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Bioethanol a by-product of agar and carrageenan production industry from the tropical red seaweeds, *Gracilaria manilaensis* and *Kappaphycus alvarezii*

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Abstract

The two red seaweeds, *Gracilaria manilaensis* and *Kappaphycus alvarezii* are cultivated in Malaysia for producing agar and carrageenan, respectively. The residues of these seaweeds after removing agar and carrageenan by dilute acid treatment were subjected to enzymatic hydrolysis using cellulase (Cellic CTec 2). In the optimization of enzymatic hydrolysis, highest glucose concentration was achieved in the sample with liquid: residue ratio of 7.5:1 and enzyme loading of 10 % w w^{-1} residue. The resulting glucose was converted to bioethanol using *Saccharomyces cerevisiae* where bioethanol yields from *G. manilaensis* and *K. alvarezii* were 56.26±1.10 g L^{-1} and 51.10±1.21 g L^{-1}, respectively, which corresponded to 91 % and 95 % of the theoretical yield. The results of this study indicate that the residues of these seaweeds can be efficiently converted to bioethanol and besides environmental benefits, additional profit can be achieved in the phycocolloid industry.

Keywords: Bioethanol, Cellulase, Agar residues, *Gracilaria manilaensis, Kappaphycus alvarezii*

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Introduction

In recent years, biofuels have attracted great attention as alternatives to fossil fuel. Successful commercial production and usage of sugarcane-based ethanol in Brazil has given hope to replace fossil fuel from sustainable resources. However, there is a drawback, as sugarcane is mainly used as a food crop. Other raw materials used for bioethanol production are sugarcane bagasse (Cardona *et al.*, 2010; Rabelo *et al.*, 2011), rice straw (Karimi *et al.*, 2006; Ko *et al.*, 2009), switchgrass (Pimentel and Patzek, 2005; Tao *et al.*, 2011), forest wood and residues (Goldemberg, 2007; Hu *et al.*, 2008), but the presence of lignin and crystalline structure of cellulose in these materials made them resistant to mild treatment conditions and pretreatment is necessary to remove lignin and increase accessibility of enzyme to cellulose (Taherzadeh and Karimi, 2007b). However, applying such treatments increases the energy consumption of the process, which would consequently increase the cost of the end product. The industrial scale of such production systems may compete for arable land and consume more fresh water. Furthermore, excessive land crop cultivation for biofuel has been shown to increase in greenhouse gas emissions which will have negative effects on the environment (Hertel *et al.*, 2010; Plevin *et al.*, 2010; Davis *et al.*, 2011).

Macroalgae such as green algae, brown algae, and red algae which basically occupy the littoral zone (Kim, 2011) have relatively simple structures and life-histories, but have efficient photosynthesis and high biomass productivity (Hillson, 1977; Bold and Wynne, 1978). Macroalgae also have very low lignin content (Wi *et al.*, 2009) or even none in some species (Ge *et al.*, 2011). They are cultivated in the sea, have no competition with terrestrial crops, arable land, food and fresh water, and may be more suitable feedstocks for biofuel production.

Dilute acid hydrolysis is a common method applied to hydrolyze seaweed biomass but this method has its drawbacks including degradation of sugar to fermentation inhibitors (Taherzadeh and Karimi, 2007a). Enzymatic saccharification is another approach with lower negative side-effects, however the bottleneck of applying enzyme is its higher cost compared to chemical hydrolysis. To conduct enzymatic hydrolysis, the enzymes must be able to access the molecules to hydrolyze them and the crystalline structure of cellulose must be reduced to increase the access of enzyme to molecules using various conditions (Badger, 2002). Cellulase enzymes are highly specific catalysts which act under mild conditions (e.g. pH 4.5-5.0 and temperature 40 to 50 °C). This allows for low corrosion of equipment, low energy consumption and also the low toxicity of the hydrolysates (Taherzadeh and Karimi, 2007b). This process is performed by the synergistic action of at least three major classes of enzymes: endo-glucanases, exo-glucanases, and ß-glucosidases. These enzymes are usually called together as cellulase or cellulolytic enzymes. The

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endoglucanases create free chain-ends. The sugar chain is degraded by exoglucanases by removing cellobiose from the chain and ß-glucosidases cleave the cellobiose disaccharides to glucose (Wyman, 1996). The liberated glucose, later can be assimilated to bioethanol by fermentation process. Currently, extensive studies have been conducted to use enzymes for hydrolyzing seaweed biomass to reduced sugars. Some of these efforts are reviewed in Table 1.

Table 1: Comparison of enzymatic treatments in the saccharification of selected seaweeds.

