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# Optimization of mannanase production by *Bacillus* sp. HDYM-05 through factorial method and response surface methodology

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***Bacillus* sp. HDYM-05 was separated from flax retting liquid and identified as *Bacillus cereus* which was a new source of microbial mannanase. To optimize the medium components and condition of shake flask experiment for a higher mannanase production, factorial method and response surface methodology were used to obtain the maximal productivity by this species originally. The optimal medium components were: konjaku flour 8.81%, yeast extract 0.5%, NaNO<sub>3</sub> 0.5%, K<sub>2</sub>HPO<sub>4</sub> 0.5%, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.02%. The optimal condition of shake flask experiment was: 75 ml medium in 500 ml flask, inoculum volume 1%, initial pH 8.0 and 140 rpm with the cultivation temperature and time at 37°C and 32.05 h, respectively. The maximum enzyme activity of *Bacillus* sp. HDYM-05 achieved 6777.4 U/ ml, 1.4 folds under the optimal culture conditions as compared to the initial control.**

**Key words:** Mannanase, *Bacillus cereus*, optimization, factorial method, response surface methodology.

## INTRODUCTION

Mannanase ( $\beta$ -mannanase; EC 3.2.1.78) participates in the degradation of hemicellulose and similar polysaccharides by hydrolyzing the  $\beta$ -1, 4-glycosidic linkages within the main chain such as galactoglucomannan, the major hemicellulose of softwood (McCleary et al., 1988; Lundqvist et al., 2002). The hemicelluloses are the second richest renewable energy substances on earth (Wyman et al., 2005). Mannan, glucomannan, galactomannan, and galactoglucomannan are the major polysaccharides that constitute hemicellulose (Yan et al., 2008). Mannanase is useful in many fields including the feed, food as well as paper and pulps industries (Gubitz et al., 1997; Sachslehner et al., 2000; Daskiran et al., 2004; Kansoh

and Nagie, 2004). Furthermore, it is employed for the preparation of manno oligosaccharides used as non-nutritional food additives for selective growth of human beneficial intestinal microflora (bifidobacteria and lactobacilli) (Alvarez-Macedo et al., 2008). Despite of the high practical potentialities, the use of mannanase is still limited due to low yield and high-production cost. Various microorganisms have been reported as mannanase producers such as bacteria, yeast and fungi. Among that, *Bacillus* sp. is recommended because of its safety, fast growth and easiness of product purification (Mendoza et al., 1994; Zakaria et al., 1998). Despite all these, seeking for new microbial source and gaining high productivity of mannanase is still an interesting field.

A culture condition optimization by the traditional 'one-factor-at-time' is not only time consuming, but also often leads to an incomplete understanding of system behavior, resulting in a bafflement and failure of predictive response. To overcome these limitations,

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**Table 1.** Experimental variables at different levels used for the production of mannanase by *Bacillus* sp. HDYM-05 using factorial method.

Variables	Units	Symbol code	Experimental values		
			-1	0	+1
Konjaku flour	w/v%	$X_1$	4	6	8
Yeast extract	w/v%	$X_2$	0.5	1.25	2
Initial pH		$X_3$	7	8	9
Culture volume	ml /500 ml	$X_4$	75	100	125
Inoculum volume	v/v%	$X_5$	1	2	3
Cultivation time	h	$X_6$	36	48	60

factorial method and response surface methodology (RSM) can be employed to optimize enzyme activity performing a minimum number of experiments (Heck et al., 2005 a).

*Bacillus* sp. HDYM-05 with a high productivity of mannanase was separated in this study and identified as *Bacillus cereus* which was a new source of mannanase. Moreover, we originally integrated statistical experimental designs to study the medium components and shake flask experiment conditions to optimize the bioprocess of this species, aiming at the maximum production of mannanase.

