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Potential Cassava Peel Waste (*Manihot esculenta* Crantz) in The Production of Bioethanol by Enzymatic Hydrolysis and Fermentation Using *Zymomonas mobilis* Bacteria

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ABSTRACT

Cassava is one of the most widely produced agricultural products in Indonesia with cassava peel waste of 20%. Cassava peel contains carbohydrates and stores a high cellulose content so that it has a potential to be a bioethanol. This study aims to utilize cassava peel waste for bioethanol production with an alkaline pretreatment process, cellulase enzyme hydrolysis and fermentation by using *Zymomonas mobilis* bacteria. Alkaline pretreatment with 14% NaOH is used to hydrolyze lignocellulose. The hydrolysis optimization process enzymatically applies the Surface Response Method (RSM) to determine the optimum conditions at hydrolysis pH in the range of 2-10 and hydrolysis temperature in the range of 30-70 °C by analyzing glucose levels using the Dinitrosalicylic Acid (DNS) method and UV-vis spectrophotometry instruments. Surface Response Method (RSM) is likewise implemented to decide the greatest conditions of the fermentation process. The pH measurement ranges 2-10, and fermentation time takes 6 to 168 hours. Based on the results of research, it results a lignin content of 6.68% (b/b), cellulose content of 58.4% (b/b), and hemicellulose content of 27.3% (b/b). The optimum conditions of the hydrolysis process obtained an optimum glucose level of 9.22mg/mL at pH 2 and a hydrolysis temperature of 50°C. The optimum conditions of the fermentation process use *Zymomonas mobilis* at pH 6 while fermentation time takes 168 hours analyzed using a refractometer produced a bioethanol content of 37.75% (v/v) and a gas chromatography produced a bioethanol content of 54.94% (v/v).

Keywords: alkaline-pretreatment; bioethanol; hydrolysis; surface-response-method (RSM); cassava-peel-waste; *Zymomonas-mobilis*

INTRODUCTION

Cassava or *Manihot esculenta Crantz* is a plant with a high source of starch. Most people process cassava into a cassava flour or as a substitute for a staple food. Indonesia is the fourth largest cassava producer in the world which is followed by Nigeria 57 million tons, Thailand 30 million tons, Brazil 23 million tons, and Indonesia 16 to 20 million tons. Cassava production in 2020 was 16.35 million tons and in 2021, it increased to 18.48 million tons [1]. Cassava is a plant that has a high source of starch, the general processes cassava to produce tapioca flour that is used as a substitute for staple food [2]. Cassava production produces 20% cassava peel waste which has not been widely utilized yet until nowadays. One of those is used as processed food, namely cassava peel chips, as an additional ingredient for an animal feed, and the rest is simply thrown away as a waste [3].

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Based on laboratory analysis, cassava bark contains 56.82% cellulose, 21.72% lignin, 8.5% glucose. Hence, it will be very potential to utilize as the renewable alternative energy sources [4]. Another advantage is the availability of large cassava peel as the raw materials, considering that Indonesia is one of the largest cassava producers in the agricultural sector. One promising alternative energy source in the future is bioethanol. One of the bioethanol raw materials can be produced from the rich organic waste that is no longer used. The utilization of organic waste containing carbohydrates and storing large enough cellulose such as cassava peel that has the potential to be raw materials for making bioethanol [5].

Bioethanol is an alternative fuel made from plants to reduce CO_2 emissions up to 18%. There are 3 groups of bioethanol source plants, including (a) starchy plants (such as cassava, oil palm, tengkawang, coconut, cottonwood, rambutan, soursop, *Malapari*, and *Nyamplung*) (b) sweet plants (such as molasses, palm sap, sugarcane sap and sweet sumac sap) and (c) cellulose fibrous plants (such as sorghum stems, banana stems, straw, wood residues and herbs). This material containing starch, glucose and cellulose fiber can be used as an alternative fuel for bioethanol production. These materials are always available in large quantities in Indonesia and easy to grow even at the critical land, moreover people are also familiar with this plant, so it has the opportunity to be used as an alternative energy production material [6]. Bioethanol has great development opportunities in Indonesia because bioethanol raw materials are easily grabbed, for example the fermentation result of biomass containing carbohydrates that is processed by microorganisms [7]. However, a bioethanol as fuel is still underutilized because it costs more than the natural fuels, because the raw materials are mostly made from the expensive cultivated plants which compete with the food availability. Meanwhile, the manufacture requires the other economical raw materials that do not compete with food, for example, cassava peel waste [8].

The use of cassava as an opportunity for the renewable electricity supply by a term of bioethanol can be processed through pretreatment (delignification), hydrolysis, fermentation, and distillation (purification) processes. A pretreatment process is required to break down the lignin structure so that cellulose and hemicellulose can be further processed to produce bioethanol. This process needs NaOH because it can degrade lignin that encloses the cellulose and hemicellulose that could rupture the lignin structure. Pretreatment cellulose will be easily hydrolyzed by acid or enzymatically and produce maximum glucose. This pretreatment process is intended to increase the ability of cellulose surface area so that capable of converting the cellulose into a glucose (fermented sugar) [9].

The process of converting the cellulose into the glucose can be taken chemically (acid or base) and enzymatically. The process of acid hydrolysis is adding sulfuric and hydrochloric acid while in the enzymatic hydrolysis process can use cellulase enzymes or other enzymes that can break down cellulose into its monomers. Enzymatic hydrolysis has several advantages over acid hydrolysis. Enzymatic hydrolysis does not occur by the degradation of hydrolyzed sugars. However, it occurs at the low temperatures that gives high glucose levels. The utilization of enzymes as hydrolysers depends on the priority of the substrate. Cellulose can be hydrolyzed enzymatically using cellulase. The hydrolysis process will produce a glucose solution that will be fermented using microorganisms to produce bioethanol [10].

The fermentation process aims to convert sugar into bioethanol by including the microorganisms, while the last stage is the purification stage carried out to obtain higher levels of bioethanol from fermentation results [11]. The fermentation process has variations of pH and fermentation time designed with Response Surface Methodology (RSM) to develop, improve, and optimize the process of determining optimal conditions for bioethanol levels. The application of the RSM method is very important, especially in the field of biotechnology to be developed and influence of the independent variable response to the fermentation process.

The advantage of RSM method is only requiring few of experimental data and it also does not take long terms [12].

