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Analysis Reducing Sugar of Enzyme Acid Hydrolysis Fermentation from Melon Peel Waste Using Clostridium acetobutylicum Bacteria

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Abstract

Regarding sustainable development, energy consumption has the potential for energy and conservation. Simple anaerobic fermentation by *Clostridia bacteria* can convert sugar such as glucose mannose, fructose, lactose, and sucrose, into aceton, butanol and ethanol. Optimization of reducing sugar is carried out by hydrolysis of powder melon peel waste, chloride acid and cellulose enzymes, followed by microbial fermentation with clostridium *acetobutylicum* bacteria. This study aims to produce and determine the optimization of reducing sugar from melon peel waste anaerobic, which is processed further to make butanol using the Clostridium *acetobutylicum* bacteria. Reducing sugar is produced through anaerobic fermentation using a substrate mixture consisting of melon peel waste powder, hydrochloric acid, an cellulose enzymes with operating condition ratios o temperature (35ºC, 40ºC, 45ºC), fermentation time (10, 12, 14) days, pH (4, 5, 6). The reducing sugar were obtained through a UV-visible spectrophotometer test at 455 nm. Optimization analysis was evaluated using Response Surface Methodology (RSM) on reducing sugar experimental data. The study results showed that the operating conditions at a temperature 40ºC, time fermentation 288 hours, pH 5, obtained the best reducing sugar yield with a conversion of reducing sugar yield of 55,94% and 54.20 %. The operating conditions obtained in the optimization calculation using RSM are at pH 4.80, Temperature 41.51ºC, and fermentation time at 273.189 hours, yielding 55.936%.

Keywords: Hydrolysis, Optimization, Fermentation, Reducing Sugar, RSM.

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1. Introduction

Melon has a sweet taste so it contains quite high nutrition and every 100 grams of melon contains 23 calories of energy, 0.6 grams of protein, 7 mg of calcium, 2400 IU of vitamin A, 30 mg of vitamin C, 0.045 mg of thiamin, 0.0065 mg of riboflavin, 1.0 grams niacin, 6.0 grams carbohydrates, 0.4 mg iron, 0.5 mg nicotinamide, 93 ml water and 0,4 grams fiber [1]. There is cellulose 19.01%, hemicellulose 22.71%, lignin 8.26%, soluble starch 17.22%, lipid 6.91%, total sugar 30.42%, total solids 7.67%, total N 0.89%, and volatile solids 6.45% [2]. Based on Agronomy 2021, melon reduction worldwide is estimated to reach 40 million tons per year, with China as the largest producer, namely 12,7 million tons per year. Commercial melon processing sources are responsible for the massive accumulation of inedible parts such as peel and seeds from 8 and 20 million tons of waste per year worldwide [3]. Organic materials such as melon peels and seeds have the potential to provide low-cost sources for creating new food products to minimize waste. Currently, melon seeds and peel are the by-products that can be utilized because they are rich in bioactive compounds, especially polyphenol (flavonoids and phenolic acid), carotenoids, β-carotene, and β-cryptoxanthin as well as fatty acids (oleic, linoleic and almi acids), among other compounds [4]. In general, melon waste meets the criteria as a substrate for biohydrogen production because it has a high carbohydrate content. Melon derivatives such as melon seeds and rinds are the fruit processing industry's most widely used by-products [5]. Lignocellulose is an organic component that is abundant in nature and consists of 3 polymers: cellulose, hemicellulose and lignin. Hemicellulose and cellulose from lignocellulose can be utilized, while lignin is removed. Hemicellulose and cellulose as polysaccharides can be used as substrates in enzyme production or chemical hydrolysis, this process is carried out to obtain simple sugars [6]

Hydrolysis converts cellulose into simple sugars, namely reducing sugar by breaking the glycosidic bonds in cellulose using enzymes, acids or bases as catalysts. Enzymatic hydrolysis is an environmentally friendly process for converting cellulose into sugar and uses cellulose enzymes [7] The initial process of hydrolysis of lignocellulose or cellulose is usually found in heterogeneous

