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# BIOETHANOL PRODUCTION FROM CELLULOSE IN RED ALGAE *Gracilaria verrucosa* BY SEPARATED HYDROLYSIS AND FERMENTATION SYSTEM USING *Trichoderma viride* AND *Zymomonas mobilis*

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## ABSTRACT

In this study, renewable marine cellulose from red algae *Gracilaria verrucosa* was utilized for the production of bioethanol. Bioethanol from the red alga cellulose was produced by the enzymatic hydrolysis and fermentation methods and the conversion value of the cellulose in *Gracilaria verrucosa* was estimated. *Trichoderma viride* fungus and *Zymomonas mobilis* bacterium were used for enzymatic hydrolysis and bioethanol fermentation, respectively. Enzymatic hydrolysis and fermentation optimization were carried out by varying pH and period of hydrolysis or fermentation. Research results indicate that the optimum condition of the enzymatic hydrolysis cellulose is obtained at pH 5.5 in 6 days, whereas the optimum condition of bioethanol fermentation is obtained at pH 6.0 in 7 days. The bioethanol concentration as measured by Gas Chromatography, showed that one kilogram of cellulose in *Gracilaria verrucosa* produces 23.01% bioethanol with the concentration of 29.60%.

**KEYWORDS:** ***Gracilaria verrucosa*, cellulose, bioethanol, *Trichoderma viride*, *Zymomonas mobilis***

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# INTRODUCTION

Today, fossil energy sources (gas and oil) face a serious problem due to energy crisis. This is reflected in the large number of research activities that aim to produce energy from green sources with guaranteed the availability. One of the energy alternatives that can solve the crisis of energy problem is bioethanol. Bioethanol is one of the alternative energy sources to replace fuel[1]. In Indonesia bioethanol is produced from materials like cassava, corn, sugar cane, forming a threat to livestock feed industry.The algae has been considered as a next generation biomass for bioethanol production [2]. The algae has high productivity per unit area per year, and there is no competition with food crops. Therefore, algae can help as alternative materials to produce bioethanol, and one such source is red algae. South Sulawesi is the largest producer of red algae in Indonesia. One of the potential red macroalgae is *Gracilaria verrucosa.* Algae that fail the export criteria can be utilized as raw materials to produce bioethanol, replacing corn, cassava and sugar cane. Cellulose biomass can be utilized to produce bioethanol[3]. And the other hand, micro and macroalgae contains about 13-19 % of cellulose[4]. In this research, we used macroalgae *Gracilaria verrucosa* as cellulose biomass source to produce bioethanol. Various kinds of these microbes are used in fermentation to produce ethanol. Yeast *Saccharomyces cerevisiae* is commonly used to produce bioethanol, but they do not have good resistance to improve concentration of ethanol[5]. Latest research focused on Gram-negative bacteria *Zymomonas mobilis* that is more prospective to produce ethanol, at large scale industry level[6]. Optimization of hydrolysis and/or fermentation was conducted at various pH and periodic uses of fungus *Trichoderma viride and* bacteria *Zymomonas mobilis. The purpose of* this research was to produce bioethanol from cellulose in red macroalga *Gracilaria verrucosa* by separated enzymatic hydrolytic and fermentation methods to find out the conversion value of the cellulose in red algae *Gracilaria verrucosa*.

# MATERIALS AND METHODS

### *Raw Materials*

The red algae *Gracilaria verrucosa* was collected in the region of Barang Lompo Island, South Sulawesi Province, Indonesian terrestrial. Algae identification was conducted in Marine Biology Laboratory Faculty of Natural Sciences Hasanuddin University, Indonesia. Macroalgae *G. verrucosa* cut into small pieces with a size of 1-2 cm, dried in the sun to dry and then ground with *cruser* until smooth powder and were utilized as substrates.

### *Pre-treatment of substrates*

The substrates were treated chemically with 4% NaOH performed by soaking for 24 hours. Residues and the filtrate separated. Residues bleached with a solution of $H_2O_2$ 3% and then washed with distilled water until neutral and then dried in an oven at a temperature of 105 $^oC$ until constant weight[7].

### *Chemical analysis of Substrates*

The substrates were subjected to the estimation of water content, lignin and cellulose content[8].

