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Application of Response Surface Method in Optimization of Medium Composition for Xylanase Production by *Bacillus halodurans* CM1 in Submerged Fermentation

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A two level factorial design was performed to optimize xylanase production by alkalothermophilic *Bacillus halodurans* CM1 using response surface method. The variables involved in this experiment were carbon (X_1), nitrogen source (X_2) concentration, and pH (X_3), corn cob and fish powder were used as carbon and nitrogen source respectively. Statistical analysis of the experimental results in the range studied, only carbon source gave significant effect on xylanase production. A second-order model was proposed to represent the enzyme activity as a function of xylan concentration (X_1) and pH (X_3). The optimum corn cobs concentration was 4.37% (w/v), and the fish powder (with high P content) concentration was 1.75% (w/v) and pH 9. These conditions were tested and validated experimentally since the maximum growth rate achieved with these parameters, and the highest xylanase activity observed was 522 U mL^{-1} after 24 h fermentation.

Key words: *Bacillus halodurans* CM1, production optimization, RSM, xylanase

Disain faktorial dua tingkat digunakan untuk optimasi produksi enzim xylanase alkalitermofilik dari *Bacillus halodurans* CM1 dengan metode statistik respons permukaan. Variabel yang terlibat dalam penelitian ini adalah konsentrasi sumber xilan tongkol jagung (X_1), konsentrasi tepung ikan dengan kandungan fosfor (P) (X_2), dan pH (X_3). Analisis statistik hasil CCD menunjukkan bahwa, dalam kisaran yang diamati, konsentrasi tepung ikan tidak memberi pengaruh yang signifikan terhadap produksi xilanase. Sebuah model orde kedua diusulkan untuk mewakili aktivitas enzim sebagai fungsi konsentrasi xilan (X_1) dan pH (X_3). Konsentrasi Xilan tongkol jagung dan tepung ikan P optimum masing-masing adalah 4,37% (b/v) dan 1,75% (b/v) pada pH 9. Kondisi ini diuji dan divalidasi secara eksperimental karena laju pertumbuhan maksimum dicapai dengan parameter ini. Hasil pengamatan aktivitas xilanase terbaik adalah 522 U mL^{-1} setelah fermentasi berlangsung 24 jam.

Kata kunci: *Bacillus halodurans* CM1, optimasi produksi, RSM, xylanase

A conventional pulping process involve application of chlorine in bleaching process of the pulp. The used of chlorine in the bleaching process not only affects the waste water released into the environment but also the final paper products. Indonesia is one of the top ten pulp producer countries using chemical pulp production method (Popp *et al.* 2011). Chemical processes in the pulp and paper industries are generally done at high temperature and pH, especially in the kraft pulping process, the process of sulfate and chlorine bleaching process. Recently, demand of elemental chlorine free (ECF) or totally chlorine free (TCF) in the world market of paper product has dramatically increased in the last decade. In order to fulfill the market demand, utilization of biotechnology might can be done by the application biobleaching (Bajpai *et al.* 1999). Biobleaching is the process of bleaching the pulp using xylanase (Buchert *et al.* 1994; Da Silva *et al.* 1994). The use of xylanase can give a good impact on the environment which replaces or reduces the amount of chlorine used in the bleaching process of pulp.

Enzymes are biocatalysts that can speed up a chemical reaction without changing its structure. It is also widely used in industry as an alternative to the use of chemicals that pollute the environment. Application of thermoalkalophilic xylanase in pulp and paper process has been studied extensively since the last two decades (Buchert *et al.* 1994; Kulkarni *et al* 1996; Zheng *et al.* 2000; Oakley *et al.* 2002; Ibarra *et al.* 2010; Paes *et al.* 2012).

