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Bioethanol production from rice and corn husks after enzymatic and microbes hydrolysis and yeast fermentation

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Abstract. *Purwoko Tj, Sari SLA, Mahadjoeno E, Sunarto. 2017. Bioethanol production from rice and corn husks after enzymatic and microbes hydrolysis and yeast fermentation. Bioteknologi 14: 19-23.* Bioethanol is a renewable resource that can be produced from fermented cellulosic biomass. The use of lignocellulosic materials from agricultural wastes provides a low-cost fermentative substrate. Lignocellulosic ethanol production involves acid or enzymatic hydrolysis. The enzymatic hydrolysis cost higher, however in environmental issue this step is favorable. The purpose of this research was to compare bioethanol production after microbes and cellulosic enzymes hydrolysis following yeast, *Saccharomyces cerevisiae*, fermentation from rice and corn husks. The rice and corn husks (1 kg) were each suspended in water until the volumes reached 5 L. The sample mixtures were treated with 2.5 mg/L cellulases, 5 g/L multienzymes, and 5 mL/L EM4 respectively. The mixtures were stirred for 24 hours at pH 5.7 and 35°C. After hydrolysis, the samples (100 mL) were treated by 1, 2, and 3 % w/v Baker's yeasts. The samples were fermented with incubator shaking for 6 days at 30°C, 90 rpm, and pH 6.5. Sugar concentrations were determined by dinitrosalicylic acid (DNS) colorimetric method. Bioethanol production and specific gravity method were then compared to IAOC Ethanol Table. Sugar concentrations and bioethanol production after multienzymes hydrolysis of rice and corn husks. There were 6.54-6.81 mg/mL and 3.17-3.54 mg/mL, respectively. Sugar concentrations of rice and corn husks after multienzymes hydrolysis treatment was higher than EM4 and cellulases hydrolysis treatments. Therefore, bioethanol productions of rice and corn husks after multienzymes hydrolysis and yeasts after 2 days fermentation were higher than the others.

Keywords: Bioethanol, corn husk, multienzymes, rice husk, sugar concentration

INTRODUCTION

With the energy crisis and increased environmental awareness there has emerged a need for more sources of renewable, the green energy around the world. Bioethanol can be produced through fermentation of sugars. Worldwide interest in the utilization of bioethanol as an energy source has its concern on the efficiency and cost of industrial processes for bioethanol production. Even though the fermentative process of bioethanol production is well known, the production costs are still an important factor for the wide use of bioethanol as a fuel. Therefore, development of fermentation processes, using cheap carbon sources, is important for commercial scale production.

Bioethanol is a renewable resource that can be produced from fermented cellulosic biomass. Bioethanol does not add to a net-CO atmospheric increase. Thus, there is no contribution to global warming. Combustion of ethanol results in relatively low emissions of volatile organic compounds, carbon monoxide and nitrogen oxides (Park et al. 2010), therefore can reduce greenhouse gasses. However, applying bioethanol on gasoline engines involves some modification (Hassan and Kalam 2013). Cost reduction is still essential for large deployment of this new technology. Since the cost of the traditionally used (sugar and starch-containing) raw materials represent a major part of the total production cost (Robelo et al. 2011). Using fewer valuable materials, like agricultural waste, could reduce the expense significantly.

The use of lignocellulosic materials from agricultural wastes provides a low-cost fermentative substrate. Using agricultural waste as lignocellulosic feedstocks for bioethanol production was greatly promising. One of the advantages of the use of lignocellulosic biomass is not interfering with food production. This implies the production of bioethanol can be done without the need of employing extensions of cultivable land for cropping sugar cane, rice or corn exclusively dedicated to the biofuel production. The development of an innovative waste management approach is focused on using agricultural and industrial waste as cheap substrates. The production of bioethanol by yeast-based sugar fermentation has already been commercially established. Crop wastes are of great interest to reduce the costs of bioethanol production process. Rice and corn are popular crops in Asian countries, including Indonesia. It is essential to hydrolyze lignocellulosic materials before fermentation because yeast cannot use lignocelluloses directly into bioethanol.