The fermentation process is carried out with microorganisms including yeast (*Saccharomyces cerevisiae*). However, *Saccharomyces cerevisiae* has long been known as a microorganism commonly used in producing a bioethanol. Therefore, it is necessary to find other Gram-negative bacteria that also have a potency to be an alternative of bioethanol production, namely *Zymomonas mobilis* bacteria. *Zymomonas mobilis* bacteria is facultative anaerobic bacteria. The use of *Zymomonas mobilis* bacteria for the bioethanol manufacturing industry has several advantages including the ability to grow anaerobically, higher production yields, and more specific fermentation capabilities compared to yeast.

Some studies using *Zymomonas mobilis* bacteria are utilizing corn cobs with acid hydrolysis to produce bioethanol levels of 20% at pH 5 with an optimum time of 96 hours [13], and utilizing rice straw with acid hydrolysis to produce bioethanol levels of 33% which has 5 days fermentation [14]. Research on the use of cassava peel as raw material by enzymatic hydrolysis method using *Saccharomyces cerevisiae* produced 10% bioethanol with 12 days fermentation [15]. This proves that the use of cassava peel waste as bioethanol is promising. The extreme amount of cassava peel as biomass waste produced by agricultural activities has risen the new efforts to utilize the biomass waste. Based on the description above, the research was conducted by utilizing cassava peel waste through an alkaline pretreatment process and enzymatic hydrolysis using commercial cellulase enzymes to produce optimum glucose levels. The fermentation was using *Zymomonas mobilis* bacteria to produce optimum bioethanol levels with the Response Surface Methodology (RSM) method.

EXPERIMENT

Cassava peel is obtained from cassava chip sellers, Sudiang, Makassar, South Sulawesi, Indonesia. This sample is cassava biomass waste for food processing or animal feed. The samples have been thoroughly washed to remove dirt, cut into small pieces, heat under the sun, and mash using a mortar tool at the Biochemistry Laboratory, Hasanuddin University. Samples in 70 mesh size were dried at ±105^{o}C for 2 hours.

Chemicals and instrumentation

The chemicals used in this study were cassava peel waste, aquades, cotton, filter paper, aluminum foil, NaOH p.a, H_2SO_4 p.a, glucose p.a, NaCl p.a, yeast extract, peptone p.a, $(NH_4)_2SO_4$ p.a, pH paper, ethanol, KH_2PO_4 p.a, $MgSO_4.7H_2O$ p.a, Rochelle salt $KNaC_4H_4O_6.4H_2O$ dinitrosalicylic acid (DNS), $K_2Cr_2O_7$ p.a, $CaCl_2$ p.a, NH_4Cl p.a, $Na_3C_6H_5O_7$ p.a, NH_3 p.a, $C_6H_8O_7$ p.a, $Na_3C_6H_5O_7$ p.a, commercial cellulase enzymes and *Zymomonas mobilis* bacteria.

The equipment used in this study was a 70 mesh sieve, furnace, vortex, a series of reflux devices, an oven (Memert), an analytical balance (Ohauss), a centrifugation (Hermle Z 366 K), a desiccator, a series of distillation device, an incubator (Memmeth 40050-IP 20), a UV-Vis Spectrophotometer (Hitachi UV-25000), a porcelain dish, a petri dish, a water bath shaker, an autoclave (NAPCO 8000 DES), a micro pipette (Gilson), Shimadzu gas chromatography 2010, Refractometers. In addition, other glassware is also commonly used in laboratories.

Procedure stages

This research consists of several stages, namely: pretreatment, hydrolysis, and fermentation.

Pretreatment

The pretreatment process was carried out by taking as much as 400 g of cassava peel powder that put into Erlenmeyer then added 4000 mL NaOH 14%. Then it incubated in a shaker at 60°C and a speed of 120 rpm for 6 hours. Meanwhile, the solution is filtered using filter paper. The filtered residue was washed with aquades until a neutral pH obtained and baking at 105°C for 6 hours. The next step is analyzing a cellulose, hemicellulose, and lignin levels by the Chesson Datta method. This pretreated cassava peel powder is then used for the enzymatic hydrolysis process.

Hemicellulose, cellulose, and lignin content analysis

The pretreatment result was analyzed for hemicellulose, cellulose and lignin levels using the Chesson Datta method. The dry sample which was weighed 2 g (a) added 150 mL of aquades and refluxed at 100°C for 1 hour. Then, it was filtered, the residue was washed with hot aquades to neutral pH. The residue was heated in an oven at 105°C and then weighed until a constant weight (b). The residue became introduced one hundred fifty mL H_2SO_4 1 N and refluxed for 1 hour at 100°C. The results were filtered and washed with aquades until it was neutral. Then, it was dried and weighed (c). The dried residue was added 10 mL of 72% H_2SO_4 and soaked at room temperature for 4 hours. It was added 150 mL H_2SO_4 1 N and refluxed for 1 hour. The residue was filtered and washed with aquades until it was neutral then heated in an oven at 105°C and the results are weighed to a constant weight (d). Then, the residue was crushed and weighed (e). The levels of cellulose, hemicellulose and lignin are calculated as follows:

$$\text{Hemicellulose levels} = \frac{b - c}{a} \times 100\%$$

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

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

a is the sample weight (g), b is the residual weight on the second weighing (g), c is the residual weight at the third weighing (g), d is the residual weight at the fourth weighing and e is the ash weight of the sample residue (g)

Moisture content analysis

The empty cups were heated in the oven for 30 minutes at105^{o}C which then cooled in a desiccator for 15 minutes and weighed (W_0). The pretreatment sample was weighed as much as 2 g put in a dish with the known weight, weighed (W_1). It then was reheated in the oven at 100 - 105^{o}C for 3 hours, cooled in a desiccator for 150-30 minutes. Furthermore, the cup and its contents weighed and heated again for ±1 hour and cooled in the desiccator while weighed again (W_2). The water content is calculated as follows:

$$\text{moisture content (\%)} = \frac{W_1 - W_2}{W_1 - W_0} \times 100\%$$

W_0 is the empty cup weight (g), W_1 is the cup weight + initial sample before heating in the oven (g) and W_2 is the cup weight + initial sample after cooling in the desiccator (g).

Enzymatic hydrolysis process

Pretreated cassava peel powder was put into 250 mL Erlenmeyer as much as 2 g. The commercial cellulase enzyme was as much as 10 mL in citrate buffer solution and ammonia

buffer at pH variation (2-10) and homogenized. It was then put into a shaker water bath and incubated at a temperature and speed variation of 105 rpm for 48 hours simultaneously that can be seen in Table 1. After stopping hydrolysis, it was separated using centrifugation at a speed of 10.000 rpm for 30 minutes. The supernatant was used for the glucose level analysis using the DNS method. Hydrolysate that has high glucose levels will be used as a substrate in the manufacture of bioethanol.