Xylanase is an extracellular enzyme that can hydrolyze xylan (hemicellulose) into short chain xylooligosaccharide (Kulkarni *et al.*1999). According to Kulkarni *et al.* (1999) in the process of bleaching paper (pulp) requires a temperature of 80 °C with a pH

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range of 6.0-8.0. Therefore, the xylanase needed is the alkalothermophilic one. *Bacillus halodurans* CM1 is a xylanase-producing bacterium and has been isolated by Ulfah *et al.* (2011) of hot springs Cimanggu, West Java. *B. halodurans* CM1 is a gram-positive bacterium that produces extracellular xylanase in submerged fermentation system. However, the optimum conditions for the enzymes production include pH, source concentration of carbon and nitrogen has not been known yet. Based on preliminary experiment, *B. halodurans* CM1 produced xylanase in the xylan containing medium (Ulfah *et al.* 2011). Therefore, the production of xylanase used xylan carbon source. This study would be planned to be continued through to the industrial scale, so inducer xylan must be substituted with a carbon source (xylan) from xylan-containing agricultural waste (corn cobs and sugarcane bagasse), as well as the source of nitrogen substitution with fish powder. Experiment optimization of xylanase production conditions implemented using response surface optimization techniques methodology (RSM). The aims of this study was to obtain the optimum condition of pH, carbon, and nitrogen source concentration for *B. halondurans* CM1 xylanase production using response surface method.

MATERIALS AND METHODS

Microorganism. *Bacillus halodurans* CM1 (culture collection of Laboratory of Bioindustry, BPPT), isolated from Cimanggu hot pond, West Java was used in this study. Stock cultures were maintained on culture medium, which was used for the submerged culture, refreshed at 55 °C in modified Luria Broth medium containing 0.5 % (w/v) bactopeptone, 0.25 % (w/v) yeast extract, 0.25 % (w/v) sodium chloride, and 1 % (w/v) beech wood xylan.

Cultivation Medium. The medium composition used in the experiment was modification of medium according to Mamo *et al*. (2006). In 100 mL medium consisted of 0.5 g corn cobs xylan, 0.5 g fish powder rich in P, 0.29 g KH_2PO_4, 1 g Na_2CO_3, 0.2 g NaCI, 0.01 g $MgSO_4$, 0.01 g $CaCI_2$. For the optimization experiments, the cultivation medium was composed of different concentration of corn cob, fish powder, and pH according to the experimental design.

Cultivation. The optimization experiments were carried out in 500 mL Erlenmeyer flasks containing 100 mL of cultivation medium. Shake flasks were seeded with inoculum, at initial concentration of 2.2 x 10^8 cells mL, which were incubated for 24 h at 50 °C 120 rpm.

Enzyme Assays. Xylanase activity was determined by dinitrosalicylic acid (DNS) method, according to the Miller (1959) for quantifying reducing sugar. 50 µL of crude enzyme extract at appropriate dilutions in tris-base buffer was mix with 450 µL of 0,5 % (w/v) beechwood xylan in 50 mM of buffer at the indicated pH 9. The mixture was incubated for 5 min in the thermomixer at the optimum temperature for enzyme activity of 70 °C. Subsequently, 750 µL DNS reagent (1% dinitrosalicylic acid, 0.2% phenol, 0.05% sodium sulfite, and 1% sodium hydroxide, 20% (w/v) potassium sodium tartrate) was added to stop the reaction. Then the mixture was boiled at 100 °C for 5 min, and kept at room temperature. Afterward 250 µL of distilled water was added. Reducing sugar released during incubation was measured as xylose equivalents at 540 nm. As blank we used the same mixture as in the above sample; however, enzymes were added following addition of DNS into the reaction mixture. One unit (U) of enzyme activity is defined as the amount of enzyme releasing 1 µmol of reducing sugar equivalent per minute under the assay conditions.

Experimental Design. A 2^3 Central Composite Design (CCD) was performed in order to determine the optimal conditions for producing xylanase by *B. halodurans* CM1. The dependent variable selected for this study was the enzyme activity, expressed in U mL^{-1}, and the independent variables chosen were corn cob concentration, fish powder (rich in P) concentration, and medium pH. The range and the levels of these variables are given in Table 1.

