Optimization of cellulase production by *bacillus sp.* BPPT CC RK2 with pH and temperature variation using response surface methodology

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Abstract. Indonesia has abundant ethanol biomass feedstocks. However the second-generation ethanol production process is still hampered by the unavailability of cellulase enzyme in the process of decomposition of lignocellulose into saccharides that can be processed into ethanol through fermentation. Cellulase is known as exozyme produced by *Bacillus sp.* in submerged fermentation. In this study, cellulase production by *Bacillus sp.* CC BPPT RK2 on natural and abundant agricultural waste substrates (rice bran and coconut water) was evaluated by investigating the optimum conditions for cellulase production in a 50 ml laboratory scale. Preliminary test using Luria Bentani (LB) medium with additional CMC (1%) were done to select optimum range of pH and Temperature. The preliminary tests results were then followed by optimization of pH and temperature, which were carried out using response surface methodology (RSM). RSM optimization model showed optimum values 6.23 for pH and 40.04 °C, with 14 terms (each with 1 degree of freedom), 4 linear effects, 6 interaction effects and 4 quadratic effects. These optimization by RSM results were slightly different compared to preliminary test, showing the effect of interactions between parameters. The characteristics of interaction among variables tested against the cellulase activity are reported in this study including: positive effects on cellulase activity of the resulting responses; negative interactions affecting the response of cellulase activity; synergistic interaction; and antagonistic interactions between each other.

1 Introduction

Second generation bioethanol is one of the alternative energy pathways that have less adverse effects to the environment [1]. The use of raw material resources of second generation bioethanol are not in conflict with the needs of food, as happened in the first-generation bioethanol. Second generation bioethanol feedstocks are organic wastes containing wood (lignocellulose) which are very abundant in nature [2, 3]. By producing second generation bioethanol, the organic wastes are used to something that is economically valuable.

Although the raw materials of second generation bioethanol are readily available, organic wastes may not be directly used as the substrates of fermentation to produce ethanol, because these wastes (lignocellulosic) need to be converted first into monosaccharides (glucose). Then the resulting glucose can be fermented into ethanol. [2]

Various methods to destroy lignocellulose has been done, both physically and chemically. One of the most effective method is through the hydrolysis reaction. The most promising method to hydrolyse cellulose is to use enzymes, e.g. cellulases [2]. The current problem to produce the second-generation bioethanol in Indonesia is the unavailability of cellulase enzymes for lignocellulose degradation process. For that, one of the efforts that need to be done is to produce cellulase enzyme in the country. Producing cellulase enzyme on an industrial scale is very expensive [2, 4, 5]. The cost of cellulase production can reach 50% of the total cost of cellulose hydrolysis by cellulase. This is related with low specific activity of cellulase, which requires the use of large amounts of cellulase to satisfy the amount of hydrolysable cellulose [5]. Therefore, it is necessary to reduce the production cost. One way to optimize the production is to optimize the parameters of the operating conditions of production in order to get the maximum production of cellulase enzyme [6].

Bacillus sp. has known as one of the microorganisms that can produce the enzyme cellulase [4, 7-9]. Although the production of cellulase by this type of bacteria is not as much as the production of cellulase by fungi (i.e. *Trichoderma reesei*), cellulase production using bacteria brings several advantages, including (i) having higher growth rate than fungi (ii) less inhibited by material already hydrolysed, and (iii) easier genetic engineering [2, 8].

The aim of this study was to find the optimum production parameters (pH and temperature) that produce cellulase in the maximum amount by using *Bacillus sp.* BPPT CC RK2 on rice bran and coconut water as

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substrates. Rice bran and coconut water are among the abundant source of bioethanol feedstocks from agricultural waste [1]. The bacteria *Bacillus sp.* BPPT CC RK2 is a collection of bacteria belonging to BPPT while rice bran and coconut water substrates were chosen as substitutes for C and N sources due to abundant availability in nature.

The optimization process is done by using statistical analysis method namely Response Surface Methodology (RSM). The conventional way to perform optimization of enzyme production parameter is very expensive and takes a long time [5, 10-14]. The optimization using RSM method is also utilized in other field of engineering [15, 16]. Therefore, in the presence of RSM method using software (Design-Expert®), it is expected that the optimization process can be much faster and accurate.