Analysis of glucose levels by Dinitro Salicylic Acid (DNS) method

A 1.5 mL of hydrolysate was put into a test tube and 1.5 mL of DNS reagent was added which then homogenized using vortexes. The tube was put in boiling water for 15 minutes and cooled at room temperature. When it is too concentrated, the sample is diluted so that being measured at a wavelength of 540 nm. The blanks measurement used distillated water. The standard curve was made using the standard glucose solution at a concentration of 0.1; 0.2; 0.3; 0.4; and 0.5 mg/mL. After obtaining measurement data, it is applied to calculate the glucose levels, as follows:

$$y = ax + b$$

where y is the average absorbance, and x is the reducing sugar concentration (mg/mL), a is the slope and b is the intercept.

Bioethanol manufacturing of fermentation media
Determination of pH and optimum time of fermentation

Erlenmeyer which contained the fermentation media is adjusted to the pH range (2-10). The fermentation time (6-168) was sterilized simultaneously based on the experimental design that has been made (Table 2). Then, it was inoculated as much as 5 mL inoculum of *Zymomonas mobilis* bacteria into each fermentation media. The Erlenmeyer was covered with cotton and aluminum foil, then fermented into a shaker water bath at 37°C and a speed of 180 rpm. The sampling was carried out under certain conditions (Table 5). Then the fermentation results were centrifuged for 20 minutes at 4°C and a speed of 10.000 rpm. The supernatant obtained from centrifugation results was then distilled and analyzed for bioethanol levels using refractometer and gas chromatography instruments.

Surface Response Method (RSM) experiment design

This study applied the surface response method to find the optimum conditions in determining the temperature and pH hydrolysis as well as the optimum conditions in determining pH and fermentation time. The predicted response is the acquisition of reduced sugar content values and bioethanol content values by RSM analysis using Minitab software. The experimental design used 13 design factors as seen in Tables 1 and 2. The experimental design of glucose level optimization with the response surface methodology (RSM) method can be seen in Table 1. ANOVA statistical results using polynomial equations:

$$Y = (X_1) + (X_2) - (X_1X_1) - (X_2X_2) - (X_1X_2)$$

Y is the content percentage (mg/mL/%), X_1 is pH and X_2 is the temperature (^oC).

Table 1. Factorial response optimization method enzymatic hydrolysis surface

Run order	Temperature (°C)	pH	Glucose levels (mg/mL)
1	50	6	
2	64	9	
3	36	9	
4	30	6	
5	50	6	
6	64	3	
7	70	6	
8	50	6	
9	50	6	
10	50	6	
11	50	10	
12	50	2	
13	36	3	

Table 2. Factorial method of surface response optimization of fermentation

Run order	Temperature (°C)	pH	Glucose levels (mg/mL)
1	50	6	
2	64	9	
3	36	9	
4	30	6	
5	50	6	
6	64	3	
7	70	6	
8	50	6	
9	50	6	
10	50	6	
11	50	10	
12	50	2	
13	36	3	

Distillation process

Centrifuged fermented hydrolysates were fed into the distillation flask. The distillation process was carried out in a temperature of 78°C to 80°C where the boiling point of ethanol is 78°C. The distillation took for 1 to 2 hours until the bioethanol did not drip anymore. The distillation result was stored in a tightly closed container. Then the bioethanol result was analyzed qualitatively using the oxidation method and potassium dichromate ($K_2Cr_2O_7$). The quantitative analysis of bioethanol used refractometers and gas chromatography.

Cassava peel bioethanol qualitative analysis

The qualitative test of bioethanol was carried out by pipette 2 mL of 2% potassium dichromate into a test tube, then added 3-5 drops of concentrated sulfuric acid and homogenized. Then it was added 1 mL of sample to be tested. The positive result of bioethanol is characterized by the change solution from orange to blue.

Quantitative analysis of bioethanol using refractometer

Measurement of bioethanol levels was carried out with a refractometer prism surface cleaned using aqueous and tissue. Then, the sample is dripped on the surface of the prism. Furthermore, it was closed and allowed the light passing by through the sample. The prism was arranged so that the light on the screen of refractometer becomes two colors with clear borders. Then the boundary mark was shifted using the regulating knob on the refractometer until it intersected the intersection point of two diagonal lines that intersect each other. It then observed and read the refractive index scale seen on the refractometer and recorded the results. After the measurement was complete, the cover was opened, and it cleaned the surface of the prism sample. It then calculated the acquisition of bioethanol levels as follows:

$$y = ax + b$$

y is the refractive index, a is the slope, x is the bioethanol content %(v/v) and b is the intercept.

Bioethanol content analysis using gas chromatography

Analysis of bioethanol levels used Gas Chromatography instruments. Meanwhile, the stages of ethanol content analysis using gas chromatography were taken 1 µl from each distillate and injected into the column through the injection site. The peak area of ethanol from

the chromatogram was calculated. The distillate ethanol content was determined by reading the chromatogram results. The calculation of bioethanol levels as follows:

$$\% \text{ Bioethanol} = \frac{\text{Sample area}}{\text{Standard area}} \text{ x Standard concentration}$$

Data analysis

The information acquired on this study had been the fermented bioethanol levels. The data obtained were analyzed using statistical analysis and Minitab 18 software.

RESULT AND DISCUSSION

Determination of cellulose, hemicellulose, and lignin Levels in cassava peel samples

The initial stage of this research was to carry out an alkaline pretreatment process with the addition of 14% NaOH to cassava peel samples. The results of lignin, cellulose, and hemicellulose analysis before and after the pretreatment process can be seen in Figure 1.

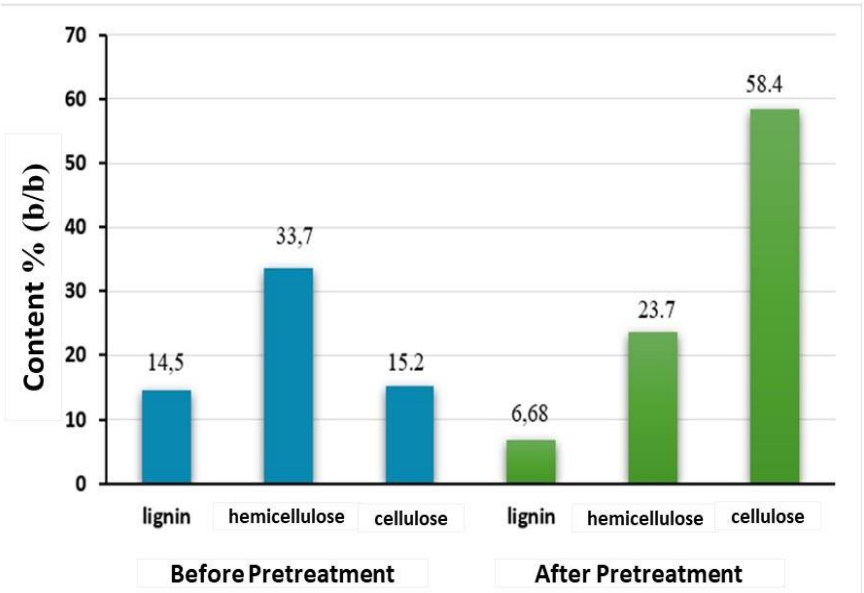


Figure 1. Lignin, hemicellulose, and cellulose levels in cassava peel samples after the pretreatment process

