during fermentation are presented in Figure 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. In nutrient supplementation, the highest ethanol yields of *K.alvarezii* and *G.amansii* was 0.47 $g.g^{-1}$ and 0.41 $g.g^{-1}$, respectively (Figure 8). Supplemented nutrient increased ethanol yield of *K.alvarezii* and *G.amansii* 28.12% and 38.23%, respectively. The highest ethanol yields both in *K.alvarezii* and *G.amansii* were achieved at 60 h of reaction time under supplemented nutrient treatment. However, the fermentation rate was decreased up to 65.2% after 48 h. Moreover, based on Tukey's Multiple Comparison Test, 48 h of fermentation time not significantly different with 60 h of fermentation time. Therefore, 48 h is the best effective time for ethanol fermentation, both in *K.alvarezii* and *G.amansii*.

In this study, the fermentation efficiency was calculated based on percent theoretical yield by the percent ratio of the average ethanol production to the ethanol theoretically (0.51 $g.g^{-1}$) produced in the biochemical conversion of the sugars consumed. 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 result of fermentation efficiency achieved in this study were higher when compared to other studies that also used *S. cerevisiae*, including 61.7% of fermentation efficiency (Li *et al.*, 2016), 78,4% of fermentation efficiency (Saravanan *et al.* (2018) and 70.3 % of fermentation efficiency (Kim *et al.* (2017) (Table 1). The higher of fermentation efficiency that was achieved in this study that was used residual biomass of *T.harzianum* as supplemented nutrient when compared to the previous study that was using commercial supplemented nutrient confirmed that residual biomass of *T.harzianum* potential for supplemented nutrient for ethanol fermentation.

The results achieved in this study indicate that the highest fermentation efficiency was achieved by treatment with supplementation. This shows that residual biomass of *T.harzianum* was able to use as a nutritional supplement for *S. cerevisiae*. As explained by Ahmed *et al.* (2017), dry biomass of *T.harzianum* contains nitrogen as much as 28.3% that higher when compare with commercial supplemented nutrient, yeast extract as much as 10.2 % (Ahmed *et al.,* 2017; Vieira *et al.,* 2016). Nitrogen was reported to protecting the cells of *S. cerevisiae* from osmotic stress due to ethanol production (Phukoetphim *et al.,* 2017*;* Appiah-Nkansah *et al.,* 2018*).*

Fermentation media represents more than 50% of the fermentation process costs (Aleman-Ramirez et al., 2019; Walker & Walker, 2018). Yeast extract gives better results for ethanol production, but they are usually expensive (Salakkam *et al.,* 2017). Therefore, an alternative nitrogen source that is inexpensive and readily available is crucial for economic ethanol production. Unlike yeast extract, residual biomass of *T.harzianum* was readily available from previous fungal pretreatment stages. Therefore, fungal pretreatment produces residual biomass that can be utilized as a supplement during fermentation. This supplemented nutrient benefit for these ethanol fermentation due to their low cost and high nutrient content.

Increased ethanol levels during the fermentation process were accompanied by a decrease in sugar content (Figure 8). At the beginning of fermentation (0 h), the sugar level ranged from 0.53 to 0.56 $g.g^{-1}$ (g sugar.g dry $algae^{-1}$). At the end of fermentation (96 h), the sugar level decreased to 0.05-0.02 $g.g^{-1}$. The sugar consumption both for *K. alvarezii* and *G. amansii* after 60 h of fermentation time was almost same of 96.1% under supplemented nutrient treatment. Sugar consumption under unsupplemented nutrient treatment both of

K. alvarezii and *G. amansii* was 70.9%. Nutrient supplementation increased sugar consumption both of *K. alvarezii* and *G. amansii* 25.2%. These results were followed by ethanol production which was also highest under supplementation treatment of *K. alvarezii* and *G. amansii* with ethanol production of 0.47 $g.g^{-1}$ and 0.41 $g.g^{-1}$, respectively.

The results of this study indicate that using fungal for cell disruption followed by enzymatic hydrolysis using *T.harzianum* using algae *K. alvarezii* and *G. amansii* was able to produce sugar 0.55 $g.g^{-1}$ and 0.53 $g.g^{-1}$, respectively. The sugar that has been obtained was then supplemented with residual biomass of *T.harzianum* and converted to ethanol using *S. cerevisiae,* which it was capable of producing ethanol with a theoretical yield reaching 96.1%. Overall results demonstrate that these methods allowed a new strategy of third-generation ethanol production from both marine algae *K. alvarezii* and *G. amansii*. This study provides a new approach to third-generation ethanol production from marine algae that lead to high yields and cost-effective than previous methods.

4. Conclusion

This study establishes the production of bioethanol from the marine algae, *K. alvarezii* and *G.amansii*, using fungal pretreatment followed by enzymatic hydrolysis and ethanol fermentation. Results indicate that fungal pretreatment followed by enzymatic hydrolysis produced a high amount of sugars compared to enzymatic hydrolysis without fungal pretreatment. The sugar that has been obtained was then supplemented with residual biomass of *T.harzianum* and converted to ethanol using *S. cerevisiae.* The highest ethanol yield was achieved after fungal pretreatment with supplemented nutrients using residual biomass of *T.harzianum*, both in *K. alvarezii* and *G. amansii*. The ethanol fermentation efficiency by using residual biomass of *T.harzianum* achieved in this study was higher

than those reported in earlier studies using commercial supplemented nutrient, yeast extract and peptone. These ethanol production results serve to illustrate the high potential of fungal pretreatment by *T.harzianum* for application in the bioethanol production fields.

