Production of Bioethanol from Seaweed, *Gracilaria verrucosa* and *Eucheuma cottonii*, by Simultaneous Saccharification and Fermentation Methods

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**Abstract.** Demand for bioethanol in Indonesia will increase with the increase of energy need. Seaweeds, *Gracilaria verrucosa* and *Eucheuma cottonii* containing high cellulose content are considered very suitable to be developed in Indonesia and can be used as a raw material for low cost bioethanol production. The seaweeds were obtained from Mandalle, Pangkep Regency, South Sulawesi, Indonesia. The method used in this research was a simultaneous saccharification and fermentation (SSF) process. The results showed that 1. *G. verrucosa* and *E. cottonii* seaweeds could be converted into bioethanol having ethanol levels of 5.7% and 6.1%, respectively after 10 days of fermentation. 2. The optimum condition of fermentation process of *G. verrucosa* and *E. cottonii* using *Clostridium acetobutylicum* bacterium to produce ethanol was 10 days fermentation at pH 6.0 with the ethanol level of 7.7% and 7.2%, respectively. 3. The conversion value when *G. verrucosa* cellulose was used as the raw material was 3.33% (33.3 g of bioethanol was produced from every kg of cellulose) with the obtained ethanol having the purity of 96.4%.

1. **Introduction**

The continuous increase of oil consumption concomitant with the exhaustion of world petroleum oil production have been raising the oil price as well as triggering global warming. Bioethanol can be an alternative answer to counter the above petroleum oil problem. Unlike petroleum oil that is considered as a limited energy resource, cellulose raw materials are abundant in Indonesia. Indonesia is a country that has abundant natural resources including organic resources that can be used as raw materials for bioethanol production such as kumbi fruit (*Voacanga foetida* (blume) rolfe) [1], sugarcane waste (*Saccharum officinarum* L) [2], durian seeds (*Duriozibethinus*) [3], palm oil (*Elaeis guineensis* Jacq) [4] coffee skin [5], kersen fruit (*Muntingia calabura*) [6], pineapple (*Ananas comosus*) [7] and seaweed and waste for *Gracilaria* sp. [8]. The opportunity to utilize bioethanol as an alternative renewable fuel in the future is increasing. Thus not only does ethanol save the country from fuel crisis but also from economic crisis. Seaweed is a source of bioactive materials to produce a range of compounds. These compounds could be derived from green algae, brown algae and red algae [9]. Cultivation of
Gracilaria verrucosa and Eucheuma cottonii seaweeds is considered as one of the featured agricultural programs in Southeast Asia, especially in South Sulawesi, Indonesia that produces an excellent raw material product containing 30-35% cellulose [10, 11]. Cellulolytic bacteria are microorganisms used to produce enzymes to hydrolyze cellulose to glucose [12, 13, 14]. Out of those Clostridium acetobutylicum is a cellulosytic ethanologenic bacterium capable to directly convert cellulosic substrates into bioethanol. The breakdown of raw material celluloses at high temperature is helpful in bioethanol production [15]. The objectives of this study were to find out the conversion value of G. verrucosa and E. cottonii seaweeds for bioethanol production by simultaneous saccharification and fermentation, to determine the optimum conditions of fermentation process using C. acetobutylicum bacterium, and to produce bioethanol from G. verrucosa and E. cottonii seaweeds based on the fermentation optimum conditions. We report here on simultaneous saccharification and fermentation from cellulose of G. verrucosa and E. cottonii as substrate to very higher ethanol yields using pure cultures of C. acetobutylicum.

2. Materials and methods
2.1 Materials
Raw materials of G. verrucosa and E. cottonii seaweeds were obtained from Mandalle, Pangkep Regency, South Sulawesi, Indonesia. Variety identification of the used seaweeds was conducted at the Laboratory of Animal Nutrition, Polytechnic of Agriculture Pangkep State, Mandalle, Pangkep, Sulawesi Selatan, 90655 Indonesia. The seaweeds were cut into small pieces of 1-2 cm size, sun-dried, and then milled using a crusher to form a fine powder and sieved to 60 mesh (0.2 mm). These fine powders were used as substrates.

2.2 Pre-treatment of fine raw materials/substrates and chemical analysis
The substrates were treated chemically by soaking the fine raw materials in 4% NaOH for 24 h. After soaking the treated substrates were filtered. The obtained residues were bleached with 3% H2O2 solution, washed with distilled water until neutral and then dried in an oven at 105°C until constant weight [16]. The hydrolysis process followed standard method [13], and water, cellulose, and lignin contents was determined according to a modified method that is based on the Chesson method [17] and Association of Official Analysis Chemists AOAC method [18].