## MATERIALS AND METHODS

### Separating and identifying of *Bacillus* sp. HDYM-05

Flax retting experiment was conducted at 37°C. The mannanase-producing bacteria were separated from retting liquid and screened according to Downie's method (Downie et al., 1994). The morphological and physiological characteristics of the isolated strain were studied. In addition, analysis of 16S rDNA sequence was employed (William et al., 1991).

### Medium and inoculum preparation

The medium (pH 7.0) for shake flask experiment was prepared by adding (w/v %) konjaku flour 2; yeast extract 0.5; NaNO<sub>3</sub> 0.5; K<sub>2</sub>HPO<sub>4</sub> 0.5; and MgSO<sub>4</sub>·7H<sub>2</sub>O 0.02. For inoculum preparation, shake flasks of seed liquids were incubated from a single colony from LB agar plate and incubated for 18 h, 37°C and 140 rpm. The basic shake flasks experiments were conducted at 37°C and 140 rpm for 48 h in 500 ml flasks containing 100 ml of the fermentation media with 2% inoculum (adjusted cell density to 10<sup>8</sup>/ml with 0.85% NaCl). After the cultivation specified for each set of experiments, the culture broth was centrifuged at 13,000 g for 10 min and the total mannanase activity in the cell-free supernatant was determined.

### Mannanase assay

Mannanase activity was assayed using a 0.5% (w/v) solution of reducing sugar-free konjaku flour in NaH<sub>2</sub>PO<sub>4</sub>-citric acid buffer as the substrate, pH 4.0. The release of reducing sugar in 30 min at 55°C was measured as D-mannose equivalents using the dinitrosalicylic acid (DNS) (Akino et al., 1988). One unit of enzyme

activity was defined as the amount of enzyme, which released 1 μmol of reducing sugar as equivalent to D-mannose per minute at 37°C.

### Selection of significant variables by factorial method

Several factors of media components and cultivation parameters affecting the shake flask experiment of mannanase production were tested via the factorial method. Low and high factors setting were coded as -1 and +1; the midpoint was coded as 0.  $X_i = (x_i - x_{i0})/\delta_i$ , where  $X_i$  was the coded value,  $x_i$  was the corresponding natural value,  $x_{i0}$  was the natural value in the center of the domain and  $\delta_i$  was the increment of  $x_i$ , corresponding to 1 unit of  $X_i$ . The response values ( $y$ ) in each trial were the average of the duplicates. The experimental design with the name, symbol code, and actual level of the variables was shown in Tables 1 and 2.  $P < 0.1$  indicated the significance of the tested variables. The linear model obtained was expressed as follow:

$$y = \beta_0 + \sum_{i=1}^k \beta_i x_i \quad (1)$$

If the mean of the center points exceeded the mean of the factorial points, the optimum would be near or within the experimental design space. If the mean of the center points had been less than the mean of the factorial points, the optimum would be outside the experimental design space thus the steepest ascent should be applied. The direction of steepest ascent was parallel to the normal of contour line of response curve of model (1) and passed through the center of factorial method.

Increment was direct ratio to regression coefficients  $\beta_i$ . Experiments were performed along the steepest ascent path until the response did not increase anymore; this point would be near the optimal point and could be used as center point to further optimization.

### Optimization by response surface methodology (RSM)

The response surface methodology (RSM), using a central composite design (CDD), was adopted for the augmentation of total mannanase production. The significant variables selected were konjaku flour and cultivation time, each of which was assessed at five coded levels (-1.414, -1, 0, +1, and +1.414), as shown in Table 5. A total of 14 experiments were conducted. All variables were taken at a central coded value, which was considered as zero. The minimum and maximum ranges of the variables were used, and the full experimental plan with regard to their values in actual and coded form was provided in Table 6. For two factors, the second-

**Table 2.** Eighteen-trial factorial method matrix for six variables with actual values along with the observed mannanase activity.