Seaweed spp.	Type	Target polymer	Enzyme/ Enzyme Conc.	Condition pH/time/temp	RS g L^{-1}	Yield	Reference
Ulva fasciata	Chl	Cellulose	Cellulase / 2% (v v^{-1})	5 / 36 h/ 45 °C	N.A	0.2 g g^{-1} SW	Trivedi *et al.*, (2013)
Ulva rigida	Chl	Starch Cellulose	amyloglucosidase α-amylase, cellulase	5/ 48 h / 37 °C	N.A	0.19 g g^{-1}SW	Korzen *et al.*, (2015)
Ulva pertusa	Chl	Cellulose, starch	Meicelase/ 5g L^{-1}	N.A/ 120h / 50 °C	43	0.82 g g^{-1} glucan	Yanagisawa *et al.*, (2011)
Alaria crassifolia	Phy	Cellulose, starch	Meicelase/ 5 g L^{-1}	N.A/ 120h / 50 °C	67	0.58 g g^{-1} glucan	Yanagisawa *et al.*, (2011)
Saccharina japonica	Phy	Starch	Termamyl 120 L (Amylase)	N.A	20.6	0.31 g g^{-1} CHD	Jang *et al.*, (2012)
Nizimuddinia zanardini	Phy	Cellulose	Cellulase b-glucosidase	4.8/ 24h / 45 °C	N.A	0.07 g g^{-1} SW	Yazdani *et al.*, (2011)
Laminaria japonica	Phy	Cellulose	Cellobiase 55 CBU g^{-1} Cellulase 45 FPU g^{-1}	4.8/ 48h / 50 °C	34	0.24 g g^{-1} SW	Ge *et al.*, (2011)
Laminaria japonica, Caulerpa sp.	Phy, Chl	Alginate	Rapidase/Viscozyme /dextrozyme	N.A	8.3	N.A	Choi *et al.*, (2009)
Gracilaria salicornia	Rhd	Cellulose	Cellulase/ 0.5 % w v^{-1}	5/ 30h / 50 °C	N.A	0.013 g g^{-1} wet Biomass	Wang *et al.*, (2011)
Gelidium elegans	Rhd	Cellulose, starch	Meicelase/ 5g L^{-1}	N.A/ 120h / 50 °C	49	0.67 g g^{-1} glucan	Yanagisawa *et al.*, (2011)
Gracilaria verrucosa	Rhd	Cellulose	Cellulase/ 20 FPU g^{-1} SW b-glucosidase/ 60 U g^{-1} SW	5/ N.A / 50 °C	40	0.87 g g^{-1} cellulose	Kumar *et al.*, (2013)
Kappaphycus alvarezii,	Rhd	Cellulose	Cellulase 45 FPU g^{-1}	5/ 24h / 50 °C	90	0.76 g g^{-1} cellulose	Hargreaves *et al.*, (2013)

Chl: Chlorophyta, Rhd: Rhodophyta, Phy: Phaeophyta, Conc: Concentration, min: minutes, N.A: Not Available, SW: seaweed biomass, Temp: Temperature, RS: Reducing sugar, FPU: Filter Paper Unit, one One international filter paper unit (FPU) was defined as the amount of enzyme that releases 1 mmol glucose per minute during hydrolysis reaction.

Kappaphycus alvarezii, which is commercially known as "cottoni" among the farmers, is the main source of kappa carrageenan, while *Eucheuma denticulatum*, recognized as "spinosum" is the main source of iota carrageenan. Both of these seaweed species are responsible for approximately 88% of raw material processed for carrageenan production, yielding about 120,000 dry tons $year^{-1}$ mainly from the Philippines, Indonesia, and Malaysia (McHugh, 2003). Generally, global distribution of *Kappaphycus* farming is between ±10° latitude, specifically from the Southeast Asian countries extending to East Africa and Brazil. The highest share of the *Kappaphycus* production occurs among the Brunei–Indonesia–Malaysia–Philippines region, known as BIMP-EAGA, the growth area that provides about 60% of the global production of *Kappaphycus* (Israel *et al*., 2010).

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Agar is another valuable phycocolloid obtained from two families of red seaweeds, Gracilariaceae and Gelidiaceae (Armisen and Galatas, 1987). This phycocolloid is widely used in the pharmaceutical, cosmetics and food industries. Agar is a mixture of the pyruvated agarose, neutral polymer agarose and sulfated galactans (Marinho-Soriano and Bourret, 2003). According to statistics (FAO, 2016), the annual cultivation of *Gracilaria* has an increasing trend in which 3.7 million tons wet weight of *Gracilaria* was produced in 2014. The world carrageenan production increased from less than 1 million wet tones in 2000 to more than 9 million wet tones in 2014. The increasing demand for phycocolloid, will promote environmental concerns over the industrial wastes from the seaweed processing. In this study, we evaluated the potential production of bioethanol from the cellulosic residues of *G. manilaensis* (agarophyte) and *K. alvarezii* (carraginophyte) after extraction of agar and carrageenan.

Materials and methods

Extracting cellulosic residues

Fig. 1 shows the two red seaweeds used in this study. Phycocolloid materials were extracted from *G. manilaensis* and *K. alvarezii* (100 g DW) according to standard procedures, where 6% KOH and 6% NaOH (2000 mL) were used to extract kappa and iota carrageenan respectively, after incubation for 2 h at 80 °C (Istini *et al.*, 1994) while agar was extracted by boiling *G. manilaensis* samples at 105°C at a pH of 6.5 for 2 h (Marinho-Soriano *et al.*, 1999). The residues from both seaweeds were collected, washed with dilute water and dried using a freeze drier (Thermo) for 48 h. The amount of residues was calculated based on 100 g initial DW of each seaweed in triplicate. Ash content was determined by combusting 2 g DW samples (triplicate) in a muffle furnace at 550 °C for 5 hours (AOAC, 2000) using the equation as below:

$$Ash\ content\ \%\ DW = \frac{(Ash+Crucible) - Crucible}{DW} \times 100$$

Figure 1: Whole seaweed plants, A: *Gracilaria manilaensis*, B: *Kappaphycus alvarezii*.