### *Microorganisms and culture*

### *Rejuvenation of Trichoderma viride*

*Trichoderma viride was* obtained from the available stock culture from Laboratory of Bioprocess, Departement Chemical Engineering, Polytechnic State Ujung Pandang. The strain was cultured and maintained on sterile Potato Dextrose Agar slants and was allowed to grow in an incubator at 30 $^oC$ for 4 days [3]. After optimum growth the culture was stored at 4 $^oC$ in refrigerator for further use.

### *Inoculum Preparation of Trichoderma viride*

The spore suspension was used as inoculum in the present studies. It was prepared from a 4 days old slant were added in 150 mL of sterilized inoculums medium consisting of bean sprouts extract, yeast extract, glucose, and $KH_2PO_4$. The flasks were incubated for 48 hrs at 30 $^oC$ untill the mycelial mat develops. This mycelial mat was used as

inoculum in further saccharification experiments.

***Hydrolysis of substrates by Trichoderma viride***

For hydrolysis of substrates locally isolated fungal culture *Trichoderma viride* was employed to determine optimum conditions with varying pH and periodic of hydrolytic. The chemically treated substrates were autoclaved and inoculated with sporulating mycelial mat of *Trichoderma viride*[3]. Hydrolysis was shaking methods for a period of 14 days at pH 5.0 and held at 30 $^{o}$C. The process was monitored every 2 days for reducing sugars released with *Nelson-Somogyi* method by Spectrophotometer UV-VIS Instrument (Shimatzu model UV-2600). For the shaking method an orbital shaking incubator was employed and shaking was performed at 150 rpm at 30 $^{o}$C temperature. The *T. viride* was selected for hydrolysis as it is cellulolytic in nature and can hydrolyze cellulose present in the substrates to simple sugars. Generally this step is carried out by commercially available cellulase enzyme which is very expensive. In our study an attempt was made to design an economical process by the use of intact fungal organism as a source of cellulase enzyme instead of commercially available enzyme. As *T. viride* grows on the cellulosic substrates hydrolyzes cellulose of the substrate and release simple sugars (glucose), which can be fermented to produce bioethanol.

***Inoculum Preparation of Zymomonas mobilis***

*Zymomonas mobilis* was obtained from the available stock culture from Laboratory of Bioprocess, Department Chemical Engineering, Polytechnic State Ujung Pandang. The strain was cultured and maintained on sterile Nutrient Agar slants and was allowed to grow in an incubator at 30 $^{o}$C for 8 days. After optimum growth the culture was stored at 4 $^{o}$C in refrigerator for further use. The suspension was used as inoculum in the present studies. It was prepared from a 4 days old slant were added in 150 mL of sterilized inoculums medium consisting of bean sprouts extract, yeast extract, glucose, urea, $KH_2PO_4$, $K_2HPO_4$, and $(NH_4)_2SO_4$ by transferring from the media for slanting. The flasks were incubated for 48 hrs at 30 $^{o}$C was used as inoculum in further fermentation experiments.

***Fermentative production of bioethanol by Zymomonas mobilis***

For fermentative production of bioethanol (stationary and shaking) *Zymomonas mobilis* was employed to determine optimum conditions with varying pH and periodic of fermentation. After 6 days at pH 5.5 of hydrolysis mycelial mat of *Trichoderma viride* was removed under aseptic conditions and 10% of *Zymomonas mobilis* inoculum culture was added to all the flasks. The process was carried out for a period of 13 days at 30 $^{o}$C. During the fermentation process every 2 days, samples were sampling for the estimation of bioethanol by refractive index with refractometre.

## RESULTS

In the present study fuel ethanol was produced by hydrolysis and fermentation of cellulosic substrates. Before starting the hydrolysis and fermentation experiments initial reducing sugars, lignin and cellulose content were determined (Table 1).

**Table 1**
***Chemical composition of cellulosic substrates***

| Chemical composition substrates | Value (%) |
|---|---|
| Water content | 8.87 |
| Lignin | 3.84 |
| Cellulose | 13.04 |

***Hydrolysis process***

***Determination of the optimum hydrolysis periodic***

In stationary fermentation the reducing sugars released increased from day 2 to day 14, highest amount of sugars released on 6$^{th}$ day of hydrolysis from all the substrates. Highest amount of

reducing sugars was released from red algae *Gracilaria verrucosa* with Nelson-Somogyi method by Spectrophotometer UV –VIS instrument (Figure 1).