Based on Figure 1, the research results using the Chesson Datta method [16] on cassava peel samples obtains lignin levels of 6.68%, hemicellulose levels of 27.3%, and cellulose levels of 58.4%. The pretreatment is carried out to reduce lignin levels and increase cellulose levels contained in cassava peel. The effectiveness of lignin degradation can be seen at the figure before and after the pretreatment levels have been decreased and the cellulose levels have been increased. The pretreatment process using alkaline solution (NaOH) is able to dissolve the lignin component and some hemicellulose components so as to increase the cellulose content [16]. Based on the results obtained by comparing the results of the study showed that the data obtained had higher cellulose levels and fewer hemicellulose and lignin levels. This is caused by the use of higher NaOH concentrations in this study (14%) compared to NaOH concentrations of 10% [17] and NaOH 2% [18]. The result also reveals that the increase of concentration of NaOH means indicating the increase of hydroxyl ions in the liquid. These hydroxyl ions will break the bonds of the basic structure forming lignin, causing lignin easily

to dissolve so that the greater of NaOH concentration use shows the greater of lignin content degradation [18].

Lignin degradation begins with the breaking of the bond between lignin and the hemicellulose part. Thus, it could create the surface area of cassava peel, decrease the degree of polymerization, crystallinity, and disturbances of the lignin structure [19]. The reaction that occurs can be seen in Figure 2.

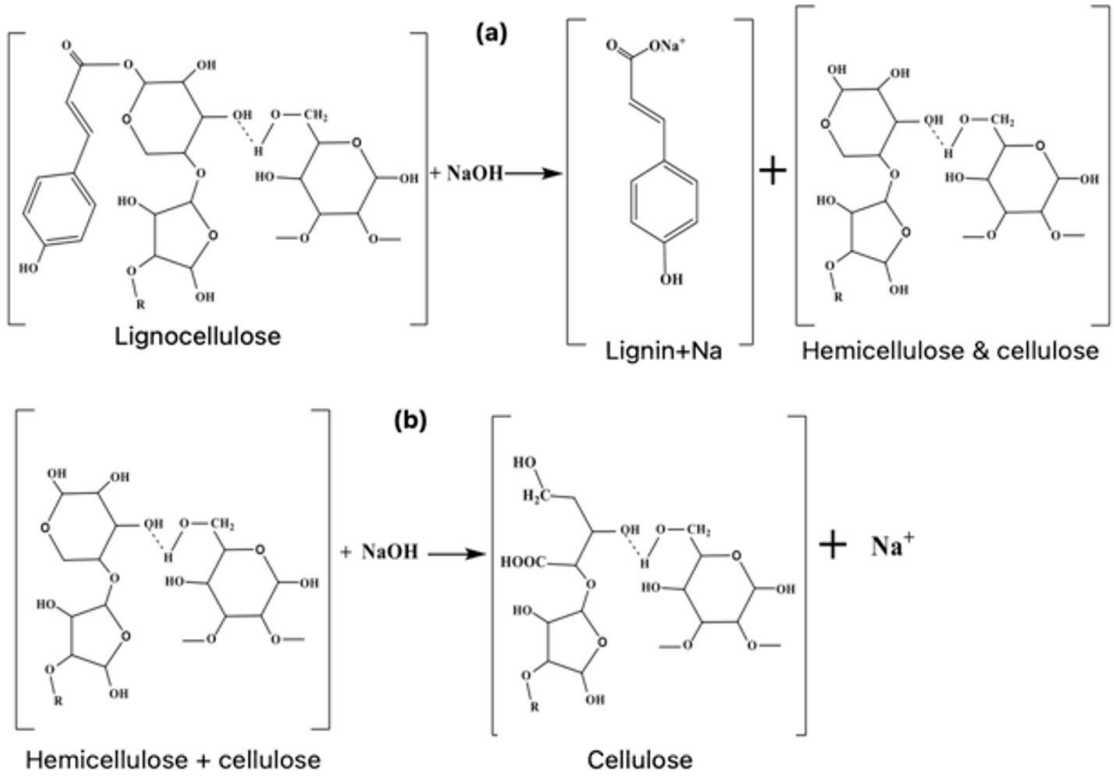


Figure 2. Lignocellulose reaction with base NaOH (a), hemicellulose and cellulose reaction in base (b).

Pretreatment process with NaOH can damage the lignin structure in crystalline and amorphous parts that separate with hemicellulose. Based on the reaction of Figure 2 shows that the dissolution of lignin at the pretreatment stage is caused by the transfer of hydrogen ions from the hydroxyl group to the hydroxyl ions NaOH. These hydroxyl ions then break the bonds from the basic structure that forms lignin, until lignin becomes easier to dissolve. The OH^- ion of NaOH will break the bonds of the lignin basic structure while the Na^+ ion will bind to lignin to form sodium phenolic. These phenolic salts are easily soluble in polar solvents. The phenolic hydroxyl group of lignin is in an ionized state and polar forming its salt so that phenolic salts are easily dissolved in water. Soluble lignin is characterized by a black color in the solution which is then referred to a black liquor. The black liquor showed the lignin layer had separated from the cellulose. This condition can increase the effectiveness of hydrolysis and fermentation [20].

Determination of water content in cassava peel powder

The water content obtained based on research that has been conducted is 5.9%. Water content analysis is important in bioethanol production. The moisture content obtained should

not exceed 10% [21]. The results obtained also support the statement that the water content sample also affects the glucose content produced. The lower water content sample would lead the higher glucose content obtained [22]. Based on the results of research conducted from bagasse, a moisture content of 7.68% is obtained [23]. from sago pulp waste that the moisture content obtained is 6.8% [24].

Optimization of glucose levels through enzymatic hydrolysis of cassava peel powder with the Response Surface Methodology (RSM)

The optimization process is carried out by experimental design and applying the surface response method (RSM), namely a centralized composite design (CCD). Data on glucose measurement results from cassava peel hydrolysis with the Response Surface Methodology (RSM) Method and analysis using Minitab18 software for analysis of variant (ANOVA) can be seen in Table 3. The results of ANOVA statistical analysis are seen in Table 3.