2.3 Reactivation of Clostridium acetobutylicum culture
C. acetobutylicum culture was obtained from the Laboratory of Microbiology, Department of Biology, Gajah Mada University, Indonesia. The strain was inoculated on sterile Potato Dextrose Agar slants and was allowed and maintained to grow in an incubator at 30°C for 4 days. After the optimum growth was reached, the fresh culture was stored at 4°C in refrigerator before further use.

2.4 Inoculum preparation of Clostridium acetobutylicum
Spore suspension from the inoculated fresh C. acetobutylicum culture was used in the present studies. It was prepared by adding the 4-day fresh culture into a flask containing 150 ml of a sterilized inoculum medium consisting of bean sprouts extract, yeast extract, glucose, and KH2PO4. The flask was incubated for 48 h at 30°C until the mycelial mat was developed. This mycelial mat was used as inoculum material for saccharification process.

2.5 Hydrolysis of substrates by Clostridium acetobutylicum
For hydrolysis of substrates, freshly isolated C. acetobutylicum culture was employed optimum conditions with varying pH and period of hydrolysis were determined. The chemically treated substrates were autoclaved and inoculated with sporulating mycelial mat of C. acetobutylicum. Hydrolysis was terminated after 14 days of incubation at pH 5.0 and 30°C incubation temperature in a
shaker at 150 rpm. The hydrolysis process was monitored every two days for its released reducing sugar content following a Nelson-Somogyi method using UV-VIS Spectrophotometer (Shimatzu model UV-2600). As C. acetobutylicum grows on the cellulosic substrates the cellulose of the substrate is hydrolyzed to release simple sugars (glucose), which can be fermented to produce bioethanol.

2.6 Fermentative production of bioethanol
For fermentative production of bioethanol, C. acetobutylicum culture was employed and optimum conditions with varying pH and period of fermentation were determined. The process was carried out for a period of 14 days at 29°C. During the fermentation process, samples were withdrawn every 2 days for the estimation of bioethanol production based on their refractive index.

2.7 Distillation and dehydration of the produced bioethanol
Distillation were conducted to separate water from the produced bioethanol using a fractional distillation apparatus. The obtained bioethanol fraction was dried using zeolite. The density was determined using a pycnometer and bioethanol content was done by a gas chromatography method.

2.8 Analysis of bioethanol levels by gas Chromatography
Bioethanol content of the sample was calculated from the curve of the relationship between the ratio of the standard bioethanol peak area and the peak area of pentane as the internal standard (A₁/A₂) to standard bioethanol concentration.

3. Results and discussion
This section discusses the research data of G. verrucosa and E. cottonii seaweeds that were converted into bioethanol through simultaneous saccharification and fermentation process using C. acetobutylicum bacterium for a maximum bioethanol production.

3.1 Determination weight of dried seaweed
The amount of G. verrucosa seaweed cellulose remained after the drying and milling processes and initial treatment was 933 grams from 12037 grams of the fresh raw material. The yield of seaweed cellulose powder was 7.7% of the initial fresh raw material weight. The weight loss of the material was mostly from its moisture content which was very high. The additional loss occurred during the milling process and due to the release of lignin after the initial treatment using 5% NaOH as shown in Table 1.

| Types of seaweed | Fresh Weight (g) | Weight after drying (g) | Weight of cellulose (g) | Yield of cellulose (%) |
|------------------|------------------|-------------------------|------------------------|------------------------|
| G. verrucosa     | 12037            | 2237.8                  | 933                    | 7.7                    |
| E. cottonii      | 12000            | 2216.7                  | 648                    | 5.4                    |

3.2 Determination of water, cellulose, and lignin seaweed contents
Seaweed as biomass is mostly composed of polysaccharides and phenol-based compounds, especially cellulose, agar, lignin and minor amount of soluble compounds (ash). The results of the analysis of water, lignin, and cellulose contents are shown in Table 2. As shown in Table 2, the cellulose content in the G. verrucosa and E. cottonii seaweed is 59.1% and 48.9% (w/w), respectively. Although, this content was differences with previously study, where the carbohydrates content in the G. verrucosa is 74.5% [19] and in E. cottonii is 35.2% [11]. This differences results, maybe using different methods and formula to calculation the carbohydrates contents (Table 2).

