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Ethanol Production from Bagasse by Simultaneous Saccharification and Fermentation (SSF) with Steaming and White Rot Fungi Pre-treatments, Combination of Enzyme Cellulase-cellubiose

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Abstract: Experiments on novel technology was used to produce ethanol from bagasse by Simultaneous Saccharification and Fermentation (SSF). Experiments were carried out with white rot fungi, steam and combination of both pre-treatments. Afterward, hydrolysis through combination of enzymes and fermentation by using *Saccharomycess cerevisia* were carried out. Combination of cellulase and cellubiase increased ethanol production. The highest conversion of bagasse to ethanol was 48.9%, which was an increase of 6.8% compared with cellulase only (42.03%). Pre-treatment with steaming increased ethanol production. The highest conversion of bagasse to ethanol was 70.0%, which was an increase of 21.1% compared to ethanol conversion through cellulase-cellubiase without pre-treatment (48.9%). Pre-treatment with several white rot fungi increased ethanol production. The highest ethanol yield was obtained from bagasse after treatment with *C. subvermispora*. The conversion of bagasse to ethanol was 61.0%, which was an increase of 13.0% compared to ethanol conversion with cellulase-cellubiase without pre-treatment (48.9%). Pre-treatment with combination of white rot fungi (*C. subvermispora*) and steam treatments (180^{o}C for 60 minutes) increased ethanol production significantly. The conversion of bagasse to ethanol was 90.0%, which was an increase of 51.1% compared to ethanol conversion with cellulase-cellubiase without pre-treatment (48.9%).

Key words: Ethanol . Bagasse . White rot fungi . Steam . Simultaneous saccharification and fermentation . Cellulase-cellubiase

INTRODUCTION

Energy and energy related problems are likely becoming more and more important in the near future. The increasing energy demand from emerging economies versus the day-by-day decreasing storage of energy resources, the rising cost of fossil fuels and the considerable environmental impact connected with their exploitation are implications that policy makers can not any further disregard [1]. The most important consideration for energy development policy in the future is how to find more alternative energy from existing resources. Developing biofuel from renewable biomass such as ethanol would provide strategic, environmental and societal benefits [2, 3].

Since ethanol has a relatively low toxicity, is highly soluble in water and readily biodegradable, the consequences of large fuel spills are far less environmentally threatening than those associated with spills of crude oil and gasoline. Perhaps most importantly, unlike fossil fuels, the use of ethanol actually mitigates the atmospheric accumulation of carbon dioxide or the so-called greenhouse effect. Substituting ethanol for gasoline as a transportation fuel would substantially reduce net emissions of carbon dioxide. When replanted in a sustainable manner, the amount and combustion of fuel ethanol would be equivalent to the amount of carbon dioxide being absorbed by replanted biomass [4]. Ethanol belongs also to the clean combustion. Its oxygen content decreases emissions in mix combustion with gasoline. Because this ethanol is originally plant matter, its use as fuel does not contribute to the net accumulation of carbon dioxide in the atmosphere [5].

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Ethanol fermentations are traditionally carried out for wine or beer production, but ethanol for transportation and industry are a large potential market and in growing use. Most ethanol produced in the world today is derived from starch or sucrose [6]. Starch is abundant in crop materials, but expansion of ethanol production for the purpose of automotive fuel requires feedstocks that do not compete for food [7].

On the other hand, lignocellulosic material is considerably the most abundant raw material such as in hardwood, softwood, grasses and agricultural residues. Further potential raw materials are newsprints, office papers, municipal solid wastes, etc.

Bagasse, the solid residue after extraction of the sugarcane juice, is rich in carbohydrate content, while in the same time has low lignin content [8]. Especially in Indonesia, the utilization of bagasse in industrial fields is still limited for energy source in a conventional manner. It has traditionally been burnt in low efficiency boilers to produce modest amounts of energy in steam and electricity production required for the cane processing plant. This practice, in certain conditions, limits the disposal problems.

Sugarcane bagasse (*Saccharum officinarum*) is a potential renewable biomass resource for ethanol production, since it is readily available and considerably inexpensive. Unfortunately, sugarcane has lignocellulose structure, which is far more complex than starch. It consists of a mixture of carbohydrate polymers (cellulose and hemicellulose), as well as lignin. The carbohydrate polymers are tightly bound to lignin mainly by hydrogen bonds but also by some covalent bonds [9]. Therefore, the utilisation of bagasse for ethanol production is still under research.

The process for converting this lignocellulosic material to ethanol requires: (1) delignification to release cellulose and hemicellulose from their complex with lignin; (2) depolymerization of the carbohydrate polymer to produce free sugars and (3) fermentation of mixed hexose and pentose sugar as monosaccharide to produce ethanol. The accurate analysis during conversion process is also important for the successful development of feasible conversion process.