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References

1. Abd-rahim, F., Wasoh, H., Rafein, M., & Ariff, A. (2014). Food Hydrocolloids Production of high yield sugars from Kappaphycus alvarezii using combined methods of chemical and enzymatic hydrolysis. *Food Hydrocolloids*, *42*, 309–315. https://doi.org/10.1016/j.foodhyd.2014.05.017

2. Ahmed, S., Mustafa, G., Arshad, M., & Rajoka, M. I. (2017). Fungal Biomass Protein Production from Trichoderma harzianum Using Rice Polishing, https://doi.org/10.1155/2017/6232793

3. Aleman-Ramirez, J. L., Pérez-Sariñana, B. Y., Torres-Arellano, S., Saldaña-Trinidad, S., Longoria, A., & Sebastian, P. J. (2019). Bioethanol production from Ataulfo mango supplemented with vermicompost leachate. Catalysis Today, (July). https://doi.org/10.1016/j.cattod.2019.07.028

4. Appiah-Nkansah, N.B., Zhang, K., Rooney, W. & Wang, D. (2018). Ethanol production from mixtures of sweet sorghum juice and sorghum starch using very high gravity fermentation with urea supplementation. *Industrial Crops & Products*. 111, 247-253. https://doi.org/10.1016/j.indcrop.2017.10.028

5. Ardalan, Y., Jazini, M. and Karimi, K. (2018) 'Sargassum angustifolium brown macroalga as a high potential substrate for alginate and ethanol production with minimal nutrient requirement', *Algal Research*. Elsevier, 36(February), pp. 29–36. http://dx.10.1016/j.algal.2018.10.010.

6. Chamnipa, N., Thanonkeo, S., & Klanrit, P. (2017). Biotechnology and Industrial Microbiology The potential of the newly isolated thermotolerant yeast Pichia

kudriavzevii RZ8-1 for high-temperature ethanol production. *Brazilian Journal of Microbiology*, *49*(2), 378–391. https://doi.org/10.1016/j.bjm.2017.09.002

7. Delabona, S., Sanchez, C., Ribeiro, M., Freitas, S., & Geraldo, J. (2012). Bioresource Technology Use of a new Trichoderma harzianum strain isolated from the Amazon rainforest with pretreated sugar cane bagasse for on-site cellulase production. *Bioresource Technology*, *107*, 517–521. https://doi.org/10.1016/j.biortech.2011.12.048

8. Goujon, A. (2019). Human Population Growth. Encyclopedia of Ecology (Second Edition). 4: 344-351 https://doi.org/10.1016/B978-0-12-409548-9.10755-9

9. Jambo, S. A., Abdulla, R., Marbawi, H., & Gansau, J. A. (2019). Response surface optimization of bioethanol production from third generation feedstock - Eucheuma cottonii. *Renewable Energy*, *132*, 1–10. https://doi.org/10.1016/j.renene.2018.07.133

10. Jankowska, E., Sahu, A. K., Oleskowicz-Popiel, P. 2017. Biogas from microalgae: Review on microalgae's cultivation, harvesting and pretreatment for anaerobic digestion. Renewable and Sustainable Energy Reviews, 75: 692-709. https://doi.org/10.1016/j.rser.2016.11.045

11. Jeong, T. S., Kim, Y. S., & Oh, K. K. (2011). Bioresource Technology Two-stage acid saccharification of fractionated Gelidium amansii minimizing the sugar decomposition. *Bioresource Technology*, *102*(22), 10529–10534. https://doi.org/10.1016/j.biortech.2011.09.017

12. Kaewkrajay, C., Dethoup, T., & Limtong, S. (2014). Ethanol production from cassava using a newly isolated thermotolerant yeast strain, *40*(52), 268–277. https://doi.org/10.2306/scienceasia1513-1874.2014.40.268

13. Keris-Sen, U. D., & Gurol, M. D. (2017). Using ozone for microalgal cell disruption to improve enzymatic saccharification of cellular carbohydrates. Biomass and Bioenergy, 105, 59–65. https://doi.org/10.1016/j.biombioe.2017.06.023

14. Kim, H.M, Hoon, C., & Bae, H. (2017). Comparison of red microalgae (Porphyridium cruentum) culture conditions for bioethanol production. Bioresource Technology, 233, 44–50. https://doi.org/10.1016/j.biortech.2017.02.040

15. Kim, S.W., Hong, C.-H., Jeon, S.-W., Shin, H.-J., 2015. High-yield production of biosugars from Gracilaria verrucosa by acid and enzymatic hydrolysis processes. Bioresource Technology 196, 634–641. https://doi.org/10.1016/j.biortech.2015.08.016

16. Ko, J.K., Um, Y., Woo, H.M., Kim, K.H., Lee, S-M., Ethanol production from lignocellulosic hydrolysates using engineered Saccharomyces cerevisiae harboring xylose isomerase-based pathway, Bioresource Technology (2016), doi: http://dx.doi.org/10.1016/j.biortech.2016.02.124

17. Lainez, M., Ruiz, H.A., Arellano-Plaza, M. Martínez-Hernandez, S.2019. Bioethanol production from enzymatic hydrolysates of Agave salmiana leaves comparing S . cerevisiae and K . marxianus. Renewable Energy 138, 1127-1133. https://doi.org/10.1016/j.renene.2019.02.058

18. Li, Z., Wang, D., & Shi, Y. (2016). Effects of nitrogen source on ethanol production in very high gravity fermentation of corn starch. *Journal of the Taiwan Institute of Chemical Engineers*, *0*, 1–7. https://doi.org/10.1016/j.jtice.2016.10.055

