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# Optimization of Bioethanol Production by Enzymatic Hydrolysis and Fermentation From Rind Cocoa Fruit (*Theobroma Cacao* L)

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## Abstract

Cocoa (Theobroma cacao L) production in Indonesia based on data from the Central Statistics Agency (BPS) for 2020 reached 720.66 thousand tons and continues to increase every year. Increasing cocoa production can cause cocoa waste to increase as well. Cocoa waste handling can be overcome by producing bioethanol as a way to reduce the amount of waste produced. This study aims to utilize cellulose compounds from cocoa fruit waste in the production of bioethanol through several stages, namely delignification, enzymatic hydrolysis by cellulase enzymes and the fermentation process with the help of Zymomonas mobilis bacteria. The results showed that the lignin level decreased by 19.5%, the hemicellulose level decreased by 6.87% and the cellulose level increased by 27.45%. Hydrolysis and fermentation stages were analyzed using the response surface method (RSM) to obtain optimum conditions. Cellulose can be optimally hydrolyzed using a pH buffer of 2 and a temperature of 30°C with a glucose concentration of 21,703 mg/mL. The fermentation process can be carried out at optimum conditions using a fermentation medium pH 10 with an incubation time of 168 hours. The bioethanol level was analyzed using a refractometer and gas chromatography (GC) with a yield of 8.43% (v/v).

## INTRODUCTION

Ethanol ($C_2H_5OH$) or commonly called ethyl alcohol is an organic solvent that has many wide applications in the perfume industry, paints, varnishes, explosives and alternative energy sources. So that the growth of the ethanol market is increasing every year (Candra et al., 2019). Ethanol can be made in two ways, namely through chemical synthesis and a fermentation process with the help of microorganisms or commonly called bioethanol (Luth et al., 2020).

Bioethanol can generally be made from raw materials such as sugarcane, wheat, potatoes, sweet potatoes and lignocellulosic such as wood and agricultural waste (Oktavianis & Sofiyanita, 2019). The bioethanol manufacturing industry in Indonesia generally uses raw materials such as molasses, cassava, sweet potatoes and wheat. However, in its use as a raw material, the manufacture of bioethanol has the potential to create competition with the use of materials for the food industry, so one alternative that can be used to overcome this problem is to utilize agricultural waste (Ridmaningrum et al., 2020). One of the agricultural wastes that can be utilized in the manufacture of bioethanol is cocoa fruit rind. Cocoa fruit rind is a lignocellulosic residue that is rarely explored as a raw material for bioethanol

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production. Usually this residue is used as organic compost (Barreto et. al., 2020).

Cocoa (*Theobroma cacao* L) is a tree that originates from Central and South America, but the highest production is found in areas with tropical climates such as Africa, Asia and South America (Meneses et al., 2021). The oval-shaped cocoa fruits are green and red and when ripe they turn yellow or purplish red (Oktavianis & Sofiyanita, 2019). According to data from the Central Statistics Agency (CSA), Indonesia's cocoa production in 2020 will reach 720.66 thousand tonnes with a plantation area of 1.508.956 Ha. And cocoa industry waste is estimated to be around 85% in the world cocoa production. For the cocoa-producing industry, this waste can be a serious problem because it causes economic losses and environmental pollution (Meneses et al., 2021). So one way to reduce the amount of cocoa waste is to utilize cocoa rind waste as bioethanol production.

Cocoa rind contains lignocellulosic compounds consisting of lignin, cellulose and hemicellulose in varying amounts depending on the biomass of origin. Based on research conducted by Darmayanti et al. (2019), cocoa rind waste contains complex lignocellulosic compounds consisting of 29.93% cellulose, 10.94% hemicellulose, and 11.64% lignin. Asiedu et al. (2019) 23.04% cellulose, 38.08% hemicellulose, and 18.19% lignin. Susanti et al. (2020) 23.8% cellulose, 8.2% hemicellulose, and 33.4% lignin.