Cellulose is normally the predominant polysaccharide ingredient in lignocellulosic materials, such as bagasse. Cellulose fractions can be hydrolyzed to sugars, which then can be easily fermented to ethanol. Hydrolysis can be catalyzed by acids or cellulolytic enzymes. Since enzymatic hydrolysis has several advantages over acid hydrolysis, it is a very promising method for saccharification of lignocellulose polysaccharides [10]. Enzymatic hydrolysis is attractive because it produces better yields than acid-catalyzed hydrolysis. Besides, enzyme manufacturers have reduced costs substantially thanks to modern biotechnology [11].

Unfortunately, since lignin hinders the access of cellulolytic enzymes to cellulose, it is necessary to decompose the networks of lignin prior to the enzymatic hydrolysis [12]. Therefore, it is known that among the key processes described above, the delignification of lignocellulosic raw materials is the rate-limiting and most difficult task to be solved [13]. Consequently, the development of cost-effective method and environmental friendly to make cellulose more accessible to enzymes is a major technological challenge for process commercialization.

Since the past decade, most of the researches have been focusing on the development of pretreatment methods, the term used for removing Lignin and releasing fermentable sugar. Significant progress has been made on thermal, mechanical, biological and chemical pretreatment.

Biological treatment using white rot fungi, which have the ability to decompose the networks of lignin with minimum losses of polysaccharides, is of great potentials. Many studies reported white rot fungi, for example *C. subvermispora* and *P. ostreatus*, as the most effective pre-treatment for bioorganosolve [14, 15]. The advantages of biological delignification over the other methods may include mild reaction conditions, higher product yields, fewer side reactions, less energy demand and less susceptible to pressure and corrosions [13].

In the last decade, steam treatment has been considered to be one of the most effective pretreatments, characterized by low use of chemical [16, 17]. Steam treatment has several advantages. For example, through this thermal treatment, the lignin is solubilised and the accessibility of the cellulose to cellulase enzyme is improved [18]. As biomass is exposed to the pressurized steam, the process recovers most pentosans and produces hydrolyzate that results in little or no inhibition of glucose fermentation [19].

Our research is aimed to obtain novel process for the production of ethanol from bagasse. The process consists of combination of cellulase-cellubiase and subsequent physical treatment with steam, biological treatments with selective lignin-degrading white rot fungi and combination of both treatments process. Hydrolysis and fermentation processes were carried out by Simultaneuous Saccharification and Fermentation (SSF). SSF is the most promising process for the production of ethanol from lignocellulosic materials. The process combines enzyme hydrolysis of cellulose with simultaneous fermentation of sugars to obtain ethanol. In the SSF process, the stages are virtually the same as in separate hydrolysis and fermentation

systems, except that stages are performed in the same reactor and simultaneously occur. Thus, the presence of yeast together with the cellulolytic enzyme complex reduces the accumulation of sugars within the reactor, thereby increasing yield and saccharification rate with respect to separate saccharification and fermentation. Another advantage of this approach is that a single fermentor is used for the entire process, thereby curbing the investment costs.

MATERIALS AND METHODS

Bagasse: Bagasse used in these experiments was originated from a sugar factory in Lampung (Sumatra Island, Indonesia). Sugarcane bagasse was milled and screened (30-60-mesh). The milled bagasse was then air-dried to a final humidity 10% and stored under dry conditions.

Fungal treatment: *P. ostreatus* ATCC 66376, *C. subvermispora* ATCC 90467 and *L. endodes* IFO 6654 were used for the fungal treatment of bagasse. The strains were precultured on 2% Potato-Dextrose Agar (PDA) plates at 28°C for 5-10 days. Before the cultivation, PDA was sterilized at 121°C for 30 minutes in the autoclave. Distilled water (15 mL) was added to the bagasse meal (5.0 g) in 300 mL of Erlenmeyer flasks. The bagasse medium was sterilized at 121°C for 30 minutes. Four pieces (approximately 30 mm^2 $piece^{-1}$) of pellets from the precultures were inoculated. The cultivation was performed stationery at 27°C with relative humidity of 70% for 8 weeks.

Simultaneuous saccharification and fermentation with enzyme cellulose: Cellulase (Meicellase from *Trichoderma viride*, Meiji Seika Co. Ltd.) and cellubiase (98%, Sigma-Aldrich, Slovakia) were used.

Saccharomyces cerevisiae AM12 was precultured on Potato Dextrose Agar (PDA) 2%, Agar (0.25 g), H_2O (50mL) and incubated 1-2 days at 28°C, then used for yeast inoculum in SSF.

S. cerevisiae AM12 was precultured in 50 mL of medium (10 g L^{-1} of glucose; 1.0 g L^{-1} of yeast extract; 0.1 g L^{-1} of KH_2PO_4; 0.1 g L^{-1} of $MgSO_4.7H_2O$; and 0.1 g L^{-1} of $(NH_4)_2SO_4$) in a 200 mL flask, at 30°C for 24 h using an orbital shaker at 100 rpm.