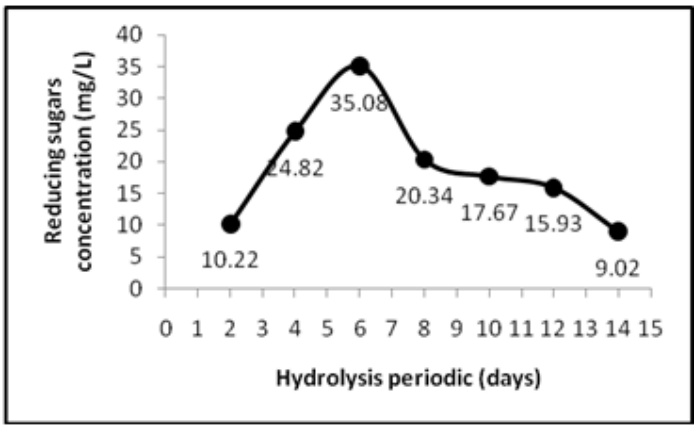


**Figure 1**
***Effect of hydrolysis periodic on reducing sugar content***

***Determination of the optimum hydrolysis pH***

In stationary fermentation the reducing sugars released increased at pH 4.5-6.5. The highest amount of sugars released from all the substrates was at pH 5.5. Highest amount of reducing sugars was released from red algae *Gracilaria verrucosa* (Figure 2).

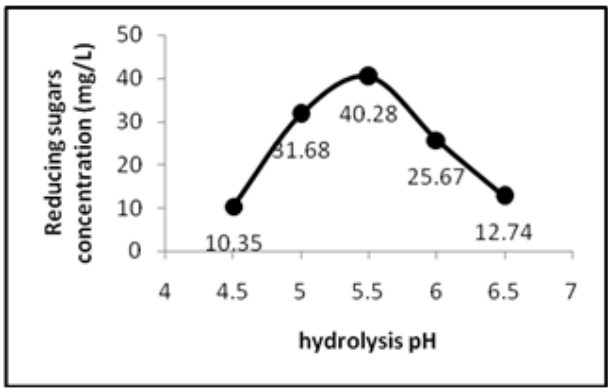


**Figure 2**
***Effect of hydrolysis pH on reducing sugar content***

***Determination of the optimum fermentation periodic***

In stationary fermentation increasing trend in ethanol production was observed from day 1 to day 13. The results of ethanol produced are presented in Figure 3. Highest amount of ethanol was produced on 7$^{th}$ day.

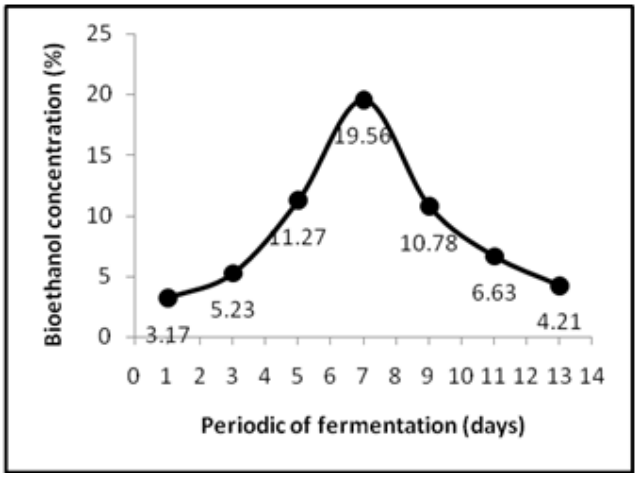


**Figure 3**
***Effect of fermentation periodic on bioethanol concentration***

***Determination of the optimum fermentation pH***

In stationary fermentation increasing trend in ethanol production was observed held at pH 4.0-8.0. The results of ethanol produced are presented in Figure 4. Highest amount of ethanol was produced at pH 6.0 with the content of 21.21%.