The production of bioethanol consists of several stages, including the pre-treatment stage which aims to reduce lignin levels which can inhibit the hydrolysis process in materials containing lignocellulosic. Most of the pretreatment steps are carried out using alkali or NaOH (Maryana et al., 2022). This is because NaOH is able to remove some of the lignin and hemicellulose that protects the cellulose molecule, and is able to break hydrogen bonds and the bonds between cellulose molecules, so that the cellulose is in an unbound state. This situation causes cellulose to become easily separated, both in terms of bonds with non-cellulose components and in the cellulose itself (Asih et al., 2018).

The second stage is hydrolysis which aims to convert cellulose into glucose, which will be the substrate at the fermentation stage. In this study using enzymatic hydrolysis because it has several advantages including, it is more environmentally friendly because it does not produce by-products and also the chain breaking process in cellulose becomes more specific (Asih et al., 2018). The enzymes used in this hydrolysis stage are cellulase enzymes. Cellulase enzyme is an enzyme that can break down cellulose into glucose. Hydrolysis can be catalyzed by a highly specific cellulase enzyme complex consisting of endoglucanase, exoglucanase, and β-glucosidase which hydrolyze complex cellulose polymers into simple glucose monomers (Weerasinghe et al., 2021). Based on research conducted by Asih et al. (2018) on samples of corn stalks with pretreatment treatment using 6% NaOH and followed by hydrolysis using cellulase enzymes were able to produce glucose levels of 16.2340 mg/mL.

And the final stage in the manufacture of bioethanol is fermentation. Fermentation is a natural process used to break down larger organic molecules into simpler ones with the aim of converting glucose into bioethanol with the help of microorganisms (Tse et al., 2021). A common microorganism for fermentation is *Saccharomyces cerevisiae* or commonly called yeast which belongs to the fungus group. But it has been found that *Zymomonas mobilis* can metabolize glucose from cellulosic biomass effectively and produce more ethanol than *S. cerevisiae*. This bacterium processes glucose via the Entner-Doudoroff pathway and provides many advantages such as less ATP produced, as well as high temperature tolerance and resistance to low pH (Yogaswara et al., 2021).

Research conducted by Billah et al. (2020) on cocoa fruit rind samples acid hydrolysis of resulted in a reducing sugar level of 9.88% so that the highest bioethanol level was obtained at optimal fermentation conditions using *Zymomonas mobilis* bacteria with a fermentation time of 192 hours at 10.62%. According to research by Nuraeni et al. (2021) cocoa rind waste has good potential to produce bioethanol with a level of 10.6% - 55.3% using *Zymomonas mobilis* bacteria. Sandi et al. (2016), on the seaweed raw material with enzyme hydrolysis and fermentation using *Saccharomyces cereviciae* produced a glucose level of 48.25% and the 5$^{th}$ day fermentation process produced bioethanol and that is level of 8.92%.

Optimization of hydrolysis and fermentation parameters plays an important role in the success of a bioprocess industry, apart from the type of microorganism used. So to determine the optimum conditions in an experimental design can be done by optimizing using a method called the

Response Surface Methodology (Bachtiar et al., 2021). The advantage of this method is that it does not require large amounts of experimental data and does not take a long time (Kusumaningrum et al., 2019). Therefore, the RSM method is expected to produce a faster optimization process and accurate optimum conditions.