Run order	Experimental values						Mannanase activity (U/ml)
	X <sub>1</sub>	X <sub>2</sub>	X <sub>3</sub>	X <sub>4</sub>	X <sub>5</sub>	X <sub>6</sub>	
1	8	2	9	125	3	60	5343.4±25.2
2	8	0.5	9	125	3	36	6431.2±30.1
3	4	0.5	9	75	1	60	3839.4±22.5
4	4	0.5	7	125	1	36	4732.6±18.4
5	4	2	7	75	3	-36	4856.8±20.1
6	4	2	7	125	3	60	4563.1±19.8
7	8	2	9	75	1	36	6854.2±40.2
8	8	0.5	7	75	3	36	6412.3±45.1
9	8	2	7	75	-1	60	5478.9±33.3
10	4	0.5	9	75	3	60	4982.4±17.5
11	4	2	9	125	1	36	5812.4±22.2
12	8	0.5	7	125	1	60	6212.4±28.1
13	6	1.25	8	100	2	48	5234.4±21.1
14	6	1.25	8	100	2	48	5267.4±20.1
15	6	1.25	8	100	2	48	5321.2±21.4
16	6	1.25	8	100	2	48	5256.3±22.5
17	6	1.25	8	100	2	48	5245.5±21.9
18	6	1.25	8	100	2	48	5301.6±19.0

order polynomial equation could be presented as follows:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_6 + \beta_{12} x_1 x_6 + \beta_{11} x_1^2 + \beta_{22} x_6^2 \quad (2)$$

in which  $y$  was the measured response,  $\beta_0$  was the intercept term,  $\beta_1$  and  $\beta_2$  were linear coefficients,  $\beta_{12}$  was the interactive coefficient,  $\beta_{11}$  and  $\beta_{22}$  were the quadratic coefficients and  $X_1$  and  $X_6$  were coded independent variables.

#### Statistical analysis and model

The statistical software package, Design-expert7.1 (Stat-Ease, Inc., Minneapolis, MN, USA) was used for regression analysis of experimental data and also to plot response surface graphs. The statistical significance of the model equation and the model terms were evaluated via the Fisher's test. The quality of fit of the second-order polynomial model equation was expressed via the coefficient of determination ( $R^2$ ) and the adjusted  $R^2$ . The fitted polynomial equation was expressed in the form of three-dimensional surface plot, in order to illustrate the relationship between the responses and the experimental levels of each of the variable for maximum response. The combination of different optimized variables, which yielded the maximum response, was determined in an attempt to verify the validity of the model.

## RESULTS

### Characteristics and identification of strain HDYM-05

A bacterium strain HDYM-05 with a high mannanase-producing ability was screened from flax retting liquid. The strain was rod-shaped, spore-forming, 1.0-1.2  $\mu\text{m}$  wide and 3.0-5.0  $\mu\text{m}$  long, gram-staining positive. The

colony on agar plate was ash-colored, rough and platode. Strain HDYM-05 could utilize gelatin, starch, glucose and fructose but could not convert arabinose, xylose and sucrose into acid. The 16S rDNA partial sequence of strain HDYM-05 was amplified, sequenced, and submitted to GenBank (Accession no. EF428235). The comparative analysis demonstrated that 16S rDNA partial sequence from strain HDYM-05 had a significant identity to a number of *B. cereus* in nucleotide database of NCBI. According to the analysis of 16S rDNA sequence, together with its morphological and physiological characteristics, strain HDYM-05 was identified as *B. cereus*.

### Screening of significant variables using factorial method

The design matrix selected for the screening of significant variables for mannanase production and the corresponding response were shown in Table 2. The adequacy of the model was calculated, and the variables evidencing statistically significant effects were screened via Student's  $t$ -test for ANOVA (Table 3). Factors evidencing  $P$ -values of less than 0.1 were considered to have significant effects on the response, and were therefore selected for further optimization studies. Konjaku flour, with a probability of 0.0127, and followed by cultivation time (0.0756) were determined to be the most significant factors. The other four factors were considered non-significant and neglected. A linear

**Table 3.** Estimated effect, regression coefficient and corresponding *t* and *P* values for mannanase activity in six variables factorial method experiment.

