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Production of Bioethanol from the Hydrolysate of Brown Seaweed (*Sargassum crassifolium*) using a Naturally β-glucosidase Producing yeast *Saccharomyces cereviceae* JCM 3012

Trianik Widyaningrum[1,2], Indro Prastowo[1,4], *
Masreza Parahadi[3] and Angga Dwi Prasetyo[3]

[1]Department of Biology Education, Faculty of Teacher Training and Education,
Ahmad Dahlan University, Kampus III, Jl. Prof. Dr.Soepomo, Janturan, Yogyakarta 55164, Indonesia.
[2]Department of Biology, Faculty of Mathematics and Natural Sciences,
Brawijaya University, Gedung Biologi, Jl. Veteran, Malang 65145, Indonesia.
[3]Department of Postgraduate Program in Biotechnology, Postgraduate School,
Gadjah Mada University, Jl. Teknika Utara, Barek Yogyakarta 55281, Indonesia.
[4]Nitidipuran Life Science Studio, Jl. Nitidipuran 26, Ngupasan,
Gondomanan, Yogyakarta 55122, Indonesia.

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The efforts to produce bioethanol using non-crop raw material are recently developed. *Sargassum crassifolium* is a promising raw material for biofuel production since containing a high concentration of polysaccharides. Pre-treatment of seaweed at working pressure of 15 Psi, temperature of 121 °C and sulphuric acid concentration of 0.2 M increased the reducing sugars concentration up to 26.68 g/l. The application of cellulase (0.5 g/ml) helped increasing the reducing sugars concentration up to 2.56 times (increasing up to 68.32 g/l). Fermentation using a naturally β-glucosidase producing yeast, *Saccharomyces cereviceae* (JCM 3012) (0.15 g/ml), produced the final bioethanol conversion up to 90.99%. The activity of β-glucosidase increased up to 20.39 U/ml during fermentation that assisted the degradation of seaweed.

Key words: Bioethanol, cellulase, seaweed *Sargassum crassifolium*, *Saccharomyces cereviceae* JCM 3012, β-glucosidase.

Petroleum oil shortage has been a global issue potentially affecting the global economic and also social conditions[1]. Studies on renewable energies (including bioethanol) have long been carried out in order to replace the petroleum oil[2-4]. Meanwhile, bioethanol production is usually faced to a classical problem associated with "food versus fuel" issues[5]. The usage of non-food raw materials for the production of bioethanol is expected to become a solution to overcome the problem[6]. Morover, the abundance and the avaibility of raw materials is also another concern in the bioethanol production[6].

The availability of brown seaweeds (*Sargassum* sp.) in some tropical regions is abundant[7-10]. The use of *Sargassum* sp as a food ingridient is still relatively limited[7]. Thus, it may potentially overcome the "food versus fuel" problem. On the other hand, seaweeds (including *Sargassum* sp) can ecologically play an important role as a biosequester that utilizes the atmospheric carbon dioxide for the assimilation as effectively as ligno-cellulosic biomass[11]. However, *Sargassum* sp have a shorter life cycle as it needs 2 – 3 months

* To whom all correspondence should be addressed.

to crop in comparison to ligno-cellulosic biomass[12]. The structure of *Sargassum* sp. is composed of a complex of unique carbohydrate polymers[8, 13]. Degradation of these polymers may provide sugars that present as a precursor for fermentations and other biochemical syntheses[14-17]. Thus, this bio-resource is potential as a raw material for industrial purposes, including for the biotehanol production.

The aim of this research was to produce a bioethanol from the hydrolysates of *Sargassum crassifolium*. The production of bioethanol utilizing raw materials containing carbohydrates needs two major steps : (1) hydrolysis of raw materials; and (2) microbial fermentation. The rigid structure of *Sargassum crassifolium* still leaves problems [8]. Thus, such preparations were carried out which involve the application of pressure, high temperature, strong acid and hydrolytic enzyme. In some cases, the combination of pressure and high temperature is effective in assisting the degradation of ligno-cellulosic materials[8, 18]. Some researchers reported that the sulphuric acid is often used in the degradation of carbohydrate polymers into sugars that shows effective results[8, 17]. Meanwhile, in many cases, the application of cellulase (E.C. 3.2.1.4) helps the degradation of cellulosic carbohydrates (the type of carbohydrate dominating algal cell walls) and produces a high concentration of sugars[8, 14, 15, 17]. Thus, those preparations are expected to hydrolyze algal polysaccharides into sugars. The production of bioethanol was carried out using a β-glucosidase producing species, *Saccharomyces cereviceae* JCM 3012, which is limited to be reported by other researchers. The presence of β-glucosidase in the fermentation process may further help the cellulolytic degradation since the enzyme degrades smaller polymers of carbohydrates (disaccharides and oligosaccharides) released from the degradation of high molecular cellulose by cellulase8[19]. The conversion of high concentration of sugars by the yeast is expected to produce high concentration of bioethanol. The yield of ethanol fermentation was later evaluated.