19. Martínez-patiño, J. C. *et al.* (2018) 'Industrial Crops & Products Application of a combined fungal and diluted acid pretreatment on olive tree biomass', *Industrial Crops & Products*. Elsevier, 121(April), pp. 10–17. https://doi.org/10.1016/j.indcrop.2018.04.078

20. Ortigueira, J., Pinto, T., Gouveia, L., & Moura, P. (2015). Production and storage of biohydrogen during sequential batch fermentation of Spirogyra hydrolyzate by Clostridium butyricum. *Energy*, 1–9; 528-536. https://doi.org/10.1016/j.energy.2015.05.070

21. Phukoetphim, N., Salakkam, A., Laopaiboon, P., & Laopaiboon, L. (2017). Improvement of ethanol production from sweet sorghum juice under batch and fed-batch fermentations : Effects of sugar levels , nitrogen supplementation , and feeding regimes. *Electronic Journal of Biotechnology*, *26*, 84–92. https://doi.org/10.1016/j.ejbt.2017.01.005

22. Qureshi, A.S., Zhang, J., Bao, J., High ethanol fermentation performance of the dry dilute acid pretreated corn stover by an evolutionarily adapted Saccharomyces cerevisiae strain, Bioresource Technology (2015), doi: http://dx.doi.org/10.1016/j.biortech.2015.04.025

23. Ra, C.H., Jeong, G.T., Shin, M.K., Kim, S.K., 2013. Biotransformation of 5-hydroxymethylfurfural (HMF) by Scheffersomyces stipitis during ethanol

fermentation of hydrolysate of the seaweed Gelidium amansii. Bioresour. Technol. 140, 421–425. https://doi.org/10.1016/j.biortech.2013.04.122

24. Rempel, A., de Souza Sossella, F., Margarites, A.C., Astolfi, A.L., Steinmetz, R.L.R., Kunz, A., Treichel, H., Colla, L.M., Bioethanol from Spirulina platensis biomass and the use of residuals to produce biomethane: an energy efficient approach, Bioresource Technology (2019), doi: https://doi.org/10.1016/j.biortech.2019.121588
25. Salakkam, A. Kingpho, Y., Aiamsonthi, K., Kaewlao, S., Reungsang, A., Kingpho, Y., Najunhom, S., Reungsang, A. (2017). Bioconversion of soybean residue for use as alternative nutrient source for ethanol fermentation. Biochemical Engineering Journal https://doi.org/10.1016/j.bej.2017.05.020
26. Salakkam, A., Kingpho, Y., Najunhom, S., Aiamsonthi, K., Kaewlao, S., Reungsang, A. 2017. Bioconversion of soybean residue for use as alternative nutrient source for ethanol fermentation, Biochemical Engineering Journal. http://dx.doi.org/10.1016/j.bej.2017.05.020
27. Saravanan, K., Duraisamy, S., & Ramasamy, G. (2018). Biocatalysis and Agricultural Biotechnology Evaluation of the sacchari fi cation and fermentation process of two di ff erent seaweeds for an ecofriendly bioethanol production, 14(March), 444–449. https://doi.org/10.1016/j.bcab.2018.03.017
28. Shirkavand, E., Baroutian, S., Gapes, D. J., & Young, B. R. (2016). Combination of fungal and physicochemical processes for lignocellulosic biomass pretreatment – A review. *Renewable and Sustainable Energy Reviews*, *54*, 217–234. https://doi.org/10.1016/j.rser.2015.10.003

29. Shokrkar, H., Ebrahimi, S., Zamani, M. 2017. Bioethanol production from acidic and enzymatic hydrolysates of mixed microalgae culture. Fuel, 200: 380-386. https://doi.org/10.1016/j.fuel.2017.03.090.

30. Sulfahri, S., Amin, M., Sumitro, S. B., & Saptasari, M. (2017). Comparison of biomass production from algae Spirogyra hyalina and Spirogyra peipingensis. *Biofuels*, *8*(3). https://doi.org/10.1080/17597269.2016.1231954

31. Sulfahri, Mohamad Amin, Sutiman Bambang Sumitro & Murni Saptasari (2016) Bioethanol production from algae Spirogyra hyalina using Zymomonas mobilis, Biofuels, 7:6, 621-626, https://doi.org/10.1080/17597269.2016.1168028

32. Trivedi, N., Gupta, V., Reddy, C., Jha, B., 2013. Enzymatic hydrolysis and productionof bioethanol from common macrophytic green alga Ulva fasciata Delile.Bioresour.*Technol.* 100, 6658–6660. https://10.1016/j.biortech.2013.09.103

33. Walker, G. M., & Walker, R. S. K. (2018). Enhancing Yeast Alcoholic Fermentations. Advances in Applied Microbiology (Vol. 105). Elsevier Ltd. https://doi.org/10.1016/bs.aambs.2018.05.003

34. Wood, N., & Roelich, K. (2019). Energy Research & Social Science Tensions , capabilities , and justice in climate change mitigation of fossil fuels. Energy Research & Social Science, 52(August 2018), 114–122. https://doi.org/10.1016/j.erss.2019.02.014

35. Yue, G., Yu, J., Zhang, X., Tan, T. (2012). The influence of nitrogen sources on ethanol production by yeast from concentrated sweet sorghum juice. *Biomass and Bioenergy*. 39, 48-52. https://doi.org/10.1016/j.biombioe.2010.08.041

Figure Captions

Figure 1. Effect of substrate concentration and reaction time on sugar yield of (A). *K. alvarezii*, (B) *G. amansii*

Figure 2. Comparison of enzymatic hydrolysis and fungal pretreatment followed by enzymatic hydrolysis on sugar yield of (A) *K. alvarezii*, (B) *G. amansii*