Statistical Analysis. Statistics, Software Design Expert 7 version 7.0.0, stat-ease Corporation Minneapolis, USA, was used for regression and graphical analysis.

RESULTS

Based on earlier studies the media selected was modified medium with corn cob was selected as a carbon source and fish powder was selected as nitrogen source. The variables involved in this study were corn cob concentration (X_1), fish powder concentration (X_2), and pH (X_3). The following experiment was to determine which factors affected the enzyme production. A two level factorial design was defined to determine factors that affected the enzyme production (data not shown). The experimental results showed that the model of corn cob concentration, fish powder concentration and pH value was significant so was the

Table 1 Values of independent variables a different levels of the CCD

Independent variables	Symbol	Levels		
		-1	0	1
Xylan	X_1	0.63	1.31	2.00
Fish powder P	X_2	0.54	0.99	1.44
pH	X_3	8	9	10

Table 2 Experimental design and result of the CCD

Run number	Coded levels			Xylanase activity (U mL)	
	X_1	X2	X_3	Observed	Predicted
1	0	0	0	345.18	505.01
2	0	0	0	97.089	90.2609
3	1	1	-1	196.26	266.453
4	1	-1	1	138.47	168.974
5	-1	-1	-1	42.496	51.88
6	-1	1	1	103.85	112.534
7	0	0	0	197.37	212.716
8	0	0	0	522.51	521.328
9	-1	-1	1	99.26	112.534
10	1	1	1	243.19	169.974
11	-1	1	-1	33.19	51.88
12	1	-1	-1	272.19	266.453
13	0	0	0	276.38	212.71
14	0	-1682	0	153.85	212.71
15	0	0	-1.682	254.61	212.71
16	-1682	0	0	67.49	43.09
17	0	0	0	198.58	212.71
18	1.682	0	0	50.34	56.74
19	0	0	1.682	234.75	212.71
20	0	1.682	0	187.32	212.71

curvature F value. In this work, these variables were statistically optimized with the help of central composite design (CCD) using Response Surface Method (Oh *et al.* 1995; Wang *et al.* 2004). The experimental design and the results are shown in Table 2. The highest xylanase activity (522.512 U mL^{-1}) was observed at run number 8, where the factors corn cob concentration and fish powder concentration was 4.37% (b/v) and (1.75% (b/v), respectively. The medium pH was 9.0 and the incubation time was 24 h. This activity was 15.7 fold higher than that of observed at run number 11, where the related factors were used at highest levels for X_2, and lowest for X_1 and X_3. Table 3 shows the regression analysis for this experiment, presenting the estimates and hypothesis test to the coefficients of regression. At the 5% probability level the linier and quadratic coefficient of X_1 (corn cob concentration), and the coefficient of the interaction X_1X_3 (corn cob concentration and pH) were found to be significant. The mathematical model representing the xylanase activity ($\hat{y}$) in the experimental region studied can be expressed by Eq.(1)

$$\hat{y} = -18.89893 + 2.07786X_1 + 4.33193X_3 - 0.19490 X_1X_3 - 0.22459 X_3^2 \quad (1)$$

The independent variables, corn cobs, pH and interaction between corn cobs and pH had a significant effect on xylanase production ($P > 0.05$) (Table 3). The statistical significance of a second-order model equation was evaluated by the F-test analysis of variance (ANOVA). The computed *F*-value (19.61) indicated that the model was significant at high confidence level. The probability *P*-value was very low

(P=<0.0001) reflecting the significance of the model (Liu *et al*. 2003). The model show lack of fit not significant and presented a fairly high determination coefficient (R^2 = 0.80), explaining 80% of the variability in the response and indicates correlation between the experimental observed and predicted values (Table 4). In general, a regression model having R^2value higher than 0.9 considered to have a very high correlation (Haaland 1989). The value of the determination coefficient (R^2 = 80%) was also high enough to indicate the significance of the model.