Lignocellulosic materials of rice and corn are: rice straw, rice husk, ccorn cobs and corn husk. Cellulose and hemicellulose, which are the principal biodegradable carbohydrate components of the husks, are found together with lignin in an intense cross-linked, rigid lignocellulose complex (Chan 2014). Most reports on lignocellulosic

ethanol production involve acid or enzymatic hydrolysis. Enzymatic hydrolysis is high cost, however this is favorable . Commercial enzymes that apply in poultry can reduce the cost. Moreover, microbes that have cellulolytic activity can also reduce the cost. The objective of this research was to compare bioethanol production after microbes and cellulosic enzymes hydrolysis following yeast *Saccharomyces cerevisiae* fermentation from rice and corn husks.

MATERIALS AND METHODS

The rice and corn husks were obtained from the farmers at Karanganyar region of Central Java, Indonesia. Baker's yeasts [Fermipan] and microbes [EM4] were obtained from a retail store at Surakarta City (Solo) of Central Java, Indonesia. The cellulases and multienzymes [Naturzime] were obtained from Sukahan, China and Macco Organique Inc., Canada, respectively.

Sample preparation

The rice and corn husks were sun-drying for 7 days. After drying, the husks were grounded. The ground samples were stored at room temperature (± 30°C), and the room humidity was controlled at 50-60% by silica gel. Each of the husk ground samples were treated by hydrolysis and followed by yeasts fermentation.

Sample hydrolysis

The ground samples (1000 grams) were suspended in water until the volumes reached 5 L. They were then mixed to form a slurry sample. The slurry samples were divided into three samples (1.5 L each). After sterilization by autoclave (121°C, 15 min), the slurry samples were treated with 2.5 mg/L cellulases (50 IU), 5g/L multienzymes (50 IU xylanases) and 5 mL/L EM4 respectively. The mixtures were stirred for 24 hours at pH 5.7 and 35°C. After 24 hours, the mixtures were filtered and stored in a refrigerator at 4-6°C prior to yeast fermentation.

Yeast fermentation

The three hydrolysis samples (500 mL each) from hydrolysis were sterilized by autoclaves (121°C, 15 min). After cooling, the samples were treated by 1, 2, and 3 % w/v Baker's yeasts. The samples were fermented at incubator shaking [Thermo Scientific] for 6 days at 30°C, 90 rpm, and pH 6.5.

Sugar determination

The hydrolysis samples from cellulosic enzymes, Naturzime, and EM4 treatments were determined the sugar concentrations by dinitrosalicylic acid (DNS) colorimetric method. The samples of two, four, and six fermentation days from each hydrolysis treatment previously were also determined the reducing sugar by DNS method.

Ethanol determination

The fermentation samples at two, four, and six fermentation days each treatment were determined the ethanol by specific gravity methods. The samples (50 mL) were distilled using a rotary evaporator until the distillate samples were reached 10 mL. The distillate samples (10 mL) were measured at 20°C and converted into bioethanol concentrations by IAOC Ethanol Table. The bioethanol concentrations of fermentation samples were calculated as 20% of bioethanol concentrations of distilled samples.

Data analysis

Data was analyzed using ANOVA with a 95% confidence level. Reducing sugar from hydrolysis samples were analyzed by 2-way ANOVA with reducing sugar before hydrolysis as covariates. Reducing sugar and bioethanol data from fermentation samples were analyzed by 4-way ANOVA.

RESULTS AND DISCUSSION

This research used enzymatic hydrolysis of rice and corn husks from microbes (EM4), commercial multienzymes and cellulases. The EM4 is microbe consortium that usually applied on composting or feed fermentation. The microbes in EM4 are *Rhodopseudomonas palustris*, *Lactobacillus casei*, and *Saccharomyces cerevisiae*. *R. palustris* is a photosynthetic gram-negative bacterium and found in wastewaters. *R. palustris* can grow on green-plant derived compounds and can metabolize lignocellulosic and acids, as well as aromatic compounds. *Lactobacillus casei* is found in the human intestine and mouth. The most common application of *L. casei* is industrial, especially for dairy production and probiotics for humans and animals. In the past few years, *L. casei* has azoreductase activity. It means they could degrade azo-bonds completely (Seesuriyachan et al. 2007). *Saccharomyces cerevisiae* is a species of yeast. It has been instrumental to winemaking, baking, and brewing. Yeast cells are found primarily in ripe fruits such as grapes. It is the microbe behind the most common types of fermentation.