In this study, the lignin in the lignocelluloses material could be reduced by the pretreatment using 5% NaOH solution. The maceration process with NaOH was quite effective in degrading the lignin. This is quite important because lignin contained in seaweed flour may block or slow the access to the
enzymes during polysaccharide hydrolysis process which in turn would reduce the amount of bioethanol produced in the fermentation process.

**Table 2.** Composition of fine powders of *G. verrucosa* and *E. cottonii* seaweeds after treatment.

| Smooth powder | Composition % (w/w) |  |
|---------------|---------------------|---|
|               | Water content | Cellulose levels | Lignin levels |
| *G. verrucosa* | 21.9          | 58.2            | 3.2           |
|               | 21.8          | 60.4            | 3.4           |
|               | 22.0          | 58.8            | 3.2           |
| Mean          | 21.9          | 59.1            | 3.3           |
| *E. cottonii* | 20.7          | 48.1            | 2.1           |
|               | 20.6          | 50.2            | 2.3           |
|               | 20.8          | 48.3            | 2.1           |
| Mean          | 20.7          | 48.9            | 2.2           |

3.3 Determination of the optimum time and pH of fermentation process

3.3.1. Production of bioethanol with SSF method

In general, the synthesis of bioethanol from biomass consists of two main stages, namely hydrolysis and fermentation. In previous method the hydrolysis and fermentation processes are done separately and called Separated Hydrolysis and Fermentation (SHF) process. However, in the most recent method, the process is carried out simultaneously and called a simultaneous saccharification and fermentation (SSF) process. One of the advantages of the SSF process is the hydrolysis and fermentation is done in a container or reactor so that it can occur efficiently. Hydrolysis takes place by a biological agent, which uses *C. acetobutylicum* bacterium that produces cellulase enzymes. Based on the above result the cellulose content in the seaweed before treatment was approximately 34.0% and after the initial treatment the cellulose content increased to 59.1% and 48.9% for *G. verrucosa* and *E. cottonii* seaweeds respectively. Cellulose is made up of hexose monosaccharides, mostly glucose. After polysaccharides are broken down into monosaccharides, then by microbes they would be converted into bioethanol. In general, the chemical equation can be written as follows:

\[
C_6H_{12}O_6 \rightarrow 2 C_2H_5OH + 2 CO_2 \]

Treatments condition by acidity (pH) and different fermentation times. The acidity in this process was controlled by adding phosphate buffer solutions. The pH used ranged from 4.0 to 6.5 by increments of 0.5, while the fermentation times were 2, 4, 6, 8, 10, 12 and 14 days. From the combination of all these fermentation times and pHs applied, the optimum fermentation time and pH for SSF process was determined based on the highest refractive index value of any treatment variations.

3.3.2. Determination of optimum fermentation time

The growth phase of the bacteria can be divided into 4 phases, namely lag phase, logarithmic (exponential) phase, stationary phase and microbial cell death phase. Bioethanol production process was done by adding the starter at the concentration of 10% into the substrate at pH 6.5 and the reactor temperature of 29°C and the mixture was harvested or withdrawn at varying fermentation times of 2, 4, 6, 8, 10, 12 and 14 days. Then, the harvested fermentation products were distilled to produce bioethanol and the refractive index values and densities of the generated bioethanol were analyzed using a refractometer and pycnometer respectively to determine the bioethanol having the highest concentration. The effect of fermentation time to the refractive index and bioethanol content of the produced bioethanol by the fermentation from *G. verrucosa* and *E. cottonii* seaweeds at pH 6.5 is shown in Table 3. From these data it can be observed that at pH 6.5 between 2 and 6 days of fermentation, the bacteria was still in the adjustment phase. At day 8 of fermentation, bioethanol levels started to rise to 4.4% bioethanol for either *G. verrucosa* or *E. cottonii seaweed* cellulose materials. At day 10, the bioethanol levels increased to 5.7% for *G. verrucosa* seaweed and 6.1% for *E. cottonii*
seaweed. At day 12 and 14 the levels of bioethanol declined to 3.0% and 2.3% respectively for *G. verrucosa* seaweed cellulose and 3.9% and 3.0% respectively for *E. cottonii* seaweed cellulose (Table 3).

