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Optimization for Novel Glucanhydrolase Production of *Lipomyces starkeyi* KSM 22 by Statistical Design

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Abstract Response surface methodology was applied to find the optimum conditions for the production of DXAMase (containing both dextranase and amylase activities) based on the cultivation variables (pH, temperature, and agitation rate). The experimental values from the model equation conceded with predicted values in which the predicted values for dextranase and amylase activities were 2.26 and 3.52 U/ml at pH 4, 28°C, 235 rpm, and the corresponding experimental values were 2.41 and 3.68 U/ml, respectively.

Key words: *Lipomyces starkeyi*, optimization, response surface methodology, Box-Behnken, dextranase

In microbial cultivation biotechnology, the productivity of the microbial metabolite is improved by genetically altering the strain and by manipulating the nutritional and physical parameters of the growth [7, 8, 11, 13, 17, 18]. External conditions play a vital role in the efficiency and economic aspect of the final process. They not only influence the growth and metabolism of the cultures, but also increase product titres and consequently process economics [1] as well. The optimal design for growth conditions is an important aspect in the development of microbial cultivation process. Conventional methods for multifactor experimental designs are time-consuming and incapable of detecting the true optima, due to interactions among factors. One of the worthwhile techniques to identify the explanatory variable in the system is experimental design technique called response surface methodology (RSM) [3]. RSM is an empirical modelization technique devoted to the evaluation of the relationship of a set of controlled experimental factors and observed results. It requires a prior knowledge of the process to achieve the statistical model. In fact, a detailed account of this technique has been outlined [4]. Basically, this optimization process involves three major steps to perform the statistically designed experiments; estimating the co-efficients in a mathematical model and predicting the response along with checking the adequacy of the model. It provides statistical models with a relatively small number of experiments. From these models, a relative influence of the various factors can be determined, and their optimal conditions are calculated for a given target, such as maximal activity or maximal metabolite production.

Lipomyces starkeyi, an ascosporogenous yeast, produces an endodextranase (EC 3.2.1.11) that cleaves the D-glucopyranosyl linkages in dextran and an α-amylase (EC 3.2.1.1) that hydrolyzes the D-glucopyranosyl linkages in starch. *L. starkeyi* dextranase has been used to successfully treat sugar process streams [10, 12]. Except for a few bacterial dextranases, microbial dextranases are generally inducible. Ryu *et al.* [9, 16] reported a novel glucanhydrolase from a mutant *L. starkeyi* KSM 22 of *L. starkeyi* ATCC 74054. This glucanhydrolase has combined activities of dextranase and amylase. They referred this glucanhydrolase as a DXAMase. It was able to inhibit or prevent plaque formation and partially remove pre-formed plaque. Furthermore, it showed elevated activity on insoluble-glucan, compared to *Penicillium funiculosum* dextranase. Degradation of insoluble glucan by DXAMase was 3.75-fold higher than that produced by the *P. funiculosum* dextranase. Also, DXAMase strongly bonds to hydroxylapatite, raising the possibility that it can persist when binding to

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teeth in the mouth. DXAMase functions as pre-brushing rinse agents to loosen plaque. It is nontoxic, nonmutagenic, and nonallergenic. This study was focused on obtaining the best temperature, pH, and agitation rate for acquiring the maximal production of a novel glucanhydrolase, DXAMase. This contains both dextranase and amylase equivalent activities which were prepared from *L. starkeyi* KSM 22, a constitutive mutant, grown on starch by the Box-Behnken design for experiments [4].

L. starkeyi KSM 22 was grown for 36 h in 8 l vessels containing 6 l LM medium with 1% soluble starch. LM medium consisted of 0.3% (w/v) yeast extract and 0.3% (w/v) KH_2PO_4. The pH of the medium was controlled with 30% NaOH and 30% HCl. 3,5-Dinitrosalicylic acid, Rochelle salt (potassium sodium tartarate), and phenol were purchased from Aldrich (Milwaukee, WI, U.S.A.); Maltose and isomaltose were purchased from Sigma (St. Louis, MO, U.S.A.); Starch was from Yakuri Pure Chemicals (Kyoto, Japan); Yeast extract was from Duchefa (Haarlem, The Netherlands). All other reagents used were analytical grade. Cell-free culture medium after centrifugation was used for determination of combined amylase and dextranase activities (DXAMase). The formation of reducing sugar from dextran or starch was determined with dinitrosalicylic acid (DNS) reagent. Assays on dextran or starch solution were carried out at 37°C by mixing 50 µl of enzyme with 450 µl of 2% dextran or starch that was dissolved in 20 mM citrate phosphate buffer, pH 5.5 [15]. The reaction was stopped after 10 min by mixing with 50 µl of reaction solution and 150 µl DNS reagent. The samples were then boiled for 10 min and cooled. Absorbance at 595 nm was read and values for reducing sugar were expressed as maltose or isomaltose equivalents. One unit of DXAMase for both amylase and dextranase equivalent activities corresponds to the formation of 1 µmol maltose and isomaltose equivalent per min, respectively.