water systems, that is in concentrated acids, it must have a strong acidity level and a high specific surface area, which is water tolerant and allows easier access of the substrate to the active sites in the solid material [8]. During the concentrated acid hydrolysis process, hemicellulose and cellulose can generally be converted into glucose and xylose, respectivel [9]. The cellulose enzyme is a protein found in living cells that functions as a catalyst in biochemical reactions. Cellulose enzyme has specific properties to hydrolysis the β (1-4) glucoside bonds of cellulose to produce cellulose, then converted into glucose monomer [10]. The cellulose enzyme generally consists of 3 central enzyme units, including Endo β-(1-4) glucanase (C1) or cellobiohydrolase, which plays a role in breaking down the crystalline part of the cellulose chain and β-Glucosidase is an enzyme unit which plays an essential role in producing glucose products from the breakdown of cellulose [11]. Sugar is the most crucial product in lignocellulose conversion. Generally, lignocellulose can be hydrolyzed into sugar using chemical catalysts, enzymes, or a combination of both. These products have a significant role in several fields such as health, food, textiles and various other industries, including using renewable energy[12]. Fermentation is changing chemical compounds in organic substrates (glucose, carbohydrates and proteins) through the activity of bacteria or enzymes. *Clostridium acetobutylicum* bacteria are most widely used in the natural fermentation process in solventogenic Clostridia. Fermentation using these bacteria will undergo an acidogenic (acid formation) and organic solvent (solvent formation) process, which leads to the production of the solvents Acetone, Butanol, and Ethanol [13]

Therefore, this research aims to find out optimal conditions for the digestion of melon peel cellulose based on digestion variations in temperature (35ºC, 40ºC, 45ºC), time fermentation (10, 12, 14) days and pH (4, 5, 6). The raw material is melon peel ripened to level 6 (yellow skin). The study was designed using a Central Composite Design (CCD), a proven method for finding optimal conditions. This study is to determine the optimal conditions that enable melon peel waste to produce the maximum amount of reducing sugar by using CCD and Response Surface Methodology (RSM). From the results, an equation for predicting the reducing sugar obtained from the fermentation of melon peel waste by cellulose was developed and tested.

2. Material and method

2.1 Materials

2.1.1 Melon waste

Approximately five kg of melon peel waste was obtained from a fruit seller, dried in an oven for 8,5 hours at 70oC, ground with a grinder, and sieved using an 80 U.K mesh. The sieved melon waste stored in the plastic bag and kept at room temperature.

2.1.2 Microorganism

The bacterium used in this research is *Clostridium acetobutylicum*, purchased from the Center of Food and Nutrition Studies, Gajah Mada Univesity, Yogyakarta, Indonesia.

2.1.2 Enzyme

The Cellulose enzyme was obtained from Nanobio Laboratory.

2.1.3 Acid

The technical grade hydrochloric acid was purchased from CV. Indrasari, Semarang, Indonesia.

2.2 Method

2.2.1 Acid hydrolysis

Ten grams of melon peel waste powder mixed with 100 ml hydrochloric acid solution. The hydrolysis process occurred at 100 C for 1 hour. After the reaction, the supernatant neutralized to pH 6.8.

2.2.2 Enzyme hydrolysis

For cellulose hydrolysis, melon peel waste powder that has been acid hydrolyzed is allowed to stand at a temperature of 40ºC and the 10/100 ml cellulose enzyme solution is added. The hydrolysis process was carried out at 45ºC for 8 hours. The mixture was cooled to room temperature using a vacuum pump to stop the reaction before separation. The supernatant was analyzed for reducing sugars.

2.2.3 Inoculum preparation

Clostridium acetobutylicum bacteria were cultured and multiplied in TSA (Tryptone Soybean Agar) media consisting of Casein peptone 15.0 grams, Soy Peptone 15.0 grams, Sodium Chloride 5.0 grams, Bacteriological agar 15.0 grams) in liters of aquadest, homogenized, then sterilized in an autoclave at operating conditions of 121ºC, 2 atm pressure for 15 minutes, then incubated at 37 C for five days. The inoculum was made using TSB (Tryptone Soybean Broth) media at a concentration of 10% (v/v) [14].

2.2.4 Fermentation

The fermentation occurred using *Clostridium acetobutylicum*, where the pH was adjusted to 4, 5, 6. The fermentation process was carried out under anaerobic conditions for 10 days, 12 days, 14 days with incubation temperatures of 35ºC, 40ºC, and 45ºC. The supernatant was analysed for reducing sugar.

