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Sugar concentration of *Gracilaria sp.* following hydrolysis using cellulase and sulphuric acid and several pretreatment methods

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Abstract. In the search for new and renewable energy, *Gracilaria* sp. was studied as the raw material for bioethanol production. This seaweed is available abundantly in the very long Indonesian coastline. This study investigates the effect of several pretreatment methods on the concentration of sugar produced from *Gracilaria* sp. when hydrolyzed using cellulase or sulphuric acid. Reducing sugar was measured by UV-Vis spectrophotometry using Nelson-Somyogi reagent and the ethanol concentration was measured by using gas chromatography. Cellulase and sulphuric acid (H_2SO_4) were used in the hydrolysis. Cellulase concentration used was 200, 400, 600 and 800 units/ml, whereas the concentration of sulphuric acid used was 1%, 3%%, 5%, and 7%. The highest concentration of reducing sugar was produced by hydrolysis using H_2SO_4 1%.

1. Introduction

Indonesia, an archipelago with a coastline length of 108,000 km [1], is rich in marine resources, e.g. seaweeds. Seaweed has many advantages as the raw material for biofuel, it can grow extensively and less land competition with agricultural crop [2]. We have collected 39 types of seaweed at Mertasari Beach, Sanur area in Bali. *Gracilaria sp.* is one of the major red seaweed found [3]. This seaweed has been also cultivated by local farmers and consumed as local culinary.

Gracilaria sp. contain polysaccharide which can be used as raw material for producing ethanol [4, 5], Sandi *et al*. [6] has conducted a hydrolysis study of *Gracilaria sp.* with catalyst cellulase, HCl, and sulphuric acid with low ethanol yield. Meanwhile, Widayanti *et al.* [7] used ammonium sulphate ($(NH_4)_2SO_4$) as a nitrogen source in bioethanol production made from *Glacilaria sp,* also with low ethanol yield. Mosier et al. [8] used *S. duplicatum* (brown algae), which is hydrolyzed with acid and fermented into bioethanol. The highest glucose level obtained was 28.051 mg/ml after soaking in 0.4 M H_2SO_4 for 120 minutes, while in fermentation, the maximum bioethanol content was reached at 72 hours incubation time, which was 0.0451%.

Red seaweed contains glucans and floridean starch. Conversion of only glucans only produced very small amount of glucose which is used for ethanol production [9], therefore it is necessary to convert other carbohydrate in seaweeds into glucose. It was found that pretreatment combined with hydrolysis can increase sugar release as substrate for ethanol fermentation.

This research aims to investigate the effect of pretreatment method prior to hydrolysis to increase the sugar content for ethanol production.

2. Materials and Methods

The red algae *Gracilaria sp.* was collected from Serangan Island, Sanur area, Bali, Indonesia in 2016. It was rinsed, sundried for three days, then it was powdered using blender and stored until further use.

2.1 Steam Pretreatment
350 g of *Gracilaria* flour was steamed at 100^0C for 15, 30, and 60 minutes in stainless steel container, drained for 1 hour at room temperature. This was done in triplicate.

2.2 NaOH Pretreatment
Pretreatment was done according to Anggriani *et al.* [10] and Ramadhina [11]. 350 grams of *Gracilaria sp.* flour was soaked in NaOH solutions (0.005; 0.01; 0.02 M) for 24 hours, then it was filtered. The residu was rinsed using hot water (80^oC). Dried residu was crushed in porcelain cup, then sifted. This was done in triplicate.

2.3 Biological Pretreatment
350 g of *Gracilaria* flour was diluted in 100 ml distilled water. The mixture was heated at 100°C for 15, 30, and 60 minutes in stainless steel container. It was then drained for one hour at room temperature. 20 ml of EM4 solution was then added to the sample.

2.4 Hydrolisis using H_2SO_4
One gram each of pretreated *Glacilaria* flour were mixed with 50 ml of 1%, 3%, 5%, 7% (v/v) H_2SO_4 in erlenmeyer flasks. The mixtures were stirred and heated at 100^0C for 1 hour, then it was filtered and used for reduction sugar determination [12]. This was done in triplicate.

