Bioethanol Production from Seaweed *Eucheuma cottonii* by Neutralization and Detoxification of Acidic Catalyzed Hydrolysate

Jaini Fakhrudin, Dwi Setyaningsih, and Mulyorini Rahayuningsih

***Abstract*—The presence of inhibitors such as hydroxymethyl furfural (HMF) and dissolved salts on the hydrolysate disrupt the growth of *Saccharomyces cerevisiae* in fermentation process. The objective of research was to increase the yield of ethanol in fermentation process through neutralization and detoxification of *Eucheuma cottonii* hydrolysate. Hydrolysate was neutralized and overliming with $Ca(OH)_2$ and NH_4OH and continued by activated charcoal adsorption. The results showed that the highest content of reducing sugars was 11.34% (w/v) on neutralization with $Ca(OH)_2$. The lowest content of HMF was 1.39 g/kg hydrolysate on overliming treatment with $Ca(OH)_2$. Overliming treatment with $Ca(OH)_2$ was able to reduce levels of HMF up to 16.77%, while the treatment with NH_4OH overliming contributed to 1.06% for the HMF reduction. The highest ethanol yield was 2.49% (v/v) through neutralization with $Ca(OH)_2$, while neutralization with NH_4OH treatment was 0.49% (v/v). The low yield of ethanol on treatment with NH_4OH due to inhibition by HMF and high levels of salinity in the hydrolysate, 1.89 g/kg hydrolysate and $160^0/_{00}$ respectively. The fermentation efficiency obtained at treatment by neutralization with $Ca(OH)_2$ was 56.14%.**

***Index Terms*—Bioethanol, *Eucheuma cottonii*, neutralization and detoxification.**

I. Introduction

The potency of natural resources that can be developed as a raw material of bioenergy especially bioethanol is seaweed. From the literature, 0.3934 kg of galactose can be obtained from 1 kg of seaweed *Eucheuma spp*, which means 1.18 kg from 1 m^2 area of the cultivation annually [1].

One of the paradigms developed for the solution is to dig more potential resources for the production of renewable and sustainable energy. Seaweed production data in 2010 showed a significant increase of 3.082 million tons, after the previous year to reach 2.574 million tons [2]. The production value of this enormous demand due to seaweed as raw material industry is huge both inside and outside the country.

Eucheuma spp. contains large amounts of polysaccharides. The polysaccharides are largely in the form of carrageenan as cell wall component. Carrageenan is a linear, sulphated polysaccharide, the primary structure being made up of alternating α (1-3)-D-galactose-4-sulphate and β (1,4)-3,6-anhydro-D-galactose residues [3].

Fermentation techniques in the production of bioethanol from *Eucheuma cottonii are* still not efficient because of low productivity. The low productivity is caused by the presence of such inhibitors on the substrate in the form of toxic compounds and the weak ability of microbes to ferment galactose. Attempt to neutralize and detoxify to reduce the limiting factor becomes very important in order to get maximum yield in fermentation process.

Inhibitor concentration and sugar content in the hydrolysate depend on the conditions of hydrolysis. Hydrolysate with high sugar level do not always give higher ethanol yield than the hydrolysate with low sugar content because there can be some inhibition of the growth of microorganism fermentation [4].

E.cottonii hydrolysate may contain toxic compounds as hydroxymethyl furfural (HMF) and furfural. Furfural is formed from the degradation of pentose sugars, and 5-hydroxymethylfurfural (HMF) from hexoses sugars [5]. HMF and furfural can decrease productivity of ethanol, and slow the growth of organisms [6]. HMF and furfural act synergistically to reduce ethanol production [7].

Detoxification is a common method used by adding alkaline compounds in the acid hydrolysate until pH 10, after which the pH is lowered to 5.5 by adding H_2SO_4. Alkaline compounds which commonly used are $Ca(OH)_2$ and NH_4OH. The addition of alkali can reduce furan and HMF found in hydrolysates, resulting in increased productivity of ethanol from fermentation [8].

The objective of the study was to assess the effectiveness of detoxification with neutralization and overliming followed by activated charcoal adsorption to remove toxic compounds in hydrolysates *E. cottonii*.

II. Methods

A. Yeast Regeneration

Growth Media used was Potato Dextrose Agar (PDA) purchased commercially. *Saccharomyces cerevisiae* was cultured on PDA media that has been sterilized at a temperature of 121° C and a pressure of 1 atm for 15 minutes, then incubated at 30° C for 3 days. Microbes on PDA media as culture stock regenerated on the Yeast extract Maltose Glucose Peptone (YMGP) media at 30° C for 24 hours before used to be mixed with hydrolysate. YMGP media was

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prepared in laboratory with composition of yeast extract 5 g/l, maltose 5 g/l, glucose 40 g/l, and peptone 5 g/l. Media was sterilized at a temperature of 121° C and a pressure of 1 atm for 15 minutes.