Table 3. Glucose measurement data from cassava peel hydrolysis

Run Order	pH Hydrolysis	Hydrolysis Temperature (°C)	Reduction (mg/mL)	FP	Actual glucose levels (mg/mL)
1	6	50	0.16	20	3.29
2	9	64	0.183	20	3.67
3	9	36	0.36	20	7.19
4	6	30	0.38	20	7.64
5	6	50	0.186	20	3.73
6	3	64	0.37	20	7.40
7	6	70	0.37	20	7.46
8	6	50	0.15	20	3.06
9	6	50	0.19	20	3.98
10	6	50	0.22	20	4.48
11	10	50	0.19	20	3.97
12	2	50	0.46	20	9.22
13	3	36	0.40	20	8.13

Table 4. Analysis of variant temperature and pH hydrolysis

Variable		Glucose Production	
		P value	Code Coefficient
Linear	temperature (°C)	0.053	-0.568
	pH	0.002	-1.220
Squares	temperature (°C) x temperature (°C)	0.001	1.905
	pH x pH	0.006	1.015
Interaction	temperature (°C) x pH	0.081	-0.698

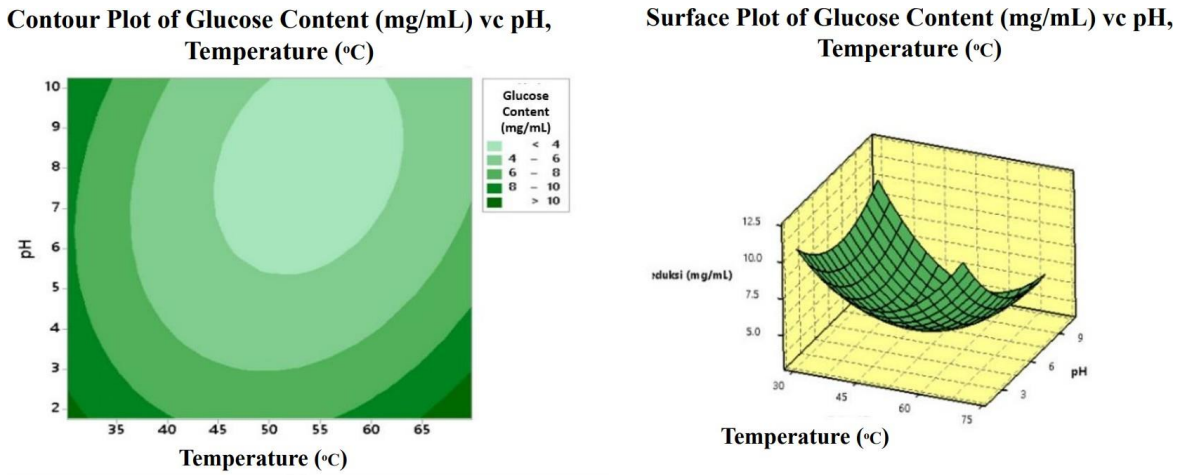
Based on the outcomes of ANOVA analysis, a correlation coefficient (R^2) of 88.51% obtains a probability value of 0.001 ($P<0.05$) which indicates that statistically the model adjusts with the design. Hence, it is considered significant in optimizing the glucose level gain. The results of the analysis shown in Table 4 that P value in the hydrolysis temperature variable is 0.053 which means it has P value of >0.05. This indicates that the hydrolysis temperature

variation does not significantly affect the glucose levels, while the hydrolysis pH variable obtains P<0.05 value of 0.002. This indicates that pH variations significantly affect the glucose levels obtained, squarely the pH and hydrolysis temperature variables, both have P<0.05 value. Thus, this variability is stated to significantly affect the glucose levels. Both interactions of temperature and pH hydrolysis obtained P value of 0.081 which means that it does not meet the conditions of P<0.05. Then statistically the two variables do not significantly provide interaction to increase glucose levels, as a result of validation of multiple regression analysis. The results obtained from the central composite design are integrated with the second order full polynomial equation. The equation showing the indication of glucose production against the temperature and pH hydrolysis variables can be expressed in the following equation:

$$Y = 31.54 - 0.913\,(X_1) - 0.930\,(X_2) + 0.00972\,(X_1X_1) + 0.1128\,(X_2X_2) - 0.01661\,(X_1X_2)$$

Y is percentage of glucose levels, X_1 is temperature (°C), and X_2 is pH.

The independent variable amount can be revealed from the absolute value listed before the equation of independent variable that explaining how independent variables (quadratic) or multiple interactions affect the acquisition of glucose levels in the hydrolysis process. The negative coefficient value shows that the independent/multiple interaction is a negative factor affecting the hydrolysis process, which is reducing the glucose level obtained. Meanwhile, the positive coefficient value indicates that the factor could increase the hydrolysis result in the range of glucose levels tested.



Gambar 3. Contour and surface plot of glucose level optimization results

The quadratic factor states that the experimental results cannot be explained by a linear model, so to ascertain whether the range of experimental data used contains the optimum region, a contour plot is made between temperature and pH hydrolysis. The contour plot displays the optimum zone formed a parabola with the optimum zone shown in the solid green plot. In addition, the contour can be seen in Figure 3. Based on Figure 3, the contour and surface plots show that the temperature range 55-70°C and pH ranges 2-5 that can be used to obtain high glucose levels. Low glucose levels are obtained at 45-50°C d and pH is between 6-10. Determination of the optimum point of glucose levels can be seen in Figure 4.