## MATERIALS AND METHODS

### Materials

Them materials used in this study were $H_2SO_4$ p.a, $K_2Cr_2O_7$ (Merck, Germany), $KH_2PO_4$ (Merck, Germany), $MgSO_4.7H_2O$ (Merck, Germany), $(NH_4)_2SO_4$ (Merck, Germany), NaOH (Merck, Germany), NaCl (Merck, Germany) $C_6H_{12}O_6$ (Merck, Germany), *dinitrosalicylic acid* (DNS) (Merck, Germany), salt *Rochelle* $KNaC_4H_4O_6.4H_2O$ (Merck, Germany), $CaCl_2$ (Merck, Germany), HCl 37% (Merck, Germany), $C_2H_5OH$ (Merck, Germany), $NH_4Cl$ (Merck, Germany), $NH_3$ (Merck, Germany), $Na_3C_6H_5O_7$ (Merck, Germany), peptone (Merck, Germany), yeast extract (Merck, Germany), distilled water, aquabides, whatman filter paper no 42, universal pH paper, aluminum foil, gauze, cotton, *Zymomonas mobilis* bacteria off the ground (ITB Collection, Indonesia), commercial cellulase enzymes 0,354 U/mL (ITB Cellection novozymes, Indonesia), and cocoa rind from Sinjai, South Sulawesi.

### Methods

The optimization process in this research uses *Response Surface Methodology* (RSM). This method was carried out using a *Central Composite Design* (CCD) composite design and analyzed using Minitab 17 software for analysis of variance (ANOVA). Optimization was carried out to find optimum conditions at the hydrolysis stage using temperature and pH variables, as well as optimum conditions at the fermentation stage using fermentation time and pH variables. The predicted responses were reducing sugar levels and bioethanol levels obtained by an experimental design using 13 factorials.

### Pretratment of Rind Cocoa

Pretreatment was carried out by preparing 15 erlenmeyer, each containing 20 g of cocoa rind powder, then adding 200 mL of 14% NaOH each, after that stirring and putting it into a waterbath shaker at 100 rpm for 6 hours at room temperature room 60°C. Then the solution was filtered and the filtered residue was washed with distilled water until the pH was neutral and then it was dried in an oven at 105°C for 2 hours.

### Analysis of Hemicellulose, Cellulose, and Lignin Level

This analysis uses the Chesson method. 1 g (a) sample was added to 150 mL of distilled water into a three neck flask and refluxed at 100°C for 2 hours. Then it is filtered, the residue is washed using distilled water until the pH is neutral. After that, the residue is dried in an oven at a temperature of 65-80°C and then weighed (b). The residue was added 150 mL of 1 N $H_2SO_4$ then refluxed for 2 hours at 100°C, then filtered and washed with distilled water until the pH was neutral, then dried and weighed (c). The dry residue was added with 10 mL of 72% $H_2SO_4$ and soaked at room temperature for 4 hours. Added 150 mL of 1 N $H_2SO_4$ and refluxed for 2 hours. The residue is filtered and washed with distilled water until the pH is neutral, then dried in an oven at 65-80°C and the results are weighed (d), then the residue is burned and weighed (e). Calculation of Hemicellulose Levels, Cellulose, and Lignin are shown in Eqs. (1) – (3). (Anggi)ani et al, 2020)

$$\frac{b-c}{a} \text{ x } 100\% \quad (1)$$

$$\frac{c-d}{a} \text{ x } 100\% \quad (2)$$

$$\frac{\boldsymbol{d-e}}{\boldsymbol{a}} \text{ x } 100\% \quad (3)$$

### Optimization Enzymatic of Cellulose Hydrolysis

As much as 3 g of pretreatment cocoa fruit powder was put into a 250 mL Erlenmeyer, then 10 mL of cellulase enzyme was added to the citrate buffer and ammonia buffer solutions with various pH (2-10) and homogenized. Then it was put into a waterbath shaker and incubated at various temperatures (30-70°C) at 105 rpm for 48 hours simultaneously. After incubation, the hydrolyzate was separated by cold centrifugation at 4°C at 10,000 rpm for 20 minutes.

### Analysis of Glucose Levels with the *Dinitro Salicylic Acid* (DNS) Method

1.5 mL of the hydrolyzate was put into a test tube, added 1.5 mL of DNS reagent, then shaken until homogeneous using a vortex. After

that it was heated in boiling water for 15 minutes and cooled at room temperature, then the reducing sugar level was analyzed using a UV-Vis spectrophotometer at a wavelength of 525 nm. The blank used is distilled water with the addition of DNS solution. The standard curve was prepared using a standard glucose solution of 0.025; 0.05; 0.1; 0.2; 0.4; mg/mL. After that, the measurement data is obtained, then calculate the reducing sugar level using the formula in Eq. (4):

$$y = ax + b \quad (4)$$

Where, y is the refractive index, a is the slope, x is the glucose level (%) b is the intercept.

