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VARIATION OF C/N RATIO AND FERMENTATION TIME IN RESPONSE SURFACE METHODOLOGY FOR CELLULASE PRODUCTION FROM *Bacillus sp.* BPPT CC RK2

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ABSTRACT

This study aimed to obtain optimum operating conditions in the production of cellulase by Response Surface Methodology using *Bacillus sp.* BPPT CC RK 2. This optimization used natural substrate that was cheap and widely available in Indonesia. This substance was also a source of carbon and nitrogen for the production of cellulose enzymes. These carbon and nitrogen sources are the emerging substance to replace carboxylmethyl (carbon source) and yeast extract (source of nitrogen) that are still expensive. The research was conducted in four stages, namely: (1) the manufacture and the selection of medium composition ; (2)enzyme production through fermentation; (3) the use of Response Surface Methodology using a software design expert in determining the optimum point of cellulase; (4) and the enzyme activity test. The results showed that the isolates *Bacillus sp*. CC BPPT optimum RK 2 produce cellulase for 12 hours in media with concentrations of rice bran 50% (w/v), and coconut water concentration of 20% (v/v).

KEYWORDS*:***Optimization, Cellulase, *Bacillus sp.*,Response surface methodology, Fermentation time**

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INTRODUCTION

The abundance of oil palm solid waste is a problem in oil palm mills. However, this lignocellulosic biomass can be utilized for cellulase production. Indonesia posses the biggest oil palm tree plantation to produce crude palm oil either for cooking oil or raw material for biodiesel production. As a consequence, this country bears huge amount of empty fruit bunch which is still rich ini cellulose materials. Some researchers investigate production of bioethanol out of this solid waste[1,2,3,4]. The important of cellulase in bioethanol production has been elaborated by many researchers, including Gozan *et al*. and Samsuri *et al*.[5,6]. Razak *et al*. used oil palm solid waste to produce cellulase[7].Enzyme is a protein which functions as a catalyze, fastening a reaction process without consumed in an organic chemical reaction. Use of enzyme in degrading polymer has been practiced in numerous kinds of industries and other sectors. A specific enzyme that is used to degrade cellulose is cellulase enzyme. Enzymatic process in hydrolyzing cellulose is a better and more beneficial way because the surrounding condition for enzyme's activity could be controlled to produce hydrolysates which contain glucose. There are three kinds of cellulase which form cellulase complex. The first one is endocellulase, which breaks internal bond in order to cut crystalline structure in cellulose and open polysaccharide chain. Exocellulase is enzyme that breaks 2-4 unit from the end of chain produced by endocellulase and generates tetrasaccharide or disaccharide. Cellobiase or beta-glukosidase hydrolizes exocellulase product to monosaccharide[8,9,10,11,12].To aim optimalized process, one of the method used is Response Surface Methodology (RSM). This method is a technique of solving problem in finding optimal condition of an operation using mathematics and statistics in a form of model analyzing the problem. This method consists of a group of statistic technique to build empirical model and exploit model 2 of an experiment involving k factors, which are x_1, x_2,..., x_k, where k factors are labelled as independent variables, predictors, or control variables, and produce Y, where Y is a dependent variable, or response variable. All of these variables could be measured and known that Y is response from x_1, x_2,..., x_k, then it is said that Y is a function of x_1, x_2,..., x_k, and generally written in the form of

$$Y = f(x_1, x_2, \dots, x_k) \quad (1)$$

This function is called as *response surface*[13,14]. Lee *et al.* (2012)and Shahriarinour *et al*. (2011) used RSM for optimization of CMCase production from rice bran and cellulase production from Oil Palm Empty Fruit Bunch Fibre[15,16].This study aimed to obtain optimum operating conditions in the production of cellulase by Response Surface Methodology using *Bacillussp*. BPPT CC RK 2. This optimization using the natural substrate that is widely available in Indonesia and cheap as a source of carbon and nitrogen sources are used as a medium for the production of cellulose enzymes to replace Carboxylmethyl (carbon source) and Yeast Extract (source of nitrogen) is still expensive.

MATERIALS AND METHODS

Medium

The nurturing medium used was liquid Luria Bertani (LB) (pH 7.0) with composition of 10 g of peptone, 5g of yeast extract (as nitrogen source), and 5 g of NaCl. LB was added with 1% CMC (as carbon source). Medium was dissolved with aquadest according to the desired production volume. In this experiment, production volume was 50 ml. Then, medium was sterilized in autoclave at 121^{o}C and pressure 1.2 atm within 15 minutes. Then, 1-2 ose of *Bacillus sp*isolate was innoculated into LB medium obliquely and incubated at 37^{o}C for 24 hours. This isolate was used as culture stock.Rice bran and Coconut water were collected from a rice paddy in Pamulang and from Serpong (Jakarta, Indonesia), respectively.Starter medium was made with inocullating 1-2 ose of *Bacillus sp.* BPPT CC RK 2 isolate into sterile LB medium (10% of production media), then was incubated at 37^{o}C for 6 hours. The agitation done along fermentation was in 150 rpm. Enzyme

production was done by inoculating starter medium into production medium, then incubated at 37^{o}C for 24 hours. The agitation along fermentation was in 150 rpm.

