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Optimization of Temperature and pH of Endoglucanase Enzyme Activities in Water Plants using the Response Surface Methodology (RSM)

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A B S T R A C T

This study aims to determine the optimal temperature and pH for the growth of cellulolytic bacteria isolates to produce high cellulase enzyme activity. This study used a potential cellulolytic bacterial isolate B2S8 which had the highest cellulase enzyme activity and the highest cellulose degradation rate in previous studies. This study used Carboxymethyl Cellulose (CMC) as a substrate in growth media and tested enzyme activity. Optimization of temperature and pH on cellulase enzyme activity using the Response Surface Methodology (RSM). The results of the analysis using the Response Surface Methodology (RSM) with the Central Composite Design (CCD) method showed that the highest cellulase enzyme activity occurred at a temperature of 36.9 ºC and pH 6.9 resulting in an endoglucanase enzyme activity value of 0.0269 IU/mL.

Keywords: Cellulolytic bacteria, endoglucanase enzymes, activity, Response Surface Methodology.

1. Introduction

Enzymes are proteins that function as catalysts for biochemical processes. An enzyme can accelerate reactions 108 to 1011 times faster than without using a catalyst (Poedjiadi and Supriyanti, 2006). Enzymes can be produced by groups of bacteria, molds and yeast (Imas, 2009). One type of enzyme that has an important role in the bioconversion of organic wastes is the cellulase enzyme. Cellulase-producing microorganisms from the bacteria group are the main choice because they have fast growth so that the time needed to produce cellulase becomes shorter (Alam et al, 2004). So it is very suitable for the production and activity test of crude cellulase enzymes from cellulolytic bacteria which can then be used as an ingredient in the manufacture of bioethanol (Chasanah et al., 2013).

Cellulase enzymes are enzymes capable of degrading cellulose with its main products namely glucose, cellobiose and cellooligosaccharides. Cellulase has an enzyme system consisting of endo-1,4-β-glucanase, exo-1,4-β-glucanase and β-D-glucosidase. These three enzymes work synergistically to degrade cellulose and release reducing sugars as the final product (Kim, 2001). The production of cellulase enzymes uses Carboxymethyl Cellulose (CMC) media because this media contains cellulose which is used as a substrate in enzymatic reactions (Meryandini et al., 2010). Carboxymethyl Cellulose (CMC) is the best substrate for inducing the synthesis of extracellular cellolytic enzymes and a concentration of 1% CMC is the optimum concentration for cellulase production (Alam et al., 2004).

Cellulase enzyme activity is influenced by several factors including temperature, pH, substrate concentration, enzyme concentration and the presence of inhibitors (Hames and Hooper, 2005). Temperature and pH are the main factors that must be known (Sari, 2008), because each enzyme will function optimally at a certain temperature and pH. The speed of the reaction decreases sharply above the optimal temperature because enzymes are proteins that will be denatured at high temperatures (Fitriani, 2003). In addition, a slight shift in pH from the optimum pH will also cause major changes in the reactions catalyzed by enzymes (Murray et al., 2003). This is because the amino acid which is the active center of the enzyme must be in a state of constant ionization in order to become active, because in essence an enzyme is a protein composed of amino acids that can ionize (Hames and Hooper, 2000).

The characterization of cellulase enzymes in this study aims to determine the optimal temperature and pH for the growth of cellulolytic bacterial isolates B2S8 in producing cellulase enzymes (endoglucanase), which are the best potential isolates of cellulolytic bacteria that have gone through the specific cellulase enzyme confirmation test from previous research conducted by Nababan (2018). . Therefore, determining the optimal conditions, especially temperature and pH, needs to be studied further. The optimization process is carried out using a statistical analysis method, namely the Response Surface Methodology (RSM). The RSM method is a set of mathematical and statistical techniques that are useful for analyzing problems where several independent variables affect the response variable and the ultimate goal is to optimize the response (Montgomery, 2001). The advantages of the RSM

method include that it does not require large amounts of experimental data and does not take a long time (Iriawan & Astuti, 2006). Therefore, with the RSM method using software (Minitab17), it is expected that the optimization process can be much faster and more accurate.