Multienzymes are enzymes that are used for increasing food digestion in poultry (Abudabos 2010). Multienzymes consist of galactosidases, galactomannases, xylanases, beta glucanases, amylases, proteases, and phosphatases. Galactosidases, galactomannases, xylanases, beta glucanases and amylases are enzymes that catalyze the polysaccharides hydrolysis of galactosides, galactomannans, xylans, glucans, and starch respectively into simple sugars. Proteases are enzymes that hydrolysis of protein, meanwhile phosphatases remove phosphate groups.

Cellulases are enzymes produced chiefly by fungi, bacteria, and protozoans that catalyze cellulolysis, the decomposition of cellulose and some related polysaccharides. Cellulases break down the cellulose molecule into simple sugars. There are five general types of cellulases based on the type of reaction catalyzed, endo cellulases, exocellulases, cellubioses, oxidative cellulases and cellulose phosphorylases.

Sugar concentrations after enzymes and microbes hydrolysis

Sugar concentrations of both rice and corn husk slurrys after enzymes and microbes hydrolysis were low (Table 1). Since the substrate complexity, both enzymes and microbes are not easy to degrade both substrates. Both enzymes and microbes could hydrolyze lignocelluloses of rice and corn husks into simple sugars. Therefore, there are increasing sugar concentrations after hydrolysis treatment compared to before hydrolysis treatment. Therefore, sugar concentrations after enzymes and microbes hydrolysis are low, not greater than 7 mg/mL. The responsible microbe in EM4, which hydrolyzed lignocelluloses into oligosaccharides and monosaccharides, was *R. palustris*. However, oligosaccharides are further hydrolyzed into monosaccharides by *L. casei* and *S. cerevisiae*. Lignocelluloses was hydrolyzed into oligosaccharides and monosaccharides by xylanases of the multienzymes. Beta-glucanases and amylases of the multienzymes were hydrolyzed oligosaccharides into monosaccharides.

Table 1 showed that sugar concentrations before hydrolysis treatment were 3.24-4.25 mg.mL. These values were not different significantly ($p \geq 0.05$). Therefore, the statistical analyzed sugar concentrations after hydrolysis treatment did not depend on hydrolysis treatment. Sugar concentrations after multienzymes hydrolysis of rice and corn husks were 6.54-6.81 mg/mL and sugar concentrations after cellulases and EM4 hydrolysis was 5.34-5.65 mg/mL and 5.03-5.45 mg/mL. The sugar concentrations after multienzymes hydrolysis were higher than after cellulases and EM4 hydrolysis ($p < 0.05$). It seemed enzymes of multienzymes were more active to hydrolyzed lignocelluloses in rice and corn husks, than enzymes of EM4 and the cellulases. Sugar concentrations after hydrolysis treatment of rice and corn husks, however, were 5.03-6.81 mg/mL and 5.45-6.54 mg/mL. These values were not different significantly ($p \geq 0.05$). It seemed that lignocelluloses of rice and corn husks had large amounts, therefore the enzyme's activity of multienzymes, EM4 and cellulases on rice and husks were not different.

Sugar concentration of rice husk after multienzymes hydrolysis was 6.81 mg/mL. This value was very low compared to 150 mg/mL and 198 mg/g sugar concentration of rice husk after H_2SO_4 hydrolysis (Novia et al. 2015) and rice straw after hydrochloride acid (HCl) hydrolysis (Hashem et al. 2013) respectively. The lowest sugar concentrations after hydrolysis were due to the small amounts of the multienzymes. However, environment consideration, using enzymes hydrolysis were favorable than acid hydrolysis. This low sugar concentration could be increased if we increased the concentration.

Sugar concentrations after enzymes and microbes hydrolysis and yeast fermentation

After enzymes and microbes hydrolysis, all samples were fermented using yeast *S. cerevisiae* for six days. Sugars are consumed by yeasts for growth and energy generation. Sterilization stopped the enzymatic and microbes activities. Therefore, there is no hydrolysis of lignocelluloses to sugar. The yeast *S. cerevisiae* lacked lignocelluloses hydrolytic enzymes. However, the yeast had disaccharides hydrolytic enzymes, galactosidases or maltases. Therefore, there is no additional sugar concentration during fermentation, except hydrolysis of disaccharides into monosaccharides. This condition was directed to the decreasing sugar concentrations following increasing fermentation days (Table 2). Sugar concentrations of two fermentation days were 2.42-7.28 mg/mL and higher than sugar concentrations of four (1.88-6.66 mg/mL) and six (1.42-5.71 mg/mL) fermentation days ($p < 0.05$). Decreasing sugar concentrations following increasing fermentation days were due to the metabolism of the yeasts. The yeasts used sugars for growth and generating energy.

