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Optimzation of enzymatic hydrolysis and bioethanol fermentation using rice straw as a substrate

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ABSTRACT

Bioethanol is the result of glucose fermentation. Due to the high demand of glucose, the fuel is transferred to the used of lignocellulosic material, where lignocellulosic materials can be processed into bioethanol by hydrolysis and fermentation. The objective of this research was to obtain the optimal conditions of hydrolysis and fermentation. The first phase was hydrolysis, carried out with two factors, the first was a blend of enzymes cellulase from Trichoderma Reesei and Aspergillus Niger by ratio of 1:0, 0:1, 1:1, 1:2, 1:3, 2:1 and 3:1 (unit/unit), the second was the hydrolysis time, observations was done every 8 hours for 72 hours. The second phase was fermentation, carried out with two factors, the first was the hydrolyzed solution ph of pH 4, 5 and 6, the second was the amount of Saccharomyces Cerevisiae inoculums of 0.5% and 0.75%.

The optimal conditions of enzyme combination from Trichoderma Reesei and Aspergillus Niger for hydrolysis is 3:1 derived optimal hydrolysis time of 48.7 hours produce an optimal glucose and D-optimal value was 10% and 1.0000, respectively. While at the fermentation phase, ethanol derived optimal level and D-optimal value was 5.5% and 1.0000, respectively at pH 3.59 and 0.7462% of Saccharomyces Cerevisiae inoculum.

KEYWORDS

Optimization;
Hydrolysis;
Glucose;
Fermentation;
Saccharomyces cerevisiae;
Ethanol.

INTRODUCTION

Fuel is very important at this age, so high demand for fuel to make ends meet. But the supply of fuel sources dwindle, because the fuel source has been widely used for the unrenewable source, or can be renewable but it takes a very long time, so it cannot achieve the target to meet the community needed. There have been many studies to find the renewable fuel source, environmental friendly as well as producing or purchasing an affordable cost. Some research resulted that the product of biofuels were biodiesel and bioethanol. Biodiesel is often derived from plant oils esterified, one type of the plant is the Jarak leaf. While bioethanol is the result of fermentation of glucose. Glucose is a material with a relatively high price, it is because of the public needed for glucose is still quite high. So that other materials used are cellulosic materials, where cellulosic materials contain cellulose that can be converted into glucose through a process

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of hydrolysis using cellulase enzymes.

Straw is one of the agricultural waste that has not been optimally used. All of this time, rice straw is used for animal feed and mushroom growing media. Nevertheless straw still abundant and sometimes have to be burned. Indonesia produced 180 million tons of rice straw every year[12]. Cellulose in rice straw can be hydrolyzed to produce glucose which can be further fermented into biofuel. Hydrolysis of rice straw can be done chemically[15], enzymatically[7,13] or used cellulase-producing microorganisms[3].

Rice straw is lignocellulosic material, which implies that there are 3 affect the final production of the production of the enzyme, glucose or bioethanol, such as lignin, hemicellulose and cellulose. Lignin is part of a growing - plants contained in the cell wall and middle lamellar as well as serve as the glue between the cells, so that lignin is the unwanted materials. So do delignification prior to destroying the lignin that binds the cellulose that can be detached and can be processed. The polysaccharide component can be decomposition through a process of degradation or fermentation using enzymes cellulase. Cellulase is a mixture of several enzymes whose composition varies, depending on the microorganism used for the manufacture and production process. Three components have been identified in the cellulase is endo-glucanase (endo-β-1,4-D-glucan-4-glukanohidrolase, EC3.2.1.4) which breaks the bond β-1, 4 on the cellulose chains at random, exo-glucanase (β-1, 4-D-glucan-selobiohidrolase, EC 3.2.1.91) that solves the cellobiose units from the ends of the chain and β-glucosidase (EC 3.2.1.21) that solves cellobiose to glucose[4]. Trichoderma Reesei has been known to produce endo-glucanase up to 80%[11], but the production of β-glukosidase is low[10]. Most of the cellulase system produced by the fungus cellulosic, β-glukosidase amount lower than that required for the hydrolysis of cellulose to glucose efficiently, so that the main hydrolysis products glucose but not cellobiose[1,10,14], which is a potent inhibitor of the endo and exo-glucanase. This problem can be overcome by adding β-glucosidase from the outside[14] or producing cellulase by combining the ability of microorganisms of Trichoderma Reesei to produce endo-glucanase and exo-glucanase strongly as the ability of microorganisms to produce β-glucosidase strong as Aspergillus Niger[14].