Figure 3. Effect of inoculum size and reaction time on sugar yield of (A). *K. alvarezii*, (B) *G. amansii*

Figure 4. Effect of supplemented nutrient and reaction time on sugar yield of (A). *K. alvarezii*, (B) *G. amansii*

Figure 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*

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

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

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

Table and Figure

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*	94.1	This study
Fungal residual biomass	*G.amansii*	*S.cerevisiae*	94.1	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.* (2015)
Yeast extract	*Sargassum angustifolium*	*S.cerevisiae*	73.0	Ardalan *et al.,* (2018)

Figure 1

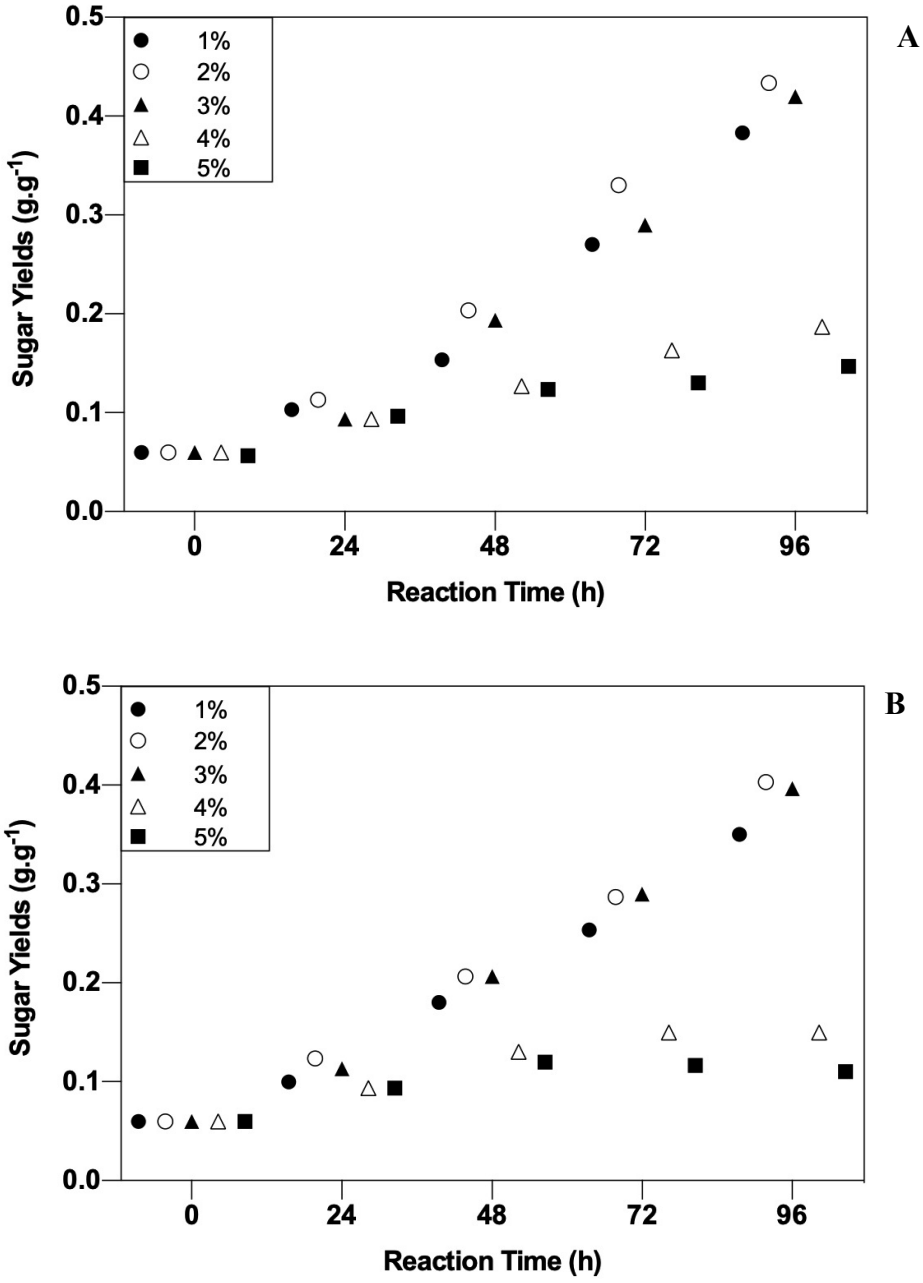

A
1%
2%
3%
4%
5%
Sugar Yields (g.g-1)
0.0
0.1
0.2
0.3
0.4
0.5
0
24
48
72
96
Reaction Time (h)
B
1%
2%
3%
4%
5%
Sugar Yields (g.g-1)
0.0
0.1
0.2
0.3
0.4
0.5
0
24
48
72
96
Reaction Time (h)

