Potential of *Kappaphycus alvarezii* Carrageenan Waste in Bioethanol Production

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Abstract
Kappaphycus alvarezii solid waste is a potential third generation biomass that can be used to produce bioethanol. The present study is to investigate the production of bioethanol by using K. alvarezii solid waste. The K. alvarezii solid waste was hydrolysed by 1.00%, 3.00% or 5.00% of sulfuric acid for different boiling time. The hydrolysate is collected undergoing fermentation by using tapai yeast, bread yeast and brew yeast. For the acid hydrolysis, at 60 minutes the glucose content (6.31±0.46%) in 1.00% of sulphuric acid is significantly higher than 3.00% (5.88±0.03) and 5.00% (5.32±0.01) sulfuric acid.

The obtained hydrolysate was used and fermented with bread yeast, tapai yeast and brew yeast. At 72 hours, 85.97% yield of bioethanol was obtained for brew yeast compared to 15.20% for bread yeast and 18.16% tapai yeast. Based on the results, acid hydrolysis and fermentation could be a good way to produce high yield of bioethanol with minimal cost and energy consumption. K. alvarezii solid waste could be a good choice of third generation biomass to produce bioethanol; at the same time it also could solve the seaweed waste issue from carrageenan processing industries.

Keyword: *Kappaphycus alvarezii*, solid waste, saccharification, fermentation, bioethanol

Introduction

Bioethanol is suggested as an alternative renewable fuel to replace fossil fuel. Bioethanol can be produced through a simple sugar fermentation by using yeast[1]. First generation bioethanol was produced from edible crops and second generation bioethanol was produced from lignocellulosic wastes[2]. The use of raw materials in the first generation bioethanol has raised the issue such as competition with food sources and the second generation bioethanol production has issue to overcome the high lignin content in the raw materials[3-5].

Thus, seaweed was suggested as a potential third generation biomass that can be used in bioenergy production[6]. Seaweed is a potential source of bioenergy due to the high availability of seaweed around the world[7]. Compared to other plant sources, seaweed has the advantage of higher carbohydrate yield and cheaper process to obtain bioethanol[8]. Seaweed was found rich in carbohydrates and phycocolloids[9]. The characteristic of having low levels of or no lignin also makes it suitable for bioethanol production[10]. The use of non-food macroalgae for bioethanol production has advantages such as reduced competition with agricultural food and feed crops, high yields per acre and non-dependence on agricultural fertilizer, pesticides, farmable land and freshwater[11,12].

Furthermore, *K. alvarezii* is one of the red seaweeds which is abundantly cultivated in countries such as Malaysia, Philippines, Indonesia and Tanzania[13]. In Malaysia, *K. alvarezii* is abundantly cultivated in the east coast of Sabah where people have demanded its carrageenan[14]. Carrageenan is a hydrocolloid which is largely used as food additive, gelling, emulsifying, thickening and stabilizing agent in dairy and pharmaceutical products[15].

The content of carrageenan is found approximately 25% which varies according to season and collection point of the seaweed. Thus after the carrageenan extraction process, it would produce approximately 75% of seaweed solid wastes. In the carrageenan processing industries, 10,800 tons of solid waste would be generated per year[16]. Furthermore, Shemberg Corporation (one of the world class carrageenan producer) has produced around 700,000 metric tons of seaweed waste per year. Therefore, converting the seaweed solid waste into a new product should not be underestimated. The 75% of *K. alvarezii* solid waste from the carrageenan extraction was proposed to be utilized to produce bioethanol.

In this study, the *K. alvarezii* solid waste was pretreated with different condition of acid hydrolysis in order to obtain the optimal condition to produce the maximum amount of fermentable sugar. Furthermore, the sugar obtained from the acid hydrolysis will be fermented by using different types of yeast (bread yeast, "tapai" yeast and brewer yeast) to produce bioethanol.

Material and Methods

Raw Materials: *K. alvarezii* which is purchased from the supplier was rinsed with distilled water and dried in the dryer until constant weight obtained. The dried *K. alvarezii* was packed and kept for future use. *K. alvarezii* was weighed and soaked in distilled water with ratio 1:100 (*K. alvarezii*: distilled water) and the mixture was boiled for 30 minutes. Next, *K. alvarezii* aqueous was then filtered and the residue was collected and dried at 40°C in a drying oven until constant weight obtained. The *K. alvarezii* solid waste was then milled and stored until further usage.