**Rejuvenation of *Zymomonas mobilis* Bacteria**

1 mL of pure *Zymomonas mobilis* was inoculated into *Luria Broth* medium (0.05 g yeast extract; 0.1 g NaCl; and 0.1 g peptone) which had been sterilized in an autoclave at 121°C for 15 minutes and pressure 15-20. Then put in a water bath shaker for 24 hours at 37°C with a speed of 125 rpm (Dompeipen & Dewa, 2015).

**Optimization of the Fermentation Process for Bioethanol Production**

Erlenmeyer containing 10 mL of hydrolyzate and fermentation medium ($KH_2PO_4$ 0,01 g; $(NH_4)_2SO_4$ 0,01 g; $MgSO_4.7H_2O$ 0,01 g; yeast extract 0,1 g; and glucose 0,5 g) the fermentation time is adjusted (6-168 hours) at a pH range (2-10) using citrate buffer and ammonia buffer, then covered with cotton and aluminum foil, then sterilized in an autoclave at 121°C for 15 minutes and 15-20. After that 5 mL of *Zymomonas mobilis* battery inoculum was inoculated into each Erlenmeyer into each fermentation medium and covered again with cotton and aluminum foil, then fermented in a waterbath shaker at 37°C with a speed of 125 rpm. Sampling was carried out under certain conditions based on the RSM design. In the next stage, the fermented product was centrifuged for 20 minutes at a speed of 10,000 rpm and a temperature of 4°C. The supernatant resulting from centrifugation was distilled at 78-80°C, then the bioethanol level was analyzed using a Refracometer and Gas Chromatography. Calculation of bioethanol level using the following Eq. (5):

$$\%\text{Bioethanol} = \frac{\text{Sample Area}}{\text{Standard Area}} \times \text{standard concentration} \quad (5)$$

## RESULTS AND DISCUSSION

**Pretreatment**

Pretreatment is the initial stage in the process of making bioethanol which aims to reduce lignin levels which can inhibit the hydrolysis process in materials containing lignocellulosic. The pretreatment process in this study used 14% NaOH solution, so that the results were obtained in Table 1. This study used 14% NaOH, because it was based on the study by Maryana et al. (2022) previously reported that the NaOH used should not exceed 17% because it would damage the cellulose structure. These results indicated that there was a decrease in lignin and hemicellulose levels and an increase in cellulose levels after the pretreatment process. The effect of alkaline solutions on lignin degradation is caused by the less stable bond between cellulose and hemi cellulose and lignin. The broken bonds are hydrogen bonds that connect lignin with cellulose and hemicellulose and bonds between ether and ester groups (Rambat et al., 2015). The released lignin then binds with alkali to form soluble phenolic salts. Dissolving lignin causes a decrease in the level of lignin produced.

Based on research conducted by Irwanto et al. (2016) delignification of cocoa fruit rind with sodium hydroxide solution reached optimum conditions at 6% NaOH concentration which caused a decrease in lignin level from 30.46 to 24.64%. Wiwinda (2022) on a sample of corn cobs using 14% NaOH produced 41% cellulose, 29.14% hemicellulose, and 14% lignin. The decrease in lignin and hemicellulose levels showed that pretreatment using alkaline solutions not only caused lignin degradation, but also hemicellulose depolymerization, this is because hemicellulose has properties that are very susceptible to high temperatures, and easily dissolves in alkalis and also has a structure that is mostly soft, so it is sensitive and easy to decompose (Larastati et al., 2019).