Figure 2

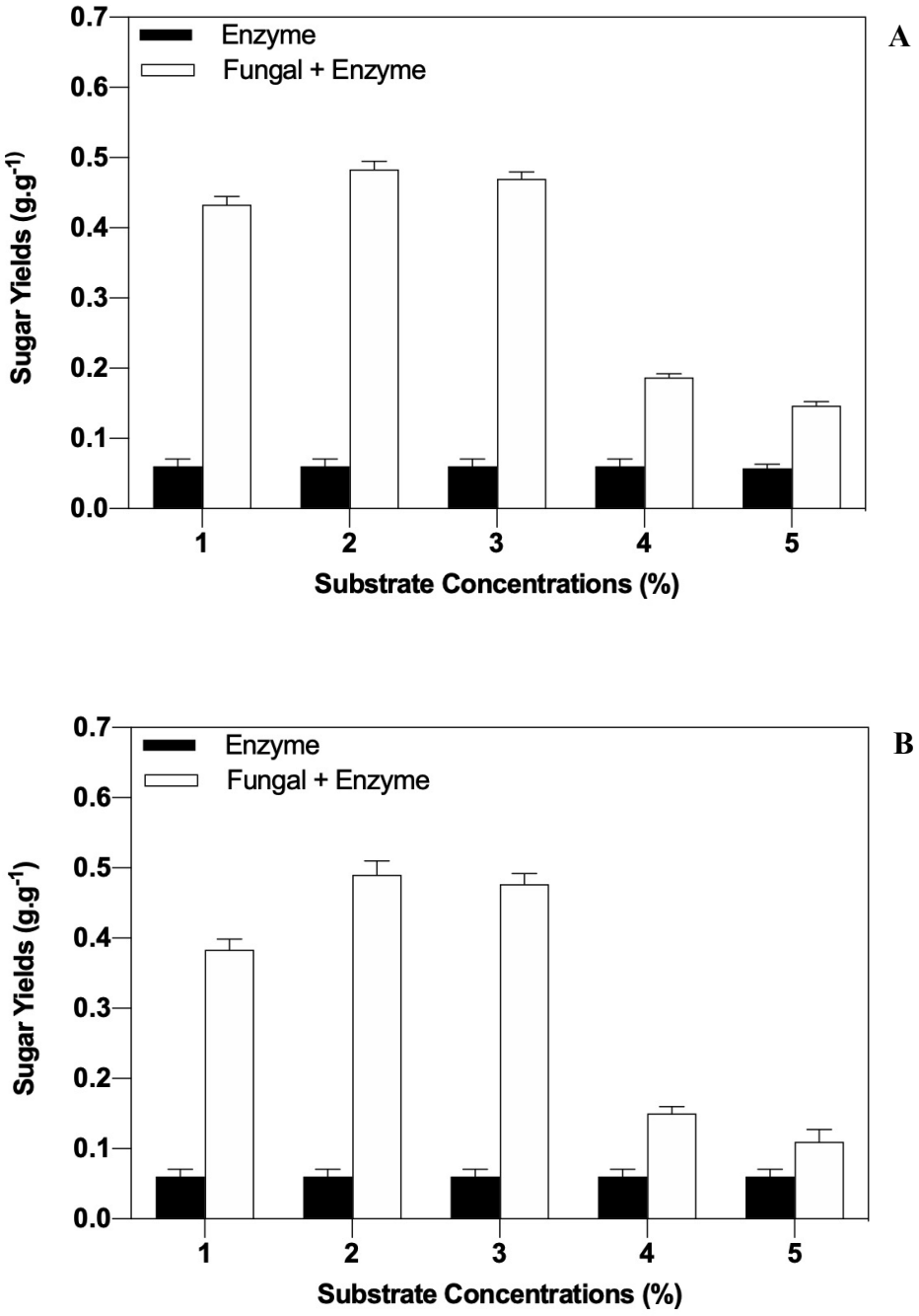

A
Enzyme
Fungal + Enzyme
Sugar Yields (g.g-1)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
1
2
3
4
5
Substrate Concentrations (%)
B
Enzyme
Fungal + Enzyme
Sugar Yields (g.g-1)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
1
2
3
4
5
Substrate Concentrations (%)