Cell Cultivation: Brew yeast, bread yeast and "tapai" yeast were used in this study. Each yeast was pre-cultured overnight in 50.00mL of YEPD (yeasts extract peptone dextrose) medium [1.00% (w/v) yeast extract, 2.00% (w/v) peptone, 2.00% (w/v) dextrose] in a 250 mL Erlenmeyer flask at pH 5.00. The prepared medium was autoclaved for 20 minutes at 121°C before use. The pre-culture was incubated at 35°C with shaking at 130 rpm for 24 h in a shaking incubator. The yeast cells were harvested by centrifugation at 10,000×g for 2 minutes. The collected cells were then washed with phosphoric acid (1.00% v/v). The washing and centrifugation steps were repeated for several times to remove the residual sugar in the medium.

Acid Hydrolysis: H_2SO_4 with different concentrations (1.00%, 3.00% or 5.00%) was poured into a flask containing 3.00% of *K. alvarezii* solid waste respectively. The flask was designed with reflux and boiled using stirrer hotplate for 0 minutes, 30 minutes, 60 minutes, 90 minutes and 120 minutes. The hydrolysis time was measured after the acid reached 100°C and the temperature was maintained along the hydrolysis process. The solution was then adjusted to pH 5.00 by adding 1.00 M NaOH to stop the reactions.

The data was collected and analysed by using ANOVA with Games-Howell post-hoc test. The mean value for glucose produced from 1.00%, 3.00% or 5.00% H_2SO_4 was analysed. The data were statistically different when *P*-value was less than 0.05. The statistical analysis conducted in this study was performed by using SPSS software version 20.

Determination of Glucose Concentration: Glucose concentration was determined by HPLC (High performance liquid chromatography) using Agilent Technologies 1200 series. The glucose content in the sample was determined by using the AOAC official method 980.13. The result was expressed in ppm.

Fermentation: After the diluted acid pre-treatment, the yeast (brew, bread or tapai) was added to the hydrolysate. The mixture was then transferred into a shaking incubator and incubated at 35°C and shaken with a speed of 130 rpm for 24, 48 and 72 hours. At 24 hours, the sample was collected and centrifuged at 10,000×g for 15 min at 5°C. The supernatant obtained after centrifugation was analysed for sugar and bioethanol. The collection steps were repeated for 48 and 72 hours samples.

Determination of Bioethanol Concentration: Bioethanol concentration was determined by GC (gas chromatography) using Agilent Technologies 7980A instrument equipped with a flame ionization detector (FID). The alcohol content in the sample was determined by using the AOAC official method 984.14. The result was expressed in %; bioethanol yield and percent theoretical yield were calculated based on the following equations:

$$\text{Theoretical conversion} = 0.51 \times \text{sugar utilized (\% w/v)} \quad (1)$$

where 0.51 is the maximum bioethanol yield per unit hexose sugar from glycolytic fermentation.

$$\text{Conversion efficiency} = \frac{\%\frac{w}{v} EtOH\ obtained}{theoretical\ conversion} \times 100\% \quad (2)$$

The data was collected and analysed by using ANOVA with Games-Howell post-hoc test. The mean value for bioethanol produced from brew yeast, bread yeast and tapai yeast was analysed. The data were statistically different when *P*-value was less than 0.05. The statistical analysis that was conducted in this study was performed by using SPSS software version 20.

Results

Extraction of *K. alvarezii* Solid Waste: In the carrageenan extraction process, 10.00g of dried *K. alvarezii* was used and 8.94±0.03g (89.40%) of *K. alvarezii* solid waste was produced. The *K. alvarezii* solid waste was then used for dilute acid pre-treatment.

Hydrolysis of *K. alvarezii* Solid Waste: Optimization of acid hydrolysis was done by using the different concentrations of H_2SO_4 and different boiling time. 1.00% of H_2SO_4 boiling at 60 minutes contributed the highest glucose content. The mean concentration of glucose produced in different conditions is shown in table 1. Biomass conversion percentage of *K. alvarezii* solid waste to glucose is shown in fig. 1.

Table 1
Glucose produced at different concentration of H_2SO_4 and boiling time.

	1.00% H_2SO_4 (ppm)	**3.00% H_2SO_4 (ppm)**	**5.00% H_2SO_4 (ppm)**
0 min	3.32 ± 0.01^{i}	$4.89\pm0.00^{e,f}$	3.29 ± 0.00^{i}
30 min	$3.91\pm0.00^{g,h}$	$5.33\pm0.01^{c,d}$	4.23 ± 0.05^{g}
60 min	6.31 ± 0.46^{a}	5.88 ± 0.03^{b}	$5.32\pm0.01^{c,d}$
90 min	5.45 ± 0.00^{c}	$5.03\pm0.01^{d,e}$	$5.31\pm0.01^{c,d}$
120 min	$5.15\pm0.01^{c,d,e}$	4.65 ± 0.01^{f}	3.80 ± 0.00^{h}

Values are mean ± SD of n = 3 replicates in each group. The values were expressed in ppm. Different superscript letter in each row indicate significant difference at $P < 0.05$.

