Effect of Varying Acid Hydrolysis Condition in *Gracilaria sp.* Fermentation Using *Sasad*

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**Abstract.** Macroalgae or seaweed is being considered as promising feedstock for bioalcohol production due to high polysaccharides content. Polysaccharides can be converted into fermentable sugar through acid hydrolysis pre-treatment. In this study, the potential of using carbohydrate-rich macroalgae, *Gracilaria sp.* as feedstock for bioalcohol production via various acid hydrolysis conditions prior to the fermentation process was investigated and evaluated. The seaweed used in this research was from the red algae group, using species of *Gracilaria sp.* which was collected from Sg. Petani Kedah, Malaysia. Pre-treatment of substrate was done using H\(_2\)SO\(_4\) and HCl with molarity ranging from 0.2M to 0.8M. The pre-treatment time were varied in the range of 15 to 30 minutes. Fermentation was conducted using *Sasad*, a local Sabahan fermentation agent as a starter culture. Alcohol extraction was done using a distillation unit. Reducing sugar analysis was done by Benedict test method. Alcohol content analysis was done using specific gravity test. After hydrolysis, it was found out that acid hydrolysis at 0.2M H\(_2\)SO\(_4\) and pre-treated for 20 minutes at 121°C has shown the highest reducing sugar content which has yield (10.06 mg/g) of reducing sugar. It was followed by other samples hydrolysis using 0.4M HCl with 30 minutes pre-treatment and 0.2M H\(_2\)SO\(_4\), 15 minutes pre-treatment with yield of 8.06 mg/g and 5.75 mg/g reducing sugar content respectively. In conclusion, acid hydrolysis of *Gracilaria sp.* can produce higher reducing sugar yield and thus it can further enhance the bioalcohol production yield. Hence, acid hydrolysis of *Gracilaria sp.* should be studied more as it is an important step in the bioalcohol production and upscaling process.

1. **Introduction**

Marine biomass comprise of macro and microalgae, and both have been proposed as potential biofuel feedstock. Microalgae are potential sources of bio-oils whilst macroalgae are potential sources of carbohydrates for fermentation or thermo-chemical based conversion. In fact, previous research on the wide range of macroalgae species was thought to be a promising next generation biofuel feedstock. Seaweeds consist of carbohydrates which can be used as main feedstock for alcohol production.
However, the conversion of those carbohydrates to fermentable sugar is the key issue for the utilization of seaweeds for the alcohol production. One major challenge in using algae biomass is its native recalcitrant structure due to their natural physicochemical barriers, which provides its a significant resistance to hydrolysis for further conversion by anaerobic fermentative bacteria. Most of the algae species present a rigid cellulose-based wall and accumulate various complex polysaccharides conferring a resistance to bacterial attack and limiting their degradation during the anaerobic fermentation process [1].

To overcome these natural barriers, several types of pre-treatment technologies have been used for bioalcohol production from algal biomass. Among them, thermal and thermo-chemical pre-treatments have gained consideration in contributing solubilisation of carbohydrate polymers into soluble sugars such as glucose, xylose, arabinose and galactose. Thus, thermo-chemical pre-treatments had been investigated to overcome the natural physicochemical barriers of such biomass and to enhance biofuel production from marine biomass (macroalgae). This particular pre-treatment used diluted acid pre-treatment by manipulating several variables such as type and concentration of acid as well as duration of thermal process.

Even through pre-treatments are often efficient in increasing the accessibility of biodegradable compounds to microorganism by weakening the physicochemical barriers of the algal biomass, they release also soluble sugar-derived by-products such as furfural, 5 HMF (hydroxymethyl furfural), or lignin-derived by-products such as vanillin, syringaldehyde and other phenolic compounds [9]. Due to their strong inhibitor effects on productivity and end products formation, these by-products may constitute a limiting factor in the feasibility of using algal biomass as substrate for biofuel conversion.

To avoid the negative effect of such by-products on alcohol, several processes for hydrolyzate detoxification, including evaporation, adsorption on active charcoal, and adsorption on ion exchangers, solvent extraction, alkaline treatment or enzymatic treatment has been proposed [2]. However, detoxification methods increase significantly the overall costs due not only to capital and chemical costs, but also to the loss of sugars from primary material [2].

Until recently, there are numerous studies focuses on production of biodiesel from microalgae but still lack of research on production of bioalcohol from seaweeds. Here, it is attempted to use Gracilaria sp, the third largest genus of class Rhodophyta or red algae for the bioalcohol production considering the pre-treatment process could contribute significantly to whole research. Due to the formation of fermentation inhibitors during the acid hydrolysis reaction, this will decrease the alcohol production from the biomass fermentation. This situation must be avoided as the fermenting agent used, Sasad is quite novel in the alcohol fermentation application which the formation of particular inhibitors could led to the deactivation of Sasad activity.

