J. Mater. Environ. Sci. 7 (7) (2016) 2526-2533
ISSN : 2028-2508
CODEN: JMESCN

Utilization of Waste Seaweed through Pretreatment with Liquid Hot Water Method and Simultaneous Fermentation using Bacteria *Clostridium thermocellum*

O.S.R. Pasanda[1], A. Azis[2], H. S. Kusuma[3]
[1,2]*Department of Chemical Engineering, Polytechnic of Ujung Pandang, Makassar, Indonesia*
[3]*Department of Chemical Engineering, Institut Teknologi Sepuluh Nopember, Surabaya, Indonesia*

Received 13 Mar 2016, Revised 22 May 2016, Accepted 25 May 2016
Email: o.pasanda@yahoo.com (O. S. R. Pasanda); heriseptyakusuma@gmail.com (H. S. Kusuma)

Abstract
Indonesia as the owner of the largest tropical waters could potentially become the largest producer of biofuels in the world. With an abundance of sunlight as a plant photosynthesis, the proliferation of marine life is much higher when compared with temperate regions. Seaweed as one of the marine life which is scientifically known as algae or algae and chlorophyll is a plant containing approximately 35.6 to 58.3% carbohydrates in the form of a polysaccharide polymer in the form of fiber. The purpose of this study was to determine the conversion of cellulosic waste from seaweed that is the rest of the crop or seaweed damaged as the material for bioethanol production through the process of saccharification and fermentation simultaneously using bacteria *Clostridium thermocellum.* The operating conditions are optimal for pretreatment with Liquid Hot Water (LHW) method was obtained at a temperature of 121°C for 60 minutes and added a solution of buffer pH 7 in the fermentation process gained 2% ethanol fermentation time for 7 days. Damage to the cell structure of seaweed analyzed by SEM. As a result of treatment with LHWmethod makes the surface layer of waste lignocellulose from *Eucheuma cottonii* the surface area of cellulose to become more open as a result of the loss of wax or lignin in microfibril.

*Keywords:*Waste seaweed, Liquid Hot Water, Fermented, Lignocellulose, *Clostridium thermocellum*

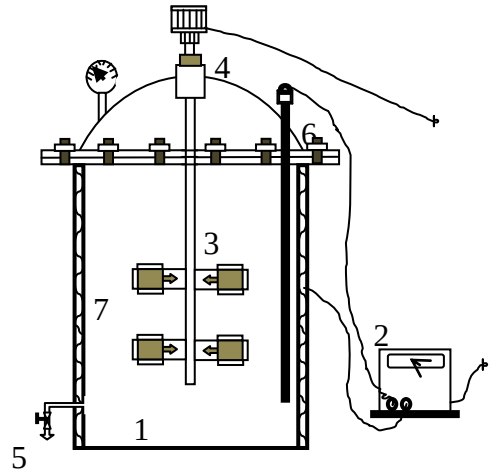
1. Introduction
In this present era, energy use is increasing, but supply of energy, especially energy dwindling fossil raw materials. Inventories of petroleum and coal is very limited and it takes millions of years to return to form. In addition, a fuel derived from petroleum and coal produces pollution and lead to global warming. Therefore, we need a renewable energy and an environmentally friendly energy so that it can overcome the problems of energy and global warming. One of the renewable energy is energy that is made from seaweed. Advantages develop energy made from seaweed that is the process of seaweed cultivation farms that do not reduce food because it does not require a landline land. This is because the availability of coastal land to cultivate seaweed is still very wide. Chemical constituents of seaweed among others karbohidarat form of polysaccharides such as carrageenan, minerals, protein, and fat. According Restiana and Diana (2004) [1] composition of dried seaweed is water (from 18.6 to 26.8%), protein (1.9 to 2.1%), carbohydrates (35.6 to 58.3%), fat (0.5-0.6%), crude fiber (0.9 to 5.3%) and ash (from 15.1 to 34.4%). While based on the research of Luthfy (1988) [2], seaweed *Eucheumacottonii* found to contain 19.92% ash, 2.80% protein, 1.78% fat, 7.02% crude fiber, and contain carbohydrates quite high which is about 68.48%.
Not all of crops from *Eucheuma cottonii* can be exported as raw material for cosmetics and foodstuffs, because there are parts that do not fit the eligibility criteria as raw materials for exported eg disease, stunted growth, as well as the presence of weeds attacks that undermine the growth of *Eucheuma cottonii*.So *Eucheuma cottonii* that do not qualify as exports, tend to be underutilized and unpunished so crumpled decay and eventually become litter the beach. So in this study, waste or leftover crops *Eucheuma cottonii* that are not utilizedis utilized as raw material for the manufacture of ethanol.