2 Methods

2.1 Enzyme Production

Luria Bertani (LB) solution was used as a standard medium for enzyme production with composition per litre are 10 g peptone, 5 g yeast extract and 5 g NaCl. This solution was added with 1% cellulose [11, 17]. The medium was then dissolved by water and sterilized in an autoclave at 121°C and 1.2 atm for 15 minutes.

Bacillus sp. BPPT CC RK2 isolate was inoculated as much as 1-2 ose into LB agar medium then incubated at 37°C for 24 hours [11]. These inoculate would be used as experiment stock. Starter medium was made by inoculating 1-2 ose Bacillus sp. Isolate was then added into the sterilized LB medium which was 10% of total production volume then incubated at 37°C for 6 hours. Agitation was done at 150 rpm. The production of enzyme was done by inoculating the starter medium to the production medium, and then incubated at 37°C for 24 hours. Agitation was also done at 150 rpm.

After 24 hours, enzyme was collected by centrifuging the broth at 6000 rpm using High Speed Refrigerated Centrifuge Himac CR21G and rotor R10A2 for 15 minutes at 4°C. Supernatant was taken as crude enzyme fraction. This crude enzyme was then analysed to investigate protein content and enzyme activity.

2.2 Temperature and pH Optimization

For preliminary experiments of pH optimization, this method was similar to the production of enzymes, but with slight modification. The modification was done by varying the pH value as well as the temperature. pH was set to 6.0; 6.5; 7.0; 7.5; and 8.0 while temperature was set to 25 °C; 30 °C; 35 °C; 40 °C; 45 °C; and 50 °C. Optimization was done here using one-variable-at-a-time method, so that in one experiment only one variable is varied.

2.3 Optimization using Response Surface Methodology

Centralized Composite Design was used to develop a mathematical model using Design-Expert® 7.1.5 software. The obtained equation would be used to calculate the optimum fermentation condition. The result was then be checked with ANOVA. The procedure was then to find the optimum conditions from the obtained equation [7, 10, 12-14].

2.4 Analysis of Enzyme Activity

The cellulase activity test performed in this experiment is a test of endo β-1, 4-glucanase (EC 3.2.1.4) or commonly referred to as CMCase. The CMCase test was chosen because it relates to the fact that the most dominant number of lignocellulose contained in cellulose is endo β-1, 4-glucose [7, 4]. Although in this test only CMCases were analysed because these enzymes were the most dominant, it does not mean that others were not produced. It was reported that for insoluble crystalline cellulose such as those possessed by bran, cellulose such as exoglycanase and betaglucosidase species were also produced [2].

CMCase activity was measured by reacting the sample in 1 mL of 1% CMC solution, 0,05 M phosphate buffer (pH 7.0) in which the enzyme sample volume was 100 ml, and the solvent volume was 900 ml [18]. The reaction lasted for 30 minutes and was incubated at 50 °C. Sugar derivatives resulting from the reaction was determined by the Miller method [19]. One unit (U) is defined as the amount of enzyme releasing 1 mol of reduced sugar from CMC every minute at 50 °C. The concentration of by-products of reduced sugar from the reaction is equivalent to 3-amino, 5-nitrosalicylic acid. This substance can be observed by spectroscopy due to high absorbance at 540 nm wavelength. The cellulase activity was calculated according to the equation

$$\text{Activity (U/ml)} = \frac{\text{mg glucose} \times 1000}{\text{Mr glucose} \times 30\,\text{min} \times 0.1\,\text{ml}} \quad (1)$$

2.5 Protein Analysis

Enzyme protein content was determined by Lowry phenol reagent method [20]. Alkaline conditions can be generated from the phosphate buffer or phosphate buffer saline (PBS). One liter of PBS solution consisted of 8g NaCl; 0.2 g KCl: 1.44 g KH_2PO_4 and 0.24 g Na_2HPO_4. PBS solution was adjusted to pH 7.4 by addition of NaOH or HCl. Lowry reagent consisted of three kinds of solution (A, B and C). One liter of solution A consisted of 20g Na_2CO_3 and 0.4 g NaOH, one liter of solution B consisted of 10g $CuSO_4$, and one liter of solution C consisted of 2g $NaKC_4H_4O_6$.