Multi-enzyme cellulase is formed by several proteins. Working converting cellulose to glucose in the enzymatic hydrolysis for ethanol production process[9]. Hydrolysis is a chemical reaction that breaks down water molecules (H_2O) into hydrogen cations (H^+) and hydroxide anions (OH^-) through a chemical process. This process is used to break down certain polymers, especially those made by polymerizing grow incrementally (step-growth polymerization).

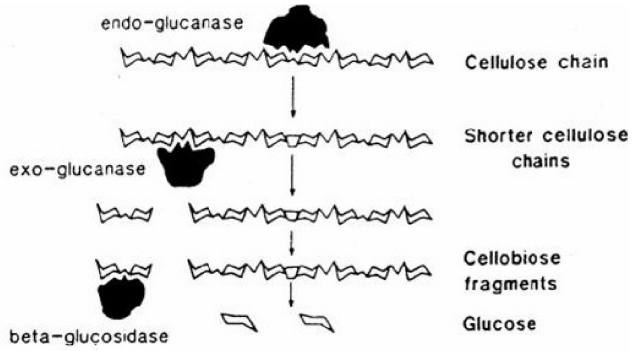

Figure 1 : Enzymatic cellulose hydrolysis reaction mechanism[5]

Having obtained hydrolyzate, fermentation can be carried out using Saccharomyces Cerevisiae. Saccharomyces Cerevisiae is a yeast species that has the very high power conversion of sugar into ethanol. Ethanol fermentation, also referred to as alcoholic fermentation, is a biological process in which sugars such as glucose, fructose, and sucrose is converted into cellular energy and also produces ethanol and carbon dioxide as byproducts.

Because this process does not require oxygen, simply by using the yeast fermentation, the ethanol fermentation is classified as anaerobic respiration. Ethanol production is mostly done biologically or through bioconversion technology, which is a technology enzymatic conversion of raw materials and biological (through fermentation). The polysaccharide component can be decomposited through a process of degradation or fermentation using yeast as a potential microbial activity such as Saccharomyces Cerevisiae for ethanol production. The process for bioethanol production from lignocellulosic materials in a simple chemical equation is as follows :

Lignocellulosic ——— Cellulase Enzyme → cellobiose and glucose ($C_6H_{12}O_6$)

cellobiose + H_2O(aq) —— cellobiase Enzyme → $C_6H_{12}O_6$ (aq) + $C_6H_{12}O_6$ (aq)

$C_6H_{12}O_6$ (aq) — Fermentation → 2 C_2H_5OH(aq) + 2 CO_2 (g)

Having obtained fermented, then distilled. Distillation is a technique to separate the solution into each component. Distillation principle is based on the difference in boiling point component substance. Distillation can be used to purify compounds having different boiling points so that it can be produced compounds that have a high purity.

Involved in the inoculum concentration greatly affect the effectiveness of producing fermentation products. If the concentration of the inoculum used too little then the fermentation process was slow[2], whereas the concentration of inoculum is too much, it will affect the competition between yeasts in taking up nutrients, so it affects the growth of yeast and alcohol levels that would be obtained. The higher concentrations of inoculum may not necessarily produce a high alcohol content[6].

Most organisms can function well in an interval of pH 3 to pH 4. Because PH is very important, most of the fermentation process is controlled by a buffer or a pH control system. Yeasts are usually more comfortable in a pH 3 to pH 6, while the fungi in pH 3 to pH 7, and the higher cariotype cells in pH 6.5 to pH 7.5[8].

This study has two objectives, the first objective of this study is to obtain optimal conditions of combined enzyme of fungi Trichoderma Reesei and Aspergillus Niger and hydrolysis time required to obtain the optimal value of glucose levels. While the second objective of this study is to obtain optimal pH conditions and the amount of Saccharomyces Cerevisiae inoculums optimal levels in order to obtain optimal values of ethanol levels.

MATERIALS AND METHODS

The research was conducted in July 2012 to March 2013 in the Mechatronics Laboratory of the Department of Agricultural Engineering Brawijaya University, Central Laboratory of Biological Sciences in Brawijaya University and Genetics Laboratory of Bio-molecular Universitas Islam Negeri Maulana Malik Ibrahim Malang.