The variables for experimental design and optimization [5, 14] of DXAMase production were pH, temperature, and agitation rate, since the composition of nutritional components was optimized previously [8]. Although the DXAMase has two enzyme activities, amylase and dextranase, the design and quadratic model equation was prepared based on dextranase equivalent activity, because dextranase and amylase equivalent activities are proportional in a DXAMase (data not shown). The significant independent variables can be approximated by the quadratic model equation [2, 4].

$$\mathbf{Y}=\mathbf{b}_0+\mathbf{b}_1\mathbf{X}_1+\mathbf{b}_2\mathbf{X}_2+\mathbf{b}_3\mathbf{X}_3+\mathbf{b}_{11}\mathbf{X}_1^2+\mathbf{b}_{22}\mathbf{X}_2^2+\mathbf{b}_{33}\mathbf{X}_3^2 +\mathbf{b}_{12}\mathbf{X}_1\mathbf{X}_2+\mathbf{b}_{13}\mathbf{X}_1\mathbf{X}_3+\mathbf{b}_{23}\mathbf{X}_2\mathbf{X}_3, \quad (1)$$

Where, **Y**=predicted response; $\mathbf{X}_1$, $\mathbf{X}_2$, and $\mathbf{X}_3$ are input variables; $\mathbf{b}_0$ is a constant; $\mathbf{b}_1$, $\mathbf{b}_2$, and $\mathbf{b}_3$ are linear coefficients; $\mathbf{b}_{12}$, $\mathbf{b}_{13}$ and $\mathbf{b}_{23}$ are cross product coefficients; $\mathbf{b}_{11}$, $\mathbf{b}_{22}$, and $\mathbf{b}_{33}$ are quadratic coefficients [2, 6]. The low, middle, and high levels of each variable, namely pH, temperature, and agitation rate, were coded as - 1, 0, and +1, respectively, and are given in Table 1, and the actual design of experiments is given in Table 2. By solving Eq. (1), a total of 17 experiments were performed in order to estimate the 10 coefficients for optimization of the DXAMase production condition [2]. A full second-order polynomial model obtained by a multiple regression technique by using the Design Expert (Statistics Made Easy, Minneapolis, MN, U.S.A.) was adopted to describe the response surface.

Table 1. Independent variables and experimental design levels for trials.

X_i*	Culture conditions	Standardized levels		
		- 1	0	+1
X_1	pH	3.5	4.0	4.5
X_2	Temperature (°C)	25	28	31
X_3	Agitation rate (rpm)	100	200	300

*Independent variables.

Dextranase equivalent activity of DXAMase from the model at each experimental point is summarized and listed in Table 2 along with experimental and theoretical values. The summaries of the analysis of variances (ANOVA) for dextranase activity are listed in Table 3. All terms regardless of their significance based on the Eq. (1) are included in the following equations:

For the dextranase equivalent activity

$$Y_1=2.21+0.13X_1\ 0.048X_2+0.22X_3\ 0.77X_1^2\ 0.68X_2^2\ 0.35X_3^2 +0.075X_1X_2+0.17X_1X_3+0.22X_2X_3 \quad (2)$$

Table 2. The Box-Behnken design for the three independent variables and experimental along with theoretically predicted values for DXAMase activity.

Trial no.	Independent variables			DXAMase activity (dextranase equivalent; U/ml)	
	pH	Temperature (°C)	Agitation rate (rpm)	Experimental value	Predicted value
1	3.5	25	200	0.60	0.75
2	4.5	25	200	0.73	0.87
3	3.5	31	200	0.64	0.50
4	4.5	31	200	1.07	0.92
5	3.5	28	100	1.03	0.91
6	4.5	28	100	0.94	0.84
7	3.5	28	300	0.90	1.00
8	4.5	28	300	1.51	1.62
9	4.0	25	100	1.27	1.23
10	4.0	31	100	0.45	0.70
11	4.0	25	300	1.48	1.23
12	4.0	31	300	1.53	1.57
13	4.0	28	200	2.20	2.21
14	4.0	28	200	2.21	2.21
15	4.0	28	200	2.18	2.21
16	4.0	28	200	2.28	2.21
17	4.0	28	200	2.20	2.21

Table 3. Regression analysis of DXAMase and quadratic RSM fitting (ANOVA).