The medium for SSF (5 mL) contained bagasse sample (0.25 g), nutrient medium (2.5 mL), 0.05 M Na-citrate buffer (pH 5.0), Meicellase (10 FPU), Cellubiase (5 FPU) and 10% (v/v) yeast inoculum. The sample, nutrient medium and buffer were heated at 121°C and 20 min, but the enzyme solution was added without sterilization. The nutrient medium composed of 1.0 g L^{-1} $(NH_4)_2PO_4$; 0.05 g L^{-1} $MgSO_4.7H_2O$ and 2 g L^{-1} yeast extract. Cultivations were carried out in a test tube with 5.0 mL of the medium using an orbital shaker at 100 rpm for 96 h at 35°C.

Hydrolysis assay: For hydrolysis with enzyme celllulase, the medium for Hydrolysis (5 mL) contained bagasse sample (0.25 g), nutrient medium (2.5 mL), 0.5 mL Na-citrate buffer (pH 5.0), Meicellase (10 FPU) and 1.25 mL aquades.

For hydrolysis with enzyme cellulase-cellubiase, the medium for Hydrolysis (5 mL) contained bagasse sample (0.25 g), nutrient medium (2.5 mL), 0.5 mL Na-citrate buffer (pH 5.0), Meicellase (10 FPU), cellubiase 5 FPU and 1.20 mL aquades.

Aliquots of the samples were taken every 6, 12, 24, 48, 72 and 96 h and assayed for glucose content content with GC.

Analytical methods: Lignin, holocellulose, a-cellulose and hemicellulose contents were analyzed by Klason lignin and Wise methods, respectively.

The ethanol concentration was determined by GC using SUPELCOWAX-10 (Supelco Inc., 0.53 mm i.d., 15 m, 0.5 mm) oven temperature at 50°C of a Shimadzu GC-14A gas chromatograph, equipped with a flame ionization detector. Before determination, sample from the cultures were taken 50 µl and added 200 µL (5 times dilution) and centrifuged at 10,000 rpm for 5 min at 4°C. Supernatant (50 µl) was taken and mixed with 50 µl. Aliquots of the samples were taken every 6, 12, 24, 48, 72 and 96 h and assayed for ethanol content.

RESULTS AND DISCUSSION

Ethanol production from bagasse using cellulase-cellobiase on SSF: Combination of enzyme cellulase and cellubiase increased ethanol production from bagasse by SSF using *Saccharomycess cerevisiae* AM12. The highest ethanol concentration was achieved after hydrolysis by enzyme cellulase-cellubiase in SSF at pH=5 up to 6.94 g L^{-1} (Fig. 1). This yield was an increase of about 16% compared with ethanol concentration using enzyme cellulase only (5.98 g L^{-1}). This result indicated that eventhough cellulase has a group of cellobiases (ß-glucosidase), it can not optimally convert cellubiose (disaccharides) to glucose. Generally, hydrolysis of cellulose is more difficult to achieve than that of the other polysaccharides. One of the difficulties involves slow complexity of interfacial heterogeneous hydrolysis influenced by various factors, for example, structure and composition of lignocellulosic material, cellulase adsorption and desorption, enzyme inhibition by cellobiose and glucose [20].

Table 1: Ethanol yields based on original bagasse and cellulose contents on SSF with enzyme cellulase and cellulase-cellubiase

Ethanol yields based on original bagasse with:		Ethanol yields based on cellulose content with:	
Cellulase (%)	Cellulase-cellubiase (%)	Cellulase (%)	Cellulase-cellubiase (%)
0.00	0.00	0.00	0.00
4.20	5.01	8.40	10.02
6.21	7.80	12.42	15.60
9.80	12.20	19.60	24.40
11.40	13.49	22.80	26.98
11.76	13.74	23.52	27.48
11.95	13.87	23.90	27.74

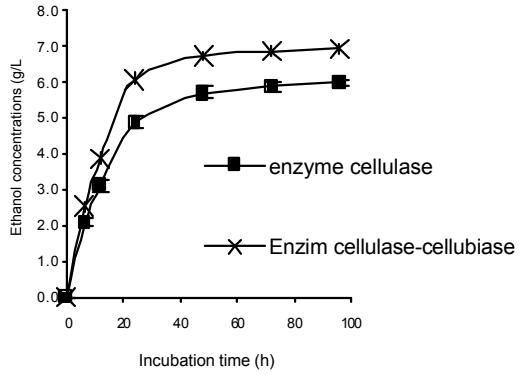

Fig. 1: Ethanol production from bagasse with enzyme cellulase-cellubiase at pH=5 on SSF

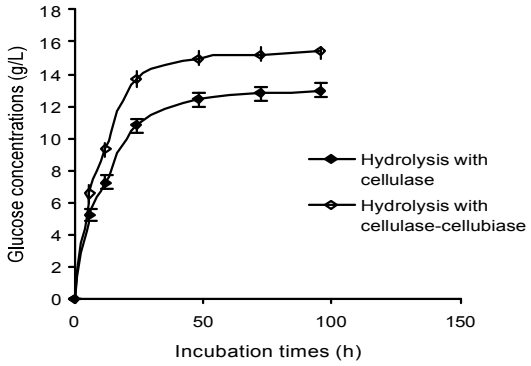

Fig. 2: Glucose concentration of bagasse after hydrolysis with enzyme cellulase and cellulase-cellubiase