The diluted acid hydrolysis is very complex, mainly because the substrate is in a solid phase and the catalyst is in a liquid phase. One of the disadvantages is the formation of undesired by products that may affect the cell growth and alcohol fermentation. Eventually, the high temperature and acidic condition during the hydrolysis process will lead to the formation of chemical inhibitors such as hydroxymethylfurfural (HMF) and levulinic acid which considered as undesired by-products [10]. Some by products such as HMF and levulinic acid might inhabit the cell growth and decrease the alcohol production. Hence, it is important to maximize the monosaccharide conversion yield and minimize the formation of by-products from this pre-treatment process.

In fact, the application of Sasad as fermenting agent in this bioalcohol production could also lead to several undesired implication during this research. Currently, Sasad application is quite well known in Sabah for its rice fermentation producing the traditional alcoholic beverages [5]. Thus, practically it’s been assumed that the seaweed could substitute the rice as a substrate for bioalcohol production. Since the content of Gracilaria sp. that has been used as substrate might slightly different from the rice, the end product probably will contain several by-products which perhaps significantly affected by the fermenting agent, Sasad.

The main objective of this research is to develop bioalcohol production technique from Gracilaria sp. through acid hydrolysis, neutralization and fermentation using Sasad.
2. Materials and Methods

2.1. Raw materials
Samples of *Gracilaria sp.* were purchased from Sg. Petani Kedah, Malaysia. The seaweed samples were soaked and washed with tap water to remove any foreign materials, salt and debris. Samples were then oven dried at 50°C until constant weight. Dried *Gracilaria sp.* was then kept in airtight container for further usage. Materials used in this research are HCl, H$_2$SO$_4$, NaOH, *Sasad*, local Borneo fermentation agent, and Benedict Reagent.

2.2. Substrate preparation
600g of dried *Gracilaria sp.* was cooked at 100°C in distilled water with volume ratio of 1:4 for 2 hours to ease the polysaccharide extraction process. Then, the substrate was size reduced using blender for 15 minutes, producing finely shredded slurry. 16 samples were prepared in 500ml flasks containing 200g of substrates. Aliquot of 2ml from substrate prepared was taken and filtered for reducing sugar concentration analysis.

2.3. Acid hydrolysis
H$_2$SO$_4$ and HCl were used in this experiment. Acid stocks solution was prepared in the range of 0.2M to 0.8M. Hydrolysis times were varied in the range of 15 to 30 minutes and it was conducted using an autoclave machine at 121°C. After hydrolysis process was done, the residues were separated by filtration. The liquid was then further analysed for reducing sugar content. Hydrolysis were done as in experimental design in table 1.

| Experiment | Acid Type | Molarity (M) | Hydrolysis Time (min) |
|------------|-----------|--------------|-----------------------|
| 1          | H$_2$SO$_4$ | 0.2          | 30                    |
| 2          | H$_2$SO$_4$ | 0.4          | 30                    |
| 3          | H$_2$SO$_4$ | 0.6          | 30                    |
| 4          | H$_2$SO$_4$ | 0.8          | 30                    |
| 5          | HCl        | 0.2          | 30                    |
| 6          | HCl        | 0.4          | 30                    |
| 7          | HCl        | 0.6          | 30                    |
| 8          | HCl        | 0.8          | 30                    |
| 9          | H$_2$SO$_4$ | 0.2          | 15                    |
| 10         | H$_2$SO$_4$ | 0.2          | 20                    |
| 11         | H$_2$SO$_4$ | 0.2          | 25                    |
| 12         | H$_2$SO$_4$ | 0.2          | 30                    |
| 13         | HCl        | 0.2          | 15                    |
| 14         | HCl        | 0.2          | 20                    |
| 15         | HCl        | 0.2          | 25                    |
| 16         | HCl        | 0.2          | 30                    |

2.4. Reducing sugar analysis
Reducing sugar concentration was analyzed using the Benedict reagent method. Glucose is used as the standard for this analysis. The ratio of Benedict reagent to standard/sample solution is 1:10. Then, the standard solution and a sample containing Benedict reagent will need to be heated using water bath at 80°C about 30 minutes. After cooled down to room temperature, the reducing sugar concentration was read at 550 nm using Jasco UV-Vis Spectrophotometer 650.
2.5. Hydrolyzate neutralization
After acid hydrolysis of Gracilaria sp, hydrolyzates were neutralized with 5M NaOH. Sodium hydroxide was added until the pH of hydrolyzate is in the range of pH 5.0 – 6.0.

2.6. Fermentation agent preparation
Sasad, was primarily size reduced using a manual mortar until it turns into powder form and stored in airtight container for further used. 10 g of Sasad was added into all samples. The anaerobic fermentative processes are allowed to take place as the conical flask will be sealed using the wool and parafilm for 10 days.