Variables	Effect	Coefficient	Standard error	<i>t</i> -value	<i>P</i> -value
$X_1$	1324.28	662.1417	174.38	-3.8	0.0127 <sup>c</sup>
$X_2$	49.75	-24.875	174.38	-0.14	0.8921 <sup>a</sup>
$X_3$	167.82	-83.90833	174.38	-0.48	0.6507 <sup>a</sup>
$X_4$	111.85	-55.925	174.38	-0.32	0.7614 <sup>a</sup>
$X_5$	-56.78	28.391667	174.38	0.16	0.877 <sup>a</sup>
$X_6$	-779.98	-389.99167	174.38	2.24	0.0756 <sup>b</sup>

<sup>a</sup>Non-significant at  $P > 0.1$ , <sup>b</sup>Significant positive effect, <sup>c</sup>Significant negative effect.

**Table 4.** Results of the steepest ascent path experiments.

Run order	Konjaku flour (w/v %)	cultivation time (h)	Mannanase activity (U/ml)
1	8.0	36	5320.3±22.4
2	8.2	35	5423.4±20.4
3	8.4	34	5512.1±25.1
4	8.6	33	5688.2±19.8
5	8.8	32	5768.5±16.3
6	9.0	31	5550.6±21.6
7	9.2	30	5423.9±22.7
8	9.4	29	5388.5±25.1
9	9.6	28	5112.1±19.0
10	9.8	27	5104.5±18.5
11	10.0	26	5098.2±18.6

regression equation could be obtained from the regression results of the experiment:

$$y = +5459.92500 + 662.14167X_1 - 389.99167X_6 \quad (3)$$

The insignificant difference between the mean of responses at all experimental points and the responses at the center points (2-tails test,  $df=11$ ,  $t=0.72$ ,  $P=0.04863$ ) indicated that optimal point was outside the experimental design space and the method of steepest ascent should be applied. The direction of steepest ascent path could be determined by Equation (3) and regression results.  $X_2$  (yeast extract),  $X_4$  (culture volume),  $X_5$  (inoculum volume) were fixed at low level.  $X_3$  (initial pH) was fixed at center point (8.0).  $X_1$  (konjaku flour) and  $X_6$  (cultivation time) were significant factors and coefficient of  $X_1$  was positive while coefficient of  $X_6$  was negative, meaning that increasing the concentration of  $X_1$  while decreasing the length of  $X_6$  had positive effects on the mannanase production. Design of experiment of steepest ascent and corresponding results were shown in Table 4. Mannanase production of Run 5 was the highest (5768.5 U/ml), then enzyme production decreased following the levels changing. The results meant that the level of  $X_1$  and  $X_6$  was near the optimal, thus Run 5 was chosen as the center point to further optimization. The results of steepest ascent also indicated that the

exceeded concentration of konjaku flour would inhibit enzyme production.

#### Optimization of significant variables using response surface methodology

Concentration of konjaku flour ( $X_1=8.8\%$ ) and cultivation time ( $X_6=32\text{h}$ ) in Run 5 were chosen as the center point to further optimization with a central composite design. Experimental design and results were shown in Table 6. The ANOVA analysis of the optimization study was shown in Table 7. The model *F*-value was 1210.82 and the *F*-value for lack of fit was 2.03. The high *F*-value and non-significant lack of fit indicated that the model was a good fit. The *P*-value for model ( $<0.0001$ ) and for lack of fit (0.2527) also suggested that the obtained experimental data was a good fit with the model.

A full second-order polynomial model was obtained from regression analysis of data of experiment of central composite design:

$$y = +6769.88333 + 43.55001X_1 + 64.23660X_6 + 50.87500X_1X_6 - 668.57916X_1^2 - 647.85416X_6^2 \quad (4)$$

The regression equation obtained from the ANOVA

**Table 5.** Experimental codes, ranges and levels of the independent variables for response surface methodological experiment.