2. Material and Method

2.1 Time and Reasearch Location

The research was conducted at the Bioindustry and Environment Laboratory, Microbiology Laboratory and Food Analysis Laboratory, Faculty of Agricultural Technology, Udayana University. The time of conducting the research is July–September 2018.

2.2 Tools and Materials

The tools used in this research are UV-Vis Spectrophotometer (Thermo-scientific), pH meter (Schott instrument), autoClave (Hirayama), laminar air flow (Kojair), vortex (Maxi Max II), waterbath shaker, measuring cup (Pyrex), Erlenmeyer (Pyrex), test tube (Pyrex), thermometer, micro pipette, dropper pipette, microtube, coolbox, magnetic stirerr, hot plate, analytical balance, Bunsen, clamp, cotton, aluminum foil. The materials needed in this study were: cellulolytic bacteria isolates B2S8 which had been screened and ranked in previous studies which were isolated from forest soil in Bali, K2HPO4, (NH4)2SO4, MgSO4.7H2O, NaCl, Yeast extract, CaCl2.2H2O , distilled water, sodium citrate buffer, NaOH, Carboxymethyl Cellulose (CMC) (MERCK), dinitro salicylic acid (DNS) reagent.

2.3 Trial Design Using Response Surface Methodology (RSM)

The Response Surface Methodology (RSM) model is used to see the optimal conditions of temperature and pH for the growth of cellulolytic bacteria in producing cellulase enzymes. The independent variable/factor x is temperature and pH to analyze the y response (cellulase enzyme activity). The experimental design used was a two-factor Central Composite Design (CCD). From the CCD results obtained a value of 1.414. The center point of temperature variations in this study refers to the best results from research conducted by Aruwajoye (2014) and Irawati (2016), namely the optimal temperature for the highest crude cellulase extract activity in CMCase is 37 °C. Determination of the optimal pH for cellulase enzyme activity refers to research from Susanti (2011) and Putri (2016) which states that the optimal pH for cellulase enzyme activity is pH 7.

This study uses a temperature of 27.1005 oC; 30 oC; 37oC; 44 oC and 46.8995 oC where 27.1005 oC and 46.8995 oC are the temperatures resulting from the formulation using the Response Surface Methodology (RSM) with a pH of 5.58579; 6; 7; 8 and 8.41421 where pH 5.58 and 8.41 is the pH resulting from the formulation using the Response Surface Methodology (RSM). The number of experimental units tested based on the CCD can be seen in Table 1. The results of the design using the CCD then became an experiment that obtained 13 experimental units. The data obtained were analyzed to obtain the shape of the response surface and determine the optimal conditions of the experiment using the Minitab 17 statistical tool. The research data are presented in the form of tables and figures.

2.4 Research Implementation Sample Preparation

The best potential isolate sample for cellulolytic bacteria was obtained from the results of the specific cellulase enzyme confirmation test from a previous study conducted by Nababan (2018). The ability of these cellulolytic bacterial isolates to produce cellulase enzymes has gone through a qualitative testing stage, namely by measuring the clear zone so that further quantitative testing is needed to determine the ability of these cellulolytic bacterial isolates to produce cellulase enzymes.

2.5 Media Creation

The media used is Basic Liquid Media (BLM) liquid media which contains CMC (Carboxyl Methyl Cellulose) substrate which is a derivative of cellulose which dissolves easily in the medium and is easily hydrolyzed (Ambriyanto, 2010). Liquid media that has been mixed with distilled water with a pH of 6, 8–7 then sterilized using an autoclave at 121 oC for 15 minutes. The media is cooled to a temperature of 50 oC. The media must be sterile (not overgrown with other unwanted microbes) so that the resulting microbial culture is not contaminated (Suriawiria, 2005).