Figure 3

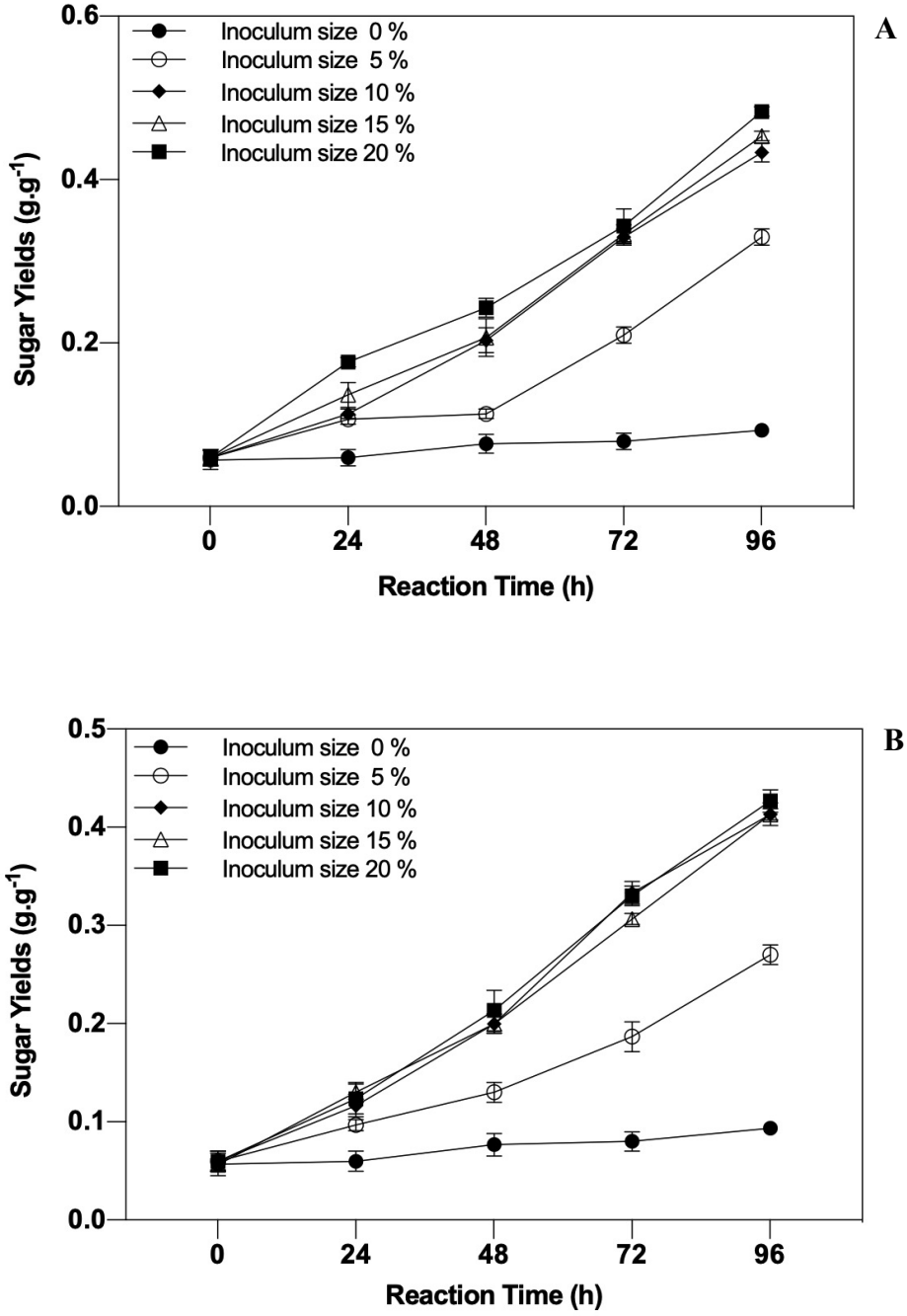
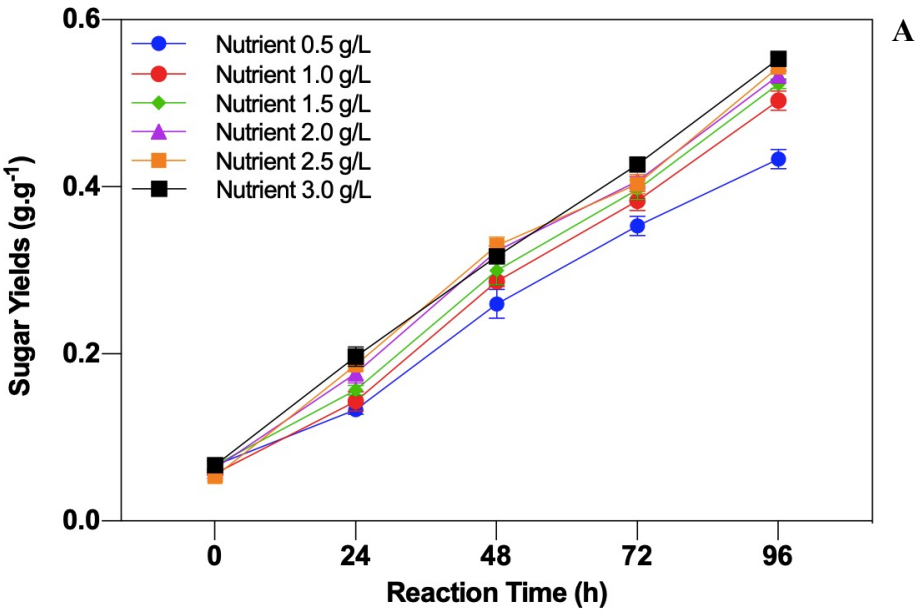

A
Inoculum size 0 %
Inoculum size 5 %
Inoculum size 10 %
Inoculum size 15 %
Inoculum size 20 %
Sugar Yields (g.g-1)
0.0
0.2
0.4
0.6
0
24
48
72
96
Reaction Time (h)
B
Inoculum size 0 %
Inoculum size 5 %
Inoculum size 10 %
Inoculum size 15 %
Inoculum size 20 %
Sugar Yields (g.g-1)
0.0
0.1
0.2
0.3
0.4
0.5
0
24
48
72
96
Reaction Time (h)

Figure 4


A
Nutrient 0.5 g/L
Nutrient 1.0 g/L
Nutrient 1.5 g/L
Nutrient 2.0 g/L
Nutrient 2.5 g/L
Nutrient 3.0 g/L
Sugar Yields (g.g-1)
0.0
0.2
0.4
0.6
0
24
48
72
96
Reaction Time (h)