Hydrolysis of cellulose to glucose is often followed by partial hydrolysis of cellulose to cellubiose. Thus, addition of cellubiase enhances the conversion of cellulose to ethanol as seen on Fig. 2. The experiments were carried out at pH = 5, the pH optimum for conversion of bagasse to ethanol using enzyme cellulase and cellulase-cellubiase (Fig. 3).

It was indicated that at pH=5 concentration of ethanol production was higher than that at other pHs

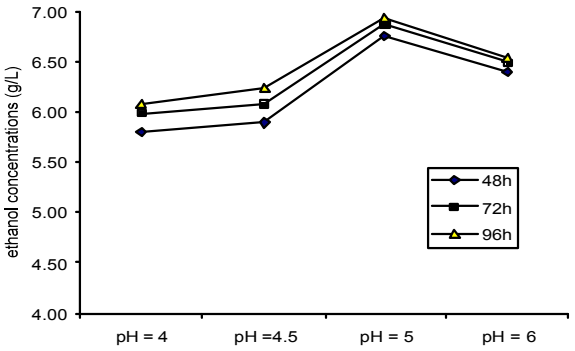

Fig. 3: Effect of pH for ethanol production from bagasse with enzyme cellulase-cellubiase on SSF

along incubation time. Our results were in line with studies, which reported that pH=5 was the optimum pH for enzimatic hydrolysis by cellulase [15, 21-23]. They agree that the structure and activity of cellulase were relatively stable at pH=5.

The ethanol production during incubation time of 0-48 h increased very significantly, whereas after 48 h incubation different result was occurred. These results indicated that incubation more than 48 h the enzyme and yeast were not so effective in converting bagasse to ethanol. Fermentation period or incubation time to achieve constant ethanol yield in this experiments was shorter compared to other studies. Ballesteros [24], reported that the maximum ethanol production from biomass using *Kluyveromyces marxianus* CECT 10875 and enzyme cellulase was done on fermentation period of 72-82 h. Other study showed 72-82 h incubation time to achieve final ethanol concentration using thermo-tolerant yeast and enzyme cellulase at 10% substrate by SSF of lignocelluloses waste (including bagasse) [21].

Calculation on the yield of ethanol production based on original bagasse and cellulose content is shown in Table 1. The highest yield of ethanol production of bagasse was 13.87% based on original

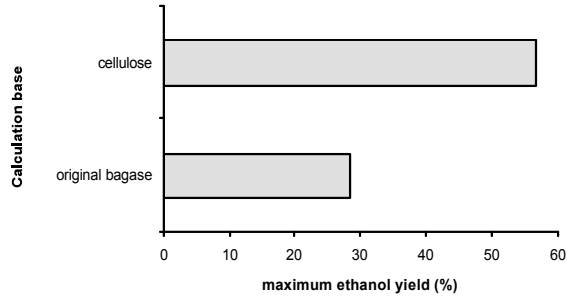

Fig. 4: Maximum ethanol yield from bagasse based on theoretical yield (based cellulose and original bagasse)

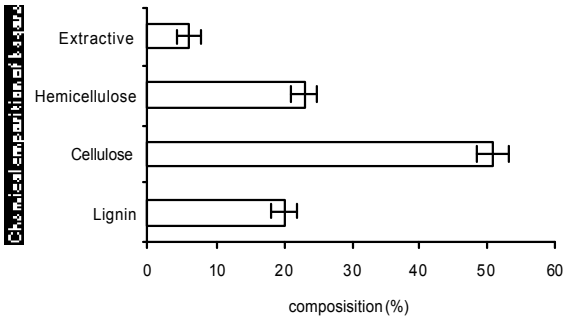

Fig. 5: Lignin, hemicellulose and cellulose content of bagasse

bagasse or 27.74% based on cellulose. Theoretically, the maximum yield of ethanol production from bagasse was 28.43% based on original bagasse or 56.86% based on cellulose (Fig. 4). Theoritical yield was calculated from cellulose composition on bagasse with assumption that all of cellulose content was converted to glucose. Chemical content analysis of bagasse showed the composition of cellulose, lignin and hemicellulose were 50%, 20% and 22%, respectively (Fig. 5).

Conversion of bagasse to ethanol with combination of enzyme cellulase-cellubiase was 48.87% and the conversion of bagasse to ethanol with enzyme cellulose only was 42.03%. The increase was about 6.8% as cellulase-cellubiase was used. Conversion was calculated from the highest ethanol yield from experiment (13.87% based on original bagasse or 27.74% based on cellulose), compared with maximum yield from theoretical (28.43% based on original bagasse or 56.86% based on cellulose).