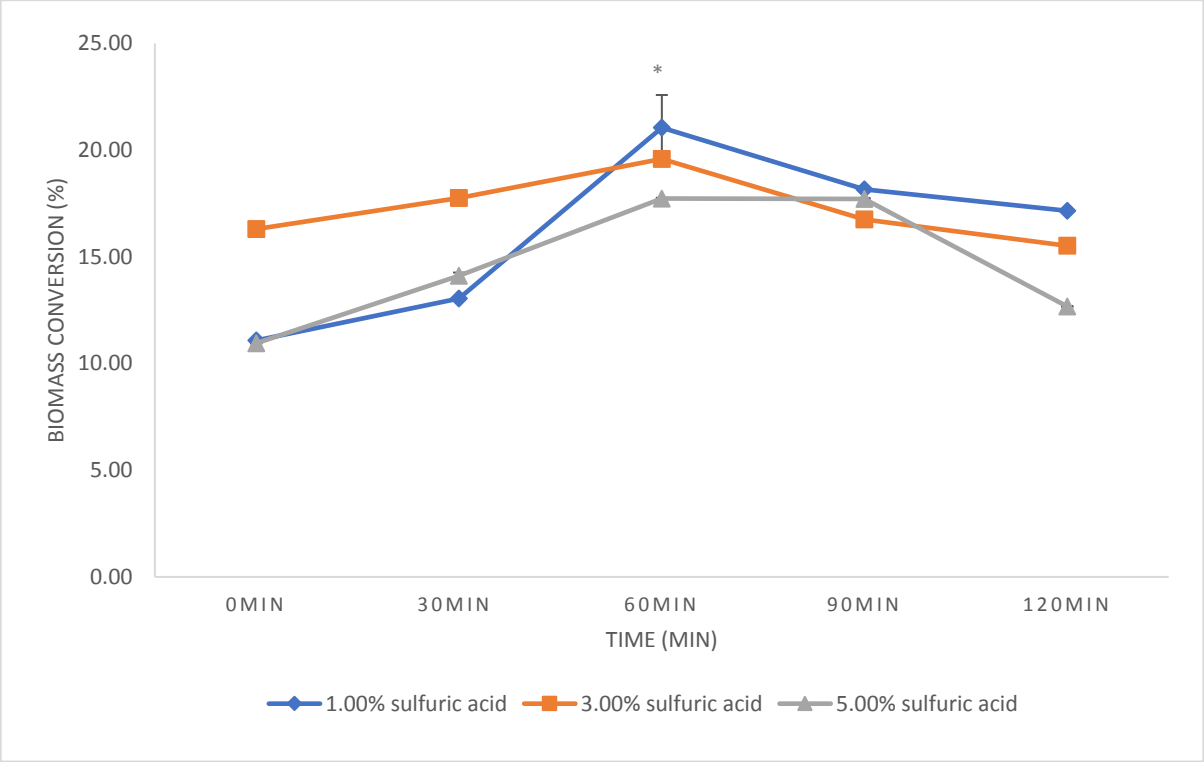

Fig. 1: Biomass conversion percentage of *K. alvarezii* solid waste to glucose. All the values are expressed as mean ± SD of triplicate determination. * indicates significance of biomass conversion of *K. alvarezii* solid waste to glucose content at 60 minutes when $P < 0.05$.

Table 2
Bioethanol and conversion efficiency for different type of yeast at different timeline.

Types of yeast	Time (hours)	Glucose content(%)	Utilized glucose (%)	Bioethanol (%)	Conversion efficiency (%)
Bread	0	6.31±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	24	5.72±0.01	9.35±0.16	0.29±0.01	10.75±0.37
	48	5.62±0.02	10.88±0.24	0.34±0.03	12.60±1.11
	72	5.49±0.01	13.15±0.16	0.41±0.01	15.20±0.37
Tapai	0	6.31±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	24	5.68±0.03	9.98±0.48	0.31±0.02	11.49±0.74
	48	5.56±0.05	11.89±0.95	0.37±0.01	13.71±0.45
	72	5.31±0.01	15.80±0.24	0.49±0.03	18.16±0.93
Brew	0	6.31±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	24	2.93±0.04	53.51±0.56	1.66±0.03	61.52±1.18
	48	1.59±0.03	74.80±0.48	2.25±0.02	83.38±0.80
	72	1.06±0.04	83.20±0.63	2.32±0.04	85.97±1.30

Bioethanol produced by Fermentation: There were three types of yeast (bread, tapai and brew) used in fermentation. Glucose content and bioethanol were determined at 24, 48 and 72 hours. The conversion efficiency was determined. Among the tested yeasts, brew yeast has the highest yield and conversion efficiency was compared to bread and tapai yeast. The bioethanol yield and conversion efficiency for the yeast are shown in table 2.