In this study, there are several materials used, such as Trichoderma Reesei and Aspergillus Niger obtained from Microbiology laboratory PAU Food and Nutrition Gadjah Mada University Indonesia, Saccharomyces Cerevisiae obtained from Bio-industry laboratory Brawijaya University Indonesia, PDA (Potato dextrose agar), Inoculum solution (yeast extract, malt extract and glucose), nutrient solution (aquades, yeast extract, Bacteriological peptone, (NH4)2SO4, KH2PO4, FeSO4·7 H2O, CMC 1 %), tween 80, NaOH, HCl, PDB (Potato Dextrose Broth), reagent control DNS (Dinitrosalicylic acid), PDB (Potato Dextrose Broth) and aseptic protective material.

The tools used include: disk mill to grind rice straw, autoclave used for delignification and sterilization, water bath shaker for hydrolysis, waterbath for fermentation and centrifuge for separation.

Preparation of raw materials rice straw

Rice straw is dried first and then cut along the ± 2 cm. Then carried out with the grinding mill and sieved to 100 mesh size. Rice straw powder divided into two parts, the first part is used for cellulose enzyme production, the second part is used for delignification which to be used in Hydrolysis.

Delignification

Rice straw size of 100 mesh and 0.5 N NaOH in the ratio 1:10 (10 grams straw : 100 ml NaOH) is inserted into the autoclave at 304.50 Kpa pressure within 60 minutes. Sludge is then dried for 24 hours at a temperature of 105 °C in the oven. Straw powder is then used in the enzymatic hydrolysis process (powdered straw pretreatment results).

Cellulase enzyme production

Fungi Trichoderma Reesei and Aspergillus Niger cultured in PDA for 7 days, then fungi inoculated in inoculums solution for 3 days. The medium used for the cultivation of cellulase enzymes as many as 5 grams of 100 mesh size powder straw without pretreatment erlemeyer put in 250 ml size with a nutrient solution was added 25 ml. Erlemeyer closed with sterile cotton, coated with aluminum foil and tied with rubber. Media sterilized using an autoclave for 15 minutes at 121 °C. Media sterilization results first cooled and then cultured Trichoderma Reesei and Aspergillus Niger inoculated into the media as much as 2%. Then the media which were filled with fungi Trichoderma Reesei and Aspergillus Niger incubated over time in accordance with the treatment. Extracting an enzyme is done by using 100 ml of a solution of 1% Tween 80. Precipitate and fermented liquid separated using centrifuge for 30 minutes at 4000 rpm. The yield of the centrifuged obtained cellu-

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lase enzyme liquid.

Hydrolysis

Rice straw which is the result of pretreatment 100 mesh fitting 5 grams added in the Erlenmeyer and as well as 50 ml of citrate buffer and 50 ml of enzyme cellulase. Then Erlenmeyer closed using aluminum foil and tied with rubber. Then placed the erlenmeyers in the water bath shaker 75 rpm stirring treated with 50^{O}C temperature. When extracting solution separated using a cold centrifuge and hydrolyzed solution obtained.

Fermentation

Hydrolyzed solution after being separated then added in to erlenmeyer according the pH condition treatment and Saccharomyces Cerevisiae inoculums was added in accordance with the treatment variable. Then incubated in a waterbath at 30 ^{O}C for 48 hours.

Research design

Research carried out by simple randomized block design. The research is divided into two phases, the first phase is the enzymatic hydrolysis with 2 factors, namely the value of the combination of cellulase enzymes by Trichoderma Reesei : Aspergillus Niger as much as 1:0, 0:1, 1:1, 1:2, 1:3, 2:1 and 3:1 (unit/unit), and observations done in 72 hours. Analysis performed every 8 hours, in order to obtain 63 treatments with 3 repetitions, in order to obtain 189 data. The third phase is fermentation with 2 factors, namely pH of hydrolyzate namely pH 4, pH 5 and pH 6 and the amount of inoculums of Saccharomyces Cerevisiae by 0.5% and 0.75%. In order to obtain 6 observations with 3 times 18 repetitions in order to obtain the data.

Research optimization

Research optimization calculations performed using Response Surface Methods using Minitab program.