In addition, the concentration of NaOH used also greatly influenced the pretreatment stage, the higher the concentration of NaOH used, the

Table 1. Comparison of cocoa fruit level before and after the pretreatment process.

| Components | Before Pretreatment (%) | After Pretreatment (%) |
|---|---|---|
| Hemicellulose | 15.04 | 8.21 |
| Cellulose | 21.42 | 48.87 |
| Lignin | 39.73 | 19.78 |

Table 2. Data on hydrolyzed glucose measurements.

| Run Order | Temperature (°C) | pH | Glucose Levels mg/mL |
|---|---|---|---|
| 1 | 50 | 2 | 22.97 |
| 2 | 50 | 6 | 12.72 |
| 3 | 50 | 6 | 12.27 |
| 4 | 50 | 6 | 13.22 |
| 5 | 70 | 6 | 12.48 |
| 6 | 50 | 10 | 4.21 |
| 7 | 30 | 6 | 20.64 |
| 8 | 50 | 6 | 11.51 |
| 9 | 64 | 3 | 16.99 |
| 10 | 50 | 6 | 16.11 |
| 11 | 36 | 9 | 15.88 |
| 12 | 64 | 9 | 8.78 |
| 13 | 36 | 3 | 23.79 |

higher the cellulose produced, this was due to the decreasing levels of lignin and hemicellulose due to the pretreatment process.

**Enzymatic Optimization of Cellulose Hydrolysis**

Delignified cocoa fruit powder is then enzymatically hydrolyzed using cellulase enzymes. The optimization process at the hydrolysis stage is carried out using RSM. The results of glucose measurement data on the temperature and pH variables can be seen in Table 2. Based on ANOVA analysis, a correlation coefficient ($R^2$) was obtained of 91.85% with a P value of 0.001 ($P<0.05$) which indicated that statistically the model was in accordance with design so that it is considered significant for the optimization of glucose levels, and these two variables influence each other on the resulting glucose levels.

Testing the optimum glucose levels resulting from hydrolysis using the DNS method. The DNS method is more widely used in measuring the activity of enzymes whose products are reducing sugars, even though the price of the reagent is relatively more expensive, but DNS has a higher level of accuracy so that it can measure reducing sugars in small concentrations, as well as in preparations its manufacture, the DNS method is easier and more practical (Pratiwi et al., 2018). Data for measuring glucose levels using the DNS method are in Table 2.

The principle of testing using the DNS method is that the aldehyde group in the polysaccharide chain is oxidized to a carboxyl group, at the same time the aldehyde group of the sugar will reduce 3,5-dinitrosalicylic acid to 3-amino-5-nitrosalicylic acid as shown in Figure 1. The reaction will continue as long as there is reducing sugar in the solution tested, the reducing sugar in the sample will react with the DNS solution, which is initially yellow in color to a reddish-orange color. DNS is an aromatic compound which will react with reducing sugar to form 3-amino-5-dinitrosalicylic acid (Putri, 2016).
To find out the value of the independent variable that causes the response variable to be optimal, the results obtained from the central composite design are integrated with a second-order full polynomial equation. This equation shows an indication of glucose levels with respect to temperature and hydrolysis pH variables which can be expressed in Eq. (6).

$$\begin{aligned} &Y\,(Glucose\ level) \\ &= 62.5 - 1.246\,(temperature) - 2.40\,(pH) \\ &+ 0.01030\,(temperature.temperature) \\ &+ 0.0594\,(pH.pH) \\ &- 0.0018\,(temperature.pH) \end{aligned} \quad (6)$$

A positive coefficient value indicates that the independent variable (squared) in this case temperature and pH has an influence on increasing