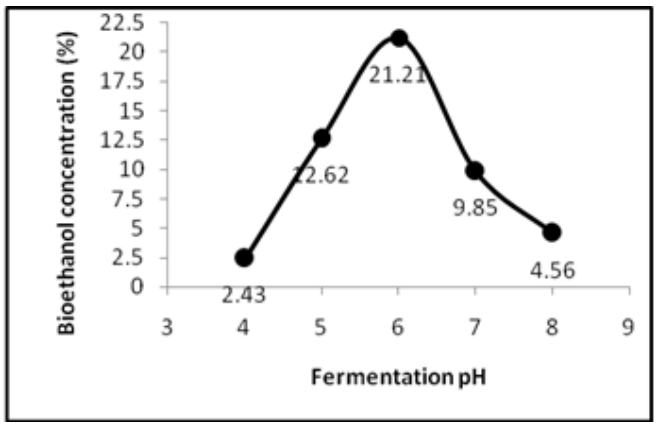


**Figure 4**
***Effect of fermentation pH on bioethanol concentration***

***Determination of bioethanol***

Research result indicates that the optimum condition of the hydrolytic cellulose is enzymatically obtained on the pH 5.5 in 6 days and the optimum condition of the bioethanol fermentation is obtained on pH of 6.0 in 7 days. The bioethanol concentration measurements is carried out by using Gas Chromatography. Purification by distillation carried out by looking at the retention time of impurities is very low ie, below the boiling point of ethanol. The boiling point of bioethanol is 78.4 $^{o}$C.

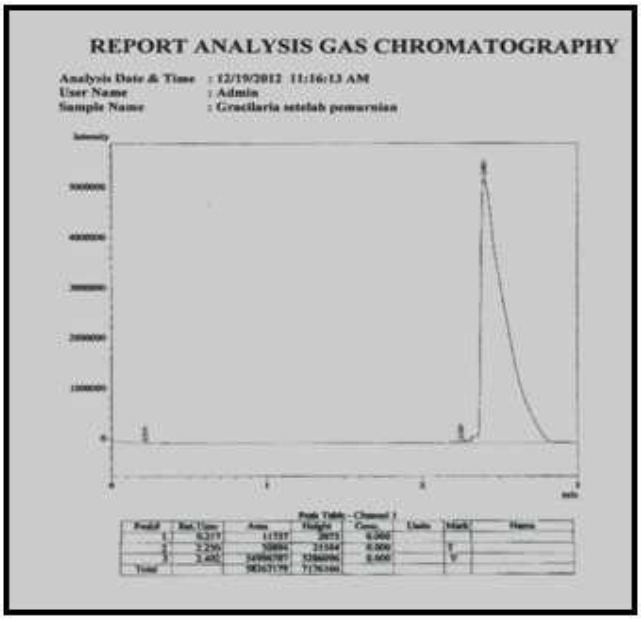


**Figure 5**
***Bioethanol analysis using Gas Chromatography***

## DISCUSSION

Today bioethanol production process was studied with hydrolysis enzymatic process by *T. viride* and fermentation bioethanol production by *Z. mobilis* [3, 6]. Substrate from *G. verrucosa* were chemically treated with 4% NaOH for a period of 24 hrs before enzyme hydrolysis to improve enzyme amenability. Normally cellulosic materials can be hydrolyzed chemically or enzymatically. After pretreatment of substrates decreased lignin content (3.84%) and increased cellulose content (13.04%). Bioethanol production is a widely studied process for biofuel production. Different workers have studied various raw materials and different methods for bioethanol production but, recently it has been observed that lignocellulosic materials are focused for bioethanol production and this research are focused potential marine resources was red algae *G. verrucosa.* Cellulosic substrates from brown macroalgae were also used by Adam *et al.,* for bioethanol production by engineered microbial extracellular enzymatic hydrolysis and fermentation was recorded [9]. Results of the influence of time of fermentation can be seen in Figure 3. In all treatments increase in levels of bioethanol is very slow from day 1 to day 5. At this stage there occurs the phase lag, a phase in which the microbes are still adjusting to environmental conditions so that microbial activity is not optimum. During this phase the cell mass increased very little without the addition of the number of cell density and therefore the rate of cell growth could be equal to zero. Long lag phase in bacteria vary widely, depending on media composition, pH, temperature, aeration, number of cells in the initial inoculum and physiological properties of microorganisms in the media earlier. When the cell has to adapt to new environment, the cells begin to divide until it reaches a maximum population. This phase is called the logarithmic phase or exponential phase. Exponential phase is marked by the occurrence of periods of rapid growth. Each cell in the population divides into two cells. The variation of the degree of bacterial growth in exponential phase is strongly influenced by genetic properties of the revelation. In addition, the degree of growth is also influenced by nutrient levels in the media, incubation temperature, pH and aeration conditions. In this study, this phase occurred on day $7^{th}$ and is characterized by elevated levels of bioethanol. On the $7^{th}$ day of the cellular reproduction began to occur, which slowly increased the concentration of biomass accompanied by increase in cell numbers. At this rate of growth, the reproductive cell reaches a maximum point,