MATERIALS AND METHODS

Materials

Brown seaweed (*Sargassum crassifolium*) was obtained from a local supplier in Gunung Kidul, the Special District of Yogyakarta, Indonesia. Commercial cellulase (with an activity of 30 U/g), available cellulose, 3,5-dinitrosalicylic acid, phenol, sodium sulphite, sodium hydroxide, potassium sodium tartrate, sulphuric acid, acetic acid, sodium acetate trihydrate, and casein were obtained from Sigma Co., (St. Louis, MO, USA). Glucose, cellobiose, cellohexose, agar, and dextrose were obtained from Merck KgaA (Darmstad, Germany). Potatoes were obtained from a local supplier. The culture of *Saccharomyces cereviceae* (JCM 3012) was obtained from The Food & Nutrition Culture Collection (FNCC), Food and Nutrition Centre, Gadjah Mada University.

Physical and Chemical Preparation of Seaweed

The fresh *Sargassum crassifolium* was firstly washed to clean the debris. About 10 g of *Sargassum crassifolium* was added into 100 ml destiled water. Concentrations of sulphuric acid (0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 M) were added into the mixture. The mixture were incubated in a pressurized chamber (Labtech LAC-5040 S, South Korea) with various working pressures (5, 10, and 15 Psi), at a temperature of 121 ^{o}C for 15 min. The treated eaweeds were later separated from sulphuric acid prior to homogenization in 100 ml de-mineralized water (25 ^{o}C) using a homogenizer (Philips 2115 P, The Netherlands) for 10 min. The reducing sugars were later calculated. Degradation products of cellulose were also calculated for both, pre-treated seaweed (*Sargassum crassifolium*) and untreated seaweed as the control.

Enzymatic Preparation of Seaweed

About 100 g/ml of the physically and chemically treated pulp of *Sargassum crassifolium* was added into various concentrations of cellulase (0, 0.1, 0.2, 0.3, 0.4 and 0.5 g/ml). The mixture was later incubated at a temperature of 30 ^{o}C, pH 5.5, 1500 rpm for 10 min. The reducing sugars and degradation products of cellulose were later calculated.

The Production of Bioethanol

Various cell concentrations (0.05, 0.1 and 0.15 g/ml) of *Saccharomyces cereviceae* (JCM 3012) was added into 100 g/l of the physically, chemically and enzymatically treated pulp of *Sargassum crassifolium*. The mixture was adjusted to anaerobic condition and incubated at a temperature of 30 ^{o}C, pH 5.5, 200 rpm for various fermentation periods (0, 12, 24, 36 and 48 hours).

The conversion of bioethanol from sugars, reducing sugars, CO_2 concentration and the activity of β-glucosidase were later calculated.

The Microbial Preparation

The cells of *Saccharomyces cereviceae* (JCM 3012) was firstly cultured in a 100 ml of CYG (Casein Yeast Glucose) medium and then incubated at a temperature of 30 °C, pH 5.5 for 24 hours. In order to make the stock, the yeast cells were streaked onto a PDA (Potato Dextrose Agar) medium and incubated a temperature of 30 °C, pH 5.5 for 3 days. The cells were later added into the fermentation process.

Analysis

The reducing sugars were determined using a Spectrophotometer (Genesys-20, USA) at 575 nm according to Miller[20], involving the 3,5-dinitrosalicylic acid as the reagent. Degradation products of cellulose were determined according to Girfoglio *et al*.,[21]. Bioethanol production was determined using a microdiffussion principle and then confirmed by gas chromatography[22, 23]. The conversion of bioethanol was determined as the percentage of bioethanol production from reducing sugars[8]. The CO_2 concentration were determined as the same amount of the formed bioethanol[8]. The activity of β-glucosidase was determined according to Nosworthy *et al*.,[19] as the release of one glucose molecule from one molecule of cellobiose.