2.6 Characterization and Production of Cellulase Enzyme Crude Extract

A total of 5 Erlenmeyer pieces containing 100 mL of Basic Liquid Medium (BLM) with a mixture of CMC (Carboxylmethyl Cellulose) substrate with a concentration of 1%, were repeated 3 times to obtain 15 experimental units, each set at pH 6.8– 7, then inoculated with selected cellulolytic bacteria isolates with an Optical Density (OD) value of 1 mL and incubated in a waterbath shaker for 3 days (Pandey et al., 2015) at various temperatures, namely 27.10 oC; 30 oC; 37oC; 44 oC and 46.89 oC. Enzyme characterization was carried out in stages, the first was to determine the optimum temperature, then the optimal temperature data obtained was used in the second characterization process, namely by varying the pH.

A total of 5 Erlenmeyer pieces containing 100 mL Basic Liquid Medium (BLM) with a mixture of CMC (Carboxylmethyl Cellulose) substrate with a concentration of 1%, were repeated 3 times to obtain 15 experimental units, each set at a varying pH of 5 .58; 6; 7; 8 and 8.41 were then inoculated with selected cellulolytic bacterial isolates with an Optical Density (OD) value of 1 mL and incubated in a waterbath shaker at the optimal temperature of 37oC for 3 days (Pandey et al., 2015). After incubation, 10 mL of each culture was centrifuged at 5000 rpm for 20 minutes at 4 oC and the supernatant served as the crude enzyme which was used to determine the enzyme activity (Immanual et al., 2006).

A total of 13 Erlenmeyer pieces containing 100 mL Basic Liquid Medium (BLM) with a mixture of CMC (Carboxylmethyl Cellulose) substrate with a concentration of 1%, were repeated 3 times to obtain 39 experimental units, each set at a varying pH of 5 .58; 6; 7; 8 and 8.41 were then inoculated with

selected cellulolytic bacterial isolates with an Optical Density (OD) value of 1 mL and incubated in a waterbath shaker at various temperatures, namely 27.10 oC; 30 oC; 37oC; 44 oC and 46.89 oC for 3 days (Pandey et al., 2015). After incubation, 10 mL of BLM which had been incubated with the culture was centrifuged at 5000 rpm for 20 minutes at 4 oC and the supernatant was the crude enzyme used to determine enzyme activity (Immanual et al., 2006). This design with 13 experimental units is the result of the formulation of the Response Surface Methodology (RSM) to predict the optimum temperature and pH of isolate B2S8 in producing cellulase enzymes which are then matched with the results of previous experiments to obtain accurate results. The use of CMC media in the production of crude cellulase enzymes functions as a substrate and as an inducer to produce crude cellulase enzymes. CMC substrates are also used by bacteria as a carbon source to produce glucose (Apriani et al., 2014). Next, an endoglucanase (CMCase) activity test was performed (Prazad et al., 2013).

2.7 Cellulase Enzyme Activity Test (Endoglucanase)

The activity of the cellulase enzyme (endoglucanase) was tested using a reaction mixture containing 1 mL of crude enzyme solution which had previously been centrifuged during the production of crude cellulase enzymes from selected isolates with 1 mL of 1% CMC solution in 50 mM sodium citrate buffer (pH 5.0) incubated at 50 oC for 15 minutes. The reaction was stopped by adding 1 mL of dinitrosalicylic acid and then boiling for 5 minutes (Dar et al., 2015).

2.8 Cellulase Enzyme Activity Calculation

Cellulase activity is expressed in international units, namely units/mL. One unit is the amount of enzyme required to break down 1 µmol of cellulose into reducing sugars per minute under the test conditions. Glucose levels resulted from the hydrolysis of cellulose with cellulase enzymes based on the absorbance value at λ 540 nm (Huang et al., 2012; Duza and Mastan 2013; Dar et al., 2015).