2.5 Hydrolisis using cellulase
One gram each of pretreated *Glacilaria* flour were mixed with 50 mL of 200, 400, 600, and 800 units/ml (v/v) cellulose (SQzyme CSR-F) in erlenmeyer flasks. The mixtures were stirred and heated at 55^0C for 1 hour, then it was filtered and used for reduction sugar determination [12]. This was done in triplicate.

2.6 Reduction sugar determination
Glucose standard solutions were prepared at 20, 40, 60, 80, and 100 ppm. 1 ml of sample solutions were mixed with 1 ml Nelson reagent in glass tubes, heated in boiling waterbath for 20 minutes, then cooled down in cold water until room temperature. 1 ml arsenomolibdate reagent and 7 ml distilled water was added and stirred evenly. Absorbans was measured using UV-Vis spectrophotometer at 540 nm [13].

2.7 Fermentation
Saccharomyces cerevisiae S-14 was incubated in glucose yeast peptone (YGP) media for 24-48 hours at room temperature at 170 rpm. It was then inoculated to Glacilaria suspension. Fermentation took place in close glass jars using 5% inoculum (57 ml Gracilaria suspension and 3 ml inoculum) and 10% inoculum (54 ml Gracilaria suspension and 6 ml inoculum) at room temperature for 5 days. The inculum contain 4,8 x 10^7 CFU/ml of *S.cereviceae.*

2.8 Determination of ethanol
Standard curve was prepared using 0%, 5%, 10%, 15%, 20%, and 25% ethanol solutions. Fermentation broth was centrifuged for 15 minutes at 3000 rpm. The supernatant was distilled and the density was determined using the method of Suartama [14, 15]. It was then verified by using gas chromatography (GC).

3. Results

3.1 Sugar content after steam pretreatment and acid hydrolysis (H_2SO_4)

Following steam pretreatment, the sugar released after acid hydrolysis is presented in Figure 1. Longer pretreatment released more sugar after hydrolysis by H_2SO_4, but the highest concentration of sugar achieved at the lowest H_2SO_4 concentration.

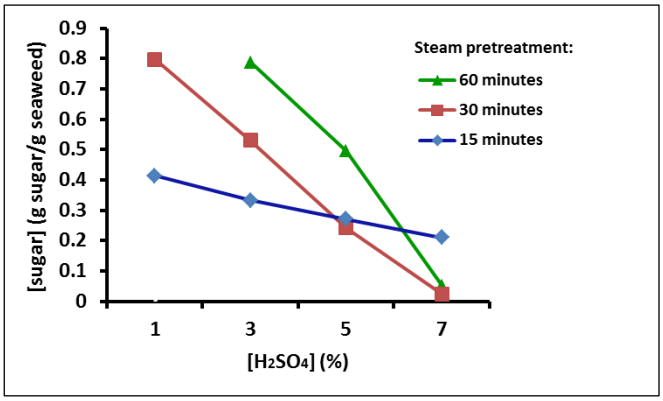

Figure 1. The concentration of reduction sugar equivalent to glucose after hydrolysed by H_2SO_4 following steam pretreatment

3.2 Sugar content using steam pretreatment and enzyme hydrolysis (cellulase)

The results in Figure 2 show that the more cellulase used release more sugar. However, the concentrations of sugar released were lower than using acid hydrolysis. Moreover, steam pretreatment longer than 30 minutes does not increase sugar concentration.

