Bioethanol Production By Utilizing Cassava Peels Waste Through Enzymatic And Microbiological Hydrolysis

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Abstract. Cassava peels waste contains, cellulose which is quite high at 43.626%, this is a potential candidate as a raw for bioethanol production. The purpose of this study was to determine the performance of the enzymatic hydrolysis, microbiological (Effective microbe) and fermentation in cassava peel waste is known from the results of quantitative measurement of multiple ethanol parameters (DNS Test, pH, ethanol concentration). This research was carried out in stages, the first stage is hydrolysis with completely randomized design with single factor variation of the catalyst, consisting of three levels ie cellulase enzymes, multienzyme and effective microbial EM4. The second stage is fermentation with factorial randomized block design, consisting of three groups of variations of catalyst, and has two factors: variations of fermipan levels 1, 2, 3% and the duration of fermentation, 2,4,6 days. The parameters in the test is a reducing sugar, pH and concentration of ethanol. The results showed that variation of hydrolysis treatment, fermentation time, and fermipan levels has real effect on the fermentation process. On average the highest ethanol content obtained from the treatment with multienzyme addition, with the addition of 2% fermipan levels and on the 2nd day of fermentation that is equal to 3.76%.

1. Introduction

One of solutions for the energy crisis is the utilization of bioethanol as alternative energy. Bioethanol can improve combustion efficiency because it contains 35% oxygen beside that, bioethanol is eco-friendly fuel because not produce carbon gas pollution[1]. In this era, bioethanol produced from staple food raw materials such as molasses from sugar cane, corn, and cassava tubers. Waste from industrial agriculture are potentially used as raw material for bioethanol because of its availability is still abundant, the cost is relatively cheap and could reduce competition with staple food[2]. Cassava production in Indonesia reach 22 million tons in 2015. From the cassava processing, cassava peel waste which generated is high and unused optimally. Utilization of cassava peels is only for animal feed or simply disposed off as trash. Lignocellulose material of cassava peels can be used as raw material for bioethanol[3]. Cassava peels waste contains 43.626% cellulose, hemicellulose and lignin 10.384% 7.646%[1]. But in Indonesia, the production of bioethanol is constrained by the high cost of production budget both in industrial scale and cottage industry scale, it causing the conversion of conventional energy to renewable energy can not be applied optimally in Indonesia.

There is two main stages of bioethanol production, first is hydrolysis process that break down cellulose into simple sugars with the help of acids or enzyme. Second is fermentation process that will convert sugar into alcohol through anaerobic respiration by microbes[4]. In this study, enzymes is used as catalysts for the break down reaction of lignocellulose into simple sugars . Enzymatic hydrolysis is relatively more efficient in producing sugar because it can minimize the formation of toxic compounds as a result of acid hydrolysis process. However, the disadvantage of these methods is it takes much longer time than acid hydrolysis process and high cost of purified enzyme material[5]. To overcome the high cost of using purified enzyme, in this study multienzyme and EM4 (effective microbe) is used,

to obtain multienzyme and EM4 performance the purified enzyme (cellulase) is used as positive comparison.

Multienzyme and effective microbial EM4 commercially known as an additional supplements for livestock. Addition of multienzyme and effective microbial EM4 on animal feed, can improve the digestibility especially for foods that contain high fiber cellulose so the nutrition could be optimally absorbed[6-7]. Based on the composition of cassava peel as lignocellulosic biomass, multienzyme has appropriate enzyme content, which can be used on enzymatic hydrolysis process such as Galactosidase, xylanase, Galaktomananase, β-glucosidase, amylase. Galactosidase enzyme will catalyze the hydrolysis of bonds α-D-galaktosidik contained in the hemicellulose galakto- oligosaccharides[8]. EM4 (Effective Microorganisms) consists of three main components, *Rhodopseumonas palustris*, *Lactobacillus casei*, and *Saccharomyces cerevisiae*. *Rhodopseumonas palustris* prove has capability to degrading aromatic compounds such as coumaroyl amide, feruoyl amide, coumaric acid and benzoic acid contained in lignin through benzoyl-CoA pathway. The degrading of lignocellulose matter will produce aromatic compunds that will inhibit hydrolysis and fermentation process. *Rhodopseumonas palustris* could degrade aromatic compounds anaerobically without damaging or consuming glucose and xylose which is needed during the fermentation process[9]. Both materials are affordable and can be obtained easily by the general public. The main objective of this study was to evaluate the usability of agricultural waste, especially cassava peel waste for bioethanol raw material, using various treatment on hidrolysis process such as: multienzyme, effective microbes in the EM4, and cellulase enzymes.