Source	DXAMase activity (dextranase equivalent)				
	S.S[a]	DF[b]	M.S[c]	*F* value	Prob>F
Model	6.38	9	0.71	19.19	0.0004
Residual	0.26	7	0.037		
Lack of fit	0.26	3	0.085	57.67	0.001
Pure error	0.006	4	0.0015		
Cor. total	6.64	16			

[a]Sum of squares.
[b]Degree of freedom.
[c]Mean square; R^2=0.9606 (dextranase equivalent).

Statistical significance of the second-order model equation was checked by an *F*-test. The fit of the model was also expressed by the coefficient of determination R^2, which was found to be 0.96 for the dextranase, indicating that the model can explain 96% of the variability in the response (Table 3). This revealed that Eq. (2) is a suitable model to describe the response of the experiment pertaining to optimal production of DXAMase. The Eq. (2) shows that the linear term of agitation rate (X_3), quadratic coefficients of X_1^2, X_2^2, and X_3^2 have remarkable effects on the dextranase activity. These model terms have less than 5% probability level (data not shown). Although there are a few less significant terms in the analysis of variances, they were not omitted from the model equation, because its adequate fit could be confirmed. This can also be seen from the 3D surface plots for these components following Eq. (2) (Fig. 1). As a result, the stationary point was observed in the surface plots of these components. The relationship between variables and responses was also investigated by examining a series of contour plots generated by holding constant one variable of the second-order polynomial equation. Figure 2 shows the contour plots holding pH. Varying pH from 3.5 to 4.0 significantly affected the activity of dextranase (a-c), but between pH 4.0 and 4.5 dextranase activity decreased, indicating that the pH for the maximum activity of DXAMase has to be kept near pH 4.0. A similar 3D surface plot was shown in the temperature (Fig. 1). The measured activity levels increased, when the temperature increased from 25 to 28°C, however, further increasing temperature to 31°C actually decreased the activity level. Thus, the optimum temperature for the

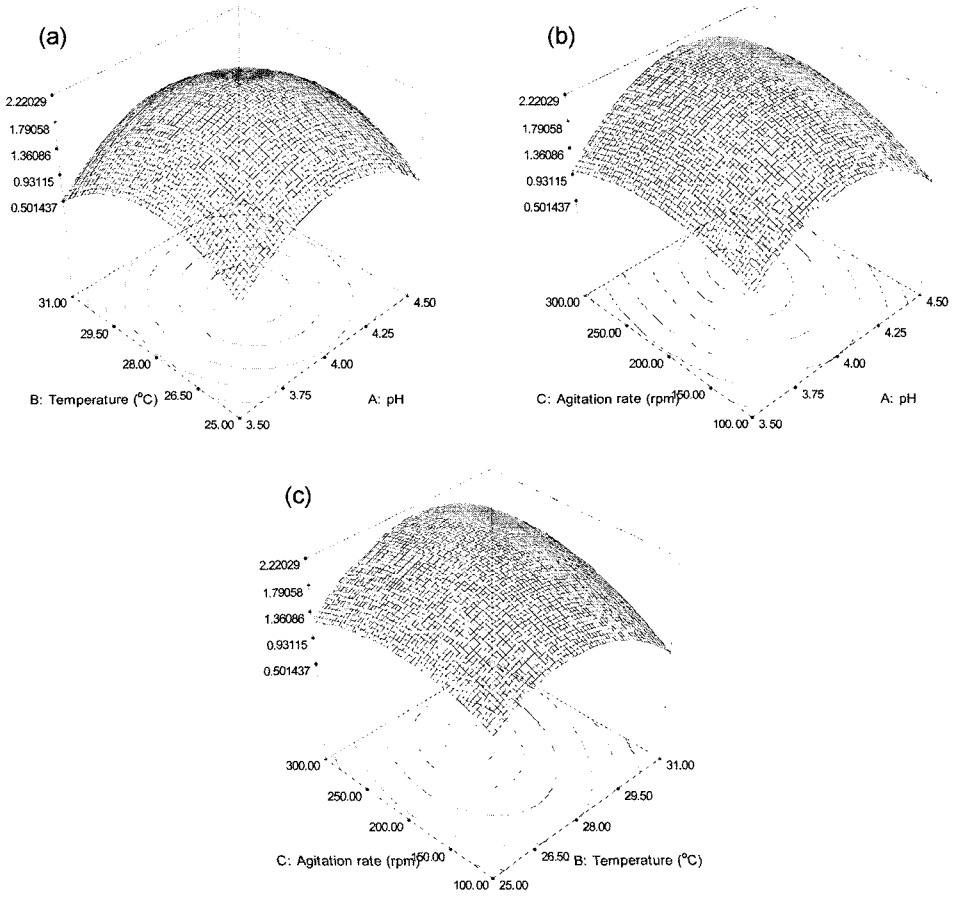

Fig. 1. DXAMase as a 3D graphics for response surface optimization on each value (a,b,c: dextranase at fixed agitation rate, temperature, and pH, respectively).