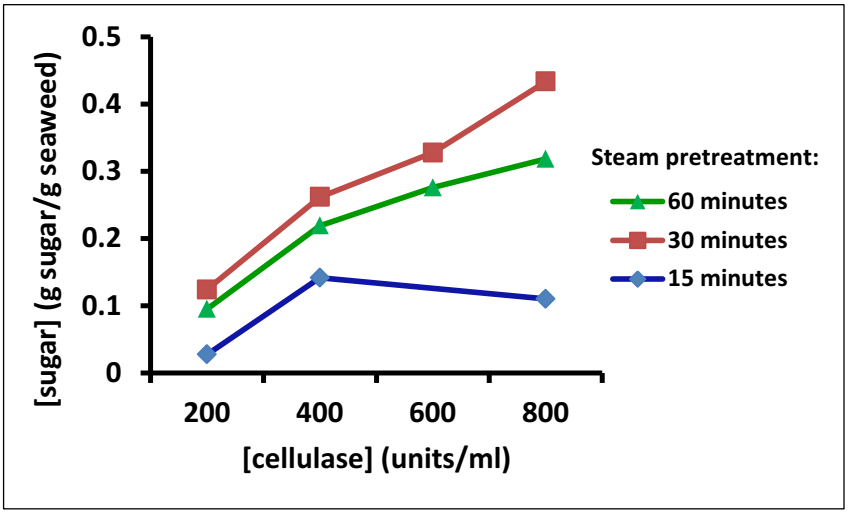

Figure 2. The concentration of reduction sugar equivalent to glucose after hydrolysed by cellulase following steam pretreatment

3.3 Sugar content after biologically pretreatment and acid hydrolysis (H_2SO_4)

EM4 contains *Azotobacter* sp., *Lactobacillus* sp., yeast, photosyntetic bacteria, and cellulolytic fungus [8]. The concentration of sugar released was presented in Figure 3. In this case, similar to Figure 1, more sulphuric acid resulted in lower concentration of sugar. However the addition of EM4 after steam pretreatment resulted in higher sugar concentration if compared to without EM4 addition (Figure 1).

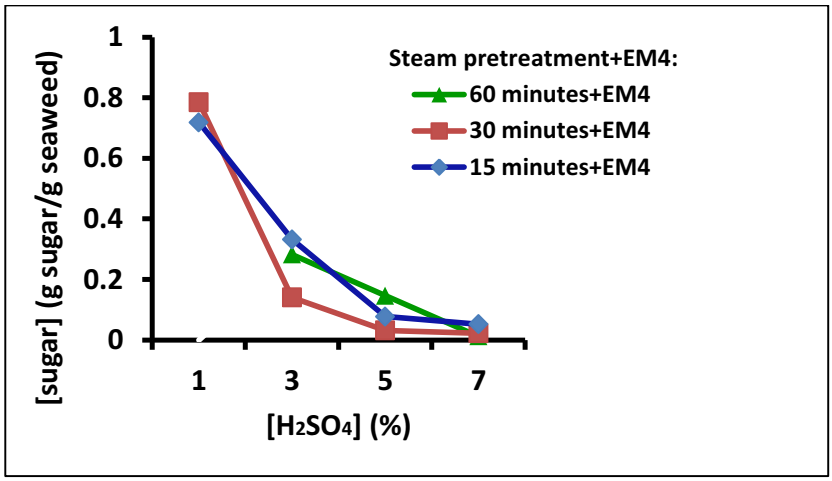

Figure 3. The concentration of reduction sugar equivalent to glucose after hydrolysed by H_2SO_4 following steam and biological pretreatment

3.4 Sugar content after biologically pretreatment and enzymatic hydrolysis (cellulase)

Longer steam pretreatment lower the concentration of sugar. On the other hand, higher cellulose concentration resulted in more sugar as presented in Figure 4. Similar to the case in Figure 3, the addition of EM4 higher the sugar released if compared to without EM4 addition (Figure 2).