2.2.5 Analytical methods

Analysis of reducing sugars using the DNS (Dinistrosalicylic acid) method. The reducing sugars react with the DNS reagent to form a brownish-yellow 3-amino-5-nirosalicyllic acid compound, then measured using a UV-visible spectrophotometer. The reducing sugar standard curve was prepared by dissolving the reducing sugar to a concentration of 0, 0.2, 0.4, 0.6, 0.8 and 1 mL of each reducing sugars solution was taken and 1 mL of DNS was added, heated for 10 minutes and then cooled distilled water was added until the volume reached 10 mL. The absorbance was measured at a wavelength of 455 nm. The reducing sugar content was calculated using a formula from the standard curve. This can be seen in Figure 2 with the following equation ($R^2 = 0.9959$):

$$y = 0.9854x - 0.0024 \quad (1)$$

Where y is the absorbance of the measured sample solution, x is the concentration of the sample solution (g/L). The results reducing sugar after enzyme acid hydrolysis obtained the absorbance concentration value of 0.572. The higher yield value, te more efficient of fermentation process [15].

Then, after obtaining the concentration, the % yield will be calculated using the equation:

$$\%\text{yield} = \frac{\triangle Produk}{\triangle Substart} x100\% \quad (2)$$

The RSM (Response Surface Methodology) method was used to study the optimum condition [16]

2.2.6 Analysis of Cellulose, Hemicellulose and Lignin in melon peel waste

Analysis of cellulose and lignin content was carried out using the Datta method. The equation is as follows:

$$\text{Cellulose content (\%)} = \frac{c-d}{a} x100\% \quad (3)$$

$$\text{Hemicellulose content (\%)} = \frac{b-c}{a} x100\% \quad (4)$$

$$\text{Lignin Content (\%)} = \frac{d-e}{a} \text{x}100\% \quad (5)$$

Where a is the initial dry weight of melon peel waste, b is dry weight of sample residue refluxed with hot water, c is the sample residue weight after adding 1N H_2SO_4, d is the sample residue weight after adding 72% H_2SO_4, e is ash weight of sample residue [17].

3.2 Model Fitting and statistical analysis

The effect of three independent variables on reducing sugar production were analyzed using CCD. The results of yield the reducing sugar can bemeasured by UV-Visible spectrophotometer and the resulting values are summarized in table 1. The analysis of variance (ANOVA) and regression coefficients for the resulting model are presented in Table 2. In the table 2, degree of freedom, sum of squares, mean aquares, significant level (P-Value) and Fisher test (F-Value) are presented. The P-value serves as a tool for checking the significance of each term. The model had a very low P-value (P<0.05), which implied that the model fitted the experimental data significantly. F-value indicates the effect of different variables on the fermentation process of reducing sugar is pH, time reaction, temperature. Table 2 shows the regression value of 0.98019 with the relationship between experimental data and predicions approaching diagonal. F the regression value is below 0.90, the diagonals may be far from each other[19]. The shadow can be seen in the figure 4. The steps that must be taken to find out the function F that does not yet exist in the experimental or analytical data are first carrying out a first-order model filtering process on the data using the following equation:

$$y = \beta 0 + \beta 1x1 + \beta 2x2 + \cdots + \beta kxk + \varepsilon 1 \quad (5)$$

Where y is the observed response, x1 is the factor or variable that influences the response, ε is the residual component (error) which is distributed randomly. However, if the model is inconsistent with several equations, modeling s needed with higher order polynomials such as order 2. With the following equations :

$$y = \beta 0 + \sum \beta iXi + \sum \beta ii\, Xi^2\, x^2 \ldots . + \sum \beta ijXiXj + \varepsilon \quad (6)$$

So, from this equation, if it is included in the experimental data, we get the following equation :

$$y = -397.892 - 6.143x1^2 + 65.332x1 - 0.069x2^2 + 6.193x2 - 0.002x3^2 + 1.237\, x3 - 0.057\, x1x2 - 0.015x1x3 - 0.001x2x3 \quad (7)$$

In equation 7, it's known that n the probability distribution calculator, by entering the value p = 0.05, get the F table value limit of 4.0990, so that by ignoring relationship we get the following equation:

$$y = -355.337 - 6.143x1^2 + 58.717x1 - 0.069x2^2 + 5.671x2 - 0.002x3^2 + 1.123x3 \quad (8)$$