B. Preparation of Raw Materials

Seaweed soaked for 2 days with the replacement of water during the soaking process. Immersion is the beginning of the desalination process to remove salt in the seaweed. Seaweed was cleaned to reduce impurities, and then crushed by drilling machine. The crushed seaweed was dried under the sun for 5-7 days. Further, seaweeds was weighed as much as 15 g for the first stage and second stage respectively.

C. Hydrolysis Process

Acid hydrolysis was carried out to elaborate a polysaccharide in the seaweed so that it becomes simple structure. The acid used in hydrolysis was sulfuric acid (H_2SO_4) with a concentration of 3% through two stages of hydrolysis. The first stage was hydrolysis for 30 minutes and proceed to the second stage by mixing 15 g crushed seaweed for 30 minutes. Hydrolysis was carried out using an autoclave at 121 °C. Hydrolysate was neutralized to pH 5,5-6 and then Reducing sugar determinated by 3,5-dinitrosalicylic acid (DNS) method [9].

D. Detoxification

The detoxification process was conducted in two ways. First, overliming done by adding $Ca(OH)_2$ and NH_4OH in the hydrolysate to pH of 10. At overliming with $Ca(OH)_2$, insoluble calcium separated by filtration. Lime solution was made by adding calcium oxide (CaO) with water (1:3 ratio). Solution was stirred for 30 minutes and filtered to remove lime and other compounds that precipitate.

The hydrolysate was lowered to pH 5,5-6, with the addition of 10% H_2SO_4. The next stage was the addition of activated charcoal. Activated charcoal used in concentrations of 2.5%, 5% and 7.5% (w / v) was added to the hydrolysate and with a stirring speed of 150 rpm for 15, 30, and 45 minutes at temperature of 40° C and followed by filtration using filter paper.

On detoxification with NH_4OH, the process was done in the same way but there was no filtering. Second, neutralization of the hydrolysate with $Ca(OH)_2$ [10] and NH_4OH to pH 6.4 to 6.8. Solution was stirred for 30 minutes and followed by activated charcoal adsorption 2.5%, 5% and 7.5% (w / v) for 15, 30, and 45 minutes at a temperature of 40° C.

The hydrolysate was analyzed to measure total dissolved solids (TDS) by TDS meter, reducing sugar content by the DNS method [9], and HMF analyzed by spectrophotometer based on AOAC official method 980.23 [11].

Five grams of hydrolysate were dissolved in 25 ml of distilled water, treated with a clarifying agent (0.5 ml of Carrez I and 0.5 ml of Carrez II solutions) and volume made up to 50 ml. The solution was filtered, and the First 10 ml discarded. The absorbance of the filtered solution was measured at 284 and 336 nm against an aliquot of the filtered solution treated with NaHSO3. HMF was determined as follow:

HMF/100 g of hydrolysate = (Abs 284 - Abs 336) × 14.97 × (5/g of sample).

E. Fermentation

The process of fermentation in this study conducted over six days at room temperature. Prior to fermented, pasteurized hydrolysate at 70° C for 15 minutes to sterilize the microbes that disrupt the fermentation process. Hydrolysates are added with urea 0.5% to enrich the substrate. Microbial fermentation used was *Saccharomyces cerevisiae*. After the fermentation process, analysis was performed to measure pH, reducing sugar [9], and ethanol content. Ethanol content analysis was determined by using Density Meter DMA 4500 M.

III. Results and Discussion

A. Acid Hydrolysis

The process of acid hydrolysis with H_2SO_4 3% continued by neutralizing and overliming used two types of base as NH_4OH and $Ca(OH)_2$. Table I showed that the highest sugar content generated in the process of hydrolysis was 11.34% (w/v hydrolysates) through the process of neutralization with $Ca(OH)_2$, while the lowest sugar content was 8.85% (w/v hydrolysates) on neutralization with NH_4OH treatment.

Acid hydrolysis and followed by neutralization of $Ca(OH)_2$ was the best treatment. The process produced reducing sugar 0.378 kg/kg dry seaweeds. The applicability $Ca(OH)_2$ also savings compared with NH_4OH in which relatively more expensive.