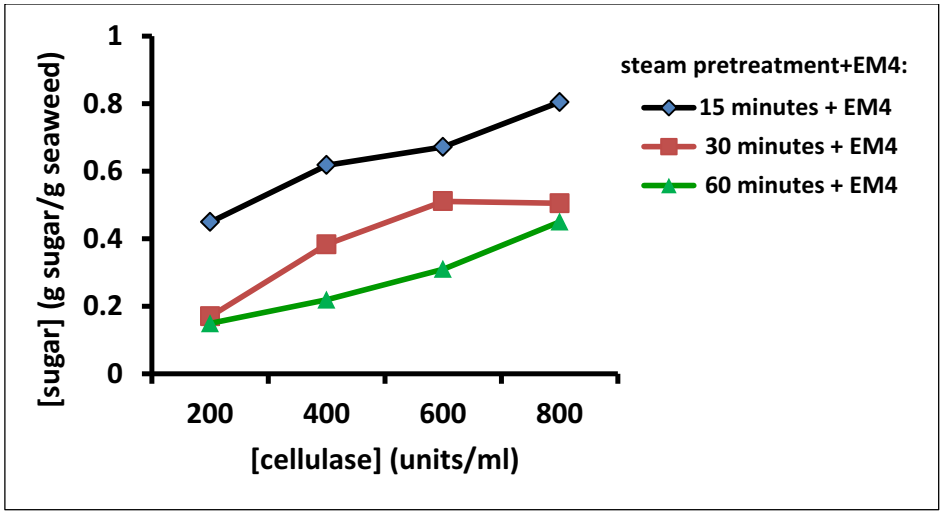

Figure 4. The concentration of reduction sugar equivalent to glucose after hydrolysed by cellulase following steam and biological pretreatment

3.5 Sugar content after NaOH pretreatment followed by acid hydrolysis (H_2SO_4)
The sugar content was lower when concentration of NaOH used was higher. Similarly, it was also lower when sulphuric acid was higher (Figure 5).

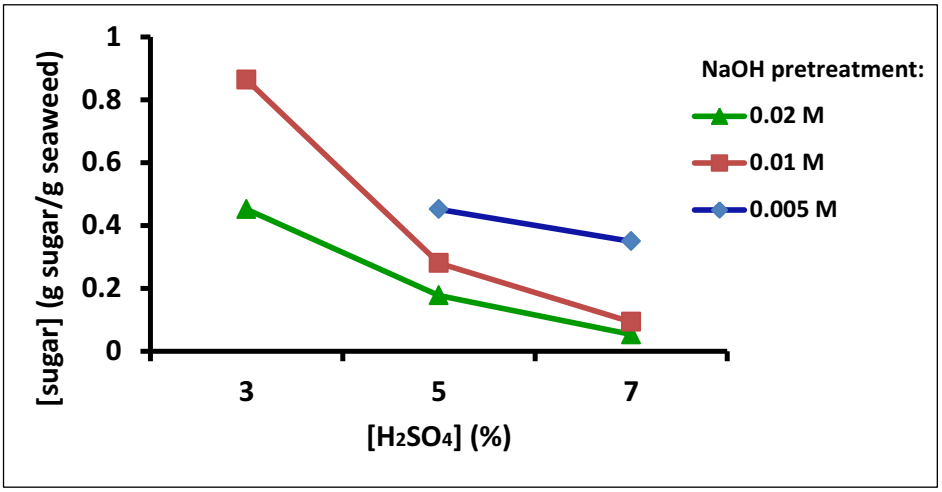

Figure 5. The concentration of reduction sugar equivalent to glucose after hydrolysed by H_2SO_4 following NaOH pretreatment

3.6 Sugar content after NaOH pretreatment followed by enzymatic hydrolysis (cellulase)
The results are presented in Figure 6. Contrary to when using sulphuric acid as hydrolyzing agent, higher concentration of cellulose results in higher amount of sugar released.