2.7. pH analysis
pH analysis is crucial in order to determine the presence of microbial activity during the fermentation process. pH was obtained using pH meter.

2.8. Distillation
The fermented product was distilled using Distillation Unit Buchi K-350. 50 ml of sample was measured at each time of distillation. The final volume of distillate was measured and recorded. It was stored in 5-8°C prior to estimation of percent alcohol in distillate.

2.9. Estimation of alcohol percentage in distillate
The determination of percent alcohol is estimated from the relationship between specific gravity and percent alcohol. The data was derived from Amerine and Ough [11].

3. Results and Discussions

3.1. pH Analysis
Table 2.0 shows pH profile for all samples in 3 different conditions:
- i. pH before substrate undergo pretreatment process.
- ii. pH after substrate undergo thermal-chemical pretreatment.
- iii. pH after hydrolysate neutralization (before adding Sasad).

Table 3.0 shows the pH profile for all samples which was obtained during fermentation. pH data was recorded once for every two days throughout the 10 days of fermentation.

It can be seen from table 2.0 and table 3.0 that most of pH samples decreased from around pH 5 to around pH 3. These results are quit significant with the pH of inoculated rice with Sasad which was rapidly drop from pH 6.6 to pH 3.4 [3]. Based on the pH analysis, we can conclude that the pH profile is acidic during fermentation. In fact, the presence of some by products (formic and levulinic acid) after been autoclaved also contributes to the acidic condition [9].

It was also believed that the correlation between acidity and pH was due to the connection with both yeasts and lactic acid bacteria (LAB). LAB were well recognized as acids producer in fermentation as well as some yeasts were also producing acid which may results in the variation of products flavor [4].

| Experiment | pH 1 | pH 2 | pH 3 |
|------------|------|------|------|
| 1          | 7.35 | 2.11 | 5.86 |
| 2          | 7.13 | 1.45 | 5.49 |
| 3          | 8.70 | 1.33 | 5.38 |
| 4          | 8.36 | 1.33 | 5.87 |
| 5          | 8.11 | 1.85 | 5.42 |
| 6          | 9.27 | 1.63 | 5.75 |
Table 3. pH profile for samples during fermentation

| Experiment | pH Decision | Fermentation Days |
|------------|-------------|-------------------|
|            |             | 2 | 4 | 6 | 8 | 10 |
| 1          | 3.43        | 3.54 | 3.23 | 2.52 | 2.58 |
| 2          | 3.85        | 3.71 | 3.63 | 2.30 | 2.23 |
| 3          | 3.84        | 3.79 | 3.62 | 3.63 | 3.63 |
| 4          | 3.68        | 3.76 | 3.51 | 3.21 | 3.24 |
| 5          | 3.40        | 3.38 | 3.24 | 3.23 | 3.00 |
| 6          | 3.35        | 3.27 | 3.11 | 2.75 | 2.50 |
| 7          | 3.35        | 3.27 | 3.11 | 2.75 | 2.50 |
| 8          | 3.52        | 3.23 | 3.60 | 3.45 | 3.36 |
| 9          | 3.84        | 3.74 | 3.55 | 3.29 | 3.22 |
| 10         | 3.90        | 3.78 | 3.64 | 3.54 | 3.41 |
| 11         | 3.84        | 3.72 | 3.63 | 3.62 | 3.36 |
| 12         | 3.65        | 3.76 | 3.54 | 3.37 | 3.18 |
| 13         | 3.23        | 3.24 | 3.07 | 3.04 | 3.11 |
| 14         | 3.35        | 3.36 | 2.94 | 3.23 | 3.03 |
| 15         | 3.41        | 3.42 | 3.35 | 2.76 | 3.21 |
| 16         | 3.84        | 3.74 | 3.55 | 3.29 | 3.22 |

3.2. Effect of acid concentration and hydrolysis time on reducing sugar yield

Based on figure 1, experiment 6 (0.4M HCl, 30min) shows the highest reducing sugar content (8.06mg/g) compared to others for the fixed duration of hydrolyzate in autoclave. As we can see from the graph, the reducing sugar contents are increasing proportionally with acid molarity. However, there are decreasing in reducing sugar content which can be noticed at 0.8M concentration for both type of acids.

Figure 2 shows that sample with sulphuric acid show an increasing amount of reducing sugar from 15min to 20min hydrolysis. However, the amount was decreasing at longer hydrolysis time. Compared to sample hydrolyzed with HCl, the reducing sugar content was increasing over the hydrolysis time, but the overall amount is lower than samples treated with H₂SO₄. Longer hydrolysis time results in the decreasing of reducing sugar concentration might be due to sugar decomposition [10].