TABLE I: Characteristic of Hydrolysates on Detoxification

Detoxification Method	Type	Brix (%)	Reducing Sugar (% w/v hydrolysates)	Reducing Sugar (% w/w dry seaweeds)	Residual Solid (g)	Salinity (‰)
Neutralization	NH_4OH	16.75	8.85	29.50	21.30	160
	$Ca(OH)_2$	11.50	11.34	37.80	28.20	125
Overliming	NH_4OH	16.25	10.74	35.78	16.15	110
	$Ca(OH)_2$	11.25	11.27	37.57	34.40	70

Hydrolysis process produces both hydrolysate and residual solid after separation process . Type of base used will affect the amount of residual solid. Generally, the higher amount of residual solid resulted in the smaller amount of hydrolysate, and vice versa. Little amount of residual solid would be better because it produces more hydrolysate. From the research, the use of $Ca(OH)_2$ resulted in the high amount of residual solid. Result showed that the highest residual solid was 34.40 g on overliming with $Ca(OH)_2$.

Neutralizing process by using alkaline is potential for salt formation. Salt was formed from the reaction of acids and bases. This salt is an ionic compound consisting of cations and anions. The high salinity as shown in the Table I indicated the presence of salt formed from the process. The highest salinity was $160^0/_{00}$ in the process of neutralization with NH_4OH. The higher the salt content in the hydrolysate will disrupt the growth of microorganisms in the fermentation process. It will lead to the low yield of ethanol.

Alriksson, [4] stated that one of the factors constraining growth *S. cerevisiae* was the salt content in the substrate. Salt $CaSO_4$ is formed as the effects of detoxification with Ca(OH)2. The addition of 1 M NaCl in the media will reduce the growth rate of *S. cerevisiae* by 70% [12]. Na^+ and Cl^- can inhibit the growth of *Z. mobilis*, glucose consumption, and ethanol production, but the inhibition of Na^+ was higher [13].

B. Detoxification of Hydrolysate

Neutralization and overliming in this study was an attempt to eliminate the toxic content on the hydrolysate. The results of the HMF analysis are shown in Table II. The highest content of HMF was the neutralization treatment with NH_4OH in the amount of 1.89 g/kg hydrolysate. HMF formation is caused by dehydration hexosa in acidic conditions and high temperatures. Liu *et al*. [14] stated that HMF was a major inhibitation to the growth of microbes that formed from hexose dehydration.

The lowest content of HMF was 1.39 g/kg hydrolysate on the treatment by overliming with $Ca(OH)_2$. Overliming treatment with $Ca(OH)_2$ can reduce HMF content of 16.77%. Generally, HMF released was lower on neutralization and overliming process using $Ca(OH)_2$ than NH_4OH. HMF had inhibitory effects on the growth of microbes [15]. HMF will interfere metabolic processes of yeast, so the yield of ethanol produced in the fermentation process was not optimal.

The use of activated charcoal for detoxification process is not only reduce toxic compounds such as HMF but also reducing sugar, because the activated charcoal is not selective. Treatment of hydrolysate with activated charcoal caused reduction of HMF and Reducing sugar, 65.18% and 25.34%, respectively (see Table III).

TABLE II: HMF CONTENT (G/KG HYDROLYSATE) ON NEUTRALIZATION AND OVERLIMING

Type	HMF Content (g/kg)		% HMF Reduction
	Neutralization	Overliming	
NH_4OH	1.89	1.87	1.06
$Ca(OH)_2$	1.67	1.39	16.77

TABLE III: REDUCING SUGAR CONTENT (% W/V HYDROLYSATE)

Activated Charcoal Concentrations	Reducing Sugar Content (% w/v)		
	15 minutes	30 minutes	45 minutes
2.50%	11.71	11.69	10.48
5.0%	11.67	11.58	10.56
7.50%	11.49	10.59	8.78
	Sugar Level Decrease (%)		
2.50%	0.43	0.60	10.88
5.0%	0.77	1.53	10.20
7.50%	2.30	9.95	25.34

Initial reducing Sugar = 11.76 % (w/v)

In Table IV showed the adsorption of activated charcoal getting stronger with higher concentrations of activated charcoal was added. The highest HMF decrease of 65.18% on the addition of 7.5% activated charcoal for 45 minutes. Activated charcoal has the ability to adsorb HMF and sugar because it has a lot of pores and large surface area. The toxic substance attaches to the surface of the charcoal.

Activated carbon is very active as it adsorbs anything include sugars. Adsorption process is related to the Van der Waals force, and it is a commuting process due to the affinity between the solute and the adsorbent. The activated charcoal has a weakness that it adsorbs sugar for it will cause the sugar content in the hydrolysate decreased.

In the Table V, it is showed that the level of salinity of hydrolysate was high which reached $125^0/_{00}$. The salinity level decreased after the adsorption proses of activated charcoal. The highest decrease occurred in the concentration of 7.50% with 30 minutes of contact time.

The use of activated charcoal did not have a significant influence on the level of salinity reduction. The highest ability of salt content reduction was only 22%. The best contact time for activated charcoal used as adsorbent was 30 minutes.