Lowry reagent was made by mixing those solutions with ratio of volume of A : B : C is 98 : 1 : 1. Protein standard solution was prepared from Bovine Serum Albumin (BSA) by varying the concentration 100-1000µg/mL, where 100.ddH_2O interval was used as blank. At first as much as 20µL of each BSA solution was reacted with 180µL PBS solution to make alkaline

conditions followed by addition of 2 ml of fresh reagent. After stirring with vortex, the analyte was incubated for 10 minutes and then reacted with 200µL Follin Ciocalteu reagent and incubated for 30 minutes. As a result, solution containing the protein will change color from clear to blue. Quantitative analysis was performed using a visible spectrophotometer instrument with 750 nm wavelength.

3 Results and Discussion

3.1 pH Preliminary Test

Bacillus sp. can live in various pH conditions. However, the pH of the production operations has an impact, not only on how the bacteria metabolize but also on how the bacteria react to existing substrates. *Bacillus sp*. produces cellulase in the pH range 4-8 [4, 6]. Because of that, variation of pH was performed for each fermentation from fairly acidic pH (pH 6) up to fairly high pH (pH 8). The adjusted pH was the pH of initial condition of the process. Figure 1 showed that *Bacillus* sp. produced cellulase with the highest activity at pH 7.5. At low pH, produced cellulase had low activity. The produced cellulase activity continued to increase as the operating pH increased until it dropped dramatically when the operating pH was 8. Higher pH might have an effect to the ionization state of acidic or basic amino acids. The basic amino acids have amine functional groups in their side chains that can readily affected [21, 22].

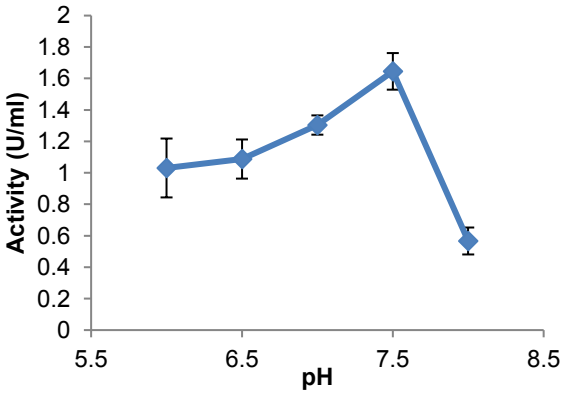

Fig. 1. Effect of pH on cellulase activity

The optimum pH condition on the preliminary test of LB + CMC media by fermentation conditions at 37°C and 150 rpm for 24 hours was 7.5. Similarly, other author [9] used the strain of *Bacillus sp.* derived from termites with fermentation conditions of 37 °C and CMC as substrate, with results not much different from thi study where the optimum pH was 7.2.

3.2 Temperature Preliminary Test

Likewise pH, the temperature also has impact on bacterial metabolism. Temperature commonly used in the fermentation of bacteria in general is 37°C. Temperature variation was performed from 25°C to 50°C with reference to the temperature where the origin of the bacteria *Bacillus sp.* CC BPPT RK2, where newspaper termite lives at room temperature (~ 30°C). From the data obtained (Fig. 2), the operating temperature resulting high activity cellulase production was not far from 37°C, which was between 35°C and 40°C. Temperature that produced cellulase production with the highest activity was 40°C. At low temperature (25°C), produced cellulase activity tends to be low, as well as at high temperature (~ 50°C), but activity remained lower at low temperature. This is related to the metabolism of the bacteria *Bacillus sp.,* although at high temperature enzyme that plays role can be better, but if it exceeds the ability it will damage the enzyme and will reduce the rate of metabolism. Even in low temperature, although enzyme in the metabolism of *Bacillus sp*. is undamaged or denatured, enzyme retains a low activity because the heat received as an activation energy in transition is small.