2. Experimental

2.1 Material preparation
Cassava peels are dried in the sun for 3 days. It is dried to remove moisture and to obtain dry biomass. To simplify the process of hydrolysis, cassava peel waste crushed using a blender. In the first step is hydrolysis with 3 different treatments, and 3 replications. To make 1 sample a ratio of 1: 4 is used , 25 grams of cassava peel waste were given 100 mL of distilled water.

2.2 Hydrolisis
Hydrolysis processes aimed to convert cellulose into glucose[5]. To catalyze the reaction, enzymes and effective microbes at EM4 is used. Each liquid sample was treated differently by adding a cellulase enzyme (Ka), multienzyme (Kb), and effective microbial EM4 (Kc). Control treatment (K0) without the addition of enzymes or other substances. To make Ka samples, 2.5 mg of cellulase enzymes were mixed into the liquid sample. Kb sample was prepared by adding 5 g multienzyme into liquid samples, two types of samples are conditioned in the range of 5- pH 5.5 using hydrochloric acid, or HCl 0.1N. Meanwhile, to make Kc sample, liquid samples were given 5 ml EM4 by conditioning the pH of 6-6.5. For a sample K0 pH is conditioned in the range of 5-5.5. Hydrolysis process is carried out at room temperature. Conditioning of pH and temperature aimed to optimize the performance of the enzyme. Hydrolysis process is carried out for 24 hours. After the hydrolysis process is finished, next step is the samples were sterilized by autoclaving at 121°C temperature for 15 minutes. Sterilization aims to stop the activities of enzymes[5]. DNS method is used to determine reducing sugar which is after the hydrolysis process.

2.3 Fermentation and distillation
The second stage is fermentation, samples which has been hydrolyzed then given inoculant in the form of fermipan that contain yeast *Saccharomyces cerevisiae*. The fermentation process aims to transform sugars into ethanol through a process of anaerobic respiration by microbiological agent[4]. Fermipan will be varied to 3 levels content ie 1%, 2% and 3% of the sample weight. The fermentation process was conducted for 6 days in room temperature conditions. Measurement of pH and reducing sugar measurements performed on day 2, day 4 and day 6 during fermentation. Ethanol content analysis performed on day 2, day 4 and day 6 during fermentation. During the fermentation process, the sample bottle is connected by a rubber hose and the hose is inserted into another bottle filled with water to minimize direct contact with air. After fermentation is complete sample were filtered and then distilled. Solution is poured into the distillation flask, heater temperature is maintained at a temperature of 70°C. The distillation process is done for 15-30 minutes. Distillate that has been obtained then measured the density of ethanol[5].

2.4. Analysis of reducing sugar content
Reducing sugar measurements using DNS method refers to the theory of Miller (1959)[10]. DNS reagent 1% was prepared by dissolving 5 g DNS, 1 g of phenol, 0.25 g Na-metabisulfite, and 5 g NaOH in 300 mL of distilled water. The solution is then diluted to 500 mL using distilled water and then filtered after 24 hours. In addition to DNS solution, rochelle salt (KNA-tartrate) 40% is produced with dissolving 40 g KNA-tartrate into 100 mL of distilled water.