TABLE IV: HMF CONTENT (G/KG HYDROLYSATE) ON ACTIVATED CHARCOAL ADSORPTION PROCESS

Activated charcoal Concentrations	HMF Content (g/kg hydrolysate)		
	15 minutes	30 minutes	45 minutes
2.50%	1.51	1.68	1.41
5.0%	1.24	1.17	1.00
7.50%	0.98	1.38	0.64
	HMF Decrease (%)		
2.50%	17.91	8.55	23.20
5.0%	32.88	36.38	45.41
7.50%	46.95	24.99	65.18
Initial HMF =	1.84		

TABLE V: SALINITY LEVELS ($^0/_{00}$) ON ACTIVATED CHARCOAL ADSORPTION PROCESS

Activated Charcoal Concentrations	Salinity Level ($^0/_{00}$)		
	15 minutes	30 minutes	45 minutes
2.50%	115	100	125
5.0%	122.5	100	110
7.50%	120	97.5	102.5
	Salinity Decrease (%)		
2.50%	8	20	0
5.0%	2	20	12
7.50%	4	22	18
Initial salinity =	$125\ ^0/_{00}$		

C. Fermentation Process

Table VI showed that the highest ethanol produced in the neutralization treatment with $Ca(OH)_2$ was 2.49% (v/v) and fermentation efficiency was 56.14%. *Eucheuma spp*. consisted of 70% carbohydrate and 56.2% galactose can be extracted from the carbohydrate. Therefore, it can be calculated that 0.3934 kg/ kg galactose obtained from seaweed [1]. While according Meinita *et al*. [16], the ethanol yield from hydrolysate *K. Alvarezii* (*cottonii*) was 0.21 g/g galactose, the fermentation efficiency of 41%.

TABLE VI: FERMENTATION PROCESS

Detoxification Method		Reducing Sugar		Ethanol (%v/v)	Ethanol (%w/w gula)	Fermentation Efficiency (%)	Subtrate Efficiency (%)
		Before Fermentation	After Fermentation				
Neutralization	NH_4OH	10.09	6.36	0.49	12.35	26.91	36.62
	$Ca(OH)_2$	12.78	3.22	2.49	14.31	56.14	74.75
Overliming	NH_4OH	8.95	6.30	0.36	13.00	28.46	30.17
	$Ca(OH)_2$	8.52	4.02	1.20	25.11	54.71	52.79

E. cottonii produced kappa carrageenan and its constituent sugars as galactose have a special path in the process of conversion to ethanol. On galactose fermentation into ethanol, galactose is converted to 6-phosphate glucose and then enter the glycolytic pathway or Embden Meyerhof - Parnas Pathway (EMP) to produce ethanol.

Enzymes work specifically to convert glucose in the glycolytic pathway. However, they are often not suitable for other types of sugar so the sugar - like galactose and fructose converted first into the intermediate glycolytic pathway. Galactose is metabolized through the Leloir pathway [17]. The Pathway produces glucose 6 - phosphate stimulated by enzymes such as galactose mutarotase, galactokinase, galactose 1 - phosphate uridyltransferase, UDPgalactose 4 - epimerase and phosphoglucomutase [18].

The use of NH_4OH on neutralization and overliming process the hydrolysate produced a lower ethanol content compared with the use of $Ca(OH)_2$. Ethanol produced of 0.49% (v/v) and 0.36% (v/v) respectively. The low content of ethanol produced due to the presence of HMF and salt. The content of HMF on neutralization and overliming with NH_4OH was 1.89 and 1.87 g/kg hydrolysate respectively. HMF at a concentration of 1 g/l was able to inhibit fermentation by *S. cerevisiae* [19], while the presence of salt resulted in increased osmotic pressure and the ions can provide resistance effect if the concentration was high [20].

In the hydrolysis of cellulose with acid to produce sugars, the process forms HMF as a form of glucose decomposition in acidic conditions, HMF will react to form organic acids such as formic acid and levulinic acid in acidic conditions and high temperatures. These compounds become inhibitors during the fermentation process so that the ethanol produced was not optimal. In addition, the soaking of *E. cottonii* with solution of H_2SO_4 allows the solution to penetrate into *E. cottonii* tissue and causes the acidity of hydrolysate, so many yeast dead and the fermentation process does not run well [21].

IV. Conclusions

Detoxification process of *E. cottonii* hydrolysate using $CaOH_2$ produced higher ethanol yields and lower HMF compared to NH_4OH. Treatment with activated charcoal adsorption could reduce the content of HMF. *E. cottonii* showed significant potential as are new able feed stock for the production of bioethanol.

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