The experimental results showed that enzyme cellulase-cellubiase were not able to achieve maximum conversion without treatment prior to SSF. Cellulose is intrinsically resistant to enzymatic attack and is protected by the surrounding matrix of lignin and hemicellulose, therefore lignocellulosic materials like bagasse must be pretreated to make the cellulose more accessible [25, 26].

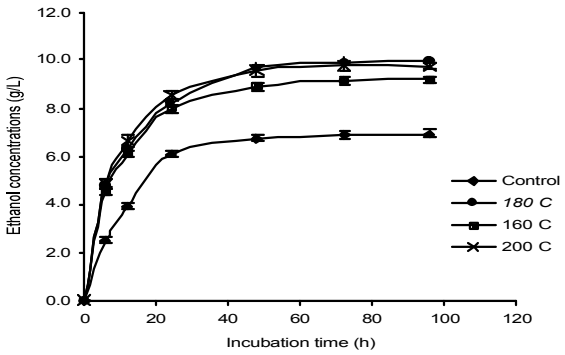

Fig. 6: Ethanol production from bagasse by SSF with cellulase-cellubiase using steam pre-treatment on pH=5

Effect of steam treatment on ethanol production with enzyme cellulase-cellubiase: Experimental results showed that pre-treatment with steam increased ethanol production from bagaase by SSF using *S. cerevisiae* and enzyme cellulase-cellubiase. The highest ethanol production after treatment with steam for 60 minutes at 160°C, 180°C and 200°C were 19.20 g L^{-1}, 19.74 g L^{-1} and 19.98 g L^{-1}, respectively (Fig. 6). This yield was an increase of 33-44% compared to ethanol production from bagasse without white rot fungi treatment (6.94 g L^{-1}). Treatment with steam at 180°C for 60 minutes was more effective if compared with the other steaming treatment.

Water pretreatment reduces the need for neutralization and conditioning chemical since acid is not added. Size reduction of the incoming biomass is not needed since the lignocellulose particles break apart when cooked in water [27, 28]. A highly digestible cellulose results when enzyme is added [19, 25, 28]. and high yields of sugars from hemicellulose occur during pretreatment. The liquid produced is then fermented to ethanol [2, 19, 25].

Steaming helps the cleavage of o-acety and uronic acid substitutions from hemicellulose to generate acetic and other organic acids. The release of these acids helps to catalyze formation and removal of oligosaccharides. However, the polysaccharides may be further hydrolyzed to monomer sugars which are subsequently partially degraded to aldehydes if acid is used. These aldehydes, principally furfural from pentoses and 5-hydroxymethyl furfural from hexose, are inhibitory to microbial fermentation [29].

Water has an unusual high dielectric constant that enables ionic substances to dissociate. Water is able to dissolve all of the lignin and hemicellulose. One half to two third of the lignin also dissolves from most biomass materials when these materials are treated

Table 2: Calculation ethanol yields based on original bagasse and cellulosese contents on SSF with enzyme cellulase-cellubiase using steam treatments

Ethanol yields based on original bagasse after treatment with				Ethanol yields based on cellulose content after treatment with			
Control	180 C	160 C	200 C	Control	180 C	160 C	200 C
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5.01	9.55	9.05	9.70	10.02	19.10	18.10	19.40
7.80	12.60	12.22	13.24	15.60	25.20	24.44	26.48
12.20	16.43	15.96	17.05	24.40	32.86	31.92	34.10
13.49	19.40	17.80	19.14	26.98	38.80	35.60	38.28
13.74	19.84	18.25	19.51	27.48	39.68	36.50	39.02
13.87	19.96	18.40	19.48	27.74	39.92	36.80	38.96

at 220^{0}C for 2 h. Steam cleaves hemiacetal linkages in biomass [30]. Sofwoods are less susceptible to solubilization for reasons that are not understood [25].

The calculation of ethanol yield (in %tage) with steam based on original bagasse and based on cellulose are shown in Table 2. The highest ethanol yield was from bagasse after treatment with Steam at 180^{o}C (19.96%, based on original bagasse; or 39.92%, based on cellulose). Theoretically, the maximum yield of ethanol production from bagasse was 28.43% (based on original bagasse) or 56.86% (based on cellulose) as shown in Fig. 4). This result indicated that 70.02% of theoretical yield was achieved if bagasse converted to ethanol with enzyme cellulose-cellubiase and steam treatment (180^{o}C) on SSF.

Other study reported that maximum ethanol production on SSF using *K. marxianus* CECT 10875 with steam explosion pretreated sweet sorghum bagasse ethanol yield about 50-72% based theoretical yield in 72-96 h incubation time [24]. Our experiments showed more effective results because ethanol was produced after pre-treatment with steam the conversion of bagasse to ethanol was 70.02% based theoretical yield in 48-96 h.