From figure 2, the highest content of reducing sugar was produced from sample treated with 0.2M H₂SO₄ in 20min. This is comparable to the previous study, whereby high reducing sugar concentration can be achieved with low reaction temperature at 120°C at shorter time [6]. It can be concluded that extreme condition during the thermal-chemical pretreatment of Gracilaria sp was not
suitable for this project as the reducing sugar might be further converted into some by-products such as furfural, 5-HMF, formic acid and levulinic acid [7].

This present work proved that mild condition of acid hydrolysis at 0.2M H$_2$SO$_4$, 20 min duration in autoclave machine (10.06 mg/g) produced the most reducing sugar. It was followed by the hydrolysis of 0.4M HCl, 30 min, (8.06 mg/g) and 0.2M H$_2$SO$_4$, 15 min (5.75 mg/g).

![Figure 1. Reducing sugar concentration for different acid concentration](image1)

![Figure 2. Reducing sugar concentration for different pre-treatment time](image2)

### 3.3. Effect of acid hydrolysis on alcohol yield

The concentration of alcohol in each fermentation sample after various hydrolysis conditions on *Gracilaria sp* can be seen in figure 3 and 4. Hydrolysis at 0.4M HCl, 121°C, 30min clearly produced the most alcohol (15.22 % v/v), followed by sample hydrolysed with 0.2M HCl, 121°C, 30min (12.52 % v/v); 0.2M HCl,121°C, 20min (7.21 % v/v); 0.2M H$_2$SO$_4$, 121°C, 20min (6.98 % v/v) and 0.8M H$_2$SO$_4$, 121°C, 30min (6.72 % v/v) with distilled products (figure 5).

Interestingly, the alcohol yield is not depending on the amount of reducing sugar produced after being hydrolyzate. For example, sample treated with 0.4M HCl, 121°C, 30min producing maximum alcohol although its reducing sugar content is lower than sample treated at 0.2M H$_2$SO$_4$, 20 min prior to fermentation process. Yet, experiment 10 still produced the fourth highest amount of alcohol which was 6.98% v/v. The reason which leads to this result is probably because the suitability of acid hydrochloric compared to sulphuric acid which can provide a mild conditions by minimizing the degradation of reducing sugar into inhibitor.

From figure 3, it shows that the alcohol production is fluctuating as the acid molarity during pre-treatment was increased. Hence, as the duration in the autoclave were fixed into 30 minutes, hydrolysis using 0.4M HCl producing the higher amount of alcohol. The effect of temperature was not significant as compared to molarity change.

Elevated temperature will produce poor fermentability hydrolysate. Sugars can be degraded to furfural which is formed from pentoses and 5-hydroxymethylfurfural (5-HMF) which is formed from hexoses. 5-HMF can be further degraded, forming levulinic acid and formic acid [8].

In addition, formic acid can be formed from furfural under acidic conditions at elevated temperatures. Acetate is liberated from hemicellulose during hydrolysis. Since the application of *Sasad* as the alcohologenic bacteria is quite novel for the bioalcohol production from macroalgae biomass, the solubilisation of inhibitory compound could lead to the significant effect which disrupting the multicellular microbial activity.

Overall, the highest production of alcohol was produced from the hydrolyzate which by using hydrochloric acid during the thermal-chemical pre-treatment. Experiment 5, 6 and 14 produced 15.21 % v/v, 12.52 % v/v, 7.21% v/v of alcohol respectively. It proves that the HCl could contribute significant yield for the mild acid hydrolysis condition which was more conducive for the microbes from *Sasad*. 

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4. Conclusion
This present’s preliminary work has shown that fermentation of *Gracilaria sp.* with *Sasad* and via acid hydrolysis pre-treatment has been done. The data has shown that the highest estimated alcohol yield obtained was 15.22% (v/v) from the *Gracilaria sp.* treated with 0.4M HCl, and 30min pre-treated at 121°C. It was followed by *Gracilaria sp.* treated with 0.2M HCl, and 30min pre-treated at 121°C which yield 12.52% (v/v) alcohol. Whereas, acid hydrolysis of *Gracilaria sp.* using 0.8M H$_2$SO$_4$ and autoclaved at 121°C for 30min yield 6.72% (v/v) alcohol.

The reducing sugar analysis shows highest amount from the hydrolysis of *Gracilaria sp.* with 0.2M H$_2$SO$_4$ and pre-treated for 20 minutes at 121°C. It was followed by other samples hydrolysis using 0.4M HCl, 30 minutes pre-treatment and 0.2M H$_2$SO$_4$, 15 minutes pre-treatment yield (8.06 mg/g) and (5.75 mg/g) reducing sugar content respectively.

However, further work in analyzing the alcohol yield would be needed to obtain an accurate result besides to detect the components in the fermentation products.

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