followed by exponentially growth. During exponential phase, increased cell growth rate is proportional to the concentration of cells at the time. At day 9, the bacteria was at a stationary phase, implying that the bacteria was not working optimally. Stationary phase occurs when the bacterial growth rate equals the death rate, so that the overall number of bacteria remained unchanged. In this study we examined the effects of varied conditions of fermentation pH from 4.0 to 8.0. Data results of the influence of pH of fermentation can be seen in Figure 4. The desired acidity is obtained by adding phosphate buffer, addition of buffer here is intended for pH in accordance with the amount of the desired pH. The highest concentration of ethanol produced at pH 6.0 treatment was equal to 21.21% while for pH 7.0 to 8.0 to produce bioethanol a lower level that is equal from 9.85% to 4.56%. Changes in pH can occur because the fermentation not only produce ethanol but also produce other compounds such as acetic acid, butyric acid and formic acid. Acetic acid can be produced by symbiotic bacteria, Acetobacter. Lactobacillus also can contribute to contamination converting glucose into lactic acid, thereby reducing the ethanol yield and inhibiting microbial growth. But there are also possibilities that hydrolyzed glucose has been fermented into other products because there is no more glucose. This can be caused by inhibitors present in biomass including lignin, a weak acid, and phenolic compounds derivatives. High or low levels of ethanol in the fermentation process can be caused by the activity of enzymes produced by bacteria. The enzyme activity is influenced by pH, ionic nature of the carboxyl group and amino group easily influenced by pH. In addition, changes in pH also cause denaturation of enzyme protein causing loss of enzyme activity[10]. *Zymomonas mobilis* bacteria can grow well at pH 6.0 and therefore the concentration of bioethanol produced higher pH than other treatments. Further studies on the improvement of bacteria and carbohydrate degradation strains are essential for the utilization of total carbohydrate in macroalgae.

## CONCLUSION

1. The optimum condition of hydrolysis enzymatic using the fungal *Trichoderma viride* was pH 5.5 for 6 days.
2. The optimum condition of fermentation bioethanol using the bacteria *Zymomonas mobilis* was pH 6.0 for 7 days.
3. Conversion value of red algae *Gracilaria verrucosa*, every one kilogram of cellulose from *G. verrucosa* produces 23.01% bioethanol with the content of 29.60%.

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## REFERENCES

1. Foody B, “Ethanol from Biomass: The Factors Affecting it’s Commercial Feasibility”. Iogen Corporation, Ottawa, Ontario, Canada, (1988).
2. Ge L, Peng W, Haijin M, Study on saccharification techniques of seaweed wastes for the transformation of ethanol. Renew Energy 36:84–89, (2011)
3. Malik, S. K., et. al., Optimization of process parameters for the biosynthesis of cellulases by *Trichoderma viride*. *Pak. J. Bot.* 42 (6): 4243-4251, (2010).
4. Rojan PJ, Anisha GS, Madhavan N, Ashok P., Micro and macroalgal biomass: a renewable source for bioethanol. Bioresour Technol 102:186–193, (2011).
5. Taherzadeh MJ, Niklasson C, Liden G, Acetic acid-friend or foe in anaerobic batc conversion of glucose to ethanol by Saccharomyces cerevisiae. Chem Eng Sci 52:2653–2659, (1997)
6. Davis, L., et. al., Evaluation of Zymomonas-based ethanol production from hydrolised waste starch stream.

*Biomass and bioenergy.* 30:809-814, (2006).

7. Seligh, M. J., Todd, B., Vinzant, T. B., Himmel, M. E., Decker, S. R., The Effect of Lignin Removal by Alkaline Peroxide Pretreatment on The Susceptibility of Corn Stover to Purified Cellulolytic and Xylanolytic Enzymes. *Appl Biochem Biotechnol*. 1:265-273, (2009).
8. Sadasivam S, and Manickam A, In: Biochemical methods for Agricultural sciences, Willey Eastern Limited, New Delhi, pp.5-11, (1992).
9. Adam JW, Effendi L, Maung NW, Drew DR, Christine NS, Peter BK, Susan RC, Ryan MR, Asael H, Alicia BS, Arun L, Yuki K, David B, Yasuo Y, An engineered microbial platform for direct biofuel production from brown macroalgae. Science 335:308–313, (2012).
10. Larsson S, Palmqvist E, Hahn-Hagerdal B, Tengborg C, Stenberg K, Zacchi G, Nilvebrant NO, The generation of fermentation inhibitors during dilute acid hydrolysis of softwood. Enz Microbiol Technol 24:151–159, (1999).