Glucose concentration converted in units of IU/mL:

IU/mL = 1 µmol/minute of glucose produced

= 0.18 mg/min glucose

So that:

$$Enzyme\ Activity = \frac{\text{Mass of glucose}}{BMG\ x\ Ve\ x\ T}\frac{\mu moll}{Minute\ ml}\left(\frac{Unit}{mL}\right)$$

Information:

BMG = Glucose Molecular Weight (180 g/mol)

Ve = Enzyme Crude Extract Volume (mL)

T = Incubation Time (minutes)(Diedit)

3. Result and Discussion

3.1 Characterization and Production of Cellulase Enzyme Crude Extract

Aulanni'am (2005) stated that cellulase enzymes from cellulolytic bacteria belong to the class of extracellular enzymes. The main function of extracellular enzymes is to convert nutrients around the cell and bring them into the cell as energy for cell growth. The crude enzyme extract is obtained by separating the bacterial suspension with the supernatant as the crude enzyme which has been characterized based on temperature and pH previously by means of centrifugation (Aulanni'am, 2005). CMC in production media functions as a substrate and at the same time as an inducer to produce cellulase enzymes. Inducer (inducer) is a compound that is needed to induce gene expression to occur, in accordance with the Jacob-Monod theory of enzyme induction. CMC substrates are also used by bacteria as a carbon source to produce glucose (Apriani et al., 2014). Enzyme production in this study used a 1% CMC substrate concentration based on research conducted by Jennifer and Thiruneelakandan (2015).

3.2 Effect of Temperature on Cellulase Enzyme Activity

Based on statistical analysis using the Response Surface Methodology (RSM) temperature affects the growth of cellulolytic bacteria B2S8 isolates in producing cellulase enzymes. Manual testing is carried out to find the optimal temperature range and prove that the results of the optimal temperature formulation according to the Response Surface Methodology (RSM) provide appropriate and accurate results. Based on the data generated in the previous treatment, the optimum temperature for the crude extract of the cellulase enzyme isolate B2S8 was at 37 °C with an enzyme activity of 0.0203 ± 0.0004 IU/mL. This study is comparable to that of Aruwajoye (2014) with the optimal temperature of 37 °C resulting in cellulase enzyme activity of 1.7 Units/mL. Irawati's research (2016) with the optimal temperature results for the highest crude cellulase extract activity in CMCase by Bacillus circulans 37 °C resulted in a cellulase enzyme activity value of 0.0219 Unit/mL.

The optimal temperature is the most appropriate temperature for a reaction that uses enzymes (Poedjiadi and Supriyanti, 1992). Temperature affects enzymatic reactions. An increase in temperature will generally increase the speed of the chemical reaction of the enzyme, but a temperature increase that is too high will cause denaturation of the enzyme, namely a change in the structure of the enzyme protein, causing a decrease in the speed of the reaction catalyzed by the enzyme (Saropah et al., 2012). Most cellulase enzymes have optimal activity in the temperature range of 20 – 50 °C. Enzymes that actively work in this temperature range belong to the mesozyme group (Saropah et al., 2012). Meanwhile, cellulase enzymes that have optimal activity at a temperature range of 50–80 °C belong to the thermozyme group or are often referred to as thermostable (heat resistant) (Meryandini et al., 2009). Based on this, the cellulase enzyme produced from cellulolytic bacteria B2S8 isolate belongs to the mesozyme group. ***Pengaruh pH terhadap Aktivitas Enzim Selulase***

Cellulase enzyme activity testing at various pH was carried out using the optimal temperature resulting from the previous treatment, namely 37 °C. Manual testing was carried out to find the optimal pH range and prove that the results of the optimal pH formulation according to the Response Surface Methodology (RSM) gave accurate results. Based on the data generated in the previous treatment, the optimal pH result of the crude extract of the cellulase enzyme isolate B2S8 was at pH 7 with enzyme activity of 0.0267 ± 0.00047 IU/mL. This study is comparable to that of Susanti (2011) who stated that the optimal pH of crude cellulase extract activity was highest in CMCase by Bacillus circulans pure culture collection ITB Microbiology Laboratory with a value of 129.97 U/mL at pH 7. Putri's research (2016) stated that the optimal pH of activity The cellulase enzyme produced by L. plantarum with a value of 0.054 IU/mL is at pH 7. The pH range for cellulase activity corresponds to the optimal pH range for CMCase activity, which is in the pH range of 4.5–7.0 (Fikrinda, 2000). Cellulase enzymes produced by bacteria work maximum at a pH close to neutral (pH 6-7) (Rasul et al., 2015). Sari (2010) explained that at pH 7 (neutral) about 90% of the amino acids making up the enzyme are in the active form. This shows that the optimum pH of the cellulase enzymes produced by isolate B2S8 is optimal at low acidic pH to close to neutral pH. Sari (2010) added that pH conditions that are too acidic or too basic can cause the amino acids that make up the enzyme to become inactive.