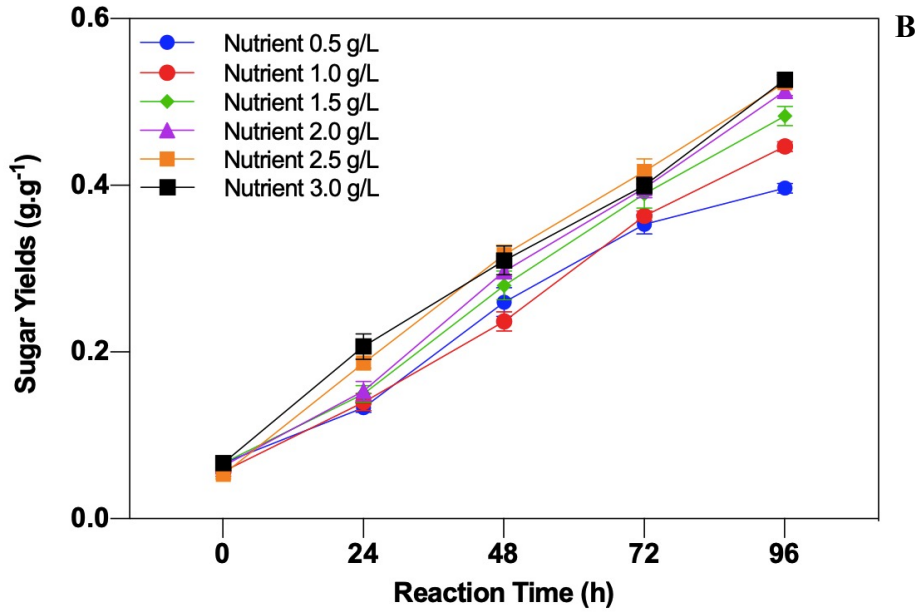
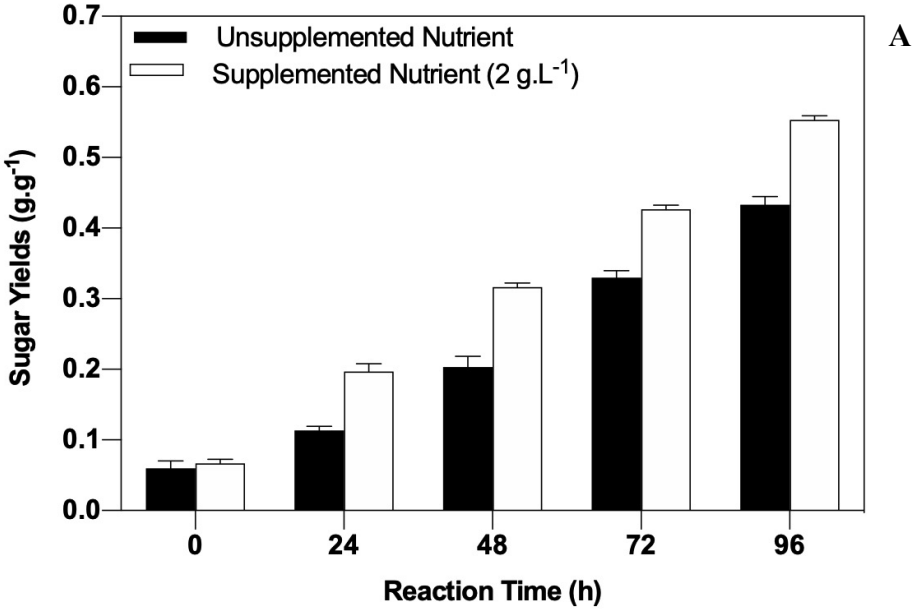

B
Nutrient 0.5 g/L
Nutrient 1.0 g/L
Nutrient 1.5 g/L
Nutrient 2.0 g/L
Nutrient 2.5 g/L
Nutrient 3.0 g/L
Sugar Yields (g.g-1)
0.0
0.2
0.4
0.6
0
24
48
72
96
Reaction Time (h)

Figure 5


A
Unsupplemented Nutrient
Supplemented Nutrient (2 g.L-1)
Sugar Yields (g.g-1)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0
24
48
72
96
Reaction Time (h)

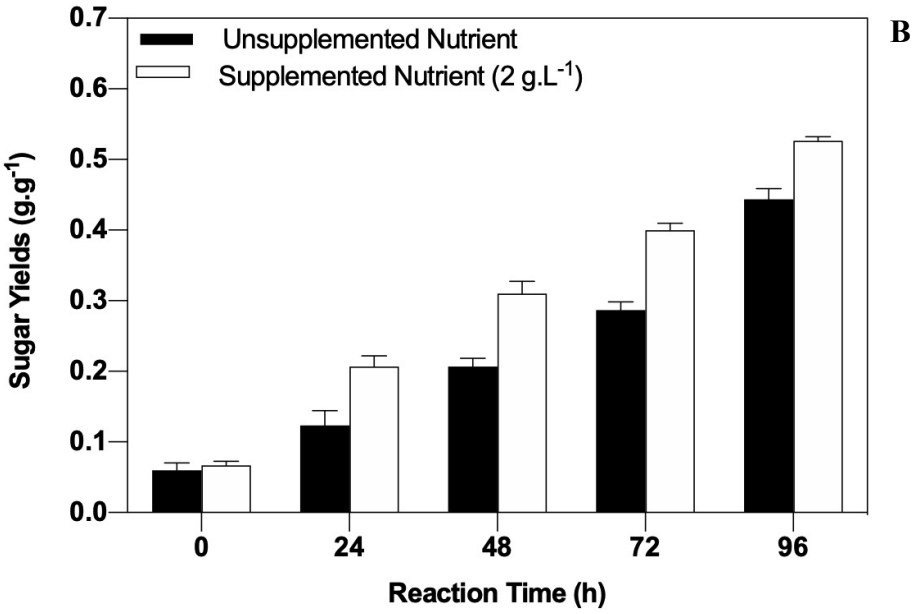

B
Unsupplemented Nutrient
Supplemented Nutrient (2 g.L-1)
Sugar Yields (g.g-1)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0
24
48
72
96
Reaction Time (h)