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Enzymatic hydrolysis

i. Optimization of the enzyme dosage

The dried *G. manilaensis* residue (2 g) was soaked in 20 mL of 0.1 M sodium citrate buffer pH 5 (in 50 mL blue cap bottles) and non-ionic surfactant Tween 80 (0.1% v/v) to prevent unproductive binding of the enzyme to the residue, which was used in all experiments (Alkasrawi *et al.*, 2003). The bottles were autoclaved for 15 min at 121 °C. Enzyme (Cellulase, Cellic, CTec2, Novozyme, Denmark) was added to each sample to make up to the concentration of 2%, 5%, 10% and 20% g g^{-1} biomass and by adding distilled water, the liquid volume was adjusted to 40 mL. This enzyme is a commercial cellulase preparation which includes two main cellobiohydrolases, five different endo-1,4-β-glucanases, β-glucosidase and also specific hydrolysis-boosting proteins (Manns *et al.*, 2015). The samples were incubated for 72 h at 50 °C on a shaker incubator at a speed of 170 rpm (Manns *et al*., 2014). The optimum pH and temperature applied in this study was according to the enzyme supplier protocol (Cellic CTec 2, Novozyme). Samples were withdrawn periodically, centrifuged for 10 min at 4000 rpm and the supernatant was subjected to analysis of reducing sugar contents using 3, 5-dinitrosalycylic acid (DNS) method (Miller, 1959).

ii. Optimization of liquid: biomass ratio

After the optimization of enzyme dosage, the liquid volume to biomass ratio was optimized. To conduct this, dried residue (2 g) was hydrolysed with the optimum enzyme dosage of 10 % w w^{-1}, at temperature of 50 °C, pH 5 and in different liquid to biomass ratios ranging from 1:2.5, 1:5, 1:7.5 to 1:10. After 48h of hydrolysis treatment, the reducing sugar contents were measured using the DNS method.

iii. Preparation of enzymatic hydrolyzate

The residues after phycocolloid extraction from *G. manilaensis* and *K. alvarezii* were washed and the pH was adjusted to 5, followed by drying for 2 days, using a freeze drier (Modulyo, Thermo, USA). Then the residues were characterized for DW and ash content.

7g of residue from each seaweed species was transferred to 100 mL serum bottle followed by the addition of 45 mL of 0.05M citrate buffer pH 5 and 0.1% Tween 80 as a surfactant. The samples were autoclaved for 15 min at 121 °C and cooled to room temperature. The cellulase was added to each sample to provide the required enzyme dosage (10 % w w^{-1} residue). To prevent unproductive binding of the enzyme to residues the non-ionic surfactant Tween 80 (0.1% v v^{-1}) was used in all experiments (Castanon and Wilke, 1981; Alkasrawi *et al.*, 2003). The final volume of serum bottles was adjusted to 50 ml by addition of 0.05 M citrate buffer. Samples were incubated on a shaking incubator (170 rpm) for 48 h at 50 °C. The hydrolysate was filtered under aseptic conditions and transferred into clean 100 mL serum bottles for further procedures.

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Fermentation study

The hydrolysates of the residues were fermented to ethanol using *Saccharomyces cerevisiae* (Ethanol Red, Fermentis, France). The medium was enriched with yeast extract 0.6% (Khambhaty *et al.*, 2012). The pH was adjusted (pH5) using 0.05 M sodium citrate buffer followed by inoculation with adapted yeast (Ethanol Red) to achieve 1.5×10^7 CFU m L^{-1} (Khambhaty *et al.*, 2012). The samples were incubated for 3 days at 30 °C and 150 rpm in a shaking incubator.

Sampling was conducted at 6, 12, 24, 48, 36 h after inoculation and taken samples were kept in a freezer at -20 °C.

Determination of bioethanol concentration using gas chromatography

The concentration of bioethanol was measured by Gas Chromatograph (GC) (Agilent 5820-A, Agilent Inc., USA) equipped with a split/splitless inlet, a flame ionization detector (FID) and a capillary column (HP-Innowax 30m, 0.32mm, 0.15um). The temperature programming of the GC analysis was as follows: 230 °C as injector and 230 °C as detector temperature, the column was held at 70 °C for 7 min and then the temperature was increased at a rate of 25°C min^{-1} to 220 °C and then held for 10 min; helium at 3 mL min^{-1} was used as the carrier gas, the flow rates for the FID were 40 mL min^{-1} for the makeup gas (He), 40 mL min^{-1} for hydrogen, and 450 mL min^{-1} for air with a split ratio of 1:100 and injection sample size of 1 µL. The ethanol yield was calculated according to the equation:

$$Y_{EtOH} = \frac{[EtOH]\,max}{[Sugar]\,ini}$$

Where Y_{EtOH} = ethanol yield (g g^{-1}), $[EtOH]_{max}$ = maximum ethanol titer achieved during fermentation (g L^{-1}), $[Sugar]_{ini}$ = total initial glucose content (g L^{-1}).

Results

Seaweed residues after extraction of phycocolloid

In this study, 13.81±0.54 g and 13.07±2.18 g residues were obtained from 100 g of dried matter of *G. manilaensis* and *K. alvarezii*, respectively. Ash content (%) and ash free DW (g) of the residue samples are given in Table 2. 7 g of the residue was used for enzymatic hydrolysis, which contained 6.90±0.73 of ash free DW of *G. manilaensis* and 6.38±0.43 g of ash free DW of *K. alvarezii*.