To break down lignocellulose into fermentable simple sugars that are ready pretreatment process is required. Enzymatic cellulose hydrolysis process provides *yield* a higher ethanolthan the acid hydrolysis method, but requires pretreatment of the raw material so that the cellulose structure ready for hydrolyzed by enzymes. While the acid hydrolysis process has the disadvantage that would be the formation of byproducts at high temperatures act as inhibitors in the fermentation process and the occurrence of corrosion problems in the equipment. In addition, acid hydrolysis process also entails a process of detoxification / neutralization before continuing on the next process, the fermentation stage. At this stage of detoxification glucose levels much missing, so the glucose levels that are ready to be fermented will become increasingly smaller.
One alternative pretreatment method that can be used is treated with Liquid Hot Water (LHW). This method makes the hemicellulose and cellulose will dissolve and break down lignin structure due to hydrothermal treatment so that the cellulose more easily processed by enzymatic treatment.
Research on pretreatment of the raw materials containing lignocellulose and hydrolysis of biomass has been conducted by several researchers. Rachmaniah (2009) [3] have proved their cell structure changes from bagasse that has been given pretreatment by LHW method. In addition, based on research conducted by Zheng (2009) [4], pretreatment with LHW methods will increase glucose in the hydrolyzate because about 80% hemicellulose can be degraded into monosaccharides and does not produce byproducts such compounds are inhibitors that act as inhibitors for the next process (fermentation). While Gozan et al. (2007)[5] has also done research to convert cellulose into bioethanol from bagasse using cellulase enzymes and selobiase. The results of these studies indicate that the use of cellulase enzymes and selobiase with optimum fermentation conditions at pH 5 resulted in the highest concentration of 13.04% bioethanol from bagasse.
Therefore in this study in the form of raw materials or residual wastecrops *Eucheuma cottonii* that are not utilized given pretreatment method LHW. In addition, this study also used cellulolytic bacteria to produce an enzyme that works to hydrolyze cellulose to glucose. The bacteria used are *Clostridium thermocellum* where the bacteriais a thermophilic anaerobic bacteria which has the ability to degrade cellulose to ethanol complex [6,7,8]. It is based on several previous studies that use cellulolytic bacteria of the species *Clostridium thermocellum* for bioethanol production with raw materials at high temperatures lignosellulosa [6,7,9,10,11,12].

2. Experimental

2.1. *Pretreatment of the seaweed*

Raw materials used for the experiment is waste seaweed(*Eucheumacottonii*)derived from Maros, Indonesia. Seaweed cleaned of impurities such as sand and rock fragments. Washing is done by spraying water into the seaweed. Washery then dried in the sun. Subsequently the seaweed is dried in an oven at 105°C for 5 hours and then reduced in size to the size of ±100 mesh.