Sugar concentrations of the rice and corn husks after multienzymes hydrolysis and yeast fermentation were 1.42-4.63 mg/mL. There were lower than sugar concentrations of the rice and corn husks after EM4 (3.82-7.28 mg/mL) and cellulases (4.49-6.66 mg/mL) hydrolysis and yeast fermentation ($p < 0.05$). The low sugar concentrations of the rice and corn husks after multienzymes hydrolysis and fermentation due to the higher sugar consumption by yeast *S. cerevisiae*. Higher sugar concentration at the beginning of fermentation would stimulate yeast *S. cerevisiae* to uptake sugars into the yeast cells. Sugar concentrations after hydrolysis and fermentation of rice and corn husks were 1.71-7.28 mg/mL and 1.42-6.95 mg/mL. These values were not different significantly ($p \geq 0.05$). This showed that sugar concentrations at beginning fermentation were similar, therefore yeast *S. cerevisiae* activities on sugar of rice and corn husks were similar too.

Bioethanol productions after hydrolysis variation and fermentation

According to Lin et al. (2012) in aerobic condition, yeast cells were initially grown in fermentative metabolism to produce ethanol, and then yeast cells were consumed ethanol in respiratory metabolism. The anaerobic condition was applied to our research, therefore there is no respiratory metabolism, instead of fermentative metabolism. Ethanol was the 'off product' when yeast consumed sugar for generating energy in fermentative metabolism. Ethanol was expelled from the yeast cell after produce; therefore, we could ease to isolate it. However, yeast *S. cerevisiae* could consume ethanol when glucose concentrations were very low (Raamsdonk et al. 2001).

Table 1 Sugar concentration (mg/mL) of rice and corn husks after enzymes and microbes hydrolysis.

Hydrolysis treatment		Rice husk	Corn husk
Before hydrolysis	EM4	3.36	3.24
	Multienzymes	4.13	3.65
	Cellulases	4.25	3.40
After hydrolysis	EM4	5.03a	5.45a
	Multienzymes	6.81b	6.54b
	Cellulases	5.34a	5.65a

Note: a,b: the numbers following different notations are different significantly at same columns ($p<0.05$).

Table 2. Sugar concentrations (mg/mL) of rice and corn husks after yeasts fermentation.

Agricultural waste types	Hydrolysis treatments	Yeast conc.	Fermentation days		
			2-days	4-days	6-days
Rice husk	EM4	1%	7.28a,x	4.23a,y	4.07a,y
		2%	6.83a,x	5.14a,y	3.87a,y
		3%	7.25a,x	4.80a,y	4.12a,y
	Multienzymes	1%	3.76b,x	1.88b,y	1.42b,y
		2%	2.42b,x	2.31b,y	1.71b,y
		3%	2.70b,x	2.29b,y	1.76b,y
	Cellulases	1%	6.64a,x	6.66a,y	5.45a,y
		2%	6.01a,x	5.45a,y	5.10a,y
		3%	6.19a,x	5.34a,y	5.71a,y
Corn husk	EM4	1%	6.87a,x	4.23a,y	3.82a,y
		2%	6.95a,x	4.83a,y	3.87a,y
		3%	6.90a,x	4.63a,y	4.12a,y
	Multienzymes	1%	4.63b,x	2.00b,y	1.42b,y
		2%	3.62b,x	2.31b,y	1.71b,y
		3%	3.33b,x	2.29b,y	1.76b,y
	Cellulases	1%	6.16a,x	5.71a,y	5.16a,y
		2%	5.64a,x	5.45a,y	4.49a,y
		3%	5.79a,x	5.26a,y	4.69a,y

Note: a,b: the numbers following different notations were different significantly at same columns ($p<0.05$). x,y: the numbers following different notations were different significantly at same rows ($p<0.05$).

Table 3 Bioethanol concentration (mg/mL) of rice and corn husks after hydrolysis and yeast fermentation.