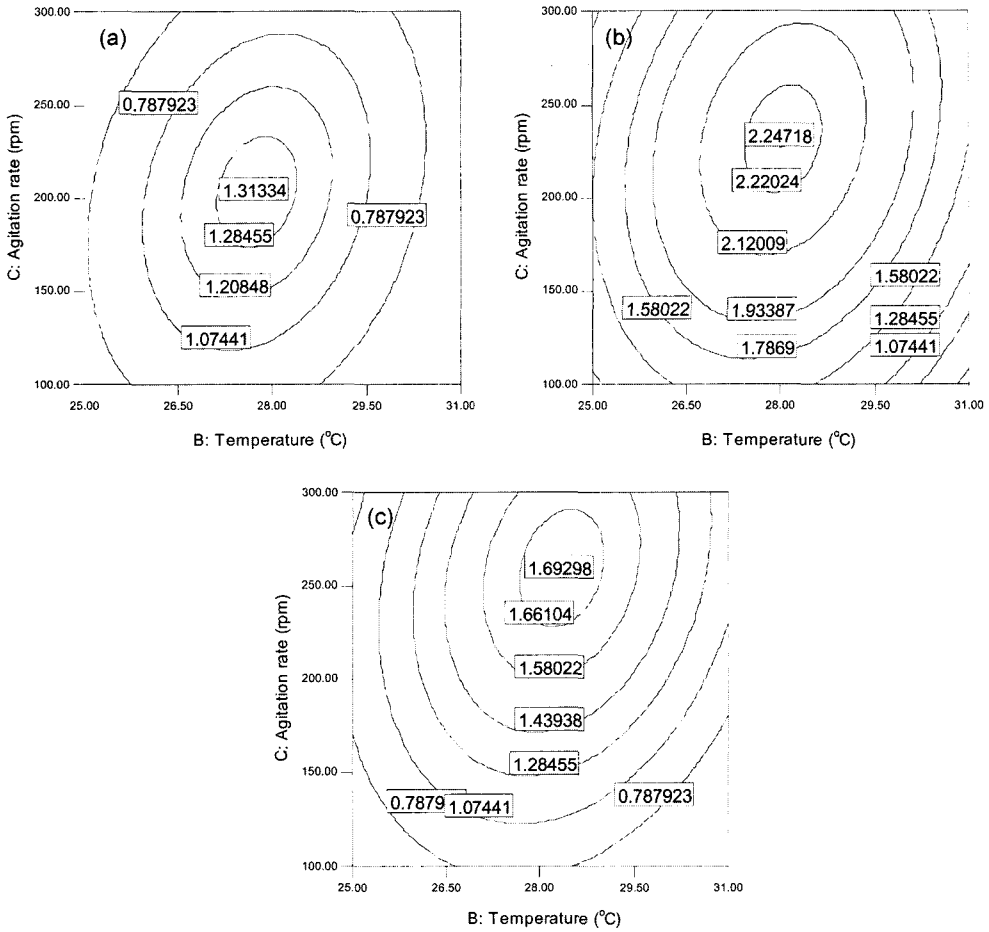

Fig. 2. Response surface contour plots of dextranase activity (a-c) on DXAMase showing effects of temperature and agitation rate under constant pH. The numbers inside plots represent activity (U/ml): pH (a) 3.5, (b) 4.0, (c) 4.5.

maximum activity in the cultivation was concluded to be 28°C. In the case of the agitation rate, the dextranase activity showed a similar pattern with the case of holding pH and temperature (Fig. 1). The method of ridge analysis computes the estimated ridge of optimum response by increasing the radii from the center of the original design. The results from the ridge analysis indicated that the optimized conditions for dextranase equivalent activity of DXAMase showed the highest predicted activity of 2.26 U/ml under the condition of pH 4, 28°C, and 235 rpm. Under this condition, the predicted amylase activity showed the highest activity of 3.52 U/ml. This estimated fermentation condition was confirmed and the dextranase and amylase equivalent experimental activities of DXAMase were slightly higher at 2.41(±0.06) and 3.68(±0.11), respectively. Thus, the response surface equation obtained was suitable to find the optimum condition of DXAMase production, which contains both dextranase and amylase equivalent activities, on the cultivation variables (pH, temperature, and agitation rate) that provide a cheaper carbon source of starch.