Optimization of the enzyme dosage

Fig. 2 illustrates the hydrolysis of *G. manilaensis* residues by different ratios of cellulase enzyme (Cellic CTech 2). In general, after 48 h of hydrolysis, the highest amount of hydrolytic conversion (87.5 %) was observed in 20% w w^{-1} enzyme treatment, which did not show any significant difference (p>0.05) with 10% w w^{-1} enzyme treatment (85.5%). The highest enzymatic hydrolytic conversion yields for 2 % (82.5 %) and 5% (87.5%) w w^{-1} of enzyme concentration were achieved after 72 h incubation.

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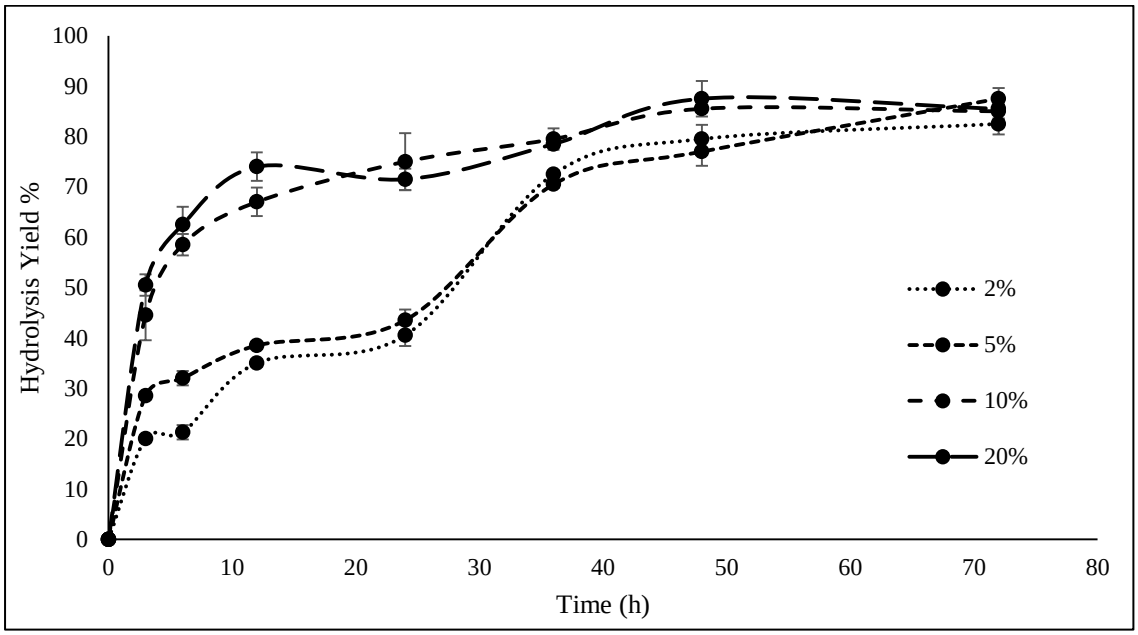

Figure 2: Enzymatic hydrolysis of *Gracilaria manilaensis* residues by different cellulase concentration (w/w residue) loading.

Optimization of liquid: residue ratio

Fig. 3 shows the effect of the liquid:residue ratio on enzymatic hydrolysis yield and glucose concentration in the hydrolysate. At the ratio of 2.5:1, no reducing sugar was produced. A total of 5.31% w w^{-1} glucose was produced after 3 days incubation in sample with a liquid:biomass ratio of 5:1. The highest hydrolysis yield was achieved in liquid:residue ratio of 10:1 where 85.12 % of residue was converted to glucose, whereas highest glucose concentration (20.89 % w v^{-1}) was achieved in the sample with liquid: residue ratio of 7.5:1.

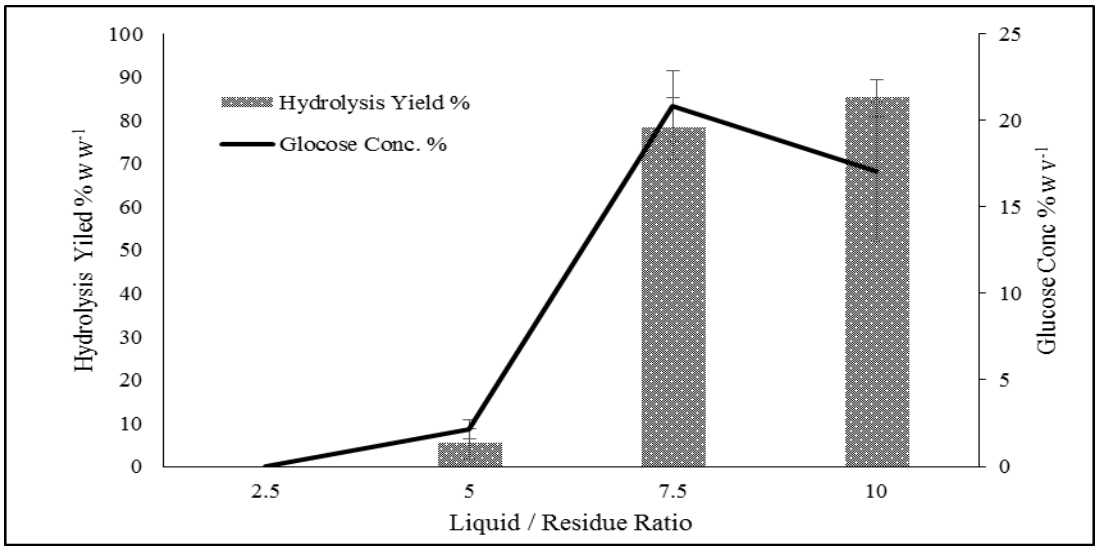

Figure 3: Effect of ratio of liquid to residue (*Gracilaria manilaensis* residues) on hydrolysis yield and final glucose concentration.