Information:
1. Stainless steel tank
2. Temperature sensor
3. Mixer
4. Mixer motor
5. Spending sample
6. Temperature control equipment
7. The heating coil

Figure 1: Schematic representation of the equipment used to pretreatment process with LHW method

2.2. *Treatment with liquid hot water method*

Process pretreatment of the raw material waste in the form of seaweed(*Eucheumacottonii*)conducted by the LHW method.Effect of operation time to damage cell structures seaweed investigated by performing a pretreatment process at a temperature of 121°C (3 atm) while maintaining the water in the liquid phase. In

addition, the study also conducted a pretreatment process with mengguanakan pressure at 50°C (1 atm) as a control or comparison conditions. Influence of operation time can be learned by doing variations of the operating time used, namely 10, 30 and 60 minutes and the effect of pH of the solution can be studied by adding a buffer solution. System equipment that serves as a hydrolysis process in which pretreatment with LHW method can be seen in Figure 1. in the system hardware consists of a heating coil that has been equipped with a sensor temperature control, stirrer, manometer, and faucets place for sampling.

2.3. *Analysis results pretreatment with Liquid Hot Water method*

The hydrolyzate obtained from pretreatment with LHW method is then filtered using a vacuum pump. Analysis performed at the beginning and at the end of the process includes the analysis of glucose levels as well as changes to the structure of the cell at the time before and after pretreatment performed using Scanning Electron Microscopy(SEM).

2.4. *Fermentation phase*

Fermentation is carried out using bacteria *Clostridiumthermocellum*.These bacteria can produce enzymes that can hydrolyze lignocellulosic lignoselulase be lignin and sugar later on by the same bacteria fermented into ethanol. Use of these bacteria provide other benefits that are easy to breed, which for ethanol production is done by giving *Clostridium thermocellum* once so that the ethanol production process can be cheaper. C*lostridium thermocellum* is a thermophilic anaerobic bacteria which has the ability to degrade cellulose to ethanol complex.

2.5. *Testing ofproducts*

2.5.1. *Analysis of cellulose and lignin levels*

Cellulose and lignin analysis was conducted using Chesson method. A total of 1 g dry sample (a) is added with 150 ml of distilled water. The mixture was subsequently refluxed at 100°C using a water bath for 1 hour. Results obtained is filtered and the residue washed with hot water (300 mL). The residue is then dried in an oven until its weight is constant then weighed (b). The residue is weighed and then refluxed by adding H_2SO_4 1 N 150 mL at 100°C using a water bath for 1 hour. Results obtained is filtered and washed with distilled water until neutral (300 mL). The residue is then also dried in an oven until its weight is constant then weighed again (c). The dry residue is then added H_2SO_4 72% as much as 10 mL and soaked at room temperature for 4 hours. The mixture is then added H_2SO_4 1 N 150 mL and refluxed at 100°C using a water bath for 1 hour. The residue obtained is filtered and washed with distilled water until neutral (400 mL) and then heated in an oven at 105°C and the results are weighed (d). After the residue diabukan and the results are weighed (e). Levels of cellulose and lignin levels can be calculated by the following equation:

$$\text{Cellulose levels} = \frac{c - d}{a} \text{ x } 100\%$$

$$\text{Lignin levels} = \frac{d - e}{a} \text{ x } 100\%$$

2.5.2. *Analysis of hemicellulose levels*

Analysis of hemicellulose was conducted by Wise method that samples first mixed with sodium chlorate, acetic acid, and distilled water. The mixture is then incubated with hot water at a temperature of 80°C. Subsequently the mixture is cooled, filtered, washed with distilled water, and finally rinsed with acetone. Section solids were obtained and then dried in an oven at 105°C for 1-2 days and then weighed.

2.5.3. *Analysis of sugar levels*

As much as 10 mL samples were taken and put in a 250 mL volumetric flask and add distilled water up to the mark. The solution was diluted further taken as many as 5 ml and put in elenmeyer for Luff.schoorl then added with a solution of 25 mL and 10 mL of distilled water as well as a number of stone boiling. The mixture is subsequently refluxed for 10 minutes. After the mixture is cooled and added to a solution of H_2SO_4 25% as much as 15 mL and 20% KI solution of 15 mL. The mixture is then titrated with sodium thiosulfate solution 0.1 N. Sugar levels can be calculated by the following equation:

$$\text{Sugar levels} = \frac{\text{mass of glucose x dilution factor}}{\text{sample volume}} \text{ x } 100\%$$

3. Results and Discussion

The first step to start the process is a waste of seaweed drying and shrinkage of up to size±100 mesh. The aim is that any raw material is processed always on the basis of the same weight (dry basis).

