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Hydrolysis of Lignocellulose by Various Microorganisms

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ABSTRACT

The objective of this study was to determine microorganism ability to hydrolysis lignocellulose to produces reducing sugar. Lignocelluloce has a strong and complex bound, so it needs pretreatment process to break lignocellulose bound to be hemicellulose, celullulose and lignin. Pretreatment carried out by biological and mild acid process, by using *Phanerochaete chrysosporium* and H_2SO_4. Substrate variables were from 1 gram to 20 grams. Biological pretreatment incubated for 10 days, and then added H_2SO_4 to concentration of 0,25% and heating. After pretreatment followed by biological hydrolysis process, conducted for 5 days and analysis of samples carried out every 24 hours. Microorganisms type tested were *Trichoderma Viridae* mixed with *Aspergilus Niger,* M16 isolated from rumen microorganisms and *cellvibrio*. Hydrolysis results showed the addition of the substrate was increased the amount of reducing sugar, but decreased reducing sugar yield per gram of substrate. M16 produces reducing sugar was higher than both of the other microorganisms. Ability of M16 to hydrolize substrate was 1.4 times larger than other microorganisms with acid addition and as larger as other microorganisms without acid addition.

KEYWORDS: acid, lignocellulose, microorganisms, M16, reducing sugar.

INTRODUCTION

Lignocellulose is potential as bioethanol raw material. One readily abundant, no price, reduce water resources quality and contain high lignocellulose is water hyacinth. Water hyacinth is an aquatic plant contains high hemicellulose amount to 48.7%, celluloce 18.2% and lignin 3.5% of dry weight [1]. Lignocellulose also a potential material, due to the technology of making ethanol from these materials is well known [2;3]. Bioethanol produced from lignocellulotic, materials of concern because it is not a food, so it does not compete with the food industry [4]. Pretreatment and hydrolysis are the signficant phases of the making of reducing sugar which coverted to bioethanol in fermentation process. Pretreatment aims to break down lignocellulose into lignin, hemicellulose and cellulose, so it is an effective way to increase the hydrolysis efficiency [5]. Pre-treatment can be conducted physically, chemically, or by enzyme. One of the microorganisms which can be used in this process is *Phanerochaete chrysosporium*, which produce lignin peroxidase and mangan peroxidase to break lignocelluloses bonds [6;7]. The advantage of using enzyme than acid is after the process there will be no acid waste which have to be treated, but enzyme takes a significant amount of incubation time. The other disadvantage is polysaccharides which formed by the process was being used by fungus for their growth [8]. In order to overcome disadvantage, the combination of acid and enzyme were used to produced optimum reducing sugar [9] and reducing incubation time.

Biologically process capable to hydrolyse the structure of hemicelluloses and cellulose to produce reducing sugar [10; 11;12;13;14]. *Trichoderma viridae* produce cellulase enzyme, endoglucanase and exoglucanase to hydrolized cellulose, but less β-glucosidase enzyme production [15;14]. *Aspergillus niger* producing high β-glucosidase enzyme but less endoglucanase and exoglucanase enzyme production [13] to hydrolyzed the hemicelluloses. Combination between *A. niger* and *Trichoderma reesei* could produced much more reducing sugar than only using one of them [15]. Cellvibrio bacteria is a gram negative aerobic bacteria, capable to degrade cellulose in plant's fiber and digest the organic compounds for growth and as a source of energy [16;17]. Compared to other microbial inoculums, rumen microorganism show a higher hydrolytic and acidogenic activity when using lignocellulosic biomass as substrates [18]. M16 used in this study consisted of 16 types of microorganisms isolated from the cow rumen [19], which there are several types of microorganisms capable to hydrolyse cellulose. This study aimed to compare the ability of variuos microorganisms to hydrolyse lignocellulose into reducing sugar in acid addition and various amounts of substrate.

MATERIALS AND METHODS

Substrate and microorganisms preparation

Raw material preparations are done by separating leaves and stems, and then washed by water. Sample chopped to small size approximately 2-2.5 cm and dried at temperature 60 + 0.3 ° C for 2-3 [20], grounded into

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small pieces. The substrate sterilized in an autoclave at a temperature of 121ºC for 15 minutes. *P. chrysosporium, T. viride and A. Niger*, *Cellvibrio* cultured on Potato Dextrose Broth (PDB) media, and ready for used after 24-36 hours. M16 can be used directly. All microorganisms purchased at Airlangga University, Indonesia.