### Table 3. Effect of fermentation time (day) on refractive index and percentage of bioethanol of the bioethanol products from *G. verrucosa* and *E. cottonii* seaweed celluloses fermented at pH 6.5

| Time (d) | Refractive Index | Percentage of Bioethanol (%) |
|---------|------------------|-------------------------------|
|         | *G. verrucosa*   | *E. cottonii*                |
| 2       | 1.3314           | 1.3312                       | 1.6 | 1.5 |
| 4       | 1.3318           | 1.3316                       | 2.8 | 2.4 |
| 6       | 1.3321           | 1.3320                       | 3.5 | 3.2 |
| 8       | 1.3325           | 1.3325                       | 4.4 | 4.4 |
| 10      | 1.3331           | 1.3333                       | 5.7 | 6.1 |
| 12      | 1.3319           | 1.3323                       | 3.0 | 3.9 |
| 14      | 1.3316           | 1.3319                       | 2.3 | 3.0 |

Additional products that are formed can be either acetic acid or other organic acids from ethanol undergoing further reaction as follows:

\[
\text{CH}_3\text{CH}_2\text{OH} \rightarrow \text{CH}_3\text{CH(OH)}_2 \rightarrow \text{CH}_3\text{CHO} \rightarrow \text{CH}_3\text{COOH} \]

From the trend of bioethanol percentages over the incubation time, at day 14 of fermentation the bioethanol concentration dropped and was kept relatively constant. At this stage the bacteria have reached their death phase characterized by an increase in mortality rate that exceeds the rate of growth, causing the overall decline in the bacterial population. This occurred for both seaweed cellulose materials, so it could be concluded that the optimum incubation time for *C. acetobutylicum* bacteria on this bioethanol fermentation process for both *G. verrucosa* and *E. cottonii* seaweed celluloses was 10 days.

#### 3.3.3. Determination of optimum fermentation pH

Acidity (pH) is one of several important factors that can affect the fermentation process to produce ethanol. The acidity of the conducted fermentation process was between 4.0 and 6.5. At the pH below 4.0 the fermentation process would decelerate. The desired acidity was obtained by adding phosphate buffers. The addition of buffers herein was carried out to correspond to the desired magnitude. The results obtained due to varying pH conditions on the levels of bioethanol from *G. verrucosa* and *E. cottonii* seaweed celluloses are as shown in Table 4.

### Table 4. The Effect of pH on refractive index and percentage of bioethanol of the bioethanol products from *G. verrucosa* and *E. cottonii* seaweed celluloses fermented for 10 days.

| pH  | Refractive Index | Percentage of Bioethanol (%) |
|-----|------------------|-------------------------------|
|     | *G. verrucosa*   | *E. cottonii*                |
| 4.0 | 1.3317           | 1.3314                       | 2.5 | 1.9 |
| 4.5 | 1.3319           | 1.3316                       | 3.0 | 2.4 |
| 5.0 | 1.3331           | 1.3333                       | 5.7 | 3.9 |
| 5.5 | 1.3335           | 1.3335                       | 6.6 | 6.6 |
| 6.0 | 1.3340           | 1.3338                       | 7.7 | 7.2 |
| 6.5 | 1.3325           | 1.3330                       | 4.4 | 5.5 |

From these data it can be seen that the highest concentration of bioethanol produced in the fermentation process with a pH of 6.0 was 7.7% and 7.2% from the cellulose materials of *G. verrucosa* and *E. cottonii* seaweeds respectively. When fermentation process occurred at pH 4.0 to 5.5,
the bioethanol content increased from 2.5 to 6.6% and 1.9 to 6.6% for *G. verrucosa* and *E. cottonii* seaweed celluloses respectively. However, at pH 6.5 the bioethanol contents were only 4.4% and 5.5% for *G. verrucosa* of *E. cottonii* seaweeds respectively.

### 3.4 Production of bioethanol in optimum condition (pH 6.0)

#### 3.4.1 Production of bioethanol in optimum condition (pH 6.0)

The level of substrates used for production of bioethanol from seaweed was 6%. This is the optimum level because when lower levels were used the yield of bioethanol produced was relatively small but when the substrate level above 6%, it would form a solid gel when cooled. In this study, for the production of bioethanol 540 grams of substrate was dispersed to make 9000 mL of dispersion. To obtain 540 g of the substrate, it needed 5.6 g of fresh seaweed raw material.

#### 3.4.2 Fermentation

A total of 9000 mL of substrate dispersion was fermented at the optimum condition which was carried out at pH 6.0 for 10 days. After fermentation followed by distillation, it resulted 27 mL bioethanol and after drying with Na$_2$SO$_4$ anhydrous the final bioethanol obtained was 25.5 mL and its density was 0.8 g/cm$^3$.