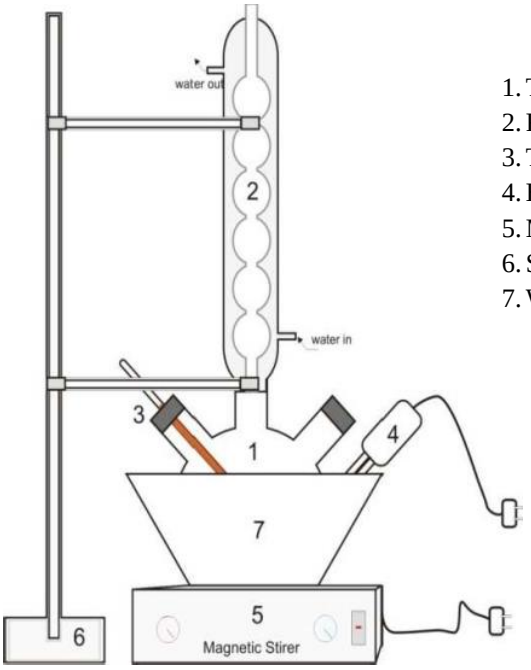
Next, analyzing the optimum results using RSM, the optimum operating condition results were obtained: pH 4.779, Temperature 41.126ºC, time reaction 273.5105 hours. The minimum operation condition is pH 3.318, temperature is 31.59ºC and time reaction 207.273 hours. The maximum operation conditions is pH 6.68, temperature 48.409ºC, and time reaction is 368.726 hours. The 3D response surface plot (Figure 5) shows the effect of tree variables on reducing sugar production. The significant levels play an important role to determine the significance of the interaction effect of variables. Whatever P value is low, the model is more valid, in statistical models, the discrepancy for pure or significant error i.e. should be 0.05% to confirm the model and demonstrate the validity of the response suface results[18].

3.3 Temperature Effect

Hydrolysis temperature, time, pH, substrate concentration, and enzyme concentration influence the yield reduction of sugar. Each enzyme has a temperature range of optimal activity. Increasing temperature will increase kinetic energy of the reactant and the reaction rate. If the enzyme exceeds the optimal limit, it denatures and no longer functions. A pH that is too acidic or alkaline can affect the reaction rate because thet enzyme molecule has an active compound whose shape does not or complements the shape of the other substrate. Small changes in pH do not cause permanent changes in the enzyme because the binding can reformed. However, extreme pH changes can cause enzymes expience denaturation and permanent loss of enzyme function [20]. The optimal pH for cellulose varies slightly, while the optimal temperature is around 20º-50ºC. If the temperature is below the optimum temperature, the enzyme works slowly. The enzyme denaturation wass occured at a temperature higher than the optimal condition[21]. The temperature about 30-45ºC in this study is the optimum condition for mesophilic bacteria in the anaerobic process. Misopolic bacteria are easy to maintain in good buffer conditions and can remain active at small temperature changes, especially if the changes are slow [19]. Glucose is the most preferred carbon source for clostridium sp, and all central carbon metabolism pathways are expressed constitutively, allowing efficient and rapid utilization of glucose. In addition, glucose is the main monosaccharide present in lignocellulosic biomass [22].

3.4 pH effect

The optimum fermentable pH for clostridium ctobutylicum bacteria is pH 5. At neutral pH of 7, clostridium acetobutylicum is inactive in producing biomass. Figure 5 shows that the optimum pH value in this experiment is 4.779, so that this value does not exceed 7 (neutral), so that the bacteria can work optimally [23].



1. Three neck pumpkin
2. Reverse cooling
3. Thermometer
4. Heaters
5. Magnetic Stirrer
6. Statives and clamps
7. Waterbath