A total of 1 mL sample were taken and put into a test tube and was given 2 mL of 1% DNS reagent, the mixture is then alloyed using vortex. Furthermore, test tubes containing samples and DNS 1% put in boiling water for 5 minutes to allow the reduction process of reducing sugar DNS to run faster and optimized, further 1 mL KNA-tartrate 40% was given, shortly before the solution start to being cool. KNA-tartrate 40% together with a solution of Na-metabisulfite is used to remove dissolved oxygen glucose so it can protect from damage caused by dissolved oxygen. Absorbance measurements using UV-VIS spectrophotometer with wavelength 540 nm.

2.5. Analysis of ethanol content with density analysis
Bioethanol content analysis using pycnometer density method at temperature of 20℃. To determine the concentration of ethanol, picnometer volume and density of ethanol is calculated using the following formula:

1. Calculate the volume of pycnometer

$$c : \frac{b - a}{\rho \text{ water}} \tag{1}$$

2. Calculating Ethanol Density

$$\text{Density} : \frac{d-a}{c} \tag{2}$$

Information
a: weight of the empty pycnometer
b: weight of pycnometer containing distilled water (g)
c: Volume Pycnometer (mL)
d: Weight Pycnometer content of solution (g)
ρ water: 0.998203 g / mL (density of water at a temperature of 20℃)

From the density number, ethanol percentage could determined by compare it with standard density of ethanol in AOAC table (2005).

3. Result and Discussion

3.1. Reducing sugar content on enzymatic hydrolysis

Table 1. Reducing Sugar Content that Resulted from Enzymatic Hydrolisis g/100 ml

Treatment	Reducing Sugar Content (g/100 ml)
Cellulose	8.0667
EM4	8.7900
Multienzyme	11.0267
Control	4.700

From Table 1 it is known that the highest levels of reducing sugars produced by the multienzyme treatment, while EM4 treatment can produce the highest reduction sugar after multienzyme i.e. 8.7900 g/L. Between reducing sugar produced by EM4 and reducing sugar produced by cellulases do not show much difference. Cellulase can produce high levels of reducing sugars of 8.0667 g/L, while control produces a low reducing sugar ie 4.770 g/L. Reducing sugar greatly affect the levels of ethanol formed, the higher the sugar content reduction of the ethanol content will also be higher.

Multienzyme has the highest reducing sugar because multienzyme consists of various types of complex enzymes that are able to do the optimumconversion process from lignocellulose into sugar,

other than that there is also an amylase enzyme that can hydrolyze the starch content cassava in the skin so the result is more optimal. Cellulase enzymes have lower glucose levels when compared to multienzyme ie 8.0667 g/L, it is because of cellulase enzymes only can hydrolyze cellulose content found on the peels of cassava. Cellulase is a complex mixture of several enzymes that work together synergistically to attack parts of the distinctive cellulose fibers[11].

EM4 has a reducing sugar which is lower than the multienzyme that is equal to 8.7900 g/L. This is because EM4 a livestock supplemen, containing effective microbes in contrast with the multienzyme consisting of different types of enzymes. When added into sample, the microbes originating from EM4 takes time for the adaptation phase until it can produce the enzyme in the exponential phase. Can be interpreted within a period of 24 hours in the process of hydrolysis, microbial EM4 not work optimally in producing sugar reduction. In the control treatment reducing sugar produced the lowest compared to the three other treatments, this indicates multienzyme, EM4 or cellulase has an influence in increasing the reducing sugar hydrolysis process on cassava peel waste. In the control treatment, the hydrolysis process still produces reducing sugar because in cassava peel contained the endogenous enzymes. The endogenous enzymes such as β-glucosidase enzyme, known as linamarase. Linamarase will hydrolyze glucoside compound linamarin and lotaustralin cyanogenik form spontaneously when the damage occurred mechanically in a network or as a result of microbial activity.