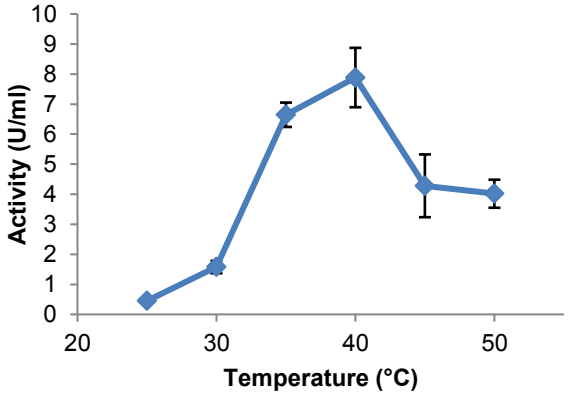

Fig. 2. Effect of temperature on cellulase activity

The optimum temperature value (40 °C) on cellulase production optimization using *Bacillus sp*. on natural substrates was also reported [22] but the pH of the research condition differed slightly from this study. This research also showed that cellulase production reached optimum when *Bacillus sp.* BPPT CC RK2 was in exponential phase in which bacteria grows rapidly in that phase. Denaturation of enzyme resulted from higher temperature was reported by many authors [10, 21, 22].

3.3 pH and Temperature Optimization Using RSM

From the results of this research, the quadratic model was chosen as the surface model of the activity response to pH, temperature and substrate (carbon and nitrogen source). The resulting model is in the form of a mathematical equation which when compiled: enzyme activity with Y, C with X_1, N with X_2, pH with X_3 and temperature with X_4, is

$$Y_i = - 18.51 - 2{,}08\, X_1 - 1.52\, X_2 + 11.17\, X_3 + 2.12\, X_4 + 1.13.10^{-2}\, X_1X_2 - 0.07\, X_1X_3 + 1.93.10^{-2}\, X_1X_4 + 0.12\, X_2X_3 - 3.91.10^{-2}\, X_2X_4 - 0.01\, X_3X_4 + 2.38.10^{-2}\, X_1^2 - 3.38.10^{-2}\, X_2^2 - 0.79\, X_3^2 - 0.05\, X_4^2 \quad (2)$$

From Fig. 3 it is shown that the highest activity response of the given model is at a pH value of 6.23 and a temperature of 40.04 ° C. From the 3D response graph (Fig. 4), it is shown that enzyme activity tends to decrease at high pH. The optimum pH value is also in acidic condition. This proves that the optimum value of pH preliminary test using pure substrate (CMC) can not be equated with the optimum value of RSM using natural substrate (rice bran) as its carbon source [4].

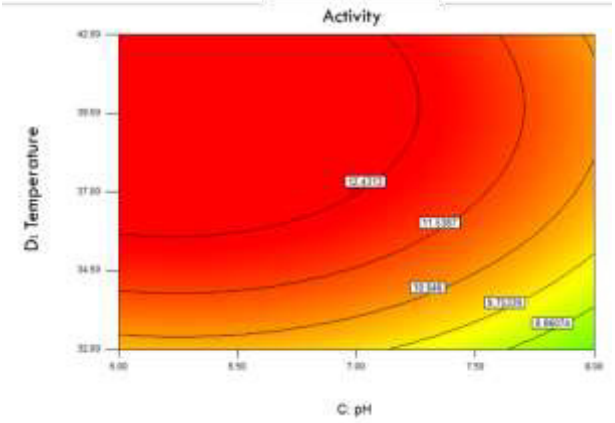

Fig. 3. Contour plot of cellulase activity on pH and temperature variations (C: 50%, N: 20%)

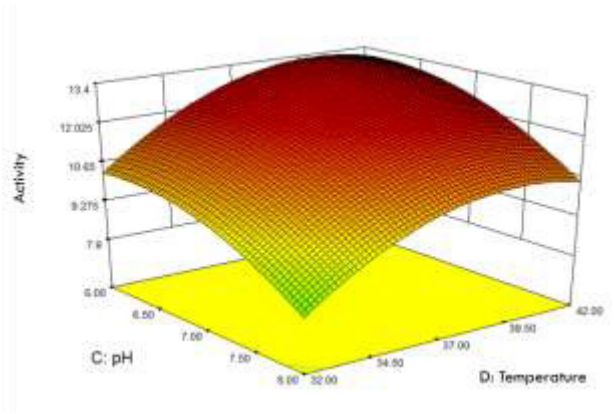

Fig. 4. 3D Graphic of cellulase activity on pH and temperature variations (C: 50%, N: 20%)

3.4 Interaction Between Response Variables

To know the interaction of responses between variables contained in the equations obtained, it is needed to refer to the result of the analysis of variance (ANOVA) model. The equation model obtained in this experiment has 14 terms with each term having 1 degree of freedom. The term consists of 4 linear effects, 6 interaction effects and 4 quadratic effects.