Fig 1. Experimental set-up of hydrolysis acid and enzyme process

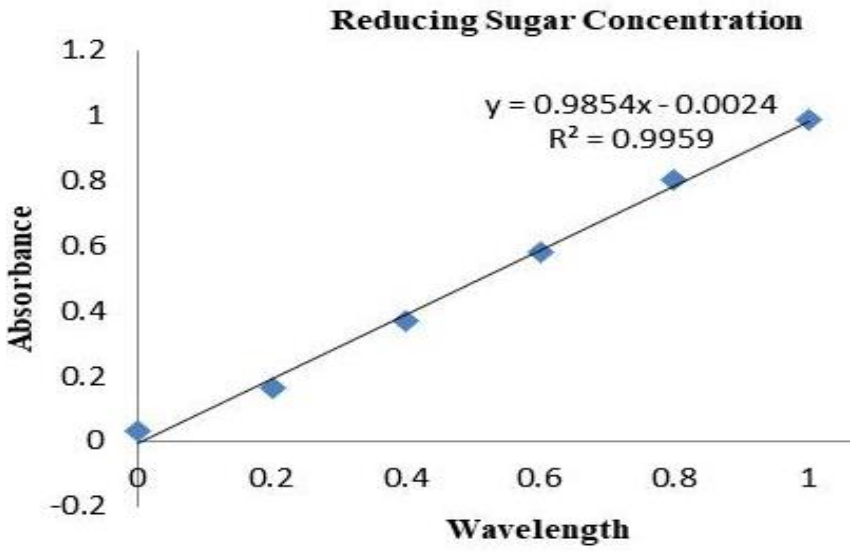


Fig 2. Curve standart of Reducing Sugar Concentration

Table 1. Analysis of variance (ANOVA) for response surface quadratic model

Factor	Sum of Squares	Df	Mean Square	F-Value	P-Value
(1)pH(L)	100.5399	1	100.5399	45.4550	0.000519
pH(Q)	349.5968	1	349.5968	158.0560	0.000016
(2)Temperature (C)(L)	8.2280	1	8.2280	3.7199	0.102026
Temperature (C) (Q)	27.5197	1	27.5197	12.4419	0.012408
(3) Time(hours)(L)	112.4369	1	112.4369	50.83338	0.000383
Time(hours)(Q)	209.2558	1	209.2558	94.6065	0.000068
1L by 2L	0.6498	1	0.6498	0.2938	0.607334
1L by 3L	4.1760	1	4.1760	1.8880	0.218544
2L by 3L	0.3120	1	0.3120	0.1411	0.720130
Error	13.2711	1	2.2119		
Total SS	669.991	6			

Table 2. Experimental factors in variable and experimental responses

Run	Independent Variable			Concentration	Dependent Variable
	X1	X2	X3	Reducing sugar (g/L)	Yield (%)
	pH	Temperature C	Time		
1	4	35	240	0.312	45.45
2	4	35	336	0.323	43.53
3	4	45	240	0.293	48.78
4	4	45	336	0.317	44.58
5	6	35	240	0.332	41.96
6	6	35	336	0.368	35.66
7	6	45	240	0.328	42.66
8	6	45	336	0.360	37.06
9	3.3	40	288	0.336	41.26
10	6.7	40	288	0.377	34.09
11	5	31.6	288	0.292	48.95
12	5	48.4	288	0.278	51.40
13	5	40	207.3	0.298	47.90
14	5	40	36.7	0.370	35.31
15	5	40	288	0.252	55.94
16	5	40	288	0.262	54.20