Discussion

In general, red seaweed contains high amount of carbohydrates, potassium and sulphated polysaccharides such as carrageenan, alginates and porphyrans[17,18]. In carrageenan processing industry, 10,800 to 700,000 tons of solid waste are produced every year and the solid waste has high content of cellulose with no hemicellulose or acid-insoluble lignin[16,19]. Since the *K. alvarezii* solid waste

contains a high amount of cellulose with low lignin, thus it can serve as a good source for bioethanol production.

Cellulose is a biopolymer of glucose unit which is connected through β-1,4-glycosidic bonds. The linkage between the units can be broken down by diluted acid through hydrolysis of cellulose polymers[20]. In this study, different concentrations of H_2SO_4 (1.00%, 3.00% or 5.00%) and different boiling time were used to hydrolyse *K. alvarezii* solid waste. In the acid hydrolysis process, 1% of H_2SO_4 (6.31±0.46ppm) shows a significantly higher yield compared to 3% (5.88±0.03ppm) and 5% (5.32±0.01ppm) at 60 minutes. This can be clearly observed from the glucose produced during the hydrolysis process.

For acid pretreatment, the researchers stated that the higher is the acid concentration, the higher the amount of glucose can be produced. But in this study, 1% of H_2SO_4 gives the best yield in glucose production. This might be because of the natural structure of *K. alvarezii* which is low or no lignin, thus it does not require a higher concentration of H_2SO_4 to break down the cell wall[21]. Furthermore, *K. alvarezii* solid waste used in this study was undergoing high heat process which assists in breakdown of the structure of *K. alvarezii*, thus low concentration of H_2SO_4 is sufficient to produce a good yield of glucose[22].

After 60 minutes of boiling time, the glucose content significantly decreased for all 1%, 3% and 5% of H_2SO_4 hydrolysis process. This is because at high temperature of boiling process, the glucose was converted to levulinic acid. As the time increased, the glucose content gradually decreased while the levulinic acid content gradually increased. The rate of conversion of glucose to levulinic acid is dependent on the temperature and the solubility component of the cellulose structure. Besides, levulinic acid is only generated in the presence of glucose and the rate of glucose conversion to levulinic acid is slower than the rate of hydrolysis of cellulose. Thus, this is the reason why the glucose content will decrease after 60 minutes[23].

The bioethanol yield is highly correlated with the type of yeast used. This is because of the different characteristics for the strain of the yeast. The characteristics such as alcohol tolerance, optimum pH and temperature and ability to ferment glucose are the reasons why different yield of bioethanol is produced from the different type of yeasts. Besides, the yield of alcohol also depends on factors such as the fermentative rate, tolerance to ethanol and SO_2, flocculent characteristics, the presence of killer factors, acetic acid production, H_2S and malic acid metabolism[24]. This could be the reason why the yield of production in brew yeast was higher than the tapai and bread yeast.

In this study, *K. alvarezii* solid waste was used as the source for bioethanol production, acid hydrolysis was used to convert cellulose to glucose. As a comparison, bioethanol yield produced by *K. alvarezii* solid waste used in this project is comparable with other macroalgae materials such as red and brown macroalgae. *K. alvarezii* solid waste fermented by using brew yeast gives the highest yield with 85.97% of production. In brief, the bioethanol produced by brown macroalgae is 67.39%, red macroalgae is 90.90% and 95.70%[9,19,25]. Thus, *K. alvarezii* solid waste could be a potential source to produce bioethanol.

Conclusion

In this study, the *K. alvarezii* solid waste was pretreated with 1% of H_2SO_4 and hydrolyzed for 60 minutes, thus contributed the highest amount of glucose as compared to the 3% and 5% of H_2SO_4 used. The longer is the acid hydrolysis time, the lower the glucose concentration can be obtained. Thus, the optimal concentration to pre-treat *K. alvarezii* solid waste is 1% of H_2SO_4 and the boiling time is 60 minutes. For the fermentation process, brew yeast, bread yeast and tapai yeast were successful in converting the hydrolysate into bioethanol.

Among the three type of yeasts used, brew yeast revealed the highest yield of bioethanol compared to bread yeast and tapai yeast. The brew yeast successfully converted 83.20% of the glucose into bioethanol and the conversion efficiency is as high as 85.97%. Since *K. alvarezii* solid waste is easy to be converted into fermentable glucose and fermentable by the yeast, *K. alvarezii* solid waste could be suggested as a potential third generation biomass in producing bioethanol. The seaweed residue after diluted acid hydrolysis is proposed to convert into biofertilizer and fish feed.

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