RESULT AND DISCUSSION

Materials preparation

Rice straw is dried and cut into pieces along approximately 2 cm in order to facilitate the work of grinding, then ground and sieved with a 100 mesh sieve size. The entire series of treatments in order to obtain rice straw powder 100 mesh. The rice straw powder then divided into two part, the first part is use to produce cellulose enzyme, and the second part is used for delignification as it is used for enzymatic hydrolysis.

The next phase is delignification which is done by using an autoclave. Delignification aims to destroy lignin to cellulose can get out of the bond and can be processed. After delignification then neutralized and dried into a 100 mesh powder of rice straw back. Then tested levels of lignin, hemicellulose and cellulose. Thus obtained:

Enzyme production sellulase

Cellulase enzyme produced in this study is the cellulase enzyme from fungi Trichoderma Reesei and Aspergillus Niger rice straw as the substrate. The phase of the cellulase enzyme production begins with the selection of

TABLE 1: Content of rice straw

Component	Content (%)
Hemicellulose	16.615
Cellulose	23.155
Lignin	8.715

Source: Analysys certificate from UGM (2012)

microbes that being used which is Trichoderma Reesei with the consideration that the type of microbes capable of producing endo-β-1.4-glucanase and exo-β-1.4-glucanase up to 80% and Aspergillus Niger to produce a high β-glucosidase. Furthermore microbes are cultured on PDA (Potato Dextrose Agar) slant in a zig-zag and incubated at a temperature of ± 30°C for 7 days. Subsequently the culture inoculated in inoculums solution for 3 days and then suspended into the media in the form of rice straw fermentation and nutrient solution, which where the sludge was sterilized first by using autoclave. Enzyme extracting process is done by separating sludge and liquid fermentation using a centrifuge with a speed of 4000 rpm for 30 min at 4°C to obtain the enzyme liquid (supernatant). Cellulase enzyme production is then performed with the enzyme activity measured using CMCase method and cellulase enzyme activity obtained in accordance the data below:

Enzymatic hydrolysis

After analyzed the cellulose enzyme activity, followed by hydrolysis. In this research, total of 5 grams of 100 mesh rice straw powder which is the result of pretreatment inserted into a 250 ml beaker glass. Unification of 100 mesh size is expected to increase the surface area for the substrate can bind to the enzyme. Further into the glass beaker was added a solution of

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TABLE 2 : Enzyme activity of the combined cellulase enzyme

Treatment	Enzyme Combination		Enzyme Activity (IU/ml)
	Trichoderma Reesei	Aspergillus Niger	
A	1	0	2.304
B	0	1	2.178
C	1	1	1.486
D	1	2	1.508
E	1	3	1.575
F	2	1	1.664
G	3	1	2.490

Source: Analysis of enzyme activity data research

citrate buffer pH corresponding variables are used, namely pH 5.5. After the acclimatization process has been reached, then cellulase enzyme added into the beaker glass accordance to the treatment with the volume of the enzyme versus citrate buffer 1:1 in order to obtain as much liquid volume 100 ml. Glass beaker covered with plastic and then covered with aluminum foil. Materials that have been prepared are then incubated in the waterbath shaker at 50°C according to the characteristics of Trichoderma Reesei and Aspergillus Niger in producing cellulase. Moreover done mixing process with shaker speed 75 rpm. Mixing process is done so that the contact between the enzyme with substrate is more common that the desired product is formed. The results of measurement of glucose levels in accordance with data below:

The highest glucose measured of enzymatic hydrolysis by using rice straw is 12,244% of the total solution or by 26.439% of the content of cellulose in 3:1 volume ratio of cellulase enzymes (Trichoderma Reesei: Aspergillus Niger). In this study, the average glucose produced ranges between 2.090% -12.244% of the total solution

TABLE 3 : Glucose levels the results of hydrolysis

Treatment	Presentage of Glucose (%)								
	8 Hour	16 Hour	24 Hour	32 Hour	40 Hour	48 Hour	56 Hour	64 Hour	72 Hour
A	2.090	2.284	3.738	4.773	5.934	6.492	7.217	7.471	7.250
B	2.278	2.798	4.054	5.359	6.492	7.455	8.306	10.485	8.168
C	2.306	3.019	4.070	5.608	6.603	8.533	8.500	7.173	6.835
D	2.395	3.158	4.087	5.746	6.752	8.970	8.909	7.709	6.371
E	2.583	3.340	4.623	5.939	6.996	9.849	9.014	7.803	6.841
F	2.632	3.617	5.868	6.127	7.267	10.507	9.180	8.323	7.128
G	2.859	3.877	7.029	7.256	10.535	12.244	9.717	8.893	7.261

Source: Analysis of glucose data research

or 4.514%-26.439% per cellulose contained.