RESULTS AND DISCUSSIONS

Physical and Chemical Preparation of Seaweed

The pre-treatment of *Sargassum crassifolium* at working pressures of 15 Psi, temperature of 121 °C and sulphuric acid concentrations of 0.2 M resulted in the increasing concentration of reducing sugar up to 26.68 g/l (Fig. 1). In general, Fig. 1 shows that the increasing working pressures and sulphuric acid concentrations resulted in the increasing reducing sugar concentrations. Meanwhile, the highest yield was obtained at a sulphuric acid concentration of 0.2 M (at all working pressures) (Fig. 1). It is suggested that the application of high temperature (121 °C) may expand glycosidic bonds linking sugar molecules that evolve seaweed polysaccharides[24]. The expanding bonds are potentially enable other substances from surroundings, including moisture (water) and sulphuric acid, to enter into the bonds[8, 14, 24]. On the other hand, the application of various working pressures (5, 10, and 15 Psi) may force water and sulphuric acid to enter into the bonds[18, 24, 25]. In the bonds, the sulphuric acid (acting as an H^+ donor) may disrupt glycosidic bonds by binding to an oxygen molecule linking two molecules of sugars[17, 24, 25, 26]. Furthermore, it creates a sugar/glucan-sulphuric acid intermediate[17, 24, 25, 26].On the other hand, the water dissociates into H^+ and OH^- [24]. The hydroxyl group (OH^-) of water may later attack the atom C of one of sugar/glucan molecules, while H^+ may attack the intermediate and release sugars/glucans and sulphuric acid[17, 24, 25, 26]. The disruption, consequently, resulted in the increasing reducing sugar concentrations (Fig. 1).

Table 1. Bioethanol production, the glucose conversion to bioethanol, the glucose consumption by cells, the remaining glucose in the reaction system of different fermentation processes incubated with different concentrations of inoculum at a temperature of 30 °C, pH of 5.5, agitated at 200 rpm for 12 h

Concentrations of inoculum	The initial concentration of glucose (g/l)	Bioethanol production (g/l)	The glucose conversion to bioethanol (g/l)	The glucose consumption by cells (g/l)	The glucose consumption by cells (%)	The remaining glucose in the reaction system (g/l)
Saccharomyces cereviceae JCM 3012 (0,05 g/ml)	68,32	30,21	30,21	17,27	25,28	20,84
Saccharomyces cereviceae JCM 3012 (0,1 g/ml)	68,32	37,24	37,24	19,19	28,09	11,89
Saccharomyces cereviceae JCM 3012 (0,15 g/ml)	68,32	43,92	43,92	23,31	34,12	1,09

The increasing sulphuric acid concentrations (0.2 – 0.6 M) resulted in a gradual decrease in reducing sugar concentrations (at all working pressures) (Fig. 1). It is suggested that the excess concentration of sulphuric acid in the reaction system may cause a sugars' dehydration that lose their functional structures[26, 27]. On the other hand, the combination of physical and chemical treatments dramatically decreased polysaccharides 2.99 times and also released relatively large amounts of oligosaccharides and monosaccharides by 29.85 % and 26.86 %, respectively (Fig. 2).

Enzymatic Preparation of Seaweed

The addition of cellulase at various concentrations (0, 0.1, 0.2, 0.3, 0.4 and 0.5 g/ml) into the pulp played a significant role in the hydrolytic reaction, dramatically increasing reducing sugar concentrations up to 2.56 times (increasing up to 68.32 g/l) (Fig. 3). The enzymatic treatment helped to reduce polysaccharides and oligosaccharides (3.5 times and 9.75 times, respectively); and furthermore released large amounts of disaccharides and monosaccharides (1.66 times and 2.56 times, respectively) (Fig. 2). It is suggested that the application of cellulase in the hydrolytic reaction may cleave ²-(1,4) glycosidic bonds within polysaccharides (cellulose) and oligosaccharides, and then release simpler sugars, in this case disaccharides and monosaccharides[14, 28].

