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Improvement of Amidase Production by a Newly Isolated *Delftia tsuruhatensis* ZJB-05174 Through Optimization of Culture Medium

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The *R*-amidase production by a newly isolated strain of *Delftia tsuruhatensis* ZJB-05174 was optimized in this paper. Effects of factors such as carbon sources, nitrogen sources, and inducers on amidase production were investigated. The medium composition was optimized using central composite designs and response surface analysis. The optimal medium components for enhanced amidase production were found to be as follows: glucose, 8.23 g/l; yeast extract, 11.59 g/l; 2,2-(*R,S*)-dimethylcyclopropane carboxamide, 1.76 g/l; NaCl, 1 g/l; KH_2PO_4, 1 g/l; and K_2HPO_4, 1 g/l. A maximum enzyme production of 528.21 U/l was obtained under the optimized conditions, which was 4.7 times higher than that obtained under initial conditions.

Keywords: Amidase, optimization, *Delftia tsuruhatensis*, 2,2-dimethylcyclopropane carboxamide

Enantiomerically pure amides and their derivatives such as (*S*)-2,2-dimethylcyclopropane carboxamide, lysergic acid, α-amino acids, and 2-arylpropionic acids are important pharmaceutical and agrochemical chiral building blocks. They can be produced *via* various processes, both chemically and enzymatic. Biocatalysis, the enzymatic way, had played important roles in the asymmetric synthesis of such chiral substances and other useful chemicals [4, 10, 23]. With the ability of enantioselective hydrolysis of amides to produce corresponding optical pure compounds, interests in amidases (E.C. 3.5.1.4) have been aroused. The increasing potential of amidase applications prompts screening for new amidase-producing microorganisms and improvement for amidase production of existing strains [2, 6–9, 14, 18, 22, 24–26].

Recently, we isolated a novel *R*-enantioselective amidase-producing strain, *Delftia tsuruhatensis* ZJB-05174, from sewage using a colorimetric screening method [27, 29]. The strain can efficiently and strictly *R*-enantioselectively degrade (*R*)-2,2-dimethylcyclopropane carboxamide (*R*-1) from (*R,S*)-2,2-dimethylcyclopropane carboxamide (*R,S*-1) to produce (*R*)-2,2-dimethylcyclopropane carboxylic acid (*R*-2) and accumulate (*S*)-2,2-dimethylcyclopropane carboxamide (*S*-1), a key intermediate in the production of cilastatin (Merck), a renal dehydropeptidase inhibitor commonly administered with penem and carbapenem antibiotics to prevent their degradation in the kidney [3]. The strain could potentially be applied in the biosynthesis of (*S*)-2,2-dimethylcyclopropane carboxamide and other optically pure substances.

In the present paper, optimization of the medium composition for amidase production by *D. tsuruhatensis* ZJB-05174 using response surface methodology was performed to improve amidase production ability.

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Materials and Methods

Materials

Compounds (*R,S*)-**1**, (*R,S*)-**2**, and (*S*)-**1** were provided by Huakang Chemicals Ltd. (Zhejiang, China). Peptone and yeast extract were purchased from Huadong Medicine Group (Zhejiang, China). All other chemicals were of analytical grade.

Microorganism and Inoculum Preparation

D. tsuruhatensis ZJB-05174 was isolated from sewage [29]. The strain was maintained on nutrient agar slants (in g/l: beef extract, 3.0; peptone, 10.0; NaCl, 5.0; agar, 15.0; pH 7.2) and was subcultured periodically at 30°C. It was stored at 4°C. For inoculum preparation, the 24-h-old slant culture was inoculated into a 250-ml flask containing 40 ml of sterilized seed medium [the original amidase production medium, in g/l: glucose, 8.4; yeast extract, 7.0; peptone, 0.7; acetamide (inducer for amidase production), 3.56; KH_2PO_4, 1.0; K_2HPO_4, 1.0; NaCl, 1.0; pH 7.5] for 20 h. The cultivation was performed aerobically on a rotary shaker at 30°C, 150 rpm.

Amidase Production

The amidase production was carried out in a 250-ml flask containing 40 ml of production medium, inoculated with 2.5% (v/v) inoculum, and incubated for 24 h at 30°C in a rotary shaker, 150 rpm. Then, the culture broth was collected to determine the amidase activity.

Selection of Best Carbon Sources, Nitrogen Sources, and Inducers

Various carbon sources (glucose, mannitol, dextrin, maltose, fructose, citric acid, sorbitol, sodium pyruvate, glycerol, lactose, xylose, and sucrose), nitrogen sources [NH_4NO_3, NH_4Cl, Peptone, yeast extract, beef extract, corn steep liquor powder, $(NH_4)_2HPO_4$, ammonium acetate, NH_4HCO_3, $NaNO_3$, and $(NH_4)_2SO_4$], and inducers [asparagine, nicotinamide, ε-caprolactam, acrylamide, acetamide, and (*R,S*)-1] were tested to select the best carbon source, nitrogen source, and inducer for amidase production. The tested carbon sources, nitrogen sources, and inducers were used as substitutes individually to the production medium with a final concentration of 10 g/l, 7 g/l, and 1.0 g/l for carbon source, nitrogen source, and inducer, respectively, while the other ingredients were kept at original concentrations. Before autoclaving, the initial pH value of the medium was adjusted to 7.5.