3.2. Reducing sugar recidue on during fermentation process

In producing ethanol at anaerobic condition, *Saccharomyces cerevisiae* will consume glucose as the main carbon source[12]. Based on Figure 1, reducing sugars is drop constantly fom the day of two fermentation until day of six fermentation. According to the several result on bioethanol research, reduction of reducing sugar during fermentation process, because the sugars in the media used by *Saccharomyces cerevisiae* as a carbon source for ethanol synthesis[13]. Reducing sugar which is decreased may indicate ethanol production, but according to Bulawayo (1996) in Meldha (2012)[14] revealed that the decrease of reducing sugar that is not accompanied with elevated levels of bioethanol, which is produced due to the sugar consumed is used only for the growth of yeast, so not all sugars can be converted into ethanol.
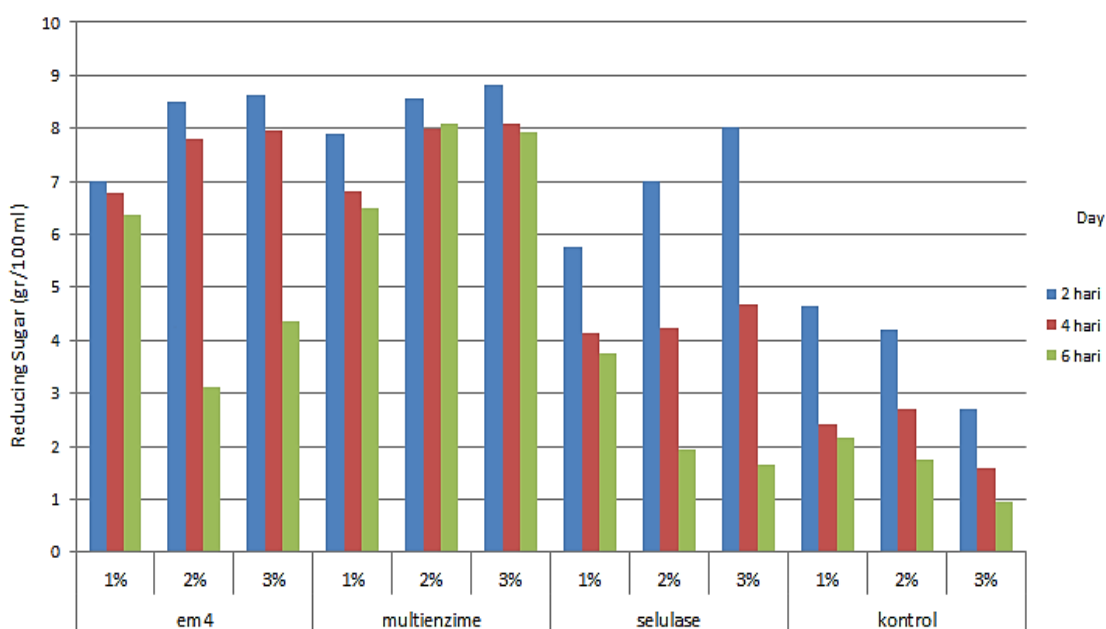

Figure 1. Reducing Sugar Fluctuations during Fermentation Process

Based on the variation of fermipan content level shows that the average usage levels of reducing sugars in fermipan 2% had the highest amount ie 1,6566 g/L, while the fermipan 3% on average ie 1,6733 g/L, and the lowest one is from fermipan 1% i.e. 0.8170 g/L (Table 2). However fermipan content level comparable to the number of cells contained in the substrate, so that the higher the number of cells, the use of a reducing sugar will also high, because the yeast have a rapid growth phase[15].

When the cell density reaches a maximum then it will trigger a stationary phase, which means the number of living cells is equal to the number of died cells, then it will followed by death phase, in this phase the amount of yeast that dies will increase until it will eventually happen death cell in bulk[12]. In contrast the number of cells that are too low will result in the reduction of sugar consumption by running slowly making yeast fermentation process is also slow.