The response surface described by the model equation (ŷ) to estimate xylanase activity over independent variables xylan concentration (X_1) and pH (X_3) is shown in Fig 1. That illustrated the relationship between the response and experimental data. There is a rather broad plateau region over which the enzyme activity changes relatively litle when xylan concentration is varied. As observed, the fish powder P concentration in the range studied (0.23-1.75% (b/v)), did not affect xylanase production therefore the fish powder concentration was maintained at 1.75 % (b/v).

The validation steps of the models using solution from regression model (Eq. 1). Based on the model obtained, the optimal working conditions were defined to attain high xylanase activity minimizing the xylan concentration and cultivation time. Thus, the point assigned as optimum corresponded to 4.37% (b/v) of corn cob concentration and 1.75% (b/v) of fish powder P concentration, pH 9.0 and 24 h cultivation time. Under this condition, the model predicted a xylanase activity of 521.238 U mL^{-1} was slightly different with the predicted value of 519.5-523.1 U mL^{-1}. As a result, the models developed were considered to be accurate and reliable for predicting the production of xylanase by alkalophilic *B. halodurans* CM1.

DISCUSSION

Although previous research regarding alkalo thermophilic xylanase production has been reported, little information on the optimization of its production is available (Heck *et al.* 2005). Some of these studies with bacteria have not been reported using statistical designs (Brodel *et al.* 1990; Subramanian *et al.* 1998). In this paper, experimental method of 2 level factorial design, and RSM design demonstrated that the CCD and regression analysis methods were effective to find the optimized corn cobs, fish powder concentration and pH for the production of alkalo-thermostable xylanase from alkalophilic *B. halodurans* CM1. The bacterium a newly isolated strain growing on some economical and

Table 3 Results of regression analysis of the CCD

Term	Coefficient	Standard error	*P*-value
Intercept	2.33	0.067	0.0060
X_1	0.22	0.045	0.0011*
X_1X_1	-0.0049	0.044	0.2939
X_2	0.053	0.045	0.2679
X_2X_2	0.037	0.044	0.4178
X_3	0.033	0.045	0.4811
X_3X_3	-0.23	0.044	0.0008*
X_1X_2	0.024	0.058	0.6938
X_1X_3	-0.13	0.058	0.0518**
X_2X_3	0.064	0.058	0.3079

*Statistically significant at 99% of confidence level
**Statistically significant at 95% of confidence level

Table 4 Analysis of variance (ANOVA) for the model regression representing xylanase activity

Source	SS	DF	MS	F-ratio	P-value
Model	1.55	3	0.52	19.61	<0.0001
Residual	0.37	14	0.026		
Lack of Fit	0.35	11	0.032	6.44	0.0759
Total (corr.)	1.93	19			