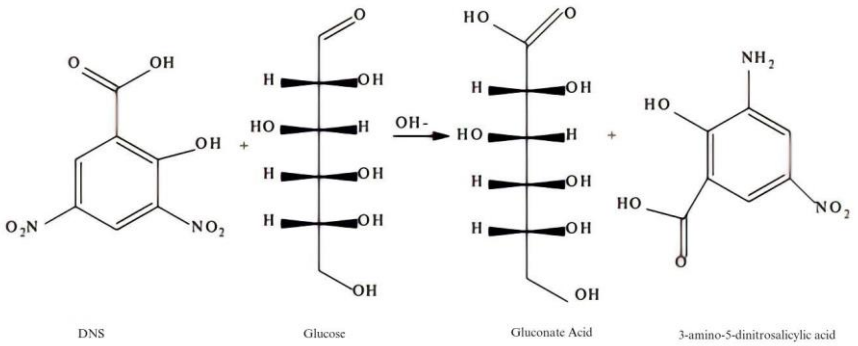


Figure 1. The DNS reaction reduces glucose (Miloski et al., 2008).

the hydrolysis process, namely increasing glucose levels, while a negative coefficient value indicates that the effect of the interaction between the two independent variables can have a large influence. negative impact on the process of hydrolysis by lowering glucose levels.

The contour plot in Figure 2 shows how the combination of variables influences each other so that the color differences can be seen according to the glucose levels produced in the hydrolysis process. The light green color which is in the temperature range of 40-70°C with a pH range of 8-10 shows the lowest glucose levels obtained, while the dark green color with a temperature range of 30-40°C in the pH range 1-4 can be used to produce glucose levels tall one. Most cellulase enzymes have optimal activity in the temperature range of 20–50°C.

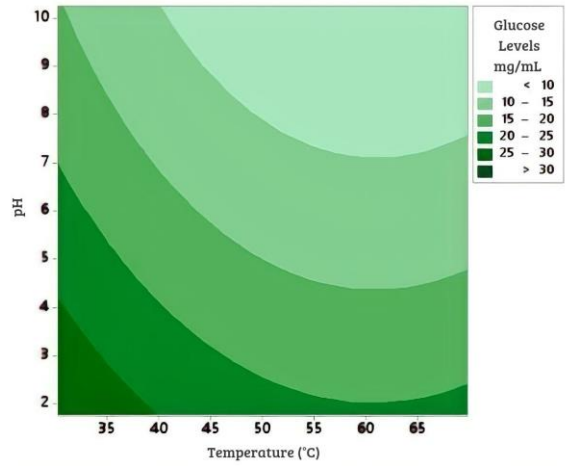


Figure 2. Contour plot of glucose level optimization results.

Based on statistical analysis using RSM, the optimum response conditions were obtained based on the hydrolysis optimization value of glucose levels at temperature and pH variables shown in Figure 2, namely 30°C at pH 2 with a maximum prediction of 30.16 mg/mL. The optimum hydrolysis conditions were then validated five times, resulting in the highest glucose level of 21.70 mg/mL with an average percentage of the five validations of 21.18 mg/mL.

Asih et al. (2018) at hydrolysis of corn stalks using the cellulase enzyme on 30°C for 3 days resulted in a glucose level of 16.2340 mg/mL. Pratiwi et al. (2010) stated that apart from the effect of temperature, the pH variable also greatly influenced the hydrolysis process in producing glucose, so that the highest glucose level was obtained in cocoa rind waste of 35.5% with a pH of 1 while the optimum condition was obtained at a pH of 4 with a level of 25.5 %.

While the research conducted by Asrianti (2017) produced the optimum temperature and time for enzymatic hydrolysis of waste paper with optimum glucose levels obtained at 30°C and hydrolysis time of 16 hours 0.67179 mg/mL. Rambat et al. (2015) regarding the hydrolysis of cocoa fruit waste, stated that the higher the acid concentration in the hydrolysis process, the higher the glucose level produced. This shows that the catalytic activity is directly proportional to the concentration of $H^+$, the more hydrogen ions in the solution, the faster the hydrolysis reaction will occur, the breaking of the glycosidic bond will result in an increase in the level of glucose formed due to the degradation of cellulose.