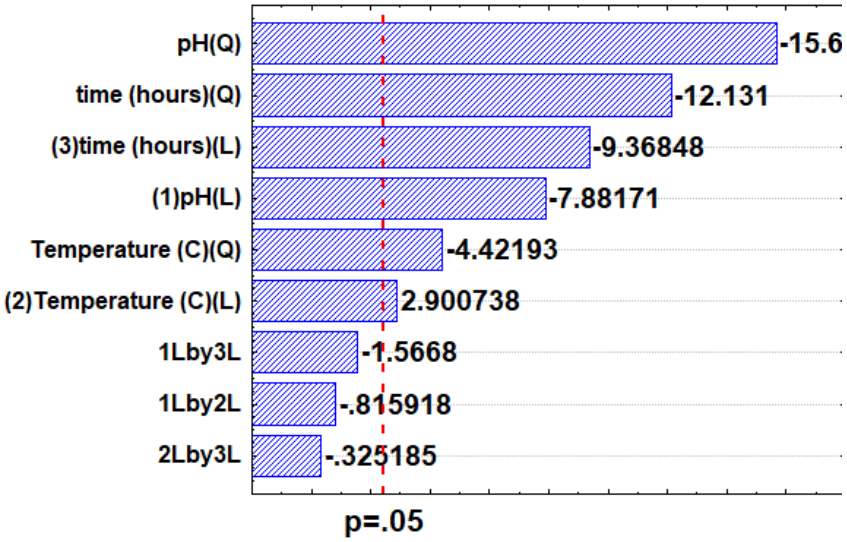


Fig 3. Pareto chart of standardized effects of sugars production

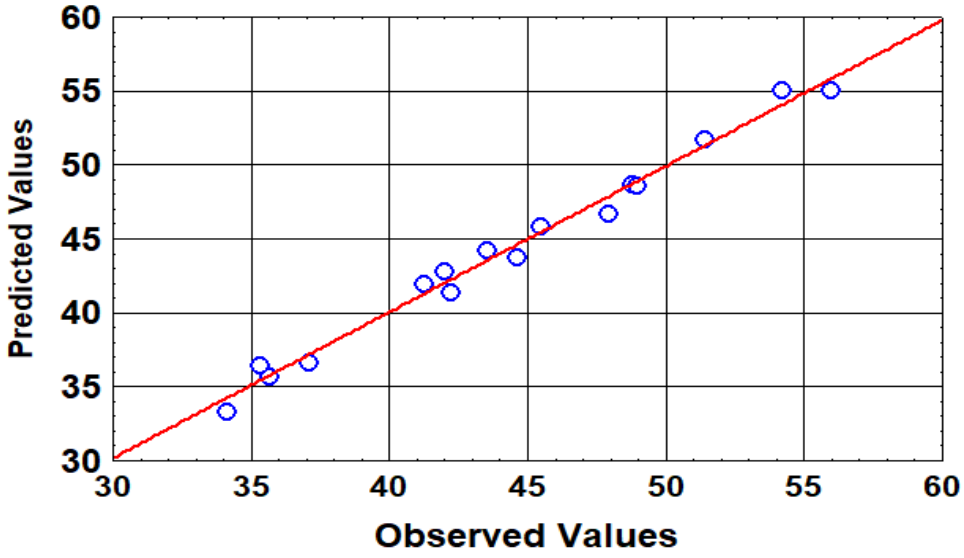


Fig 4. Predicted values vs observed values

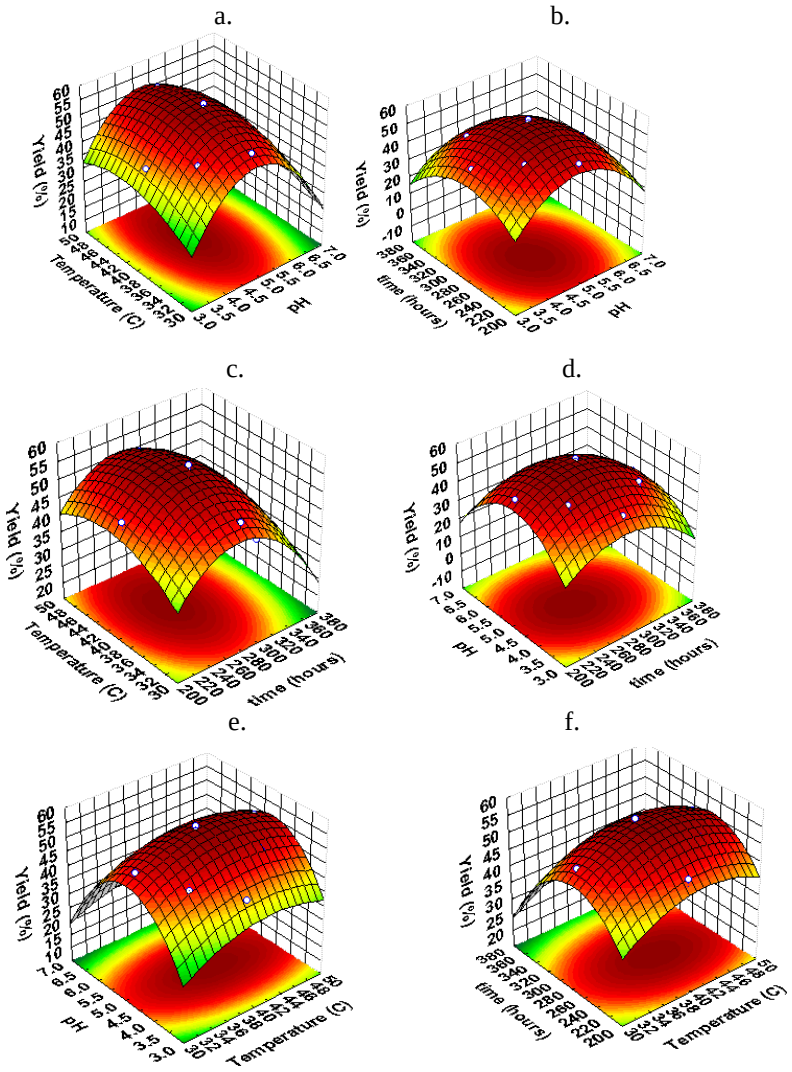


Fig 5. Response surface plot in optimum conditions

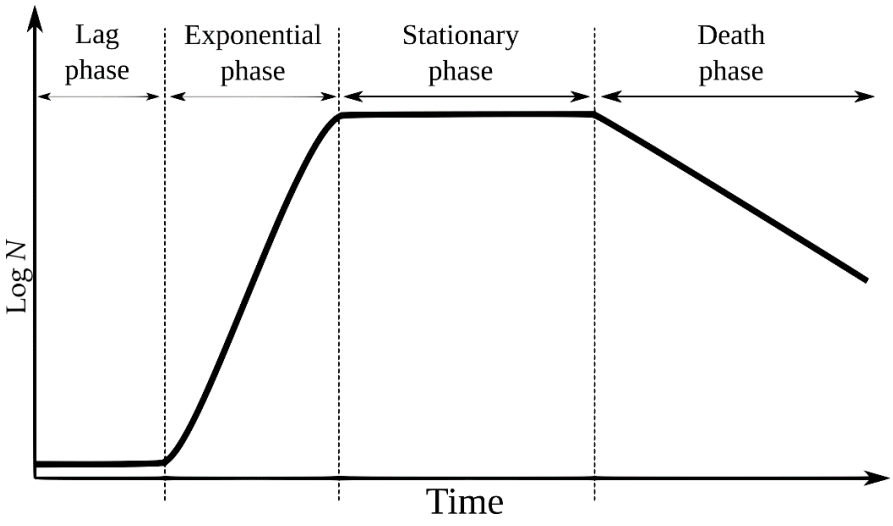


Fig 6. Microbial growth curve in a closed system, where N represents the number of bacterial cells [24]

3.5 Time reaction effect

The yield of reducing sugars increases with the length of hydrolysis time but then decreases after optimum conditions. The yield of sugar in enzymatic hydrolysis 8 hours, this shows that the higher the enzyme used, the higher the rate of formation of reducing sugar. The higher the growth rate of microorganism reaches the maximum, the increased digestion time will affect the reaction time. After 14 days or more, the yield tends to decrease because the optimal reaction time has exceeded what is known as the dead phase. For the picture can seen at figure 6

3.6 Ezymatic effect

The enzyme dose increases the sugar concentration to an optimum point, which tends to decrease. The hydrolysis reaction is inhibited when the solid content is high and the enzyme dose is low. This is related to issues of mass transfer and use of available water. Reaction inhibitors affect the kinetics of the reaction, causing longer hydrolysis time and a decrease in glucose productivity, which significantly affects the resulting product. However, inhibition can be avoided by using higher enzyme doses. Considering the economic feasibility on an industrial scale, low doses are often chosen compared to processes that require higher amounts of enzyme [21][25]