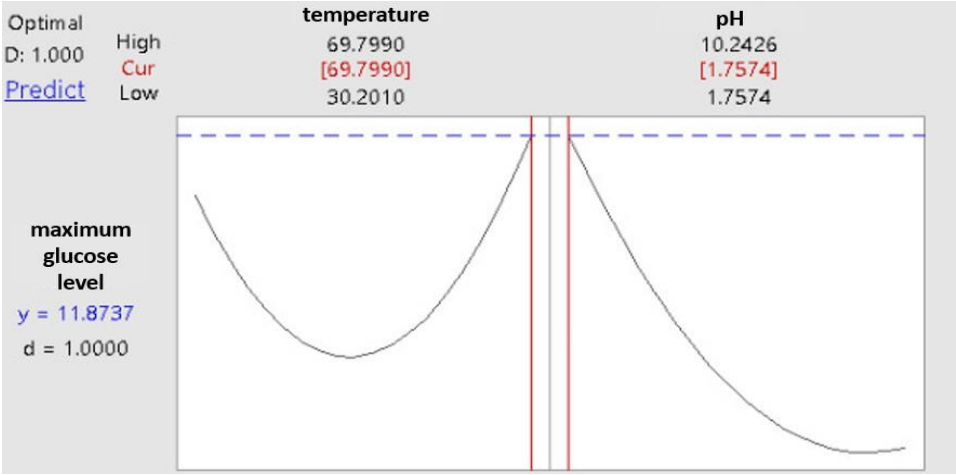


Figure 4. Determination of the Optimum Point of glucose levels

The determination of the optimum point is finished by determining the zero-gradient value in the contour plot at Figure 4 and obtaining a predictive value with a maximum glucose content of 11.87 mg/mL at pH 2 and a hydrolysis temperature of 70°C. Low pH (acidic pH) can be a catalyst that helps the process of breaking disaccharides into monosaccharides, thus causing the faster hydrolysis reaction that occurs. the greater temperature would lead the greater reaction speed constant so that the reaction can be faster, and the glucose levels obtained are higher. Based on this study, maximum glucose levels of 9.22 mg/mL are obtained at pH 2 and hydrolysis temperature of 50°C. The optimum result was validated five times. Based on the validation results, the average percentage of ± SD glucose levels was 9.15 ± 0.13 mg/mL. This proves that the experimental design model is considered feasible or significant to be used in determining the influence of variables on glucose levels.

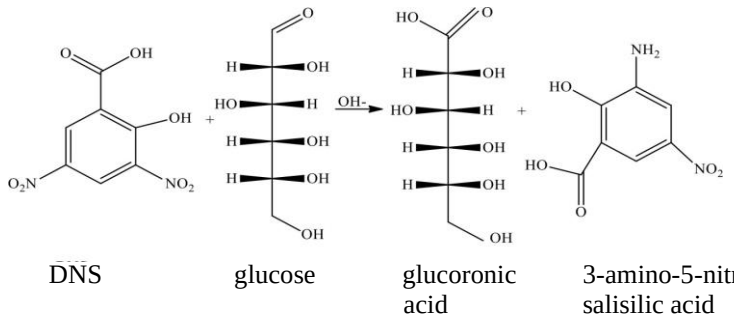


Figure 5. Schematic reaction DNS reduces glucose

Determination of optimal levels is carried out by measuring glucose levels. This aims to determine the highest glucose levels at the hydrolysis stage using cellulose enzymes. The glucose level test is carried out by Dinitro Salicylic Acid (DNS) method. Because the use of DNS reagents to measure glucose levels has high accuracy, it was applied to even small levels of glucose [25]. Glucose produced through the process of hydrolysis is the result of the synergistic work of cellulolytic enzymes group. The cellulolytic enzyme system consists of three main groups namely *endoglucanases, exoglucanases, and β-glucosidases*. The endoglucanase enzyme hydrolyzes randomly on the amorphous part of the cellulose fiber to produce oligosaccharides of different lengths and the formation of new chains of cellulose. The

exoglucanase enzyme works on the ends of the polysaccharide chain and produces cellobiose which is a disaccharide. Then, the β-glucosidase enzyme breaks down cellobiose into two glucose molecules which are the main products of cellulose hydrolysis [26]. The glucose-reducing DNS reaction can be seen in Figure 5.

Testing glucose levels is carried out using the DNS method because it has a higher level of accuracy so that it can measure the reducing sugar produced even in small concentrations. The principle of the test with dinitro salicylic (DNS) method is that the aldehyde group in the polysaccharide chain is oxidized to a carboxyl group, at the same time, the sugar aldehyde group will reduce 3,5-dinitrosalicylic acid to 3-amino-5-nitrosalicylic acid. The reaction will continue as long as there is glucose in the solution tested. The sample glucose will react with the DNS solution which was originally yellow to brownish orange. Dinitro Salicylic Acid (DNS) is an aromatic compound that will react with glucose to form 3-amino-5-dinitrosalicylic acid to absorb strongly electromagnetic wave radiation at a wavelength of 540 nm [27].

Table 5. Bioethanol data from cassava peel fermentation using *Zymomonas mobilis* determined using refractorometer

Run order	Fermentation time (h)	Fermentation pH	Refractive Index	Bioethanol levels (% v/v)
1	168	6	1.3439	37.75
2	87	6	1.3433	36.25
3	87	6	1.3433	36.25
4	30	9	1.3425	34.25
5	6	6	1.3424	34.00
6	87	2	1.3429	35.25
7	30	3	1.3425	34.25
8	87	6	1.3432	36.00
9	144	9	1.3433	36.25
10	87	10	1.3430	35.50
11	87	6	1.3435	36.75
12	144	3	1.3433	36.25
13	87	6	1.3432	36.00

Tabel 6. Analysis of variant (ANOVA) pH and fermentation time

Variable		Bioethanol Production	
		P Value	Code Coefficient
Linear	Fermentation time (Hours)	0.001	1.163
	pH	0.735	0.044
Quadratic	Fermentation time (Hours) x Fermentation time (Hours)	0.075	-0.281
	pH x pH	0.006	-0.531
Interaction	pH x Fermentation time (Hours)	1.000	-0.001

Optimization of bioethanol production through fermentation process using *Zymomonas mobilis* bacteria with Response Surface Methodology (RSM) Method

The optimization process is carried out by experimental design and applying the surface response method (RSM), namely a centralized composite design (CCD). Data on

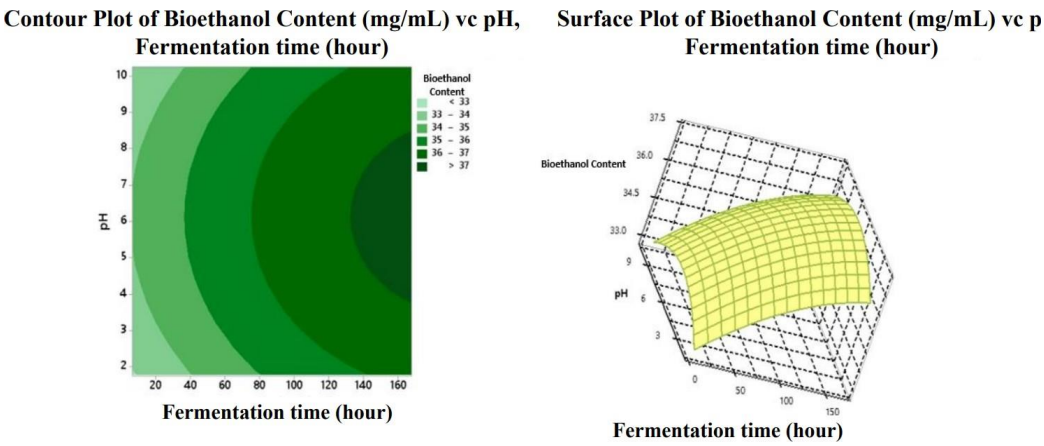
glucose measurement results from cassava peel hydrolysis with the Response Methodology (RSM) Method can be seen in Table 5 and analysis using Minitab18 software for analysis of variant (ANOVA). The results of the statistical analysis of ANOVA are seen in Table 6.