The Production of Bioethanol

The production of bioethanol is shown in Fig. 4A, 4B and 4C and described as an ethanol conversion from reducing sugars. The final ethanol conversions increased as the increasing of yeast cells concentration (0.05, 0.1 and 0.15 g/ml) (Fig. 4A, 4B and 4C). It is suggested that the increasing concentration of yeast cells increases individual yeasts that naturally convert sugars into ethanol in the fermentation process[8, 29-33]. Figure 4A, 4B and 4C show a drastic increase in the ethanol conversion (44.22 – 64.28%) in the first 12 hours of the fermentation process (Fig. 4A, 4B and 4C). In this period, yeast cells consumed a high concentration of monosaccharides and converted to bioethanol (Fig. 4A, 4B and 4C)[29-33]. On the other hand, the activities of β-glucosidase were still low (1.09 - 1.81 U/ml) in the first 12 h of the fermentation process (Fig. 5). It is suggested that the yeasts

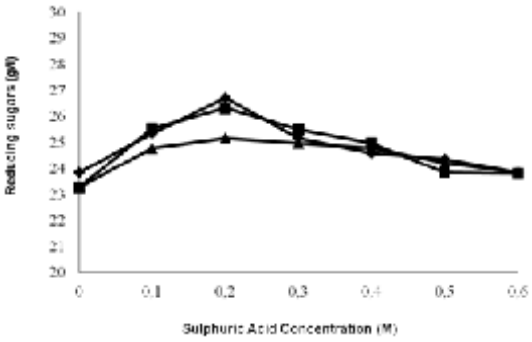

Fig. 1. Reducing sugars concentrations resulted from the preparation of seaweed (*Sargassum crassifolium*) using a combination of various sulphuric acid concentrations and various working pressures: 5 Psi (▲); 10 Psi (■) and 15 Psi (◆) at a temperature of 121 °C for 15 min

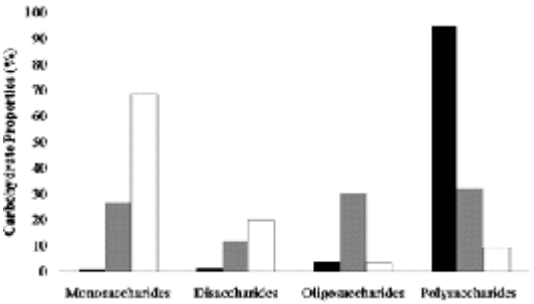

Fig. 2. The properties of degradation products of carbohydrates after such treatments : without treatment (black), physical and chemical treatments (grey) and physical, chemical and enzymatic treatment (0.5 g/ml) (white)

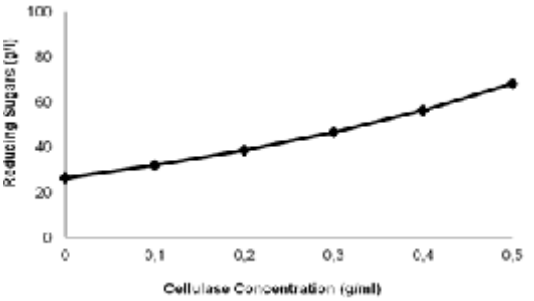

Fig. 3. Reducing sugars concentration produced from the application of various cellulase concentrations to seaweed pulp at a temperature of 30 °C, pH of 5.5, 1500 rpm for 10 min. The pulp was previously treated at a working pressure of 15 Psi, temperature of 121 °C and sulphuric acid concentration of 0.2 M for 15 min