3.3 Effect of pH on Cellulase Enzyme Activity

The results of the analysis of cellulase enzyme activity against temperature and pH treatments can be seen in Table 2. The value of the enzyme activity used in the calculation using the Response Surface Methodology (RSM) is the average value of the results of the replicates, while the center point is at 37 ºC and pH 7 used in the calculation is the repeat value of the enzyme activity. Analysis of cellulase enzyme activity obtained regression results with the regression equation model, namely: Y = -0.495 + 0.011540, Temperature + 0.08881, pH – 0.000152, Temperature 2 – 0.006245, pH2–0.000048 The RSM equation shows that changes in pH have the greatest effect on the results of the cellulase enzyme activity test. This influence can be seen from the pH coefficient which has the largest value compared to the incubation temperature. This might happen because the determination of the range of pH code values is too narrow so that the difference in the results is not too much different from the incubation temperature. Statistical calculations show that the coefficient of determination (R2) = 98.08%. The value of the coefficient of determination gives the understanding that the correlation between temperature and pH on cellulase enzyme activity is 98.08% while the remaining 1.92% is influenced by other factors which cannot be explained by the response.

The results of the lack of fit test can be used to test the suitability of the model. Lack of fit data on cellulase enzyme activity has a p-value of 0.094 where the p-value of lack of fit is greater than the value of α (5%), meaning that the model made is appropriate (Myers and Montgomery, 1995). Thus, the model equation is very valid and able to predict responses, because an RSM equation is valid or not determined from the data regression test. The curve formed in Figure 1 resembles an inverted parabola that forms the maximum response. The increase in cellulase enzyme activity occurred up to a temperature of 36.9 ºC with a pH of 6.9, but after passing a temperature of 36.9 ºC and a pH of 6.9 the activity of the cellulase enzyme decreased. The increase in the value of cellulase enzyme activity is directly proportional to the increase in incubation temperature and pH until it reaches the optimal point (Fig.1).

The contour plot graph of cellulase enzyme activity is seen in Figure 2. The color change on the contour plot graph shows that there are differences in the values of cellulase enzyme activity with different combinations of temperature and time. The green contour plot graph shows that the cellulase enzyme activity value is greater than 0.025 IU/mL, while the blue color indicates the oleoresin yield value is less than 0.000 IU/mL. Based on statistical analysis using the Response Surface Methodology (RSM), the optimal condition for the response obtained based on the optimization value for cellulase enzyme activity as shown in Figure 3 shows that the optimal temperature is 36.9 ºC with a pH of 6.9. The predicted enzyme activity at that point is 0.0269 IU/mL.

3.4 Results of Cellulase Enzyme Activity Data Analysis Using RSM

The results of the analysis of cellulase enzyme activity against temperature and pH treatments can be seen in Table 2. The value of the enzyme activity used in the calculation using the Response Surface Methodology (RSM) is the average value of the results of the replicates, while the center point is at 37 ºC and pH 7 used in the calculation is the repeat value of the enzyme activity. Analysis of cellulase enzyme activity obtained regression results with the regression equation model, namely: Y = -0.495 + 0.011540 Temperature + 0.08881 pH – 0.000152 Temperature 2 – 0.006245pH2–0.000048 Temperature. pH. The RSM equation shows that changes in pH have the greatest effect on the results of the cellulase enzyme activity test. This influence can be seen from the pH coefficient which has the largest value compared to the incubation temperature. This might happen because the determination of the range of pH code values is too narrow so that the difference in the results is not too much different from the incubation temperature. Statistical calculations show that the coefficient of determination (R2) = 98.08%. The value of the coefficient of determination gives the understanding that the correlation between temperature and pH on cellulase enzyme activity is 98.08% while the remaining 1.92% is influenced by other factors which cannot be explained by the response.