Figure 6

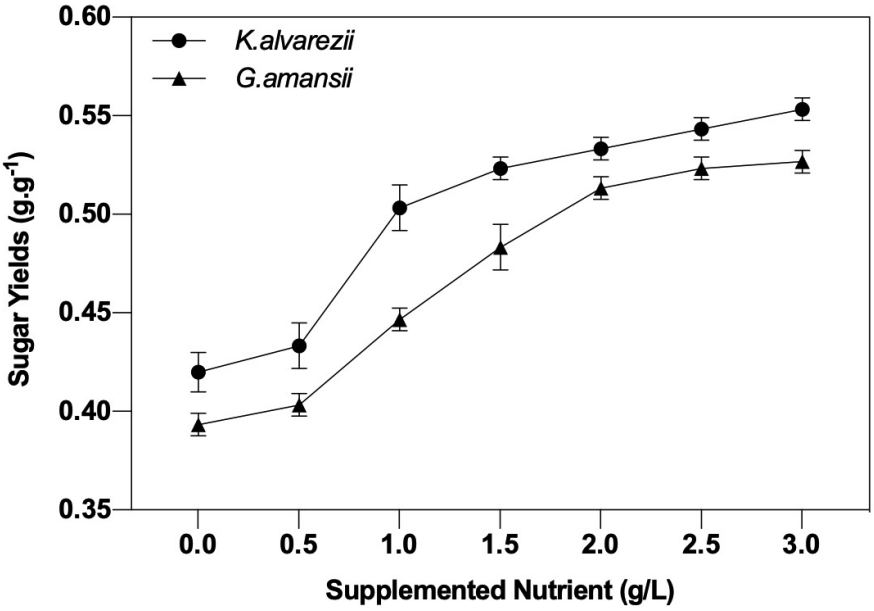

K.alvarezii
G.amansii
Sugar Yields (g.g-1)
0.60
0.55
0.50
0.45
0.40
0.35
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Supplemented Nutrient (g/L)

Figure 7

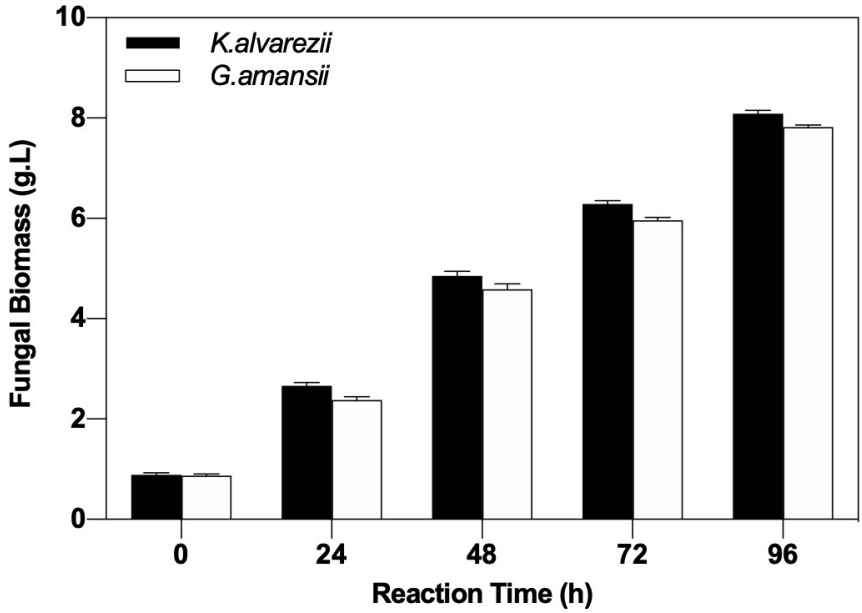
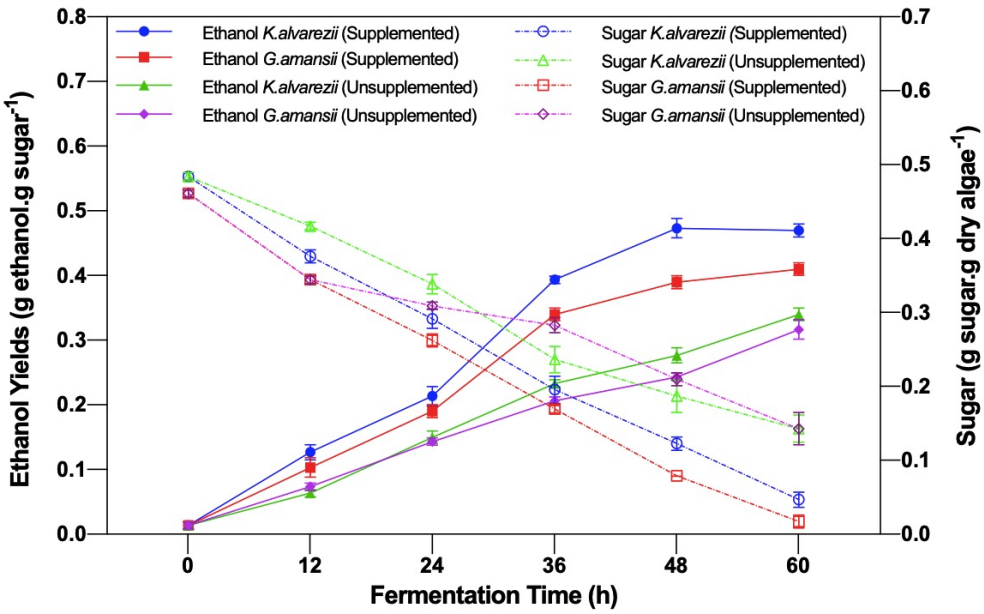

K.alvarezii
G.amansii
Fungal Biomass (g.L)
10
8
6
4
2
0
0
24
48
72
96
Reaction Time (h)

Figure 8


Ethanol K.alvarezii (Supplemented)
Ethanol G.amansii (Supplemented)
Ethanol K.alvarezii (Unsupplemented)
Ethanol G.amansii (Unsupplemented)
Sugar K.alvarezii (Supplemented)
Sugar K.alvarezii (Unsupplemented)
Sugar G.amansii (Supplemented)
Sugar G.amansii (Unsupplemented)
Ethanol Yields (g ethanol.g sugar-1)
Sugar (g sugar.g dry algae-1)
Fermentation Time (h)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0
12
24
36
48
60