3.1. Optimum time for pretreatment with Liquid Hot Water method

The results of the analysis of glucose levels of waste seaweed used in this study after a pretreatment with LHW method at 121^{o}C with a variety of time can be seen in Tables 1 and 2. Table 1 and 2 shows that the longer heating the collisions that occur between particles of carbohydrate is also getting old. As a result, the number and energy of the collision becomes larger. With the increasing frequency of collision with rising temperatures lead to molecules split carbohydrates into glucose. This is according to research conducted by Barry and Abtokhi (2014) [13]. From Table 1 and 2 can be seen that in a time of 60 minutes, the glucose levels obtained in the substrate is of 1.54% for those without the addition of buffer and 2.59% for that with the addition of buffer. Glucose levels of 2.59% is soluble glucose formed in the substrate. In this study was not done warming periods longer than 60 minutes because it was feared glucose that is formed will be degraded and dissolved in water that could eventually become a component of which is an inhibitor to the fermentation process. This is because one wants is the complete hydrolysis when they are fermented. One purpose of the pretreatment with LHW method for dissolving hemicellulose that separate from the residual of the solid materials while reducing the formation of inhibitors. However, reactive cellulose fibers can also be formed during the disruption of the entire matrix at the time of the lignocellulosic biomass cell penetration by water, which at the same time become soluble hemicellulose and lignin. There are two products that are formed in this process, namely pulp which many solid fraction containing hemicellulose and cellulose that many contain. Where the product in the form of slurry and solid fractions are separated from one another. Porridge which many contain hemicellulose will further break down into simple sugars such as glucose, galactose, mannose, hexose, pentose, xylose and arabinose. Further compounds tersebutlah simple sugars that will be fermented by microorganisms to produce ethanol [14].

Table 1: Results of pretreatment with LHW method at a temperature of 121°C without the addition of buffer

No.	The heating time (minutes)	The average of glucose levels (%)
1	10	1.37
2	30	1.47
3	60	1.54

In the compressed condition along with an increase in temperature at pretreatment with LHW method causes the dissociation constant of water (Kw) to be increased. At a temperature of 200°C, the dissociation constant of water (Kw) has a value of 6.10^{-12} (pH = 5.61). So with the increasing temperature, the pH of the solution will be lower as a result of the growing value of the dissociation of water (Kw). Therefore, the high temperature water can act as a result of acid decomposition of H_2O form the ion H^+ and OH^- and the high value of the dissociation constant of water (Kw) [15]. So the use of buffer solution in this study aims to maintain the pH of the solution so as not to fall or not acidic. Acidic conditions arise not because of the addition of acid but because their Hions^{of+} ionizationfrom the water due to water compressed [16]. In the dilute acid conditions, complex furan (furfural and hydroxymethyl furfural) can be formed in the hydrolyzate as a result of the various reactions of pentose and hexose. Where these furan groups include one group of inhibitors of the most significant effect in reducing the level of ethanol production and the growth of microorganisms in the media through a mechanism that occurs at the level of enzymatic hydrolysis process. Furfural degradation resulting from pentose such as xylose, while hydroxymethyl furfural (HMF) produced from the degradation of hexoses such as glucose [17].

Table 2: Results of pretreatment with LHW method at a temperature of 121°C which added with buffer pH 7

No.	The heating time (minutes)	Average of glucose levels
1	10	1.37
2	30	1.99
3	60	2.59

Table 2 it can be seen that with the addition of a pH 7 buffer solution, then the productivity of the formation of glucose to be increasing in the amount of 2.59 % with heating time for 60 minutes. So that the heating time for 60 minutes and with the addition of a pH 7 buffer solution can be said is that the optimum conditions for

pretreatment with LHW method. The optimum conditions have been obtained is then used for the later stages because it is considered more effective in lignocellulose degradation process.