Based on the outcomes of ANOVA analysis, a correlation coefficient (R^2) of 89.17% obtains P value of 0.001 (P<0.05) which indicates that statistically the model adjusts with the design. Thus, it is considered significant for optimization of bioethanol level acquisition. The results of the analysis shown in Table 6 that P value in the fermentation time variable is 0.001 which means P<0.05 indicates that the fermentation time is significant in affecting the acquisition of bioethanol levels. Meanwhile, the fermentation pH variable obtains a P value of 0.075 which means a P value of >0.05 indicates that the fermentation pH is not significant in affecting the acquisition of bioethanol levels. Squarely, the fermentation time factor has a P value> 0.05 means that it has a statistically insignificant effect on the level of bioethanol obtained. Meanwhile, squarely the fermentation pH factor obtained a P value of 0.006 which meets the requirements of P<0.05 means that statistically it has a significant influence on the bioethanol levels obtained. Both interactions between pH and fermentation time have a P value of 1.000. In this case, a P>0.05 value means that statistically the two variables do not significantly interact with each other on the acquisition of bioethanol levels. As a result of analysis validation Multiple regression, the results obtained from the central composite design are integrated with the full polynomial equation of the second order. The equation showing the indication of bioethanol production against the temperature and pH hydrolysis variables can be expressed in the following equation:

$$Y = 31.606 + 0.03546\,(X_1) + 0.723\,(X_2) - 0.000087\,(X_1X_1) - 0.0590\,(X_2X_2) - 0.00000\,(X_1X_2)$$

Y is percentage of bioethanol content (% v/v), X_1 is fermentation time (hours) and X_2 is pH level.

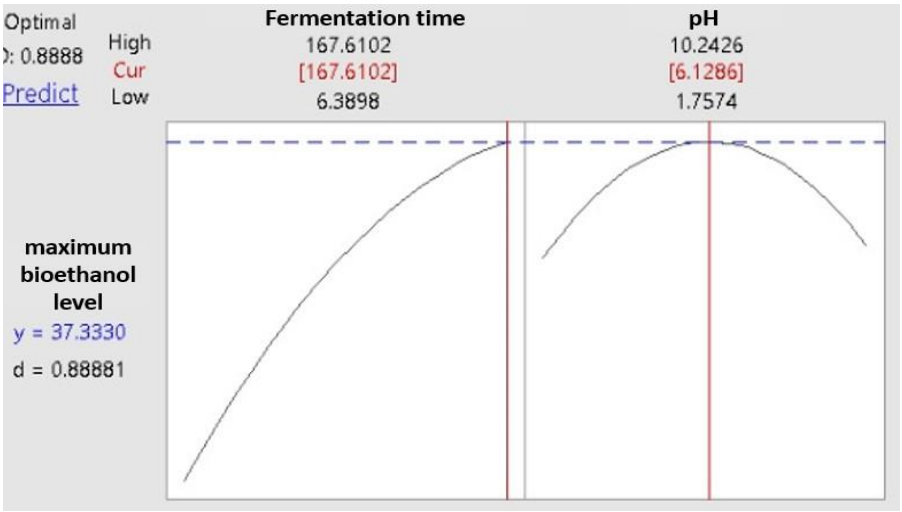
The amount of the independent variable can be determined from the absolute value listed before the independent variable equation of 10 explaining how independent variables (quadratic) or multiple interactions affect the acquisition of glucose levels in the hydrolysis process. The negative coefficient value shows that the independent/multiple interaction is a negative factor affecting the hydrolysis process, which is reducing the glucose level obtained. Meanwhile, the positive coefficient value indicates that the factor can increase the hydrolysis result in the range of glucose levels tested.



Gambar 6. Contour and surface plot results of bioethanol content optimization

The quadratic factor states that the experimental results cannot be explained by a linear model, so to ascertain whether the range of experimental data used contains the optimum region, a contour plot is made between the time and pH of fermentation. The contour plot displays the optimum zone formed a parabola with the optimum zone shown in the deep green plot. In addition, the contour can be seen in Figure 6.

Based on contour plots and surface plots, it shows that the fermentation time range of 80-168 hours and the pH range of 4-8 are the ranges that can be used to obtain high bioethanol levels. Low bioethanol levels were obtained in the fermentation that takes 10-75 hours at pH 2-4 and pH 9-10. Determination of the optimum point of bioethanol levels can be seen in Figure 7.



Gambar 7. Determination of the Optimum Point of Bioethanol Levels

To determine the optimum point, it was carried out by selecting the zero-gradient value in the contour plot at Figure 7 that obtained a prediction value with a maximum bioethanol content of 37.33 mg/mL at pH 6-, and 168-hours fermentation. The result of research conducted using *Zymomonas mobilis* bacteria is that the acquisition of bioethanol levels from rice straw produced 33% with 5 days fermentation and an optimum pH at pH 5 [28]. This shows that the longer fermentation time produces the higher bioethanol content. The results obtained also support the statement that the use of *Zymomonas mobilis* bacteria fermentation is very safe and productive in producing high bioethanol levels in a fairly wide pH range of 3.5-7.5. In addition, ethanol productivity is higher by using *Zymomonas mobilis* bacteria compared to *Saccharomyces cereviseae* bacteria, because *Zymomonas mobilis* has a high specific cell surface area and consumes glucose faster than *Saccharomyces cereviseae* [29]. Based on this study, the bioethanol content obtained was 37.25% at pH 6 and the optimum fermentation time was 168 hours. The optimum result was validated five times. Based on the validation results, the average percentage of ± SD bioethanol levels was 37.2 ± 0.40% (v/v). This proves that the experimental design model is considered feasible or significant in determining the influence of variables on bioethanol levels. Based on the results of research utilizing corn cobs, it produces bioethanol of 20% at pH 5 with 96 hours fermentation [13]. Accordingly, it was found that the acquisition of bioethanol levels from sugarcane waste resulted in 16.99% at pH 6 with 3 hours fermentation [30]. Accordingly, it displays that the bioethanol content of sugarcane bags yields

28.5% with a fermentation time of 120 hours and a pH range of 5-6 [31]. Based on the results obtained by comparing the results of the study, it shows that the research data has a higher percentage due to the selection of microorganisms use in producing bioethanol and fermentation optimization treatment carried out with pH variations and fermentation time that affect the fermentation process. This study used *Zymomonas mobilis* bacteria which is fermented for 168 hours compared to the use of *Saccharomyces cereviseae* microorganisms that takes 3 hours fermentation [30]. The use of microorganisms *Zymomonas mobilis* takes 120 hours fermentation [31].