Pretreatment

Experiments were conducted in two stages, pretreatment and hydrolysis. Pretreatment process was carried out by using *P. chrysosporium* 2 mL/g substrate. *P. chrysosporium* is a fast-growing white rot fungus, only requiring a fewdays to weeks for vigorous degradation of lignocellulose. Inoculation was performed for 10 days [20]. After pretreatment, H_2SO_4 was added to concentration 0.25% [8]. Other experimental variables were without acid addition. Then heated by $\pm$100ºC temperature in 30 minutes [21].

Hydrolysis

Three major groups of cellulases is involved in the hydrolysis process: (1) endoglucanase (endo-1,4-glucanohydrolase) attacks regions of low crystallinity in the cellulose fiber, creating free chain-ends; (2) exoglucanase degrades the molecule further by removing cellobiose units from the free chain-ends and (3) *β*-glucosidase hydrolyzes cellobiose to produce glucose [29] *T. viride* produces endoglucanase and few β-glucosidase, otherwise *A.niger* produce more β-glucosidase. Therefore hydrolysis process was carried out by using *T. viride* and *A. Niger* mixture with ratio of 2: 1 (TVAN). Other microorganisms used in the hydrolysis process were *Cellvibrio* (CV) and M16.

Test substrate variables from 1 gram to 20 grams. All variables incubated for 24 hours. Experiments were conducted by using *T. viride* and *A. Niger*, need buffer addition to maintain pH stayed 5, regard to optimum pH of microorganism growth. *Cellvibrio* and M16 growth at pH 7-8, so it is no buffer addition needed [22]. Reducing sugar production was measured by Nelson Somogy method [23].

RESULTS AND DISCUSSION

Pretreatment by biological process

Lignocellulose consists of lignin, hemicellulose and cellulose. Lignin is further linked to both hemicelluloses and cellulose forming a physical seal around the latter two components that is an impenetrable barrier preventing penetration of solutions and enzymes [24], therefore it needs to break lignocellulose prior to hydrolysis process. Without any pretreatment, the conversion of native cellulose to reducing sugar is extrimely slow, since cellulose is well protected by the matrix of lignin and hemicellulose in macrofibril [25]. Initial hemicellulose, cellulose and lignin content of water hyacinth in this research is similar to Gunnarsson and Petersen [26] research, but lower than Nigam [1]. Its because water hyacinth characteristics depend on their environment growth. Table 1 showed hemicelulose, cellulose and lignin content initial and after pretreatment.

Table 1. Hemiceluloce, cellulose and lignin content initial and after pretreatment

Parameters	Initial	After 10 days Pretreatment By *P. chrysosporium*	% removal	Pretreatment H_2SO_4 0,25 % and heating	% removal
hemicelulose	33.95	24.15	28.9	12.26	49.2
cellulose	12.38	9.72	21.5	7.26	25.3
lignin	8.76	6.55	25.2	4.59	29.9
Reducing sugar (mg/L)	1.22	20.12		48.82	
pH	7.43	6.89		6.81	

Decrease of hemicellulose content is influenced by the amount of inoculum and incubation time. Currently hemicellulose decomposition process earlier than the cellulose and lignin. This is because the hemicellulose chains shorter than cellulose, so it can be dissolved. Hemicellulose content was deacreased from 33.95% to 24.15%, cellulose content decreased from 12.38% to 9.72% and lignin reduced from 8.76% to 6.55%. Reducing sugar content is increased from 1.22% to 20.12% or 16.8 times after pretreatment by *P. chrysosporium*. Hemicellulose, cellulose and lignin content reduction was similar to Singh and Bisnoi research [27] that use acid as pretreatment process. This means biological pretreatment in this reaserch was reliable. At initial incubation, the fungus got nutrients from materials available in the growth medium. Once growth medium gradually exhausted, fungus utilized other available organic materials available, the water hyacinth. Next process was delignification of water hyacinth substrate. Lignin degradation will stop if organic materials that are

easier to digest formed such as reducing sugar. Therefore, pretreatment process to be stopped at the right time, so there is no loss of reducing sugar produced in the process. Biological process was stopped by acid addition and heating.