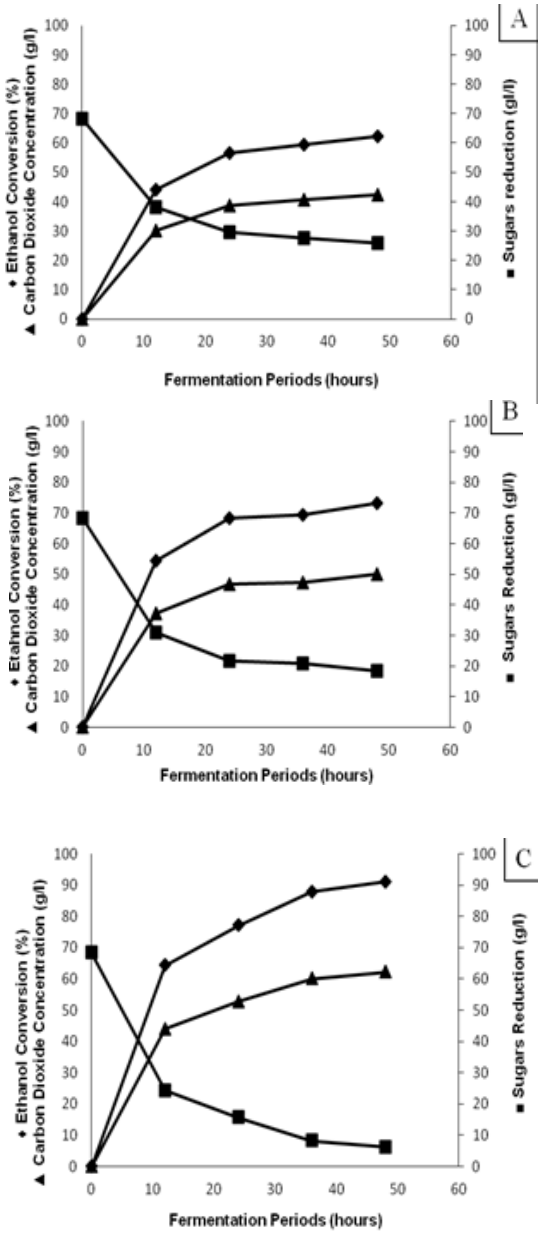

Fig. 4. Ethanol conversion (◆), carbon dioxide (▲), and reducing sugars concentration (■) during a fermentation process incubated with a β-glucosidase producing yeast *Saccharomyces cereviceae* (JCM 3012) at various concentrations: 0.05 g/ml (A), 0.1 g/ml (B) and 0.15 g/ml (C) at 30 °C, pH 5.5, 200 rpm for various fermentation periods. Seaweed (*Sargassum crassifolium*) pulp was previously pre-treated at a working pressure of 15 Psi, temperature of 121 °C, sulphuric acid concentration of 0.2 M for 15 min; followed by the application of cellulase (0.5 g/ml) at a temperature of 30 °C, pH of 5.5 for 10 min

may still use monosaccharides and may have not secreted the higher amount of β-glucosidase to degrade higher molecular sugars (disaccharides, oligosaccharides, and polysaccharides) in the fermentation process.

A 12 hours fermentation of glucose was separately carried out to determine the glucose conversion to bioethanol and glucose consumption by cells (table 1). The increasing amount of yeast cells (0.05 – 0.15 g/ml) converted glucose to bioethanol (30.21 – 43.92 g/l) and consumed glucose for their cells (25.28 – 34.12%) (table 1). The consumption may consequently impact the amount of the remaining glucose in the reaction system at the end of 12 hours fermentation process (table 1)[29-33]. Thus, yeasts may show different responses to the remaining glucose concentration in the system. The lowest remaining glucose concentration (1.09 g/l) was obtained from the fermentation of glucose (68.32 g/l) at a yeast incubation of 0,15 g/ml for 12 hours (table 1). On the other hand, disaccharides (19.61 %), oligosaccharides (3.06 %) and polysaccharides (9.01 %) are type of sugars (other than monosaccharides) produced after the enzymatic treatment (Fig. 2). In this condition, yeast may start to use another forms of sugar (disaccharides, oligosaccharides and polysaccharides) and secrete β-glucosidase (E.C. 3.2.1.2.1) to help the degradation. Thus, the activity of β-glucosidase in the fermentation at a yeast incubation of 0.15 g/ml was drastically increasing in a period from 12 to 24 hours (increasing from 1.81 U/ml to 20.39 U/ml) (Fig. 5). As β-glucosidase degraded, yeast

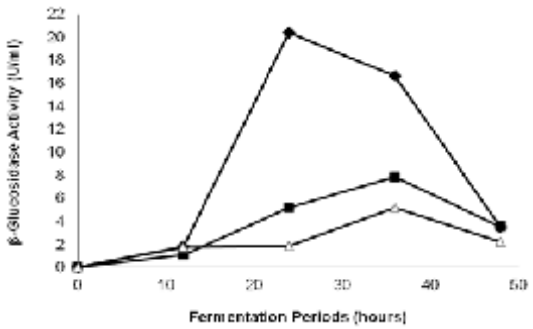

Fig. 5. The activity of β-glucosidase in the fermentation process incubated with 0.05 g/ml (Δ); 0.1 g/ml (■) and 0.15 g/ml (◆) yeast cells at a temperature of 30 °C, pH of 5.5, agitated at 200 rpm for 48 h.