**Optimization of the Fermentation Process for Bioethanol Production**

Hydrolyzate at optimum conditions then proceed to the fermentation stage, while the process of optimizing bioethanol production at this fermentation stage is carried out using RSM to obtain a correlation coefficient ($R^2$) of 90.41% with

Table 3. Results of data on measuring bioethanol levels using a refractometer

| Run Order | Fermentation Time (Hours) | pH | Bioethanol Levels (% v/v) |
|---|---|---|---|
| 1 | 87 | 2 | 10.25 |
| 2 | 87 | 6 | 13 |
| 3 | 6 | 6 | 9 |
| 4 | 87 | 6 | 10.5 |
| 5 | 144 | 3 | 13.25 |
| 6 | 30 | 9 | 10.5 |
| 7 | 30 | 3 | 10.25 |
| 8 | 87 | 10 | 10.75 |
| 9 | 144 | 9 | 18.5 |
| 10 | 168 | 6 | 22 |
| 11 | 87 | 6 | 11 |
| 12 | 87 | 6 | 13 |
| 13 | 87 | 6 | 10.75 |

a P value of 0.00 (P<0.05) which shows that statistically the model is in accordance with the design so that it is considered significant for optimizing the bioethanol level. Data on the results of measuring bioethanol levels at temperature and pH variables can be seen in Table 3.

To find out the value of the independent variable that causes the response variable to be optimal, the results obtained from the central composite design are integrated with a second-order full polynomial equation. This equation shows an indication of bioethanol production in the variable fermentation time (hours) and the pH of the fermentation can be expressed in Eq. (7).

The positive coefficient value indicates that the independent variable (quadratic) or double interaction in this case the fermentation time (hours) and pH can have an effect on increasing the fermentation process, which is able to increase the bioethanol level, while the negative coefficient value indicates that the effect of the interaction between the two independent variables can be has a negative impact on the fermentation process, namely by reducing levels of bioethanol.

$$\begin{aligned} &Y\,(Bioethanol\ levels) \\ &= 10.69 - 0.0842\,(Fermentation\ time) \\ &+ 0.348\,(pH) + 0.000602 \\ &(Fermentation\ time.Fermentation\ time) \\ &- 0.0604\,(pH.pH) \\ &+ 0.00731\,(Fermentation\ time.pH\ ) \end{aligned} \quad (7)$$

Level which shows a combination of variables, namely fermentation time and pH influence each other. There are different colors on the contour plots indicating different levels of bioethanol obtained. The dark blue color is in the range of fermentation time at 20-100 hours with a pH range of 2-7 indicating the lowest bioethanol level, while the dark green color with a range of fermentation time of 160-168 hours with a pH of 6-10 is the range that can be used to obtain high levels of bioethanol.

Determination of the optimum point needs to be done to determine the prediction of optimum conditions on the independent variables. Based on the results of statistical analysis using RSM, the optimum response condition was obtained, namely the fermentation time for 168 hours at pH 10 with a maximum prediction of 23.26%. The optimum fermentation conditions were then validated five times and obtained a bioethanol levels of 8%.

Glucose level is not only the factor that most precisely influences reaction kinetics but also other factors such as temperature, pH, microorganisms, and fermentation time (Yogaswara et al., 2021). The difference in pH obtained by researchers with previous studies is suspected because previous studies only used a pH range of 4-7, while this study used variations obtained in the RSM design, namely with a pH range of 2-10. In addition, the environmental factors of the fermentation media used by microorganisms play a very important role in determining the control of metabolic activity and the optimization of the observed metabolic end products. Yogaswara et al. (2021) the difference in levels obtained could be because due to bioethanol or other by-product compounds accumulating in the fermentation media so that it can inhibit the growth of *Z. mobilis* cells.