Variables	Units	Symbol code	Levels				
			-1.414	-1	0	+1	+1.414
Konjaku flour	w/v%	$X_1$	8.52	8.6	8.8	9.0	9.08
Cultivation time	h	$X_6$	30.58	31	32	33	33.41

**Table 6.** Central composite design matrixes for the experimental design and observed response for mannanase.

Run order	Block	$X_1$	$X_6$	Mannanase activity (U/ml)
1	1	-1	1	5423.6±23.1
2	1	0	0	6749.4±24.6
3	1	1	1	5589.1±33.1
4	1	-1	-1	5436.2±19.2
5	1	0	0	6789.1±36.2
6	1	0	0	6798.8±26.2
7	1	1	-1	5398.2±17.5
8	2	0	0	6754.2±28.2
9	2	0	0	6738.2±29.4
10	2	0	-1.414	5347.2±24.1
11	2	-1.414	0	5346.3±17.9
12	2	0	1.414	5584.5±18.6
13	2	1.414	0	5202.5±16.4
14	2	0	0	6789.6±18.4

**Table 7.** Analysis of variables (ANOVA) for the parameters of response surface methodology fitted to second-order polynomial equation.

Source	SS	DF	MS	F-value	P-value>F
Model	6.002×10 <sup>6</sup>	5	1.2×10 <sup>6</sup>	1210.82	<0.0001
Residual	6939.69	7	991.38		
Lack of fit	4185.80	3	1395.27	2.03	0.2527
Pure error	2753.89	4	688.47		
Total	6.010×10 <sup>6</sup>	13			

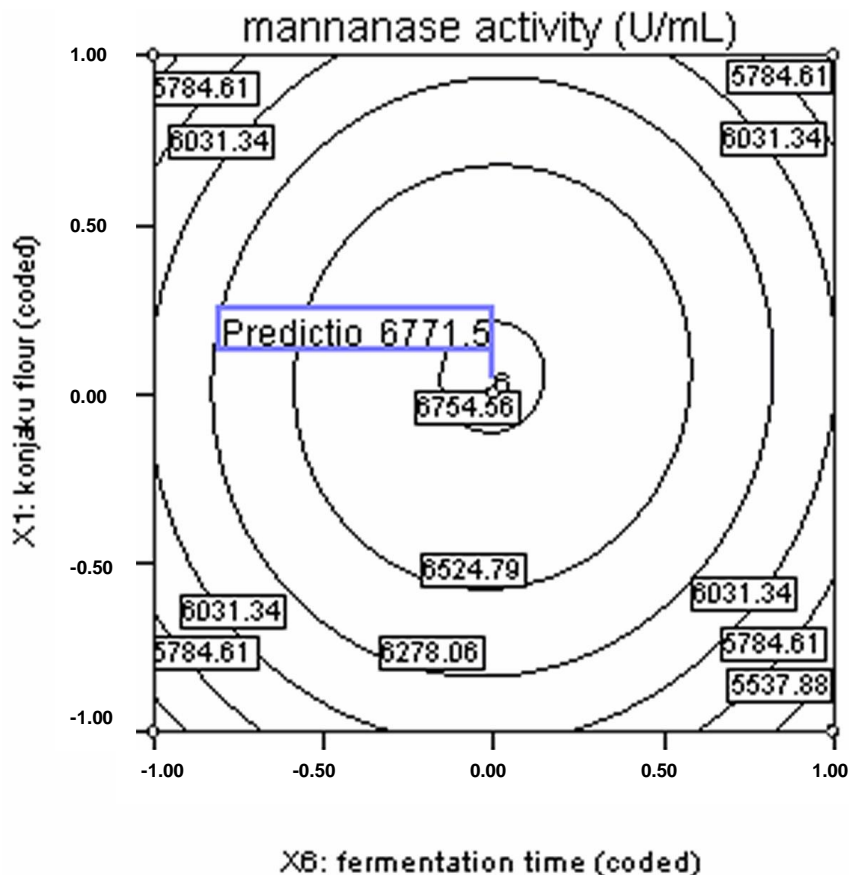
$R^2=0.9988$ ,  $CV=0.52\%$ ,  $Adj-R^2=0.9980$ ,  $Pred-R^2=0.9917$ , SS, sum of squares, DF, degree of freedom, MS, mean square.