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Preparation of seaweed hydrolysate for fermentation study

The results of enzymatic hydrolysis of the two seaweed sample residues are shown in Table 2. About 7 g of residue from each seaweed sample was used to calculate the potential amount of sugar produced but the final calculation was based on 100 g DW of seaweed (Table 3).

Table 2: Results of enzymatic hydrolysis of residues of two seaweeds.

	K. alvarezii	***Gracilaria manilaensis***
Residue used (g)	7.00±0	7.00±0
Ash %	8.75±0.54 ns	7.03±0.19 ns
Ash Free DW (g)	6.38±0.43 ns	6.90±0.73 ns
Used Buffer (mL)	50±0	50±0
Recovered Hyd (mL)	44.17±2.25 ns	45.77±1.66 ns
Hyd Sugar Conc (g L^{-1})	120.33±10.97 ns	105.67±4.16 ns

ns: Not Significant at t-Test analysis $p>0.05$

Table 3: Calculated values of enzymatic hydrolysis of residues form two seaweeds obtained from 100 g DW biomass.

	Kappaphycus alvarezii	***Gracilaria manilaensis***
Residues (g)	13.07±2.18 ns	13.81±0.54 ns
Produced Sugar (g) *	9.94±1.38 ns	9.54±0.32 ns
Sugar Yield (g g^{-1} Residue AFDW)*	0.82±0.01 ns	0.74±0.01 ns

ns: Not Significant with t-Test analysis at p>0.05, AFDW: Ash free dry weight
*The values are calculated based on saccharification yield of 7.00 g DW residue in Table 2

Fermentation of enzymatic hydrolysate

In the fermentation of seaweed hydrolysate, yeast or bacteria consume reduced sugar to produce bioethanol through an anaerobic process. *S. cerevisiae* is the most well established microorganism used in anaerobic fermentation. Fig. 4 shows the profile of reducing sugar consumption and bioethanol production in *G. manilaensis* (Fig. 4a) and *K. alvarezii* (Fig. 4b). The initial reducing sugar concentration in *K. alvarezii* hydrolysate was 120.33±10.97 g L^{-1} and after 48 h of incubation, the highest (56.26±1.10 g L^{-1}) bioethanol concentration was achieved. While the highest bioethanol content of 105.67±4.16 g L^{-1} of *G. manilaensis* was achieved after 36 h of incubation (51.10±1.21 g L^{-1}). The fermentation efficiency was 91 % and 95 % of theoretical yield in *K. alvarezii* and *G. manilaensis,* respectively.

In the present study, all sugar contents of both seaweed species were consumed to produce bioethanol but for *K. alvarezii* this happened after 48 h, while no reducing sugar was detected in *G. manilaensis* at the 72 h sampling time.

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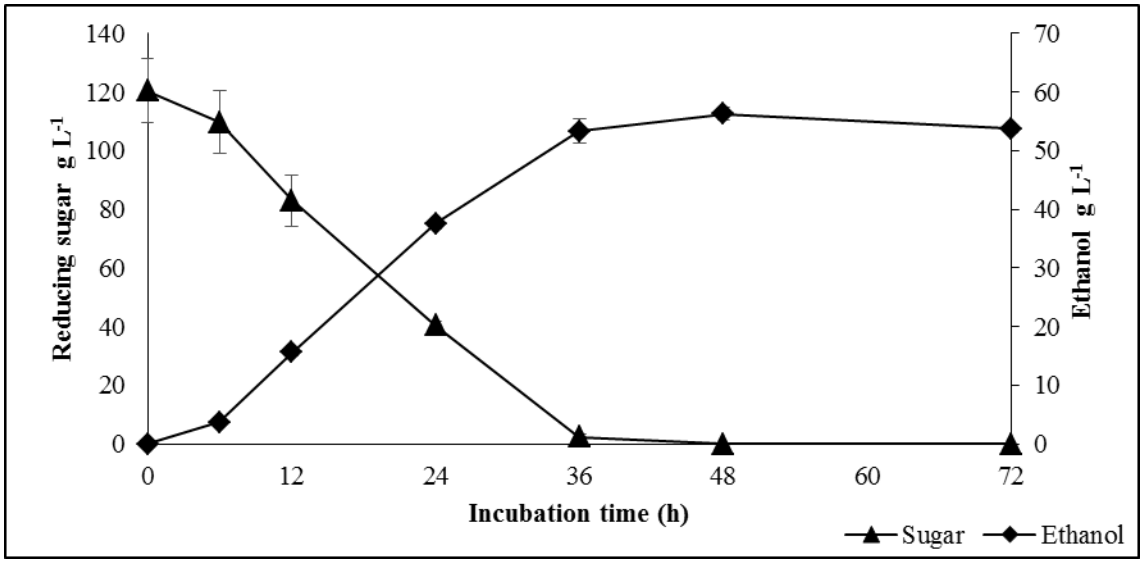