3.2. The pretreatment process with Liquid Hot Water method

Pretreatment process of lignocellulose is very important and is the first step in facilitating the breakdown of cellulose into glucose. Pretreatment of lignocellulosic biomass needs to be done to obtain maximum results. The process is also indispensable in the development of bioconversion technology to commercial scale [14]. Where pretreatment is a stage that will greatly affect the cost of the whole process. Pretreatment can increase the yield of sugars obtained. Theoretically sugar obtained without pretreatment is less than 20%, whereas if the pretreatment is done then the sugars obtained can be increased to 90% [18]. In addition, the purpose of the pretreatment is to open up the structure of lignocellulose so that the cellulose more accessible to enzymes that will break down the polysaccharide polymers into sugar monomers. The process can be illustrated as shown in Figure 2.

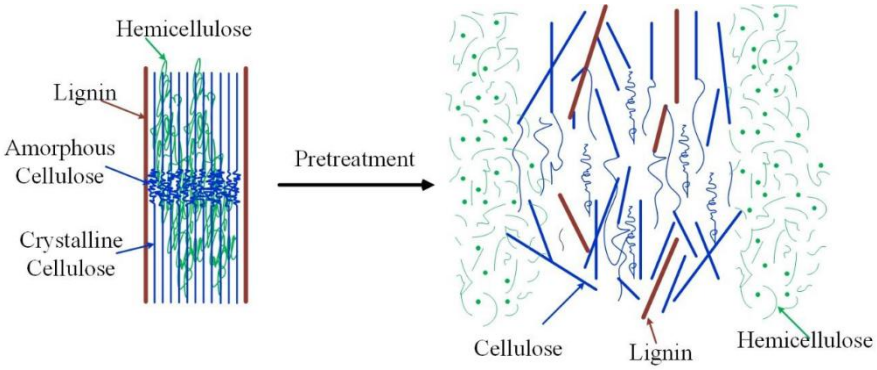


Figure 2: Effect of pretreatment with LHW method on the structure of biomass [4]

Pretreatment with LHW method necessary to change the size or macroscopic and submicroscopic structures of lignocellulosic biomass. Lignocellulosic biomass is usually arranged in the form of microfibrils, with a diameter of about 3-6 nm and contains up to 36 glucan chains and have thousands of glucose residues. Cellulose can be broken down into glucose either enzymatically by a cellulolytic enzyme or chemically by sulfuric acid or other. Hemicellulose is a branched polymer consisting of pentose (5-carbon) and hexoses (6-carbon) can be hydrolyzed by hemicellulases or acid to release its sugar components, including xylose, arabinose, galactose, glucose and / or mannose. Hexoses such as glucose, galactose and mannose easily fermented into ethanol by many natural mikorganisme [4].

Table 3 shows that there has been a change in the levels of the lignocellulosic wastes *Eucheuma cottonii* doafter pretreatment with LHW method. This is evident from the percent composition of each component that has changed. Changes in the composition is due partly dissolved lignin along with hemicellulose. Based on the degree of crystallinity, classified into crystalline cellulose and paracrystalline (amorphous) resulting cellulose into soluble [4]. Results from pretreatment with LHW method is then processed to the next stage of the fermentation stage.