Contour of the above showed that the greater the cellulose enzyme activity used, the greater glucose levels were obtained. But in terms of the time it can be seen that the optimal conditions for the production of glucose is from 40 to 70 hours.

In the optimization calculations obtained optimal treatment conditions hydrolysis is cellulose enzyme with enzyme activity 2.6979 IU/ml with 48.7 hours long hydrolysis time with D-optimal value 1.000. By enzymatic hydrolysis obtained an equation :

$$Y = 6.49267 - 0.35321X_1 + 1.14658X_2 + 2.39506X_3 + 0.68794X_2^2 - 1.74706X_3^2 - 0.03175X_2X_3$$

From the equation, the value of X_1 is a group of simple randomized block design calculations, X_2 is a blend of cellulase enzymes treatment of fungus Trichoderma Reesei and Aspergillus Niger, while the X_3 is the time of treatment. Based on the analysis of variance, there was no significant difference between treatments in the process of hydrolysis.

Bioethanol fermentation

Hydrolysis solution was inserted in the Erlenmeyer

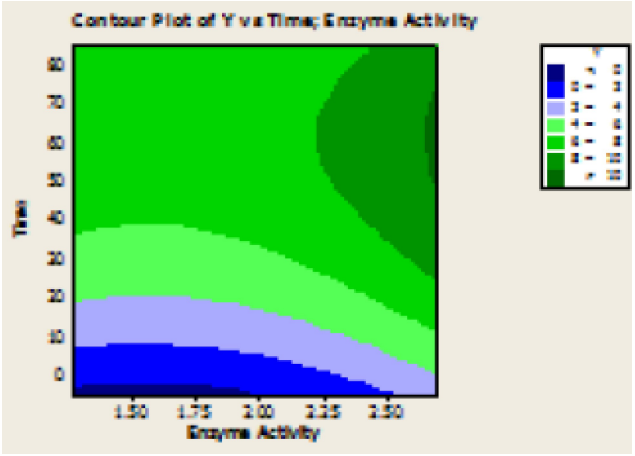

Figure 2 : Contour plot of glucose level

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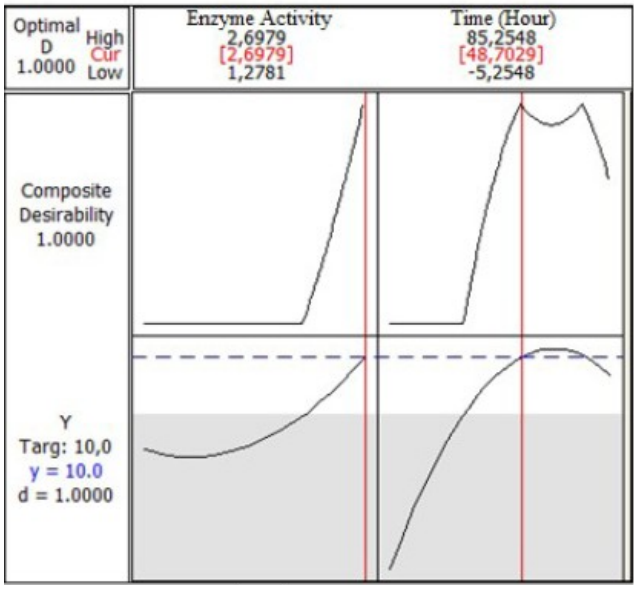

Figure 3 : Hydrolysis optimization

and set the acidity level at first accorded to the variable, which were pH 4, pH 5 and pH 6. Then added with inoculum of Saccharomyces Cerevisiae was done in accordance with the treatment, is 0.5% and 0.75%. Fermentation was done by using waterbath with a temperature of 30^{O}C for 48 hours. After fermentation, ethanol content obtained in accordance with the table below:

Thereunder by Judoamidjojo[8] that yeast can produce well at pH 3 to pH 6, which in this study as TABLE 4 shows that pH 4 produced the highest levels of ethanol, amounting to 5.664%. Also in line with the statement Astawan and Mita[2], the inoculum concentration greatly Affect the effectiveness of producing fermentation products. If the concentration of the inoculum used too little then the fermentation process was slow. Which in this study suggests that as many as inoculum 0.75% Saccaromyces cerevisiae produces ethanol levels higher than fermentation with Saccharomyces cerevisiae inoculum as 0.50%.