consumed and converted sugars to bioethanol. Thus, bioethanol conversion increased gradually, although not as drastically as the conversion in the first 12 h of fermentation. The conversion later culminated at 90.99% at the end of fermentation (Fig. 4C). On the other hand, yeasts consumed less glucose and left higher remaining glucose concentrations in the system in the fermentation of glucose (68.32 g/l) at a yeast incubation of 0.05 and 0.1 g/ml for 12 hours (table 1). Thus, yeast may still consume the remaining glucose and secrete a little amount of β-glucosidase. Fig. 5 shows that the activities of β-glucosidase in the fermentation at a yeast incubation of 0.05 and 0.1 g/ml from 12 to 24 hours were much lower than that of at 0.15 g/ml. The highest β-glucosidase activities in the fermentation at a yeast incubation of 0.05 and 0.1 g/ml were obtained at 36 hours as yeast may start to use another forms of sugar (disaccharides, oligosaccharides and polysaccharides). The ethanol conversions gradually increased and culminated at 62.22% and 73.16% for the yeasts incubation at 0.05 and 0.1 g/ml, respectively (Fig. 4A and 4B).

On the other hand, the activity of β-glucosidase decreased up to 5.77 times (from 20.39 U/ml to 3.53 U/ml) from 24 to 48 hours in the fermentation at a yeast incubation of 0.15 g/ml. It is suggested that the decrease was presumably caused by a collaborative effect of the reducing concentration of disaccharides in the reaction system and the presence of high concentration of ethanol in the reaction system. The activity of β-glucosidase was calculated based on the conversion of disaccharides to monosaccharides by β-glucosidase. As the reducing concentration of disaccharides in the reaction system, β-glucosidase may naturally start to degrade larger polymers of carbohydrate, such as oligosaccharides and polysaccharides[19,34]. However, oligosaccharides and polysaccharides are structurally more complex than disaccharides[34]. Thus, β-glucosidase may take a relatively longer period to release monnosaccharides from oligosaccharides or polysaccharides rather than from disaccharides. Furthermore, it may decrease the rate of sugars production from those polymers and also consequently decrease the activity of β-glucosidase[34]. On the other hand, the high accumulation of product (ethanol) in the medium may inhibit metabolic enzymes, including β-glucosidase, by forcing the water within enzymes to diffuse to the outside[4]. The mechanism may further cause β-glucosidase unstable and decrease its activity [4]. Meanwhile, the activities of β-glucosidase at yeast incubation of 0.05 and 0.1 g/ml from 24 hour to 36 hour were increasing and both culminated at 36 h. Although increasing and then culminated at 36 hour, the activities of β-glucosidase at yeast incubation of 0.05 and 0.1 g/ml at 36 hours were much lower than that of at 0.15 g/ml at 36 h (Fig. 5). It is suggested that the accumulation of ethanol may influence the stability of β-glucosidase.

The final bioethanol conversion (90.99%) is slightly higher than that of Borines *et al.*,[8]. The final ethanol conversion (89%) was achieved from a 48 hours fermentation process using *Sargassum* hydrolysate which was previously prepared using sulphuric acid, heat and in combination with the application of cellulase and β-glucosidase (E.C. 3.2.1.2.1) (Borines *et al*., 2013). The application of β-glucosidase may provide a higher concentration of monosaccharides which more readily to covert into ethanol by yeasts[8]. There is a principal difference in employing β-glucosidase. Borines *et al.*,[8] added the enzyme at beginning of the reaction. Meanwhile, this research used a natural β-glucosidase producing yeast, *Saccharomyces cereviceae* (JCM 3012), during the fermentation. The application of microbial cells producing particular enzymes in the production of bioethanol is expected to be more efficient since the pre-treatment and the fermentation may work simultanously[35-38]. The propagating cells may continuously produce a higher amount of enzymes in the reaction system that may potentially reduce the cost for the additional enzymes in the biomass pre-treatment[35-38]. On the other hand, this research produced a higher final ethanol conversion (90.99%) than that of in some other works using seaweeds and lignocellulosic materials[25, 29-33, 39-41].

CONCLUSIONS

The application of working pressure (15 Psi), high temperature (121 ^{o}C) in combination with sulphuric acid concentration (0.2 M) in the pre-treatment of *Sargassum crassifolium* increased the reducing sugars concentration up to 26.68 g/l. The

addition of cellulase (0.5 g/ml) into the physically and chemically treated *Sargassum crassifolium* pulp increased the reducing sugars concentration up to 2.56 times (increasing up to 68.32 g/l). The fermentation using *Saccharomyces cereviceae* (JCM 3012) (0.15 g/ml) produced the highest bioethanol conversion (90.99%). The highest bioethanol conversion was achived due to the help of β-glucosidase activity that increased up to 20.39 U/ml during fermentation.

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