Effect of white rot fungi treatment on ethanol production with enzyme cellulase-cellubiase on SSF: Pre-treatment with white rot fungi *C. subvermispora, L. edodes* and *P. ostreatus* increased ethanol production from bagasse by SSF using *Saccharomycess cerevisiae* and enzyme cellulase-cellubiase. The highest ethanol production after treatment with *C. subvermispora, L. edodes* and *P. ostreatus* were 8.68 g L^{-1}, 8.23 g L^{-1} and 8.10 g L^{-1}, respectively (Fig. 7). This yield was an increase of 16.78% compared to ethanol production from bagasse without white rot fungi treatment (6.94 g L^{-1}).

White rot fungi like *C. subvermispora*, *L. edodes* are selective lignin degrading fungi that are able to decompose lignin in bagasse without intensive damage

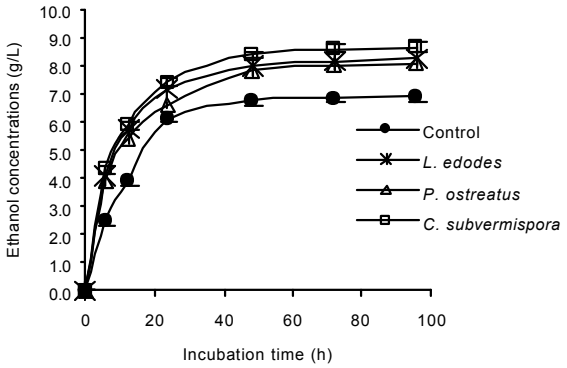

Fig. 7: Ethanol production from bagasse with enzyme cellulase-cellubiase and whiter rot fungi treatment at pH=5 on SSF

to cellulose [31]. Decomposition of chemical lignin structure and lignin-corbohydrate complexes is very important. Bagasse is a family of grasses, whose lignin comprises of guaiacylprpanoid, syringylpropanoid and *p*-hydroxyl-benzylpropanoid structures and linked with polysaccharides by cinnamamic acid bridges. This makes lignin in bagasse (or grasses) is more difficult to decompose from its lignocellulosic materials compared to that in coniferous trees. Lignin in coniferous trees primarily comprises of guaiacylpropanoid structures and directly linked with hemicelluloses by benzyl ether and ester bonds [32]. Yuan-ZongLai has discussed intensively the results of many researchers, which conclude that the reactivity and accessability of cellulose and lignins depend on many factors such as cristallinity and supramolecular structrure [33].

The increase in ethanol yield by the fungal treatments ascribes to the increase in accessibility of cellulolytic enzymes to cellulose by decomposing Lignin surrounding the polysaccharide. The changes in lignin, holocellulose and cellulose content during fungal treatment for 8 weeks are shown in Table 3. Lignin losses of bagasse after treatment with

Table 3: Lignin, holocellulose and cellulose content and weight loss of bagasse during fungal treatment for 8 weeks

Chemical composition	Without fungal treatment	Treatment with *C. Subvermispora*		Treatment with *P. ostreatus*		Treatment with *L. edodes*	
		Composition (%)	Weight loss (%)	Composition (%)	Weight loss (%)	Composition (%)	Weight loss (%)
Lignin	20	16.86	15.70	17.11	14.45	17.2	14.00
Cellulose	51	49.50	2.94	48.50	4.90	49.1	3.73
Hemicellulose	23	21.60	6.09	21.20	7.83	21.4	6.96

Table 4: Ethanol yield from bagasse (based on original bagasse and cellulose content) on SF with enzymes cellulase-cellubiase and treatment with several white rot fungi

Ethanol yields based on original bagasse after treatment with (%)				Ethanol yields based on cellulose content after treatment with (%)			
Control (cellulase-cellubiase)	*L. edodes*	*P. ostreatus*	*C. subvermispora*	Control (cellulase-cellubiase)	*L. edodes*	*P. ostreatus*	*C. subvermispora*
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5.01	8.20	7.84	8.60	10.02	16.40	15.68	17.20
7.80	11.42	10.80	11.82	15.60	22.84	21.60	23.64
12.20	14.40	13.21	14.82	24.40	28.80	26.42	29.64
13.49	16.00	15.60	16.80	26.98	32.00	31.20	33.60
13.74	16.38	16.00	17.20	27.48	32.76	32.00	34.40
13.87	16.45	16.20	17.35	27.74	32.90	32.40	34.70

P. ostreatus, L. edodes and *C. subvermispora* for 8 weeks were 14.45%, 14.00% and 15.70%, respectively. The losses of a-cellulose from bagasse were 4.90%, 3.73% and 2.94%, respectively. The losses of hemicellulose from bagasse were 7.83%, 6.96% and 6.09%, respectively. This yield indicated that biological treatments with these white rot fungi decomposed the network of lignin. *C. subvermispora* is characterized as one of the most effective for biological pulping process and biorganosolve material [14, 34]. In this research *P. ostreatus, L. edodes and C. subvermispora* were also effective for biorganosolve to convert biomass (especially bagasse) to ethanol. Improvement in digestibility of bagasse, shown by the weight loss of cellulose, is greater with *C. subvermispora*, *L. edodes* and *P. ostreatus* compared to non selective white rot fungi [35]. Besides, the selective white rot fungi like *C. subvermispora* have high ability to decompose aryl ether bonds of lignin, which in turn promote ethanol fermentation of softwood [36]. Those explanations are affirmative to the results from experiments which showed that among those three white rot fungi treatments, the one with *C. subvermispora* produced the highest ethanol.