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References

1. Anindya, S., S. Padma, and K. D. Parichay. 1998. Optimization of solid state medium for the production of clavulanic acid by *Streptomyces clavuligerus*. *Proc. Biochem.* **33:** 283-289.
2. Annadurai, G. and R. Y. Sheeja. 1998. Use of Box-Behnken design of experiments for the adsorption of verofix red using biopolymer. *Bioproc. Eng.* **18:** 463-466.
3. Bing-Lan, L. and T. Yew-Min. 1998. Optimization of growth medium for the production of spores from *Bacillus thuringiensis* using response surface methodology. *Bioprocess Eng.* **18:** 413-418.
4. Box, G. E. P., D. W. Behnken, and J. S. Junter. 1957. Multifactor experimental design for exploring the response surfaces. *Ann. Math. Statist.* **28:** 195-214.

5. Chan, L., B. Jinghua, C. Zhaoling, and O. Fan. 2002. Optimization of a cultural medium for bacteriocin production by *Lactococcus lactis* using response surface methodology. *J. Biotechnol.* **93:** 27-34.
6. Cochran, W. G. and D. W. Cox. 1968. *Experimental Designs*. New York: John Wiley and Sons Inc. pp. 611-626.
7. Kim, C. Y., J. H. Lee, B. H. Kim, S. K. Yoo, E. S. Seo, K. S. Cho, D. F. Day, and D. Kim. 2002. Production of mannitol using *Leuconostoc mesenteroides* NRRL B-1149. *Biotechnol. Bioprocess Eng.* **7:** 234-236.
8. Kim, D. and D. F. Day. 1995. Isolation of a dextranase constitutive mutant of *Lipomyces starkeyi* and its use for the production of clinical size dextran. *Lett. Appl. Microbiol.* **20:** 268-270.
9. Kim, D., S. J. Ryu, E. J. Son, H. J. Chung, S. H. Kim, D. W. Kim, and D. F. Day. 2002. Glucanhydrolase from *Lipomyces starkeyi* KSM22 as a potential mouthwash ingredient. *J. Microbiol. Biotechnol.* **12:** 993-997.
10. Kim, D., H. C. Seo, and D. F. Day. 1996. Dextran production by *Leuconostoc mesenteroides* in the presence of a dextranase producing yeast *Lipomyces starkeyi*. *Biotechnol. Techniq.* **10:** 227-232.
11. Kim, Y. M., H. J. Ryu, S. O. Lee, E. S. Seo, S. Y. Lee, S. K. Yoo, D. L. Cho, D. Kim, A. Kimura, S. Chiba, and J. H. Lee. 2001. Production of maltopentaose and biochemical characterization of maltopentaose-forming amylase. *J. Microbiol. Biotechnol.* **11:** 636-643.
12. Koenig, D. W. and D. F. Day. 1989. Induction of *Lipomyces starkeyi* dextranase. *Appl. Environ. Microbiol.* **55:** 2079-2081.
13. Lee, H. S., S. H. Park, J. H. Lee, and H. K. Lee. 2001. Effect of aeration rates on production of extracellular polysaccharides, EPS-R by marine bacterium *Hahella chejuensis*. *Biotechnol. Bioprocess Eng.* **6:** 359-362.
14. Lee, J., L. Ye, W. O. Landen, and R. R. Eitenmiller. 2000. Optimization of an extract procedure for the quantification of vitamin E in tomato and broccoli using response surface methodology. *J. Food Comp. Anal.* **13:** 45-57.
15. Miller, G. L. 1959. Use of dinitrosalicylic acid reagent for the determination of reducing sugars. *Anal. Chem.* **31:** 426-428.
16. Ryu, S. J., D. Kim, H. J. Ryu, S. Chiba, A. Kimura, and D. F. Day. 2000. Purification and partial characterization of a novel glucanhydrolase from *Lipomyces starkeyi* KSM 22 and its use for inhibition of insoluble glucan formation. *Biosci. Biotechnol. Biochem.* **64:** 223-228.
17. Yoo, S. K., D. Kim, and D. F. Day. 2001. Highly branched glucooligosaccharide and mannitol production by mixed culture fermentation of *Leuconostoc mesenteroides* and *Lipomyces starkeyi*. *J. Microbiol. Biotechnol.* **11:** 700-703.
18. Yoo, S. K., D. Kim, and D. F. Day. 2001. Co-production of dextran and mannitol by *Leuconostoc mesenteroides*. *J. Microbiol. Biotechnol.* **11:** 880-883.