Experiments

After 24 hours, enzyme was harvested by centrifuging with agitation of 6000 rpm using High Speed Refrigerated Centrifuge HimacCR21G and rotor R10A2 for 15 minutes at 4^{o}C. Then, supernatant was separated as crude enzyme fraction. The crude enzyme then was analyzed on the bases of protein concentration and enzyme activity.First, preparing 900 µL CMC 1% dissolved in 0.05 M phospate buffer pH 7.0. Each test tube which will be tested was filled upto 1 ml of mixed solution of 900 µL glucose + 100 µL enzyme for sampling, while for controlling, enzyme was added after DNS was poured in. After that, incubating them for 30 minutes at 50^{o}C, then add 1ml DNS for each test tube. All the test tube then was heated in a water bath for 5 minutes so that reaction of glucose and DNS occurs. Test tube then was cooled and shaken so that it was mixed completely. Absorbance of each solution then was measured at 540nm [17], which the result was in U/ml, where 1 unit (U) was defined as the amount of enzyme that releases 1 µmol reduced glucose from CMC per minute at 50^{0}C and pH 7.

Analysis

Enzyme protein concentration was determined with modified Lowry method[18]. This condition was produced by phosphate buffer saline(PBS). One liter of PBS consists of 8 g NaCl; 0.2 g KCl; 1.44 g KH_2PO_4 and 0.24 g Na_2HPO_4. PBS solution was adjusted into pH 7.4 by adding NaOH or HCl. Besides, reagent Lowry was produced, consisting of three kinds of solutions (A, B dan C). One liter of solution A consists of 20 g Na_2CO_3 and 0.4 g NaOH, one liter of solution B consists of 10g $CuSO_4$, and one liter of solution C consists of 2g $NaKC_4H_4O_6$. Production of reagent Lowry was done by mixing those three solutions with ratio of volume A : B : C was 98 : 1 : 1.

RESULTS AND DISCUSSION

Table 1 explains Analysis of Variance (ANOVA) from the experiment done, where the four factors subjected to optimizing process, which are carbon source (A), nitrogen source (B), pH (C), and temperature (D), show linkage between each other through quadratic model suggested to be used.

Table1
ANOVA Model 2nd Order RSM

Source	Std. Dev.	R-Squared	Adjusted R-Squared	Predicted R-Squared	Precision	Model Suggested
Model	181.82	14	12.99	91.62	< 0.0001	Significant
A-Carbon	51.77	1	51.77	365.22	< 0.0001	
B-Nitrogen	7.85	1	7.85	55.39	< 0.0001	
C-pH	7.42	1	7.42	52.31	< 0.0001	
D-temperature	3.27	1	3.27	23.10	0.0002	
AB	1.28	1	1.28	9.00	0.0090	
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	0.06	1	0.06	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	0.06	not significant
Pure Error	0.21	5	0.04			
Total Cor	183.95	29				

Table 1 shows that value of calculated F is 91.6 indicating significant model of cellulase production. If the value of P (Prob > F) less than 0.05; then it is significantly influencing. According to the data, it is known that concentration of rice bran, concentration of coconut water, pH, and temperature show significant impact to cellulase production with equation obtained using design expert is:

$$Y_i = -18{,}51 - 2{,}08X_1 - 1{,}52X_2 + 11{,}17X_3 + 2{,}12X_4 + 1{,}13.10^{-2}X_1X_2 - 0{,}07X_1X_3 + 1{,}93.10^{-2}X_1X_4 + 0{,}12X_2X_3 - 3{,}9.10^{-2}X_2X_4 - 0{,}01X_3X_4 + 2{,}38.10^{-2}X_1^2 - 3{,}38.10^{-2}X_2^2 - 0{,}79X_3^2 - 0{,}05X_4^2 \quad (2)$$

Where:
X_1 = Carbon source (rice bran)[% w/v]var 30 to 50
X_2 = Nitrogen source (coconut water) [% w/v]var 10 to 30
X_3 = pH[scale from 1-14], var 5 to 9
X_4 = temperature[oC], var 32 to 47
According to ANOVA test on Table 2, coefficient of determination value R^2=0,988, which shows that 98.8% of sample variables on cellulase production are influenced by independent variables.