Table 3: Component of the results of pretreatment with LHW method at a temperature of 121°C which added with buffer pH 7 for 60 minutes

No.	Component	Levels (%)	
		Without Heating (*Eucheuma cottonii*flour)	With Heating
1	Glucose	-	5.86
2	hemicellulose	14.80	3.82
3	Cellulose	18.98	21.35
4	Lignin	4.24	0.64

Bacteria used in the fermentation process is the bacteria*Clostridium thermocellum.* These bacteria is the anaerobic bacteria, thermophilic, and form spores and produce complex cellulosome which serves to hydrolyze

cellulose or oligodextrin (cellobiose, cellotetrose, and cellopentose) which is then processed to produce ethanol, organic acids (acetic acid, formic acid), hydrogen, and carbon dioxide [19,20]. Cellulose is hydrolyzed into sugars, especially into glucose. Glucose, galactose, mannose, and sugar with six other carbon atoms and hexoses a sugar easily fermented into ethanol by organisms such as *Clostridium thermocellum* [21].
From the research results can be seen that the variation of fermentation time to waste biomass *Eucheuma cottonii* using bacteria C*lostridium thermocellum* gives the ethanol levelshigher on day 7. The levels of several components in a wide variety of fermentation time can be seen in Table 4.

Table 4: Results offermentation *Eucheuma cottonii* using bacteria*Clostridium thermocellum*

Fermentation time (days)	Levels (%)				
	Cellulose	Hemicellulose	Lignin	Glucose	Ethanol
3	1.64	1.32	0.33	0.82	0.04
4	1.98	1.65	0.33	0.65	0.05
5	2.01	1.68	0.34	0.53	0.14
6	1.94	1.62	0.32	0.42	0.71
7	1.61	1.29	0.32	0.35	2.00

When the fermentation, bacterial cells do not consume oxygen to produce energy. Instead, the bacteria use the simple sugar molecules such as glucose derived from the breakdown of cellulose to obtain energy during fermentation. During fermentation, other products are also produced such as hydrogen, carbon dioxide, acetic acid and ethanol which are its main products. The concentration of ethanol obtained from the fermentation process can not be determined only by the concentration of glucose reductions early because the fermentation process is influenced by many factors such as: cultural factors inoculum used, length of fermentation, temperature, pH media, and the number of macro and micro nutrients in the fermentation. Based on the results of fermentation in Tables 3 and 4, it can be seen that the initial glucose levels by 5.86% after fermentation for 3 days glucose levels dropped to 0.82% and after fermentation for 7 days glucose levels go down again so that it becomes as big as 0.35%.

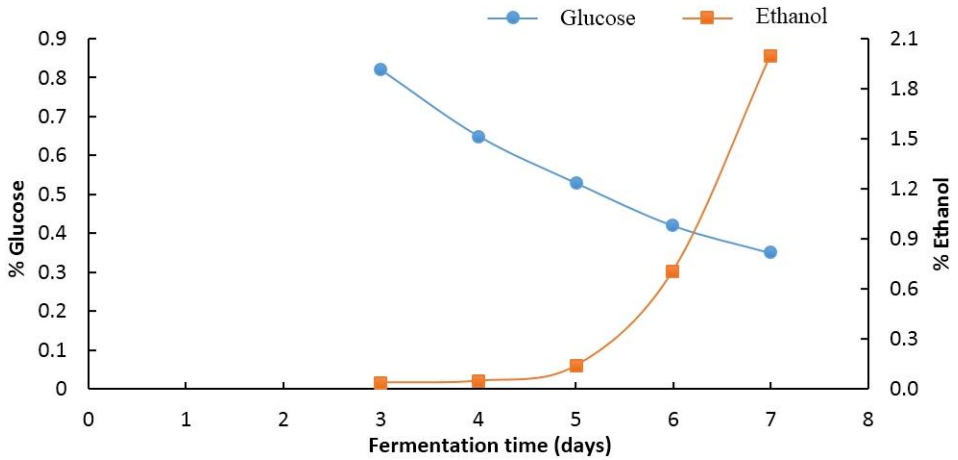


Figure 3: The relationship between glucose to ethanolin thefermentation of *Eucheuma cottonii* using bacteria *Clostridium thermocellum*