Acid addition prior to hydrolysis by biological process

After bilological preteratment hemicellulose content was 24,15%, and after addition of 0.25% H_2SO_4 and heating 100 + 3 ° C decreased to 12.26% or removed 49,2%. Based on other research the heating could degrade hemicellulose content of up to 90% [5]. Cellulose from 9.72% to 7.26 %, or reduced 25,3% slightly higher than other research which stated heating can reduce the cellulose content up to 22% [5]. Lignin from 6.55% to 4,59 % or reduced 29,9%. Heating could reduced lignin content up to 60% [5]. The dilute acid pretreatment can achieve high reaction rates and significantly improve cellulose hydrolysis [25]. This is indicated in experiment by acid addition, which is produced reducing sugar 1.6 to 3 times greater than without acid addition for all variables, as can be seen in Table 2. Hydrolysis aims to break down hemicellulose and cellulose polymers into its monomer. Hemicellulose is generally composed by polymer of pentose, hexose and acid, while cellulose is composed by polymer of glucose. Cellulose is more resistant to hydrolysis than hemicellulose, because it has highly crystalline structure, therefore it is necessary acid addition to break down the β-1,4 bond chains of glucose or xylose [25]. It is showed that pretreatment by combined biological, acid addition and heating could increased substrate hydrolysize.

Tabel 2. Reducing sugar produced by various microorganisms with and without acid

Water Hyacinth (gram)	Reducing sugar product Non-H_2SO_4 addition		Reducing sugar product H_2SO_4 0,25%		
	T.viridae + *A.niger* (%)	M16 (%)	*T.viridae* + *A.niger* (%)	M16 (%)	Cellvibrio (%)
1	14.87	33.01	38.75	42.86	39.35
2.5	20.84	45.21	40.91	54.01	43.91
5	25.08	50.24	46.43	75.66	48.83
10	34.29	51.08	56.62	79.65	56.14
20	29.65	44.90	59.50	88.67	60.82

Acid breakdown products is easier to hydrolyzed by microorganisms. Acid addition was affected reducing sugar product significantly, indicated by statistical analysis results in which $p < 0.000$ and R^2 was 0.815. The advantage of using acid is a reducing sugar production efficiency up to 90% [5]. However there are some disadvantages due to formation of toxic components, such as furfural, hydroxyl-methyl furfural, acetic acid, levulinic acid and formic acid [28], which can inhibit the fermentation process by microorganisms [29]. Table 1 showed acid addition on M16 is able to hydrolyze substrates was higher than other variables. In experiments with the substrate as much as 20 grams on M16 with and without acid addition, reducing sugar production decreased by 10 and 13% respectively. In addition to the number of larger substrates, the heating time for acid hydrolysis require greater time also.

Effect of substrate concentration on reducing sugar production

Effect of substrate concentration on reducing sugar product can be seen in Figure 1, where reducing sugar product increased by addition of the substrate. This is due to the biological breakdown process, mixing microorganisms should be evenly distributed contact with the substrate. It is difficult to mix the microorganisms on a large number of substrates. Beside, the addition of substrate also increase toxic materials concentration as side product, which inhibits the growth of microorganisms. At 10 grams substrate incubation by TVAN reducing sugar is 34,29%,which decreases to 29,65% when the substrate amount increases to 20 grams. All experiment showed reducing sugar decreased on substrate 20 grams compare to others experiment, exept for M16.The effect of the substrate on the amount of the reducing sugars was significant (p value<0.0001) with R^2 value 0.,755. Reducing sugar production compared to reducing sugar yield showed in Figure 2. Reducing sugar yield decreased with increasing number of substrates.