Table 2. The Average of Reducing Sugar Utilized by Yeast

	Fermipan Level		
	1%	2%	3%
	0.2304	0.7171	0.6530
	0.4192	4.6561	3.6048
	1.0927	0.5808	0.7295
	0.3193	-0.0953	0.1556
	1.6097	2.7553	3.3472
	0.3915	2.3102	3.0287
	2.2375	1.5211	1.1104
	0.2359	0.9411	0.6237
Average	0.8170	1.6733	1.6566

3.3 Ethanol yield content

Table 3. Ethanol Yield Percentage (%)

Day of Fermentation	EM4			Multienzyme			cellulose			Control		
	1%	2%	3%	1%	2%	3%	1%	2%	3%	1%	2%	3%
2 day	2.50	3.15	3.27	3.14	3.76	3.66	2.38	2.63	3.17	1.72	2.00	1.70
4 day	2.46	2.89	3.30	2.63	3.24	3.42	2.43	2.45	2.61	2.38	2.57	2.34
6 day	2.48	2.22	2.68	2.73	3.21	3.45	2.09	2.22	2.50	1.81	2.23	1.90

As a result of primary metabolism, ethanol is produced along with the growth of cells. In fermipan content level variation, the use of fermipan 2% or 3% could increase ethanol production rather than the use of fermipan 1%. Tthehe tabtable le le 3 show that ethanol yield percentage with 2% and 3% fermipan is higher rather than fermipan 1%.This point out that the rise of fermipan content level on the substrate it will followed by the increasing of ethanol rate production by yeast. According to the research by Zely (2014)[16], that the number of yeast cells affect the content of ethanol formed. Increasing the number of yeast on the substrate will accelerate the fermentation process, especially if the sugar content in the substrate is high. Yeast activities in sugar fermentation will increase so that the ethanol content could increased.

In the three variation of the hydrolysis treatment the average value of the highest ethanol content generated on the 2nd day of fermentation, after fermentation time passing day of 2, the ethanol concentration tends to decrease as shown in Table 3. It is directly proportional to the decrease of reducing sugar levels at the time fermentation, the possibility is reducing sugar which is used by the yeast is not converted to ethanol. The decrease in reducing sugar levels are not accompanied by the increase of ethanol content because the sugar used by yeast for cell growth or survival without can be converted into ethanol. On the 2nd day, ethanol reach the highest percentagr because yeast cells are in the exponential phase, in this phase the yeast cells will produce ethanol and growth rapidly. After the 2nd day of fermentation, the increasing of cells density number because of rapid growth trigger cells to enter stationary phase cells so that the cells no longer produce ethanol makes the optimum concentration of ethanol drop slowly. The level of ethanol production is influenced by the activity of the cells, whereas cell activity is influenced by the type of media, and environmental conditions (eg temperature and pH).

The different result show from control treatment, ethanol yield percentage increase in the day of four fermentation and then drop at sixth day it is because the slow consumption of reducing sugar by yeast or different type of sugar which is produce from hydrolysis process with control treatment. Cassava peels wastes contain cyanogenic glucose named linamarin and lotaustralin that will be degraded by endogenous enzyme linamarase (β-glucosidase enzyme) .

Based on the average value of ethanol content in Table 3, the highest ethanol content is generated by multienzyme ie 3.76% using 2% fermipan. In EM4, the highest ethanol content produced from by fermipan 3% ie 3.27% and the cellulase highest levels of ethanol produced at the levels of use fermipan 3% at 3.17%, control treatment could produce 2,57% ethanol at fourth day of fermentation using fermipan 2%. These results are appropriate with reducing sugar obtained from hydrolysis process presented in Table 7, the highest reducing sugar produced by multienzyme amounted to 11.0267 g/L. The highest reducing sugar content will produces high percentage of ethanol yield.

4. Conclusion

The highest reducing sugar which produce from enzymatic hydrolisis is deliver from Multienzyme treatment (11,0267 g/100ml) it proves that multienzyme has a better performance compare to purified enzyme cellulase. The highest of ethanol yield precentage that is produce from fermentation process is 3,76 % with the addition of 2% fermipan level; on the second day fermentation using multienzyme treatment.

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