Figure 4a: Fermentation with enzymatic hydrolysate of *Gracilaria manilaensis.*

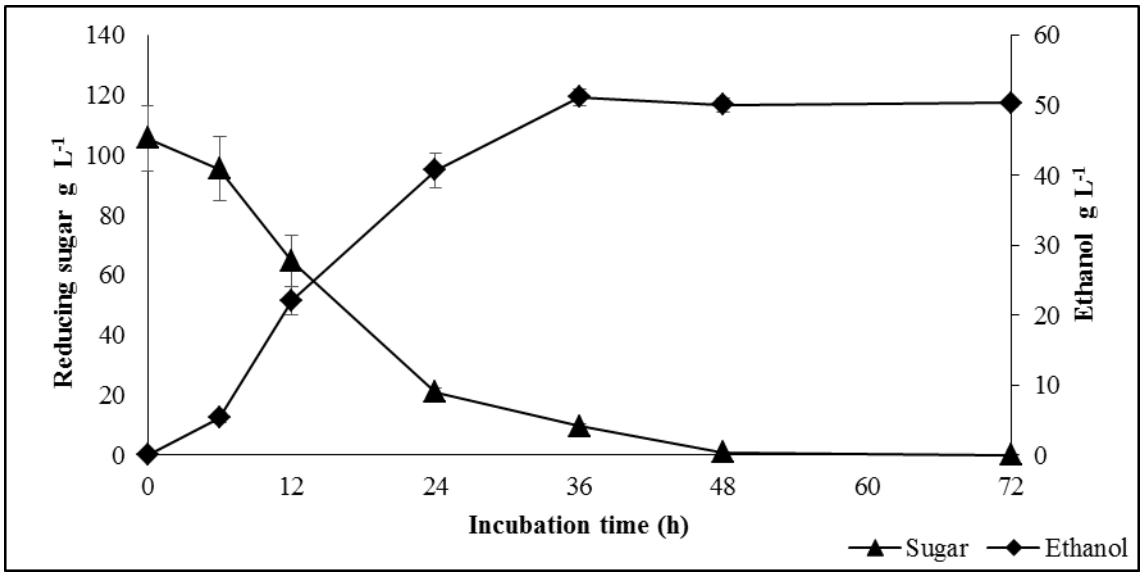

Figure 4b: Fermentation with enzymatic hydrolysate of *Kappaphycus alvarezii.*

Discussion

Phycocolloid production is a growing industry, dominated by a few Asian countries, namely China and Indonesia which are producing over 80 % of the total seaweed production (FAO, 2014). The residues after phycocolloid extraction can be an environmental threat if the rich organic waste material is not properly managed and discharged to rivers or coastal waters (Rabiei *et al.*, 2014). In this study, we used residues of the two main seaweeds, *G. manilaensis* (agarophyte) and *K. alvarezii* (carageenophyte), used for phycocolloid production in Malaysia.

The mean percentage of residues obtained after phycocolloid extraction from *K. alvarezii* and *G. manilaensis* were 13.07± 2.18 and 13.81± 0.54 % w w^{-1} DW, respectively and no significant differences was seen (p>0.5). It is reported that 125 k tons/year of *K. alvarezii* is produced in Malaysia (Galid, 1999).According to Tan and Lee (2014), 81.28 k tons of residue is

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produced annually which corresponds to 65 % w w^{-1} DW, which is far higher than residues obtained in the present study. In another study on *Gracilarila verrucosa,* 23-26 % pulp after agar extraction was achieved (Kumar *et al.*, 2013) although it was reported that about 15% w w^{-1} of seaweed biomass is holocellulosic material which is in accordance with our residue yields (13.07±2.18 and 13.81 ±0.54 % w w^{-1} DW).

The amount of ash in both seaweed residues was not high (7.03±0.19 - 8.75±0.54 %DW) in comparison with the ash content in whole plants of *K. alvarezii* (19-27 % DW) (Abd-Rahim *et al.*, 2014; Kumar *et al.*, 2015) and ash content of *Gracilaria* (19- 22.7 % DW) (Norziah and Ching, 2000; Krishnaiah *et al.*, 2008). Because much of the salts and debris are removed during the phycocolloid extraction process.

Dilute acid conversion is the most feasible technology for generating reduced sugars. This technology represents the best commercialization option (Kaylen *et al.*, 2000). Moreover, dilute-acid hydrolysis is an inexpensive process compared to the enzymatic process. However, the dilute acid hydrolysis may result in the generation of fermentation inhibitors, low efficiency and involves expensive equipment to withstand acidic conditions and high temperature (Taherzadeh and Karimi, 2007a). Thus, we applied enzymatic hydrolysis in this study.

One of the challenges in producing bioethanol is obtaining a high final concentration in the fermented mash, as the distillation of low content of ethanol (<5%) is not economically feasible (Fan *et al.*, 2003; Lu *et al.*, 2010). Therefore, in order to achieve acceptable ethanol content, optimization of the enzymatic hydrolysis is conducted through the manipulation of enzyme to biomass ratio, and the liquid to residue ratio, during hydrolysis. This is advantageous to lower the enzyme consumption or to recycle the enzyme to make the enzymatic hydrolysis an economically feasible process for industrial applications (Tu *et al.*, 2009; Weiss *et al.*, 2013; Jordan and Theegala, 2014).