The calculations of ethanol yield (in %-tage) with white rot fungi treatment based on original bagasse and based on cellulose are shown in Table 4. The highest ethanol yield was from bagasse after treatment with *C. subvermispora* (17.35%, based on original bagasse; or 34.70%, based on cellulose). Theoretically, the maximum yield of ethanol production from bagasse was 28.43% (based on original bagasse) or 56.86% (based on cellulose) as shown in Fig. 4). This result indicated that 61.02% of theoretical yield was achieved if bagasse converted to ethanol with enzyme cellulose-cellubiase and white rot fungi treatment on SSF.

Other study reported ethanol production by SSF of beech wood after pretreatment with *C. subvermispora* and ethanolysis gave high yield, i.e. 62% of theoretical yield [23]. Our experiments showed more effective results because ethanol was produced after pre-treatment with *C. subvermispora* without ethanolysis and the conversion of bagasse to ethanol was 61.03%. This was an increase of 12,96% compared to ethanol conversion with enzim cellulase-cellubiase without fungal treatment (48.87%). The conversion of bagasse to ethanol with enzyme cellulose was 42.03%, which was an increase of about 21% as cellulase-cellubiase with *C. subvermispora* was used. Conversion was calculated from the highest ethanol yield from experiment (17.35% based on original bagasse or 34.70% based on cellulose), compared with maximum yield from theoretical (28.43% based on original bagasse or 56.86% based on cellulose). The result of conversion from experiment indicated that white rot fungi treatment was effective to increase ethanol conversion with ezyme cellulase-cellubiase.

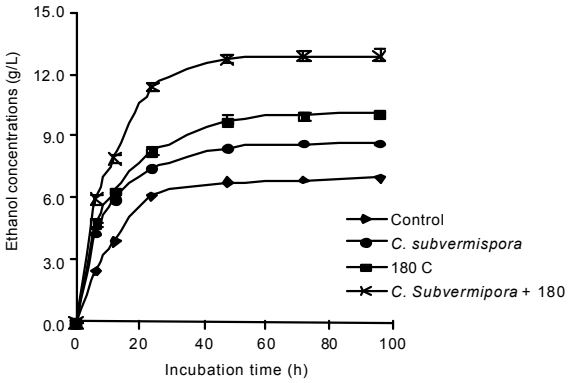

Fig. 8: Ethanol production from bagasse by SSF with enzyme cellulase-cellubiase using combination steam at 180°C for 60 minutes dan white rot fungi treatment for 8 weeks

Effect of combination of steam and white rot fungi treatment on ethanol production with cellulase-cellubiase: Combination white rot fungi *C. subvermispora* and steam (180°C) increased ethanol production from bagasse by SSF using *Saccharomycess cerevisiae* and enzyme cellulase-cellubiase. Ethanol production after incubation time 48, 72 and 96 h was about 12.73, 12.82 and 12.86 g L^{-1}, respectively (Fig. 8). This yield was an increase of 83-85% compared to ethanol production from bagasse without white rot fungi and steam treatment (6.94 g L^{-1}).

The increasing of ethanol yield by combination of fungal treatments and steam treatments were ascribed to increase in accessibility of cellulolytic enzymes to cellulose by decomposing Lignin surrounding the polysaccharide and solubilised of lignin and hydrolytic degradation of hemicellulose in steam. *C. subvermispora* is known as a selective white-rot fungus that degrades lignin in wood cell walls without penetration of its extra cellular enzymes into the cell wall region [32]. Accessible cellulose-cellubiase more effective after treatment with steam because after decompose of lignin with *C. subvermispora,* lignin continuously solubilised by steam. This yield indicated that biological treatments with these white rot fungi decomposed the network of lignin. steaming treatment make hydrolytic degradation of dissolved xylan likely contributed to incomplete xylan recovery. It is also possible that xylan and other material (e.g. furfural) were lost through volatilization and recondensation on the interior of the reactor to degrade of product [37]. Degradation of xylan and other products (e.g furfural) trough hydrolytic degradation, volatilization and recondensation makes access of enzyme cellulase-cellubiase to convert cellulose to became ethanol easier.