Agricultural waste types	Hydrolysis treatments	Yeast conc.	Fermentation days		
			2-days	4-days	6-days
Rice husk	EM4	1%	3.35a	3.17a	4.22a
		2%	3.49a	3.19a	3.08a
		3%	3.54a	3.28a	3.14a
	Multienzymes	1%	3.17b	3.33b	3.45b
		2%	3.31b	3.54b	3.17b
		3%	3.31b	3.71b	3.24b
	Cellulases	1%	3.43a	3.24a	3.38a
		2%	3.14a	3.10a	3.19a
		3%	3.21a	3.26a	3.52a
Corn husk	EM4	1%	3.28a	3.42a	3.21a
		2%	3.40a	3.49a	3.28a
		3%	3.39a	3.40a	3.24a
	Multienzymes	1%	3.14b	3.28b	3.26b
		2%	3.47b	3.59b	3.40b
		3%	3.28b	3.54b	3.42b
	Cellulases	1%	3.24a	3.24a	3.28a
		2%	3.33a	3.24a	3.24a
		3%	3.28a	3.26a	3.19a

Note: a,b: the numbers following different notations are different significantly at same columns ($p<0.05$).

Bioethanol productions after hydrolysis and fermentation of two (3.14-3.54 mg/mL), four (3.10-3.71 mg/mL) and six (3.08-4.22 mg/mL) days were similar and not different significantly ($p \geq 0.05$). It seems that yeast *S. cerevisiae* fermented sugar into ethanol quickly and gave maximum bioethanol productions. Then yeasts slow their fermentation and consumed ethanol and convert into esters (Peddie 1990), therefore decreasing but not significantly bioethanol production. These esters were responsible for wine flavor.

Bioethanol production after hydrolysis and fermentation of rice husks were 3.08-4.22 mg/mL and did not differ significantly with after hydrolysis and fermentation of corn husks (3.14-3.59 mg/mL; $p \geq 0.05$). This showed bioethanol production was not different at rice and corn husks. These showed that similar sugar concentrations at beginning fermentation (see Table 2) would lead similar bioethanol productions of rice and corn husks.

Bioethanol production after multienzymes hydrolysis of rice husk was 3.17-3.54 mg/mL. However, the highest bioethanol production of rice husk was 4.22 mg/L and obtained after EM4 hydrolysis. These bioethanol productions were lower than bioethanol obtained by Hashem et al. (2013) and Rabah et al. (2014). They were obtained 6.31 mg/L and 5.8 mg/L bioethanol from rice straw and rice husk respectively. This showed bioethanol productions mainly depend on the sugar concentrations. Sugar concentration in our research was lower than Hashem et al. and Rabah et al., therefore bioethanol production was lower too.

Bioethanol production after multienzymes hydrolysis of corn husk was 3.59 mg/mL and lower than 10.08 mL/L that obtained by Itelima et al. (2013) who used co-fermentation *Aspergillus niger* and .*S cerevisiae* on corn cobs. However, when corn cobs were hydrolyzed by *Aspergillus niger* and then fermented by *Saccharomyces cerevisiae*, the ethanol production was only 0.64 mg/mL (Zakpaa et al. 2009). This showed hydrolysis by *A. niger* at the same time with and fermentation by yeast *S. cerevisiae* would give continuous sugar to yeast and then yeast ferment sugar to bioethanol. However, this was not evident when hydrolysis by *A. niger* was at a different time with and fermentation by yeast *S. cerevisiae*.

We could also compare sugar consumption and bioethanol production. We did assume that when sugars consumptions were high, then bioethanol productions were high too. This assumption was true when we compared Table 2 and Table 3. In Table 2, yeast at multienzymes hydrolysis treatment consumed sugars higher than at EM4 and cellulases hydrolysis treatments. In Table 3, yeast at multienzymes hydrolysis treatment produced bioethanol higher than at EM4 and cellulases hydrolysis treatments. If we compared sugar consumption and bioethanol production, we then saw that sugar consumption was two times higher that bioethanol production. This comparison corresponded with the theoretical calculating of producing bioethanol from sugar.

In conclusion, sugar concentrations of rice and corn husks after multienzymes hydrolysis treatment was higher than EM4 and cellulases hydrolysis treatments were 6.54-6.81 mg/mL. Therefore, bioethanol productions of rice and corn husks after multienzymes hydrolysis and yeasts after 2 days fermentation were 3.17-3.54 mg/mL.

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