An enzyme concentration of 10% w w^{-1} residue, produced significantly ($p \leq 0.05$) higher sugar conversion yield (75 %) (Fig. 2) compared to the treatment with 2 and 5% enzyme after 24 h of incubation, and was selected for further studies. However, all enzyme concentrations produced the same amount of glucose after 72 h incubation. Other studies reported lower dosage of the enzyme, where 2% enzyme was used for a period of 36 h and temperature of 45 °C, to hydrolyze *Gracilaria dura* and obtain 0.91 g glucose g^{-1} substrate (Baghel *et al.*, 2015).While in another study, 2%, 5%, 10%, 15% and 20% cellulolytic enzyme (Cellic CTec 2) was used to investigate optimum dosage of enzyme (Manns *et al.*, 2015). They reported that, 10% enzyme is the optimum dosage to release maximum glucose (47.5 % of DW) in *Laminaria digitata* and further increase in enzyme dosage to 15 and 20 % did not increase the glucose yield. The ratio of liquid:solid of 7.5:1 gave

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the highest glucose concentration (20.82% w v^{-1}), which is 78.11 % of biomass converted to glucose. Hargreaves *et al.*(2013) used a 5.5:1 ratio and obtained a glucose concentration of 9.23 % w v^{-1} which is lower than our result. Mechanical agitation during enzymatic hydrolysis provided better contact between substrate and enzyme (Radhakrishnaiah *et al.*, 1999). The addition of more surfactants, such as Tween 80 or Tween 20 (Börjesson *et al.*, 2007), may also increase the process efficiency when substrate loading is high (Taherzadeh and Karimi, 2007b).

Using the optimum conditions of 10% enzyme to substrate loading and 7.5:1 ratio of liquid to solid, after 48h of incubation resulted in an efficiency of 82.97±4.23 and 74.15±1.73 (% g glucose g^{-1} residue) for *K. alvarezii* and *G. manilaensis*, respectively. The glucose concentration was 120.33±10.97 g L^{-1} and 105.67±4.16 g L^{-1} for *K. alvarezii* and *G. manilaensis*, respectively. Our results for *K. alvarezii* in accordance with other studies (Table 1) Hargreaves *et al.* (2013) who investigated the optimization of enzymatic hydrolysis by cellulosic residue loading (% w v^{-1}) and enzymatic load (FPU g^{-1}) using a central composite rotational design. They found that using the condition of 18% w v^{-1} cellulosic residue loading and 45 FPU g^{-1}, produce the highest glucose concentration (92.3 g L^{-1} glucose) with 77.3% enzymatic efficiency. The highest glucose concentration in their study is lower than our values. Hargreaves *et al.* (2013) reported that 84.1% as the highest enzymatic efficiency when 13 % w v^{-1} residue slurry and the enzyme loading of 80.3 FPU g^{-1} was used. To ensure a complete hydrolysis of the biomass, a 48-72 h treatment is recommended by the enzyme provider. In the current study, overall, after 48 h of incubation, further enzymatic hydrolysis did not significantly increase the concentration of glucose.

There are no published reports pertaining to the enzymatic hydrolysis of *G. manilaensis*. Instead, other species of *Gracilaria* have attracted more attention. *G. dura* was investigated for enzyme treatment at the optimum condition of 2% enzyme and a hydrolysis period of 36-h at a temperature of 45 °C (Baghel *et al.*, 2015). They reported about 910 mg glucose/ g cellulosic matter. The dry weight of this species was 12.24±0.09 %, while the cellulosic matter was 3.57±0.10 % DW. In another study, *G. salicornia* was hydrolysed by enzyme, and 2.99 g L^{-1} glucose was achieved after 26 h incubation (Wang *et al.*, 2011).

G. verrucosa was hydrolysed by adding 16 U ml^{-1} of single and mixed enzymes using Spirizyme Fuel, Viscozyme L, and Celluclast 1.5 L, and 0.87 g glucose g^{-1} cellulose was obtained (Ra *et al.*, 2015). Using a slurry with 60 g DW of total carbohydrate L^{-1}, 21.7 g L^{-1} glucose was generated after 24 h of treatment with only two enzymes (Viscozyme L and Celluclast 1.5 L).

The data obtained from enzymatic hydrolysis, seems to imply that the

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higher hydrolysis efficiency and concentrated reduced sugar (glucose) can be generated by the enzymatic approach, while faster hydrolysis can be done with dilute acid treatment. It is also believed that the higher cost of enzymatic hydrolysis is a limiting factor, and efforts are ongoing to reduce the cost via recycling of the enzyme.

In the present study, fermentation of glucose obtained from the enzymatic hydrolysis of the residues was conducted to illustrate the potential of ethanol production from the waste of the phycocolloid production by *K. alvarezii* and *G. manilaensis.* The ethanol production in the present study was 56.26±1.10 g L^{-1} and 51.10±1.21 g L^{-1} from *G. manilaensis* and *K. alvarezii*, respectively which are comparable with the yield observed by Hargreaves *et al.* (2013). They used whole plants of *K. alvarezii* and conducted combined acid treatment and enzymatic hydrolysis followed by fermentation and yielded 53 g L^{-1} ethanol.

Kumar *et al.* (2013), used the agar extraction residues of *G. verrucosa* and produced an ethanol yield of 14.89±0.24 g L^{-1} which is lower than our result. But in another study, Yanagisawa *et al.* (2011) achieved a high concentration of ethanol using brown, red and green seaweeds. They reported that 59 g L^{-1} ethanol can be produced from *Ulva pertusa* with high fermentation yield of 0.46 g ethanol per g of cellulose. In another study, fermentation yield of 0.34 g ethanol g^{-1} cellulose was achieved from *Ulva rigida* after enzymatic hydrolysis (Korzen *et al.*, 2015).

In the present study the ethanol concentration produced was in an acceptable range (>5%) and upon extrapolation, 4.61 and 4.65 g of ethanol can be produced from residues of 100 g DW of *G. manilaensis* and *K. alavarezii*, respectively. The mass balance of bioethanol production from these two red seaweeds in this study is illustrated in Fig. 5.