showed that the  $R^2$  (multiple correlation coefficient) was 0.9988 (a value >0.75 indicates fitness of the model). This was an estimate of the fraction of overall variation in the data accounted by the model, and thus the model was capable of explaining 99.88% of the variation in response. The 'adjusted  $R^2$ ' was 0.9980 and the 'predicted  $R^2$ ' was 0.9917, which indicated that the model was good (for a good statistical model, the  $R^2$  value should be in the range of -1.0, and the nearer to 1.0 the value was, the more fit the model was deemed to be).

Figure 1 showed the effect of konjaku flour and cultivation time on the mannanase production. This result demonstrated that the response surface had a maximum point. The maximum production of mannanase by *Bacillus* sp. HDYM-05 was obtained under the optimized

condition when the concentration of konjaku flour and cultivation time was 8.81% (w/v), 32.05 h, respectively. The maximum response predicted from the model was 6771.54 U/ml. Repeated experiments were performed to verify the predicted optimum. The result from ten replications (that is, 6775.3, 6780.4, 6788.2, 6785.5, 6782.1, 6768.9, 6765.4, 6788.2, 6772.3, 6767.5 U/ml) was coincident with the predicted value and the model was proven to be adequate (T-test,  $P=0.0948$ ).

The final results optimized with RSM were: media contained (w/v %) konjaku flour 8.81; yeast extract 0.5;  $NaNO_3$  0.5;  $K_2HPO_4$  0.5;  $MgSO_4 \cdot 7H_2O$  0.02. Shake flask experiment conditions were: 75 mL medium in 500 ml flask, inoculum volume 1%, initial pH 8.0 and 140 rpm with the cultivation temperature of 37°C. Compared with



**Figure 1.** Response surface plot for the effect of konjaku flour ( $X_1$ ) and cultivation time ( $X_6$ ) on mannanase production.

original condition, the mannanase activity increased from 4885.5 to 6777.4 U/ml and cultivation time shorted from 48 h to 32.05 h.

## DISCUSSION

The use of statistical models to optimize culture medium components and cultivation conditions has increased in present-day biotechnology, due to its ready and applicability and aptness (Reddy et al., 2008). In this work, the factorial method and response surface analysis was originally proved to be effective to determine optimal shake flask experiment condition of mannanase production by *B. cereus*. A high degree of similarity was observed between the predicted and experimental values that reflected the accuracy and applicability of RSM to optimize the process for enzyme production. Similar improved production was reported in other RSM experiments, most notably in the case of  $\alpha$ -amylase from *Bacillus circulans* GRS313 (Dey et al., 2001) and in the case of protease production using *Bacillus* sp. RGR-14 (Chauhan and Gupta, 2004). In addition to establishing optimal cultivation medium compositions, the present

methodology also makes it possible to predict both yield and productivity when the system is disturbed in some way (Tang et al., 2004). This is useful not only for the additional knowledge supplied about the process, but also for the potentials of process control. A successful and significant improvement (1.4-fold) in the production of mannanase by *Bacillus* sp. HDYM-05 was accomplished within a shorter cultivation period.

*Bacillus* species normally generate extracellular enzymes, which perform a central function in present-day biotechnology (Priest 1977), several of which have been reported as mannanase producers (Feng et al., 2003; Heck et al., 2005 b; Jiang et al., 2006). This study obtained a mannanase-producing strain *B. cereus* HDYM-05 which extended the microbial source of the industrially widely-used enzyme. The purification and characterization of the new mannanase need further studied in following research.

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