Conversion value of the numbers of reduced glucose into ethanol. However, data resulting from the conversion of simple sugars into ethanol which is calculated based on the reduced amount of glucose is not accurate, because the amount of glucose used kosumsi bacteria to multiply ignored. Whereas on any fermentation process, glucose consumption is used for two things: to grow and breed bacteria and some will be converted into a metabolite products such as ethanol. The analysis showed that when the resulting ethanol levels of 2.00%, it seems that glucose levels become increasingly fell to 0.35%. The resulting ethanol levels greater with increasing fermentation time. This indicates that the pattern of growth of microorganisms that are in the exponential phase, meaning that if the fermentation continued for a little time longer then there is the possibility of ethanol obtained also increased in view of the curve found in Figure 3 shows a positive trend.

3.3. *Microstructure surface of biomass Eucheuma cottonii*

In Figure 4b and 4c shown that an increase in operating temperature due to pretreatment with LHW method making structure microfibrils of the fiber seaweed widened more broken and open than the structure of the microfibrils of the fiber seaweed is not given pretreatment with LHW method which can be seen in Figure 4a. This indicates that most of the lignin and hemicellulose components have been degraded (Figure 4b and 4c), which later resulted in the surface area of cellulose into increasingly open [22].

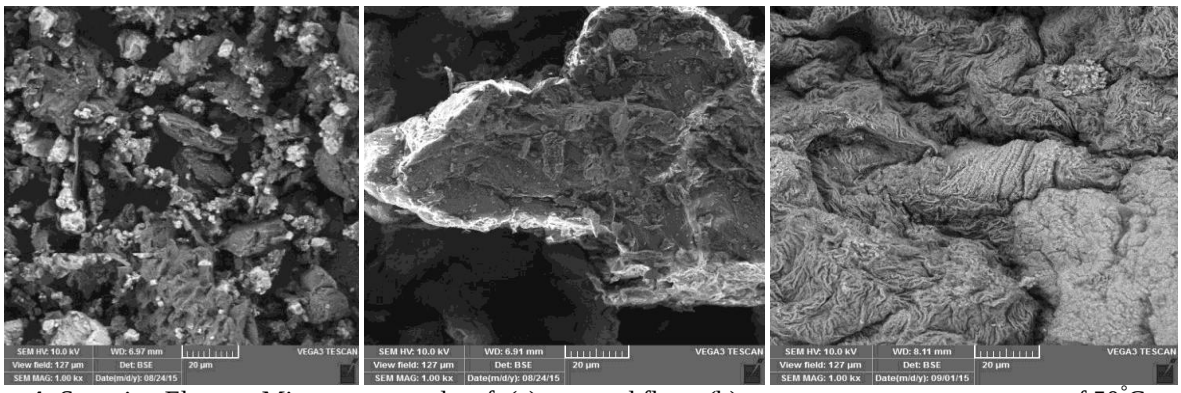


Figure 4: Scanning Electron Microscopy results of: (a) seaweed flour, (b) pretreatment at a temperature of 50°C, and (c) pretreatment at 121°C ditambahan with pH 7 buffer (60 minutes)

Also in Figure 4a can still be seen the surface layerthe microfibrils, wherein the coating is made microfibrils become whole and looks smooth. But in Figure 4b, the layer somewhat lost due to heating at a temperature of 50°C and in Figure 4c, while the coating is lost as a result of the pretreatment with LHW method. In operating conditions with a temperature of 50°C and at a temperature of 121°C, the shape of the inner surface of the parenchyma cells seen more clearly. So it can be said that the higher the operating temperature used, the inside of the parenchyma cells become increasingly apparent as shown in Figure 4c. This is possible due to the loss of wax or lignin in microfibril.

Conclusions

Bacteria*Clostridium thermocellum* able to perform the complex process of bioconversion of lignocellulose from *Eucheuma cottonii* as indicated by a decreased lignin, cellulose and hemicellulose levels and increased reducing sugars levels. Pretreatment withLiquid Hot Watermethod is performed at a temperature of 121°C for 60 minutes and the fermentation time was obtained for 7 days at 2.00% ethanol levels.