The results of this study indicate that the enzymatic hydrolysis is an effective approach to generate bioethanol as a value added product for the phycocolloid industry. However, the use of seaweed for biofuel production is still not economically feasible, because of several limitations. Our studies show that *G. manilaensis* and *K. alvarezii*, as two very important commercial seaweeds in the tropics, can be potentially important feedstocks for bioethanol production through use of enzymatic hydrolysis on the residues. This approach has a dual purpose, namely bioconversion of the seaweed waste residues to a biofuel, and thereby contributing to environmental sustainability. We strongly suggest that the biofuel industry produce bioethanol as a petroleum additive to replace methyl tert-butyl ether (MTBE), which is a highly carcinogenic compound, currently added to petroleum in developing countries such as Malaysia. Many investigations have confirmed its carcinogenic attributes (Mehlman, 1996; Mehlman, 1998). MTBE is an oxygenate compound that is added to petroleum to raise its octane

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number. Its production and consumption has been banned in the USA since 2004 (Metcalf *et al*., 2016), and now been replaced by ethanol. This could present as an additional incentive for replacing MTBE with bioethanol from renewable resources such as seaweeds. Seaweed cultivation can also remove nutrients from wastewaters (Rabiei *et al.*, 2014; Rabiei *et al.*, 2016) as well as reduce the content of carbon dioxide from the atmosphere (Hughes *et al*., 2012; Kader *et al*., 2013; Liu, 2013).

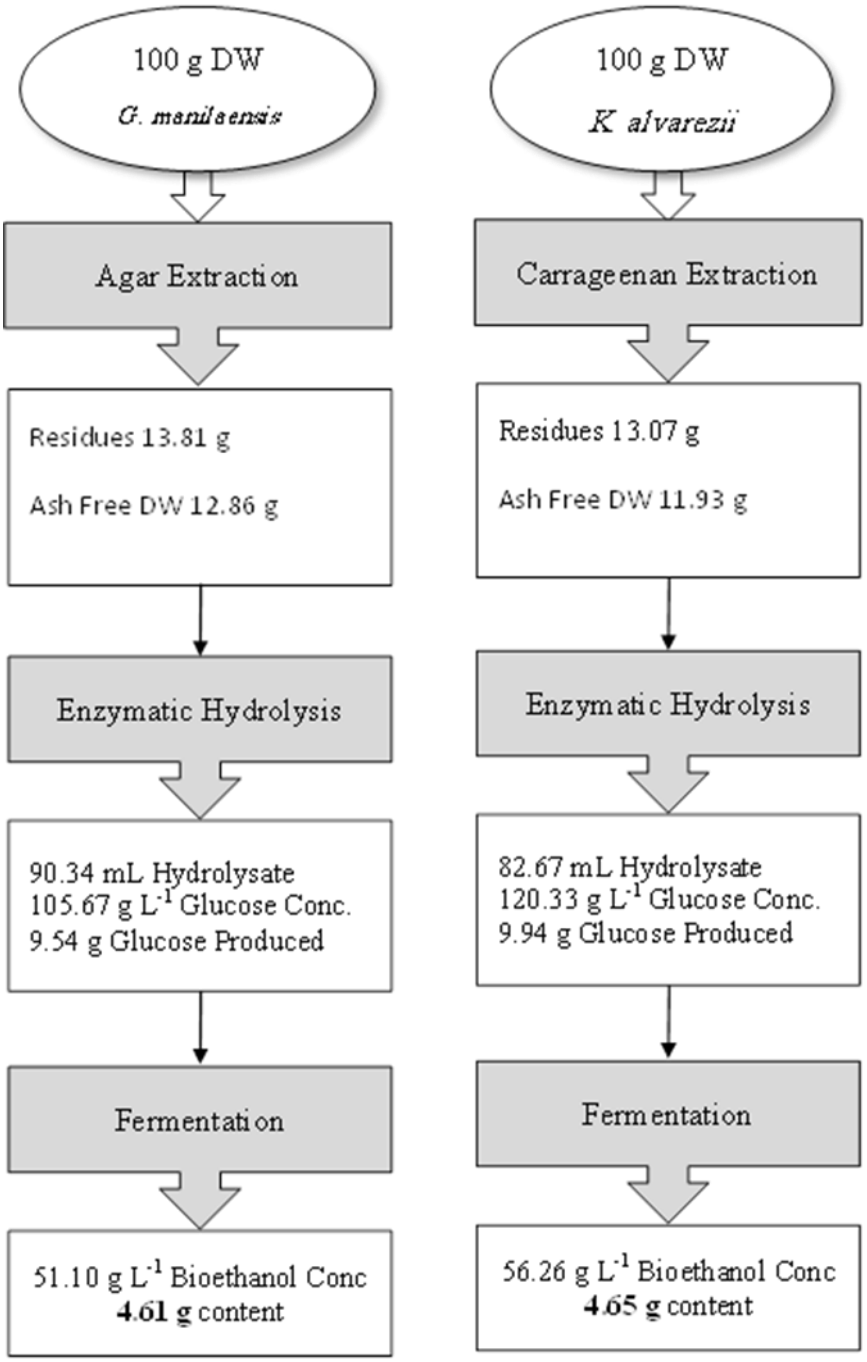

Figure 5: Mass balance of *Kappaphycus alvarezii* and *Gracilaria manilaensis* during bioethanol production.

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