R^2 = 0.80;SS, sum of squares; DF, degrees of freedom; MS,mean square

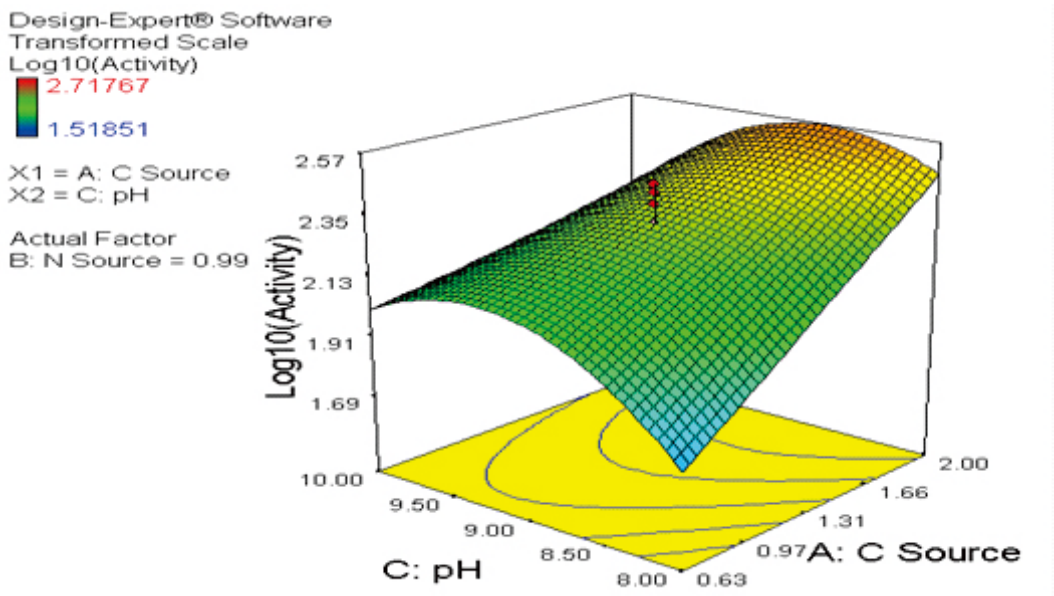

Fig 1 Response surface described by the model ŷ, which represents xylanase activity (U mL) as a function of xylan concentration and pH.

abundant substrate as corn cobs replaces the expensive carbon source-xylan and fish powder replaces the expensive nitrogen source-pepton. Using the RSM analysis it was found that maximum production xylanase activity was obtained at 4.37% (w/v) corncobs (w/v), 1.75% (w/v) fish powder P , pH 9.0 after 24 h incubation the activity was increased nearly twice compared to the original medium. According to Lin *et al.* (2007) experiment alkalophilic *Bacillus* sp under high pH conditions around 9.0-10.0 the cell growth and the production of xylanase were comparatively desirable, it corresponds to *B. halodurans* optimum pH 9.0. Statistical method for optimizing fermentation process could overcome the limitations of classic empirical methods and was proved to be a powerful tool to optimize the production of alkalothermostable xylanase from alkaliphilic *B. halodurans* CM1. In this work, RSM model was proposed to study the combined effects of culture media composition. Under optimal composition, the predicted production of xylanase after 24 h incubation was 52 U mL^{-1}.

Optimization study of xylanase production by *Bacillus circulans* D1 using RSM by Bocchini *et al.* (2002), showed that optimum xylanase activity of 19 mL^{-1} was gained at the optimum xylan concentration and cultivation time of 5 g L^{-1} and 48 h respectively. In our work the xylanase production was much higher and the highest xylanse activity was reached in a shorter period of time than that of Bocchini *et al.* (2002) report.

Response surface methods were also used to optimizing a culture medium with regards to xylanase production by a *Schizophyllum commune* strain (Haltrich *et al.* 1993), the optimum concentration of 73.4 g L^{-1} avicel, 55.5 g L^{-1} yeast extract, and 1.38 g L^{-1} NH_4NO_3 were determined by a central composite design. The highest xylanase production (5.74 U mL^{-1}), resulted in an increase of 330% compared initial medium and was reached within 11 days fermentation. The cultural conditions for thermostable xylanase production of *Thermomyces lanuginosus* was also studied by Purkarthofer *et al.* (1993), an experiment using CCD was performed to optimize the medium component concentrations. When the fungus was grown in the optimized medium 31.2 g L^{-1} corn cob, 30.2 g L^{-1} yeast extract, and 5.0 g L^{-1} KH_2PO_4, the highest xylanase production observed within 7 days was 2.70 U mL^{-1}.

Validation experiments were also carried out to verify the availability and the accuracy of the models, and the result showed that the predicted values were well agreed with the experimental values. It strongly suggested that the production of alkalo-thermostable xylanase by wild-strain alkaliphilic *B. halodurans* CM1 could improve in a large-scale fermentation process. The result of this study also provides some useful information for other extremozymes fermentation processes. These facts are important in making the whole process economically more feasible, in view of the high cost of pure xylan and the difficulty of its extraction from agricultural waste material. The alkalophilic pH of the fermentation medium 9.0 and the

high temperature used 50 °C also reduced the chances of contamination by opportunist microorganisms.

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