3.7 Cellulose, hemicellulose and Lignin content

This calculation is to determine the content of cellulose, hemicellulose and lignin. Lignin is one of the main factors that inhibits he enzymatic hydrolysis of lignocellulose. Lignin is physically able to inhibit enzymes in opening cellulse components, and is permannt in nature absorb cellulose there by reducing enzymatic hydrolisis activity[8] Enzymatic hydrolysis of lignocellulosic biomass begins with adsorption enzymes on the fiber surface. The morphology of the lignocellulosic substrate has a large influence on the initial hydrolysis rate[26] . The after intial treatment with alkaline can improves the efficiency of enzymatic hdrolysis by effectively removing lignin [27]. The content of hemicellulose is 14.2 %, cellulose s 2.9%, and lignin is 5% abu.

4. Conclusions

This study optimizes the analysis of reducing sugar as the main ingredient in biogas especially biobutanol, because it uses clostridium acetobutylicum bacteria with melon peel waste as raw material by acid hydrolysis followed by cellulose enzymes. The best results from this research were operating conditions of pH 5, temperature 40^{o} C, and fermentation time of 288 hours with a yield value of 55.94% and 54.20 % with a regression value is 0.98019. Based on the RSM optimization, the optimum operating condition at 41.126^{o}C for 273.51 hours at pH 4.7792, with a predicted yield of 55.887%.

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