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Cellulase enzyme activity test was carried out with Carboxymethyl Cellulose (CMC) substrate. Cellulase enzyme activity on Carboxymethyl Cellulose (CMC) substrates is an endo-β- gluconase enzyme activity by hydrolyzing β-1,4 glucosidic bonds randomly, especially in the amorphous region of cellulose fiber and does not attack cellobiose but degrades cellodextrin and cellulose that has been softened with acid phosphates and substituted cellulose such as CMC which forms glucose and cellooligosaccharides. This enzyme is generally known as CMCase or cellulase Cx. Enzyme activity measurements were also carried out using the DNS method based on the estimation of the amount of glucose (reducing sugar) as a result of hydrolysis of cellulose (Jennifer and Thiruneelakandan, 2015). DNS reactions take place in alkaline conditions and at high temperatures or in boiling water. The presence of reducing sugar in the sample will react with the DNS solution which is initially yellow to reddish-orange. The size of the enzyme activity will affect the level of reducing sugar produced (Kusmiati and Agustini, 2010 in Novitasari, 2014). The darker the color of the DNS mixed, the greater the amount of reduced sugar (Rosyada, 2015).

3.5 Cellulase Enzyme Activity Test (Endoglucanase Enzyme)

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4. CONCLUTION

Based on the results of this study it can be concluded that the optimal temperature and pH conditions for the growth of cellulolytic bacterial isolate B2S8 to produce the highest cellulase enzyme activity occurred at 36.9 ºC with a pH of 6.9 so as to produce cellulase enzyme activity (endoglucanase) of 0.0269 IU /mL.

6. Table 1 Optimization of temperature and pH for endoglucanase enzyme activity using RSM with the CCDB method. Bulleted lists may be included and should look like this:

No	Code		Trial	
	X1	X2	Temperature (^{0}C)	pH
1	-1	-1	30	6
2	+1	-1	44	6
3	-1	+1	30	8
4	+1	+1	44	8
5	-1,414	0	27,1005	7
6	+1,414	0	46,8995	7
7	0	-1,414	37	5,58579
8	0	+1,414	37	8,41421
9	0	0	37	7
10	0	0	37	7

11	0	0	37	7
12	0	0	37	7
13	0	0	37	7

7. Table 2. Results of analysis of endoglucanase enzyme activity

No	Temperature (ºC)	pH	Enzyme Activity (IU/mL)
1	30	6	0,0141
2	44	6	0,0149
3	30	8	0,0137
4	44	8	0,0132
5	27,10	7	0,0117
6	46,89	7	0,0108
7	37	5,58	0,0140
8	37	8,41	0,0132
9	37	7	0,0260
10	37	7	0,0266
11	37	7	0,0274
12	37	7	0,0268
13	37	7	0,0276

8. Illustrations

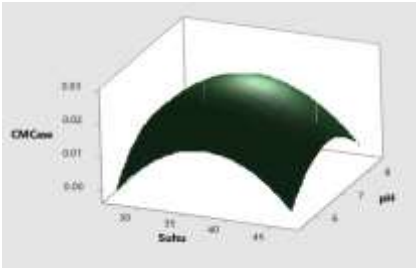

Fig. 1 - Graph of surface plot of cellulase enzyme activity against temperature and pH.

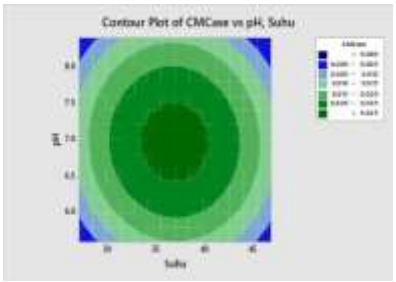

Fig. 2 - Contour plot graph of cellulase enzyme activity against temperature and pH.

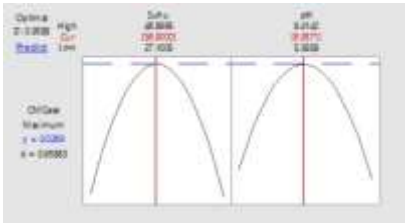

Fig. 3 - Graph D-Optimally cellulase enzyme activity against temperature and pH

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