The result is also in line with the research results using *Zymomonas mobilis*. In the fermentation process, it shows that the optimal fermentation time is 4-6 days. The longer the fermentation time would produce the higher bioethanol levels to reach a certain point. If the fermentation time is still continued, there will be a decrease of bioethanol. The fermentation time reduction of bioethanol production that continues after reaching the optimal point of bioethanol production is caused by the reduction of substrate in the media for bioethanol formation so that there is no significant bioethanol formation. According to studies that have been conducted, the longer fermentation time would lead to the lower pH value [32]. The high and low rate of bioethanol fermentation process is also caused by enzyme activity. Enzyme activity is affected by pH. The improper pH changes will cause catalytic regions and the change of enzyme confirmation [33].

Qualitative test of bioethanol levels

Qualitative tests were conducted to ensure the bioethanol content sample using 2% potassium dichromate reacted with concentrated sulfuric acid. The positive result of bioethanol sample test obtains blue solution [34]. Based on the results of this study, the whole bioethanol sample test changed color from orange to blue after the addition of 2% potassium dichromate and concentrated sulfuric acid. The faster change of color would obtain the higher bioethanol levels. The results also support the statement that the higher rate of color change produces the higher concentration of bioethanol. As the law of reaction rate, the higher concentration of a solution also leads to the faster reaction rate [34].

Quantitative test of bioethanol levels using Gas Chromatography

Distilled bioethanol is then quantitatively analyzed using gas chromatography instruments. Quantitative measurement of bioethanol levels is determined using bioethanol content calculations. Fermented distillate obtained a bioethanol content of 54.94%. The results is calculated from ratio of chromatogram area of the sample divided by the area of the bioethanol standard multiply by concentration of bioethanol standard (Figure 8).

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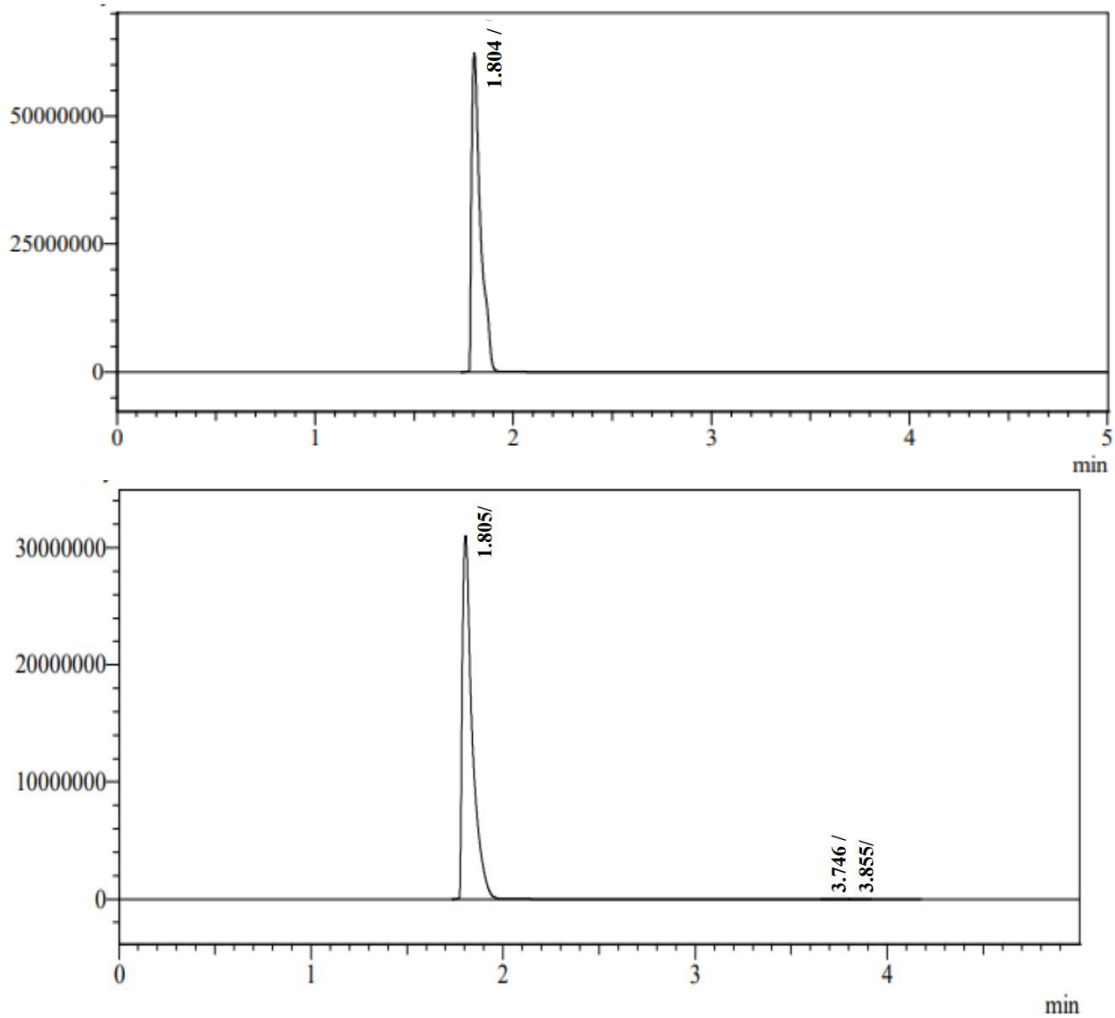

Figure 8. Chromatogram of bioethanol standard (top) and the sample isolated from fermentation (bottom).

CONCLUSION

The levels of lignin, hemicellulose and cellulose were obtained after the alkaline pretreatment process using 14% NaOH are 6.68% (b/b), 27.3% (b/b) and 58.4 (b/b) respectively. The optimum condition for glucose hydrolysis from cassava peel using commercial cellulose enzyme was obtained at pH 2 and temperature of 50°C with glucose content of 9.22 mg/mL. The optimum condition of bioethanol fermentation from cassava peel using *Zymomonas mobilis* bacteria was obtained at pH 6 fermentation time 168 hours with analysis using a refractometer produced bioethanol content of 37.75% (v/v) and analysis using gas chromatography produced bioethanol content of 54.94% (v/v).

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