Table 2
Accuracy value of experiment

Accuracy Parameter	Value
Std. Dev.	0.38
Mean	5.35
C.V. %	7.04
Precision	11.33
R-Squared	0.99
Adj R-Squared	0.98
Pred R-Squared	0.94
Adeq Precision	**42.01**

Experiment using response surface methodology obtains result with optimum condition of fermentation for highest cellulase activity achieved at a concentration of rice bran 50, concentration of water coconut 20%, pH 7, and production temperature at 37^{o}C(Figure 1). Under this condition, value of activity was obtained at 12.23 U/mL. On the model portrayal above, range of activity value concentrated on the center of the figure ranging from 6 to 8 U/mL with center point of 7.31 U/mL while optimum point was at the most upper point of the graph. This point was the value of alpha plus 1 from Central Composite Design (CCD).

Response Surface Methodology relationship of carbon source (rice bran) and nitrogen source (water coconut)in 3 dimensions and 2 dimensions

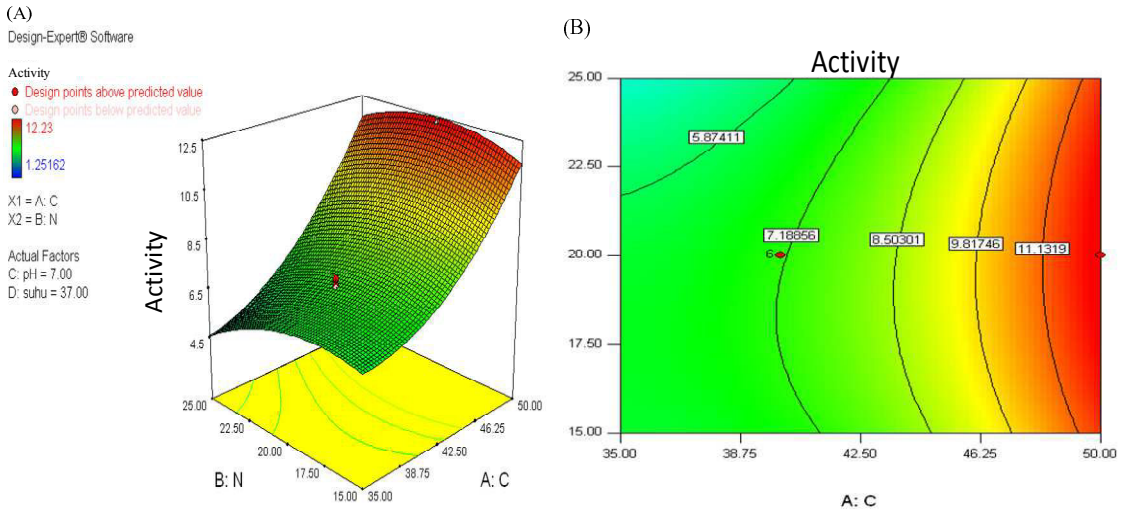

Figure 1
Response Surface Methodology relationship of carbon source (rice bran) and nitrogen source (water coconut)in 3 dimensions and 2 dimensions

Activity was observed by analysing the glucose content reduction. Glucose content was measured by DNS method. In the other hand, protein was measured by Lowry method.During observation of enzyme activity for each 3 hours in 24 hours, enzyme activity was obtained increasing until fermentation time of 12 hours and at the 15th hour, enzyme activity begins to decrease (Fig.2). At the peak of cellulase activity, bacteria secrete cellulase enzyme maximumly to outer surrounding. Highest cellulase activity occurs at 12th hour with cellulase activity was 13.25 U/mL and protein concentration 17.05 mg/mL shown at figure2. The occurence of fast production process at the 12th hour was caused of feed back inhibition received by enzyme, thus it inhibits cellulas activity through quite concentrate amount of substrate at the succeeding hours after highest phage at 12th hour, thus bacteria are in saturated phage faster in producing cellulase. At optimizing cellulase production, protein concentration was increased as production time goes. This was caused by growing bacteria to stationery phage, producing a lot of protein instead of cellulase. In general, cellulolytic microorganism from bacteria group has faster growth level, thus time used for enzyme production was shortened. Beside of optimum condition determination, observation on other parameter (amount of cells) was also done in each 3 hours for 24 hours (Fig. 3).