References

1. Restiana W.A., Diana R., Analysis of the Nutritional Composition Seaweed(*Eucheumacottonii*)on the Island of Karimun with Different Drying Process (in Bahasa Indonesia), Diponegoro University, (2009).
2. Luthfy S., Learning the extraction of Carrageenan with Semi-refined Method of *Eucheuma cottonii* (in Bahasa Indonesia), Bogor Agricultural University, (1988).
3. Orchidea R., Lisa F.S., Khoir L., Effect of Liquid Hot Water to change cell structure of Bagasse (in Bahasa Indonesia), Proceedings of the National Seminar XIV - FTI-ITS (2009) xxx-1.
4. Zheng Y., Pan Z., Zhang R., *Int. J. Agric. Biol. Eng*. 2(3)(2009)51.
5. Gozan M, Nasikin M, Wijanarko A, Hermansyah H., Research Fuel Conservation (Bioethanol and Biodiesel) (in Bahasa Indonesia), University of Indonesia, (2007).
6. Balusu R., Paduru R.M., Seenayya G., Reddy G., *Appl. Biochem. Biotechnol*. 117 (2004) 133.
7. Demain A.L., Newcomb M., Wu J.H., *Microbiol. Mol. Biol. Rev*.69(1) (2005) 124.
8. Riyanti E.I., *J. Agric. Res. Dev*. 28(3) (2009) 101.
9. Weimer P.J., Zeikus J.G., *Appl. Environ. Microbiol*. 33(2) (1977) 289.
10. Sparling R., Islam R., Cicek N., Carere H., Chow H., Levin D.B., *Can. J. Microbiol*. 52 (2006) 681.
11. Carere C.R., Sparling R., Cicek N., Levin D.B., *Int. J. Mol. Sci*. 9(7) (2008) 1342.
12. Sato K., Goto S., Yonemura S., Sekine K., Okuma E.,Takagi Y., Hon-Nami K., Saiki T., *Appl. Environ. Microbiol*. 58 (1992) 734.
13. Mayangsari V., Abtokhi A., *J. Neutrino*. 7(1) (2014) 16.

14. Mosier N., Wyman C., Dale B., Erlander R., Lee Y.Y., Holtzapple M., Ladisch M., *Bioresour. Technol*. 96(6) (2005) 673.
15. Orchidea R, Andi K.W., Dedy R.P., Lisa F.S., Lazuardi K., Pahlevi R., Cakra D.M., Effect of Pretreatment Methods in Lignocellulose Materials on the Quality of the Produced Hydrolyzate (in Bahasa Indonesia),National Conference Papers on Chemical Engineering Soebardjo Brotohardjono, Resilience of Food and Energy (2010) E10-1.
16. Baig M.N., Zetzl C., Brunner G., Conversion of Extracted Rice Bran & Isolation of Pure Bioethanol by means of Supercritical FluidTechnology,Universität Hamburg, (2006).
17. Banerjee N., Bhatnagar R., Viswanathan L., *Eur. J. Appl. Microbiol*. 11 (1981) 226.
18. Hamelinck C.N., van Hooijdonk G., Faaij A.P.C., *Biomass Bioenergy*. 28 (2005) 384.
19. Maki M., Leung K.T., Qin W., *Int. J. Biol.Sci*. 5(5) (2009) 500.
20. Shihui Y., Richard J.G., Lezlee D., Zamin K.Y., Nancy L.E., Timothy J.T., Robert L.H., Steven D.B., *BMC Genomics*. 13 (2012) 336.
21. Gong C.S., Claypool T.A., McCracken K.D., Mann C.M., Ueng P.P., Tsao G.T., *Biotechnol. Bioeng*. 25 (1983) 85.
22. Hu Z., Wen Z., *Biochem. Eng. J*. 38(3) (2008) 369.

(2016) ; http://www.jmaterenvironsci.com