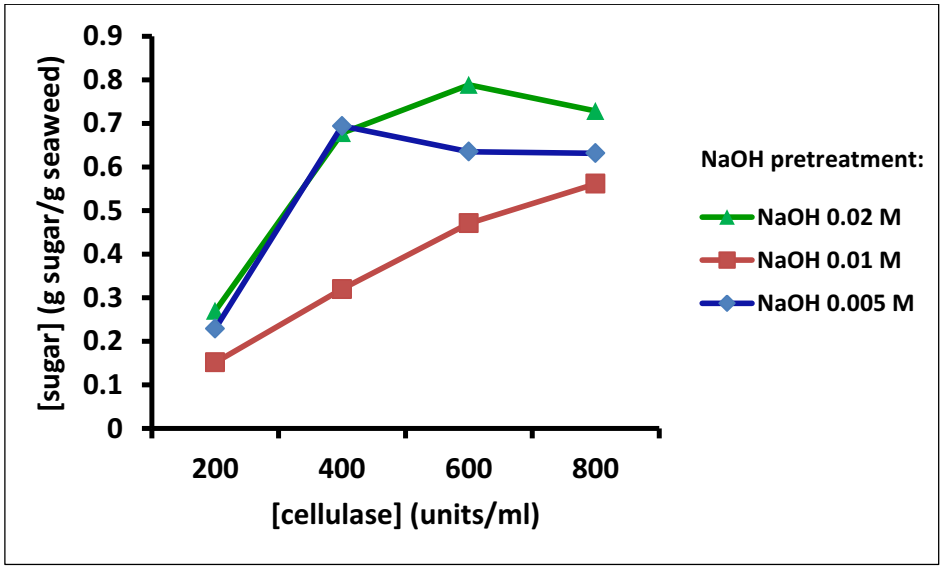

Figure 6. The concentration of reduction sugar equivalent to glucose after hydrolysed by cellulase following NaOH pretreatment

3.7 Fermentation
The concentration of ethanol produced was 0.51% v/v (using 5% inoculum) dan 1.55% v/v (using 10% inoculum) after 5 days incubation.

4. Discussion
In all results, saccharification using sulphuric acid resulted in more sugar than using cellulose. It seems that cellulase only act at certain sugars whereas acid hydrolysis can release more monosaccharide that can be fermented into ethanol as red seaweed consist of not only glucans, but also carrageenan [16], which is not the substrate of cellulase. Probably when a cocktail of enzymes that can hydrolyse both cellulose and this sugar were used, higher amount of monosaccharides can be released for ethanol fermentation. In green seaweed, *Ulva lactuca*, the use of cellulose cocktail released more more than 90% sugars [17].

In the other hand, the length of hydrolysis probably affects the amount of sugar released in this study. It was reported that the maximum amount of reducing sugar produced by hydrolysis of *Gracilaria verrucosa* using cellulase was 6 days [18], whereas in this study the hydrolysis was performed for only one hour.

In this study, the maximum amount of sugar released by the lowest concentration of H_2SO_4. Higher concentration of acid may degrade the product of saccharification into furfural [9, 19]. Carrageenan consist of agarose and agaropectin [8], whereas agarose consists of repeating units of β-D-galactose and 3,6-anhydro-α-L-galactose, which can be degraded by acid into furfural and hydroxymethylfurfural, which can inhibit subsequent fermentation [20, 21]. In contrast, when enzyme was used in this study, it was found that higher amount of cellulose used, more sugar was released to the medium. It was found that maximum sugar released from Gracilaria when 1200 units/ml cellulase was used as the hydrolising agent [22].

Generally, steam pretreatment increases the amount of sugar released, except when hydrolizing using cellulose, steam pretreatment more than 30 minutes actually decreases the sugar content released. After adding EM4 containing bacteria that are expected to produce enzymes that can break down carbohydrates into monomers, it turns out that sugar levels are actually higher than before adding EM4. Apparently, the microbes contained in EM4 secrete enzymes that can break down carbohydrates in seaweed into their monomers eventhough the existing sugar is also likely to be consumed by these bacteria.

The higher the NaOH level in the pretreatment decreases the sugar level after it is hydrolyzed with H_2SO_4, while in hydrolysis with the enzyme there is no specific trend shown. NaOH is known able to breakdown amorphous structures of hemicellulose [23].

The ethanol production was still very low (1.55%) although *S.cerevisiae*, which was used in this study, was reported able to ferment galactose as well as glucose into ethanol [9].

5. Conclusion
Maximum amount of glucose released when hydrolysed using dilute H_2SO_4 (1%) with longer steam pretreatment (60 minutes) plus EM4 addition. Higher concentration of acid may destroy the resulted monosaccharides. Use of enzymes which is able to convert cellulose and agar will probably increase the monomers (glucose and galactose) yield.

Acknowledgement
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