### 3.5 Determination of bioethanol concentration

The concentration of the dehydrated bioethanol was assayed by gas chromatography (GC 2010 Shimadzu). The peak of bioethanol sample presented as the 7$^{th}$ peak with a retention time of 2.28 min (Figure 1). This retention time was the same as the retention time of standard ethanol which was 2.29 min. Peak number 5 was the internal standard pentane with a retention time of 1.78 min, while peak numbers 3, 4 and 6 having the retention times of 0.79, 1.06, and 2.00 min respectively are the peaks of the impurities. There was no peak of water in the chromatogram anymore. This means that the water contained in the obtained bioethanol had already been completely dried with Na$_2$SO$_4$. However, the bioethanol sample still contained some impurities or other byproducts, so this needed further purification. The impurities caused by the byproducts might exist as acetic acid or other organic acids formed from ethanol that underwent further reaction during fermentation process. The additional peak may also come from the left over compounds in either the injector or the column used (Figure 1).

![Figure 1. Chromatograms of bioethanol sample before distillation purification by gas chromatography analysis.](image)
Purification was done by re-distillation of the anhydrous bioethanol (25.5 mL) at a temperature of 60°C. The resulted purification was 22.5 mL. This purification by distillation was done to remove the impurity compounds that have the boiling point lower than that of ethanol. The boiling point of ethanol is 78.4°C. After further purification by re-distillation, the final bioethanol could be considered as pure ethanol because there was no other peak observed from gas chromatography analysis as shown in Figure 2.

From the chromatogram it could be seen that the present of bioethanol was shown as the 3rd significant peak with a retention time of 2.29 min and the 2nd significant peak was the internal pentane standard.

![Chromatogram of bioethanol sample (unknown) after distillation purification by gas chromatography analysis.](image)

To determine the amount of the bioethanol in the sample by GC analysis, the content was calculated based on the ratio value between the peak area of the standard solution and the peak area of the sample. The results of GC analysis are shown in Table 5.

**Table 5.** Peak areas and peak ratio values of standard ethanol and sample from gas chromatography analysis.

| No. | Type | Standard Bioethanol(%) | Peak area | A_i/A_{IS} Ratio |
|-----|------|------------------------|-----------|-----------------|
|     |      |                        | Bioethanol (A_i) | Internal Std (A_{IS}) |
| 1.  | Standard | 20                     | 37556383 | 23081740          | 1.63 |
|     |        | 40                     | 64896218 | 24051936          | 2.70 |
|     |        | 60                     | 101916058 | 15376745          | 6.63 |
|     |        | 100                    | 178837092 | 13648829          | 13.10 |
| 2.  | Sample | -                      | 191286559 | 15651653          | 12.22 |

In the analysis above pentane was used as the internal standard. The internal standard was used as the correction factor to minimize errors that may occur during analysis by a gas chromatography method. Such errors may take place due to a variation of the injection time during the injection of standards and samples. From the results in Table 5 above a graph demonstrating the relationship between the standard ethanol concentration and A_i/A_{IS} ratio value as shown in Figure 3.
Figure 3. Relationship between standard ethanol concentrations versus $A_I/A_{IS}$ ratio values.

The regression equation could be expressed as: $Y = 0.150X - 2.241$. From this equation the $A_I/A_{IS}$ ratio value of the sample (12.2215) would give 96.4% as the bioethanol content of the obtained bioethanol sample. The result was very high bioethanol content and spectacular different to the one just given by bioethanol content was of 29.6% from cellulose of the same seaweed *G. verrucosa* by separated hydrolysis and fermentation system using *Trichoderma viride* and *Zymomonas mobiles* [20].

4. Conclusion
Seaweeds from *G. verrucosa* and *E. cottonii* could be converted into bioethanol having the bioethanol levels of 5.7% and 6.1%, respectively after 10 days of fermentation. The optimum conditions for fermentation process of *G. verrucosa* and *E. cottonii* seaweed celluloses using *C. acetobutylicum* bacterium to produce bioethanol was 10 days of incubation time carried out at pH 6.0 resulting the bioethanol levels of 7.7% and 7.2%, respectively. When *G. verrucosa* seaweed was used, the conversion value was 33.3 grams (3.3%) of bioethanol having 96.4% purity after a multi-level distillation process from every kg of cellulose.

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