Relationship of production time and activity and protein concentration given cellulose

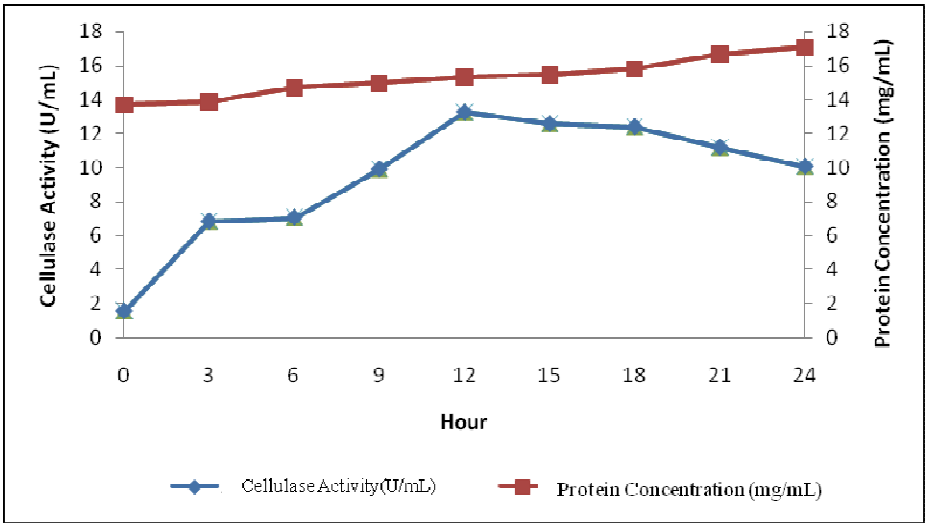

Figure 2
Relationship of production time and activity and protein concentration given cellulase

On Figure 3, the amount of bacteria was shown increasing since the 0th hour of incubation. The growth continued after 24 hour, and has not reach stationery phage yet. On the contrary, the control culture, i.e. culture of bacteria without carbon and nitrogen sources added, showed no growth at all.

Amount of cells to time

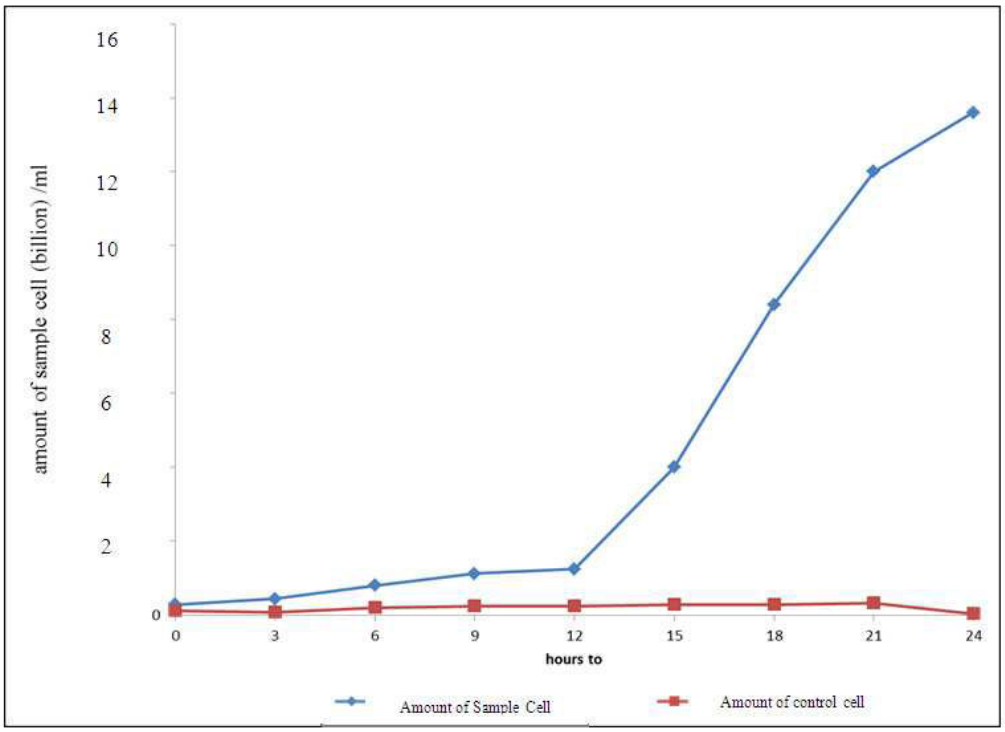

Figure 3
Amount of cells to time

CONCLUSION

Based on the result of experiment and discussion, then it was concluded as below :

1. Optimizing cellulase production process from *Bacillussp*.BPPT CC RK 2 was obtained by substrate composition of 50% (b/v) rice bran (carbon source) and 20% (v/v) coconut water(nitrogen source) in modified medium with enzyme activity of 12.23 U/mL.
2. Optimum time for producing cellulase from *Bacillus sp*.BPPT CC RK 2 was obtained at 12th hour with activity of 13.25 U/mL and amount of bacteria was1.24 x 10^9 cells/ml.

CONFLICT OF INTEREST

Conflict of interest declared: none.

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