White rot fungi like *C. subvermispora*, *L. edodes* are selective lignin degrading fungi that are able to decompose lignin in bagasse without intensive damage to cellulose [31]. Yuan-ZongLai has discussed intensively the results of many researchers, which conclude that the reactivity and accessability of cellulose and lignins depend on many factors such as crystalline and supramolecular structrure [33]. The other aspect water has an unusually high dielectric constant that enables ionic substances to dissociate. Water is able to dissolve all of the lignin and hemicellulose. Steam cleaves hemiacetal linkages in biomass, so that access of cellulase-cellubiase to polysaccharides is easier [30].

The calculations of ethanol yield (in %-tage) with combination of steam (180°C) and white rot fungi (*C. subvermispora)* treatments based on original bagasse and based on cellulose are shown in Table 5. The highest ethanol yield was 25.72%, based on original bagasse; or 51.44%, based on cellulose). Theoretically, the maximum yield of ethanol production from bagasse was 28.43% (based on original bagasse) or 56.86% (based on cellulose) as shown in Fig. 4). This result indicated that 90.02% of theoretical yield was achieved if bagasse converted to ethanol with enzyme cellulose-cellubiase and combination of steaming and white rot fungi treatment on SSF. Conversion was calculated from the highest ethanol yield from experiment (25.72% based on original bagasse or 51.44% based on cellulose), compared with maximum yield from theoretical (28.43% based on original bagasse or 56.86% based on cellulose). The result of conversion from experiment indicated that white rot fungi treatment was effective to increase ethanol conversion with ezyme cellulase-cellubiase.

Other study reported ethanol production by SSF of beech wood after pretreatment with *C. subvermispora* and ethanolysis gave high yield, i.e. 62% of theoretical yield [23]. Other research reported that ethanol production on SSF with steam explosion pretreated sweet sorghum bagasse ethanol yield about 50-72% based theoretical yield in 72-96 h incubation time [24]. Our experiments showed more effective and higher results because ethanol was produced after pre-treatment with combination of *C. subvermispora* and steam (180°C for 60 min) and the conversion of bagasse to ethanol was 90.02%. This was an increase of 51.11% compared to ethanol conversion with enzim cellulase-cellubiase without fungal treatment (48.87%).

CONCLUSIONS

Ethanol was produced from bagasse by SSF with combination of enzyme cellulase and cellubiase using

Table 5: Ethanol yields based on original bagasse and cellulosese contents on SSF with enzyme cellulase-cellubiase using combination steam and white rot fungi treatments

Ethanol yields based on original bagasse after treatment with:				Ethanol yields based on cellulose content after treatment with:			
Control	*C. subvermispora*	*180 C*	*C. subvermispora + 180 C*	Control	*C. subvermispora*	*180 C*	*C. subvermispora + 180 C*
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5.01	8.60	9.55	11.90	10.02	17.20	19.10	23.80
7.80	11.82	12.60	15.77	15.60	23.64	25.20	31.54
12.20	14.82	16.43	22.61	24.40	29.64	32.86	45.22
13.49	16.80	19.40	25.45	26.98	33.60	38.80	50.90
13.74	17.20	19.84	25.64	27.48	34.40	39.68	51.28
13.87	17.35	19.96	25.72	27.74	34.70	39.92	51.44

pre-treatments: white rot fungi, steam and combination both. Combination of cellulase and cellubiase increased ethanol production from bagasse by SSF using *Saccharomycess cerevisiae*. The highest ethanol concentration was 6.94 g L^{-1}, this was an increase of 16% if compared with ethanol concentration using enzyme cellulase only (5.98 g L^{-1}). The conversion of bagasse to ethanol was 48.87%, It was an increase of 6.84% compared with cellulase only (42.03%).

Pre-treatment with steaming increased ethanol production ethanol production from bagasse by SSF using *S. cerevisiae.* The highest ethanol production after treatment with steaming at 180^{o}C for 60 minutes was 9.98 g L^{-1}. The yield was calculated as 19.96% (based on original bagasse) or 39.92% (based on cellulose). The conversion of bagasse to ethanol was 70.02%, which meant an increase of 21.11% compared to ethanol conversion with cellulase-cellubiase without pre-treatment (48.87%)

Pre-treatment with white rot fungi *C. subvermispora, L. edodes* and *P. ostreatus* increased ethanol production from bagasse by SSF using *S. cerevisiae* and combination cellulase-cellubiase enzymes. The highest ethanol yield was obtained from bagasse after treatment with *C. subvermispora*, i.e. 17.35% (based on original bagasse) or 34.70% (based on cellulose). The conversion of bagasse to ethanol was 61.03%, which meant an increase of 12.96% compared to ethanol conversion with cellulase-cellubiase without pre-treatment (48.87%).

Pre-treatment with combination white rot fungi (*C. subvermispora*) and steam treatments (180^{o}C for 60 minutes) increased ethanol production very significantly. The highest ethanol production was 12.86g L^{-1}. The highest ethanol yield was 25.72%, based on original bagasse or 51.44%, based on cellulose. The conversion of bagasse to ethanol was 90.02%, which meant an increase of 51.11% compared to ethanol conversion with cellulase-cellubiase without pre-treatment (48.87%).

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