TABLE 4 : Ethanol level

Treatment		Ethanol Level (%)
pH	Saccharomyces Cerevisiae Inoculum (%)	
4	0.50	4.88
	0.75	5.664
5	0.50	4.555
	0.75	5.563
6	0.50	3.76
	0.75	4.414

Source: Analysys ULP certificate from the faculty of pharmacy, Airlangga university (2013)

Figure 4 showed that the lower the pH level and the higher amount of inoculums, the higher levels of ethanol produced.

Figure 5 showed that the optimal condition of bioethanol fermentation is at pH 3.5862 with the amount of inoculums 0.7462%, with the value of D-optimal 1.0000, obtained the ethanol level of 5.5%. By fermentation treatment, obtained an equation :

$$Y = 4.76667 - 0.02305X_1 - 0.38468X_2 + 0.35663X_3 - 0.14850X_2^2 + 0.10175X_3^2 - 0.03258X_2X_3$$

From the equation, the value of X_1 is a group of simple randomized block design calculations, X_2 is the pH, while the X_3 is the amount of inoculums. Based on the analysis of variance, there was no significant difference between treatments in the treatment of fermentation.

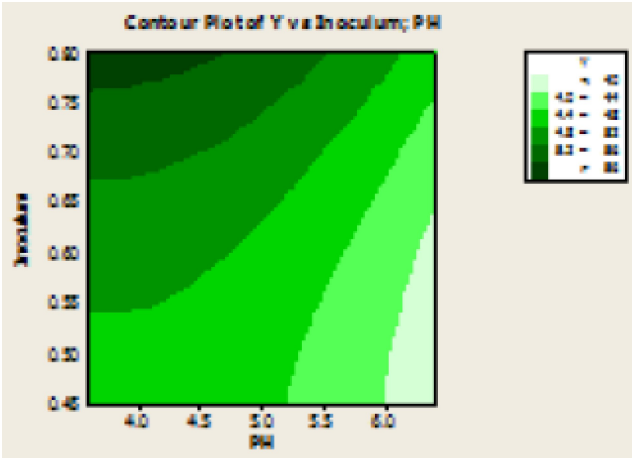

Figure 4 : Contour plots of ethanol level

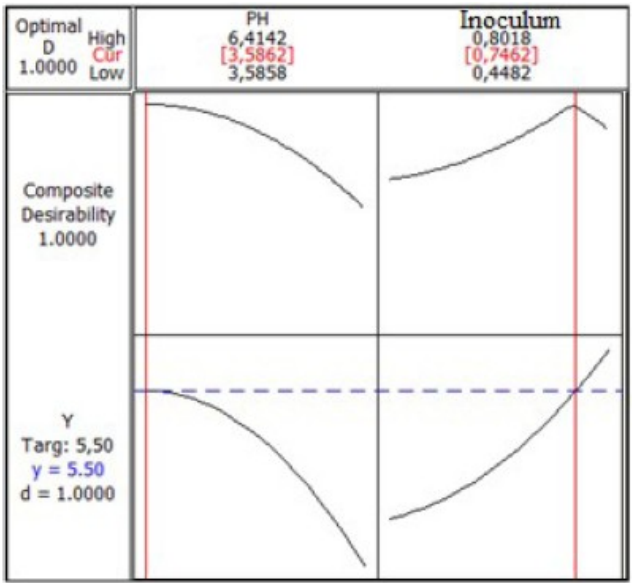

Figure 5 : Fermentation optimization

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CONCLUSION

From the results of the research which has been done, can be drawn some of conclusions among others:

1. In the hydrolysis phase, the optimal condition is with a combination of cellulase enzymes from Trichoderma Reesei and Aspergillus Niger with the value of enzyme activity at 2.6979 IU/ml with 48.7 hours long hydrolysis of treatment in this study with an average percentage of glucose 10% with D-optimal value of 1.0000.
2. At fermentation phase, the optimum condition is at pH 3.59 with 0.7462% of inoculum by optimal ethanol concentration of 5.5% with D-optimal value of 1.0000.

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