In Table 1, A denotes the concentration of carbon source, B denotes the concentration of nitrogen source, C denotes pH and D denotes temperature. The term consisting of a single letter (single variable) denotes a linear effect and a two-letter term (two variables), denotes the interaction effect.

The interaction between pH and temperature has no significant effect on the resulting cellulase activity. However, the interaction between pH and rice bran concentration, pH and nitrogen concentration, bran temperature and concentration, as well as the temperature and concentration of nitrogen, still have a significant effect on the response of the resulting cellulase activity. And to know what the effect of each term is, it is needed to look at the estimated coefficients of each term.

Table 1. ANOVA of cellulase activity RSM model

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	181.82	14	12.99	91.62	< 0.0001
A-C	51.77	1	51.77	365.22	< 0.0001
B-N	7.85	1	7.85	55.39	< 0.0001
C-pH	7.42	1	7.42	52.31	< 0.0001
D-suhu	3.27	1	3.27	23.1	0.0002
AB	1.28	1	1.28	9	0.009
AC	1.79	1	1.79	12.65	0.0029
AD	3.73	1	3.73	26.33	0.0001
BC	6.07	1	6.07	42.81	< 0.0001
BD	15.26	1	15.26	107.65	< 0.0001
CD	0.058	1	0.058	0.41	0.5303
A^2	9.68	1	9.68	68.31	< 0.0001
B^2	19.53	1	19.53	137.76	< 0.0001
C^2	17.13	1	17.13	120.84	< 0.0001
D^2	38.69	1	38.69	272.92	< 0.0001
Residual	2.13	15	0.14		
Lack of Fit	1.91	10	0.19	4.52	0.0549
Pure Error	0.21	5	0.042		
Cor Total	183.95	29			

Estimated coefficients contained in the table is the coefficient of each factor contained in the equation of coded model as follows

$$\text{Activity} = 7.13 + 1.47A - 0.57B - 0.56C + 0.37D + 0.28AB - 0.33AC + 0.48AD + 0.62BC + 0.98BD - 0.06CD + 0.59A^2 - 0.84B^2 - 0.79C^2 - 1.19D^2 \quad (3)$$

Table 2 shows that the interaction effect between pH and temperature has an insignificant effect because the value of the estimated coefficients is very small when compared with the estimation of coefficients of other factors. Of factors that exist, which have positive effects on cellulase activity of the resulting responses include: The linear effect of rice bran concentration, the linear effect of temperature, and the quadratic effect of substrate concentrations.

Factors that negatively affect the response of cellulase activity include: the linear effect of concentration of coconut water, the linear effect of pH, the quadratic effect of coconut water concentration, the quadratic effect of pH, and the quadratic effect of temperature.

Table 2. The estimated coefficient of each factor

Factor	Coefficient Estimate	df	Standard Error
Intercept	7.13	1	0.150
A-C	1.47	1	0.077
B-N	-0.57	1	0.077
C-pH	-0.56	1	0.077
D-temperature	0.37	1	0.077
AB	0.28	1	0.094
AC	-0.33	1	0.094
AD	0.48	1	0.094
BC	0.62	1	0.094
BD	0.98	1	0.094
CD	-0.06	1	0.094
A^2	0.59	1	0.072
B^2	-0.84	1	0.072
C^2	-0.79	1	0.072
D^2	-1.19	1	0.072

The two factor interactions which are synergistic interaction include: the interaction between the concentration of rice bran and coconut water, the iinteractions between bran concentration and temperature, the interaction between concentration of coconut water and pH, and the interaction between the concentration of coconut water and temperature. Meanwhile, the interaction of two factors which are antagonistic interactions between each other are: the interaction between concentration and pH of the bran and the interaction between pH and temperature.

4 Conclusion

RSM optimization model showed optimum values 6.23 for pH and 40.04 °C, with 14 terms (each with 1 degree of freedom), 4 linear effects, 6 interaction effects and 4 quadratic effects. The optimization by RSM results were slightly different compared to preliminary test, showing the effect of interactions between parameters. The interactions were shown to be: positive effects on cellulase activity of the resulting responses; negative interactions affecting the response of cellulase activity; synergistic interaction; and antagonistic interactions between each other.

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