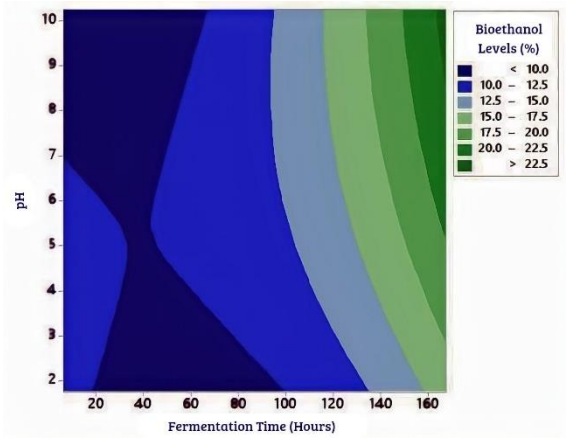


Figure 3. Contour plot of bioethanol level optimization results.

Environmental factors in this case pH and temperature have an important influence on the growth of fermenting microorganisms. Setting the pH of the growth media (fermentation) needs to be done properly to maximize bioethanol production by organisms. To maximize the production of bioethanol, it is necessary to adjust the pH of the fermentation media appropriately and the characteristics of microorganisms (Ogbonda & Kabari, 2013).

Based on the validation data in this study, the yield of bioethanol is very low, this is presumably because the substrate used, namely the glucose hydrolysate, is not fresh and unstable due to long storage resulting in a decrease in glucose levels in the hydrolysate and some of it will be oxidized freely. Based on the research of Kristianti et al. (2010) there are several factors that directly or indirectly affect fermentation including the substrate, temperature, pH, oxygen, and the microbes used so that the bioethanol level produced in this study is still very low when compared to the sugar composition found in cocoa pulp liquid where bioethanol is only 4.85%, the level of bioethanol produced from glucose conversion is 8-13%.

The level of bioethanol formed in the fermentation process of cocoa fruit rind is on average quite small, this is influenced by several factors, one of which is the unstable sugar level. However, several factors that affect the synthesis of bioethanol, including the concentration of bacteria that are not the same and the extraction time of the starter were analyzed simultaneously. In addition, the growth rate of microorganisms and the rate of production must be in line. And after delignification, the lignin level must be analyzed so that it can be known what percentage of the remaining lignin level can interfere during fermentation (Billah et al., 2020).

Bioethanol under optimum conditions was then analyzed for its level using gas chromatography, as shown in the chromatogram in Figure 4 which shows that the peak of the bioethanol sample from cocoa fruit rinds was at peak 13 with a retention time of 1.911. This retention time is almost close to the standard 15% ethanol retention time resulting in a bioethanol level of 8.43%.

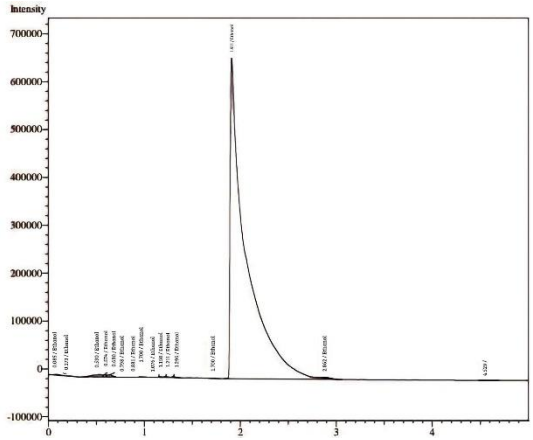


Figure 4. Chromatogram of bioethanol at optimum condition.

## CONCLUSION

Based on the research that has been done, it can be concluded that the delignification process can reduce the level of lignin compounds from 39.73% to 19.78% and hemicellulose from 15.04% to 8.21% and increase the cellulose level from 21.42% to 48.87%. Then proceed to the hydrolysis stage, in order to obtain optimum conditions at a temperature of 30°C and pH 2 with a glucose level of 21.70 mg/mL. 43%, then the optimum conditions were obtained at the fermentation stage, namely at a fermentation time of 186 hours and a pH of 10 which produced a bioethanol level of 8.43%.

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