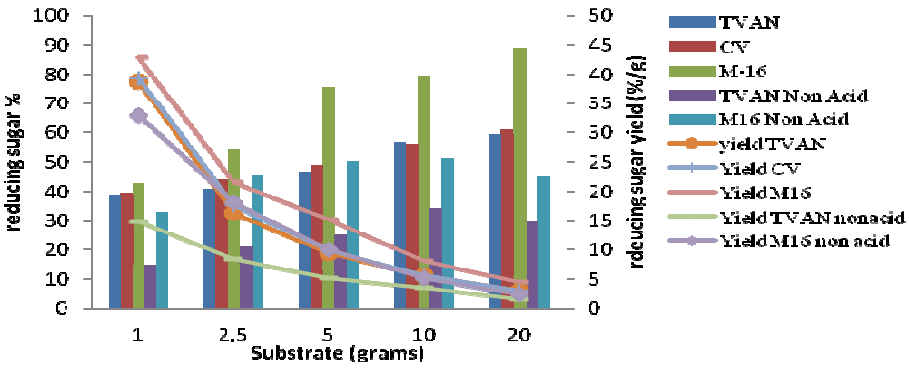

Figure 2. Reducing sugar product compare to reducing sugar yield

Increase substrate concentration will increase reaction rate as can be seen in Table 3. It is based on Michaelis-Menten formula, which states that reaction rate will increase with increasing substrate concentration. However, at a certain concentration limits, reaction rate will not increase even though substrate concentration increase, because the ratio substrate to microorganisms has been exceeded [30]. The reaction rate will continue to increase until it reaches a point where the limit of the enzyme is saturated with substrate. This point is called the maximum rate, and then the reaction rate will be constant with the addition of time. Table 2 showed maximumum rate obtained on 72 hours and after that reaction rate constant until the end of experiment. It is conclude inoculation time on hydrolysis by *T.viridae* and *A. niger* was 72 hours.

Table 3. Reducing sugar formation rate by P. *chrysosporium* and 0.25% H_2SO_4 pretreatment, hydrolysis by *T.viridae* and *A. niger*

Water Hyacinth (gram)	24 hours (ppm/h)	48 hours (ppm/h)	72 hours (ppm/h)	96 hours (ppm/h)	120 hours (ppm/h)
1	0.215	0.320	0.590	0.515	0.510
2.5	0.265	0.380	0.784	0.659	0.629
5	0.295	0.450	0.809	0.749	0.724
10	0.490	0.649	1.049	1.029	0.949
20	0.610	0.934	1.139	1.094	1.059

Hydrolysis of lignocelluloce by various types of micro-organisms

Hydrolysis of lignocellulose by microorganism depends on the cellulose crystal structure, cellulose enzyme composition, surface area, innoculum-substrate ratio and substrate pureness [17]. Acid addition will deconstruct lignocellulose, and microorganism activity will increase due to decreasing of complicated bonds and also increasing the surface area of the substrate. In this research substrate preparation by chopping, drying and grinding will expand the contact area, so that the microorganism is easier to hydrolyze a substrate.

All microorganisms type capable to hydrolysze substrate, but M16 has the highest ability. M16with acid addition is able to hydrolyze cellulose and hemicellulose into sugar reduction 1.4 times greater than other microorganisms, within twenty-four hours. Even on M16 without acid addition when compared to *T.viridae* and *A.niger* mixed and *Cellvibrio* with acid addition still produce reducing sugar almost same amount. This is possible because M16 contains consortium of microorganisms, while other variables using pure cultures. Somehow, each microorganism support other microorganisms in producing necessary enzymes to hydrolyze substrates. Species of cellulolytic bacteria isolated from cow rumen fluid, among others *Nitrosomonas europae*, *Bacillus sphaericus*, *Cellulomonas cellulans, Cytophaga hutchinsoi, Acidothermus cellulyticus, Lactobacillus acidophilus* and *Cellvibrio mixtus* [31]. But then, in line with increasing time reducing sugar product is decreased, it may be because other bacteria utilized reducing sugar to grow. Other advantages M16 required shorter contact time than other types of microorganism and easily obtained at a low cost. Statistic analysis showed p-value of 0.000 is less than the value of the significance level $\alpha = 5\%$, so that decisions can be taken is to reject H0. There is the influence of microorganisms with R^2 value 0,781.

Cellvibrio showed similar capability with *T.viridae* and *A. viger* mixed to hydrolyze a substrate. It was shown by reducing sugar produced almost identical, as can be seen in Table 2 and Figure 2. During incubation time the acid degree (pH) measured about 7-7.9, the cellvibrio could live because the pH for cellvibrio to live about 6,4-8,2 [32]. Cellvibrio growth showed by the biofilm layer. and cellvibrio genus

produced catalase that could transform cellulose into glucose. Cellulose degradation bacteria ability indicated by the transparent area around the colony [33]. In this research cellvibrio growth as colony covered all substrate.

CONCLUSIONS

In conclusion, this investigation has shown various types of microorganisms tested has the potential to hydrolyze hemicellulose and cellulose. Type of microorganism that produces the largest reducing sugar with or without acid addition was M16 and incubation time was 24 hours.

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