Optimization of Medium with Response Surface Methodology (RSM)

In this study, a central composite design was used. According to this design, 20 experiments were performed containing 6 replications at the center point in triplicates. Three key variables with three concentration levels were adopted. The production of amidase was the response variable. The individual and interactive effects of the variables were determined by fitting the second-order polynomial equation to data obtained from 20 experiments using mean values of the triplicates:

$$Y=B_0+\sum B_iX_i+\sum B_{ii}X_i^2+B_{ij}X_iX_j \quad (1)$$

where Y is the predicted amidase production, i and j take values from 1 to number of independent variables, the B_0, B_i, B_{ii}, and B_{ij} represent, respectively, the interception, the linear, quadratic effect of X_i, and the interaction effect between X_i and X_j on amidase production.

The data obtained were analyzed statistically using the statistical software Data Processing System (DPS) version 3.01 and the level of significance was 95%.

Enzyme Assay

For amidase assay, 10 ml of the resulting culture broth was centrifuged for 8 min at 9,000 ×*g*, 4°C. The cells were collected for measurement of amidase activity. The resulting cells were washed once with 5 ml of 0.85% (w/v) NaCl solution and centrifuged at 9,000 ×*g* for 8 min at 4°C. Then, the cell pellets were suspended in 5 ml of 20 mM potassium phosphate buffer containing 0.5% (w/v) (*R,S*)-**1**. Reaction was performed at 35°C for 5 min with a reciprocal shaker (112 rpm). The amidase activity was determined using an established gas chromatography method [28]. One unit of the enzyme activity was defined as the amount of enzyme required to produce 1 μmol of (*R,S*)-**2** per minute at 35°C, 112 rpm.

RESULTS

Selection of Best Carbon Source, Nitrogen Source, and Inducer for Amidase Production

For selection of best carbon source, nitrogen source, and inducer for amidase production, a series of experiments were conducted to investigate the effect of these medium components on amidase production.

Effect of Carbon Source on Amidase Production

As shown in Fig. 1A, glucose and fructose were better for amidase production than other tested carbon sources. The maximal amidase production (82.06 U/l) was obtained when glucose was tested. Therefore, glucose was chosen for further study. In order to determine the optimum concentration of glucose for amidase production, glucose concentrations of 8 to 23 g/l were used in the medium. The amidase production progressively increased with the increase of glucose concentration up to 17 g/l, and declined thereafter (Fig. 1B).

Effect of Nitrogen Source on Amidase Production

As can be seen in Fig. 2A, amidase production obtained from complex nitrogen sources (Peptone, yeast extract,

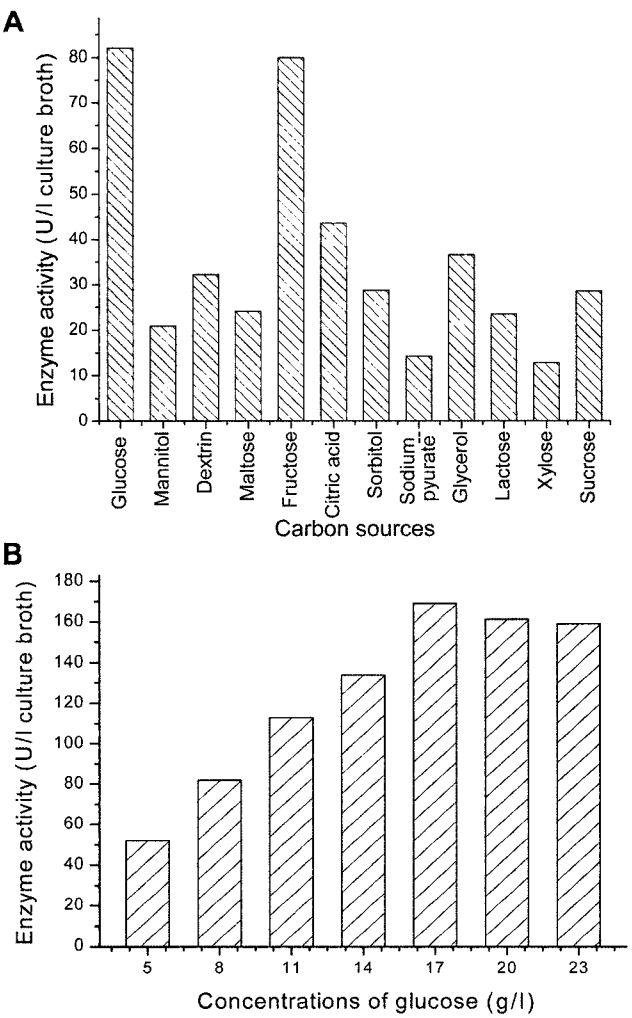

Fig. 1. Effects of different carbon sources (**A**) and different glucose concentrations (**B**) on amidase production of *D. tsuruhatensis* ZJB-05174.

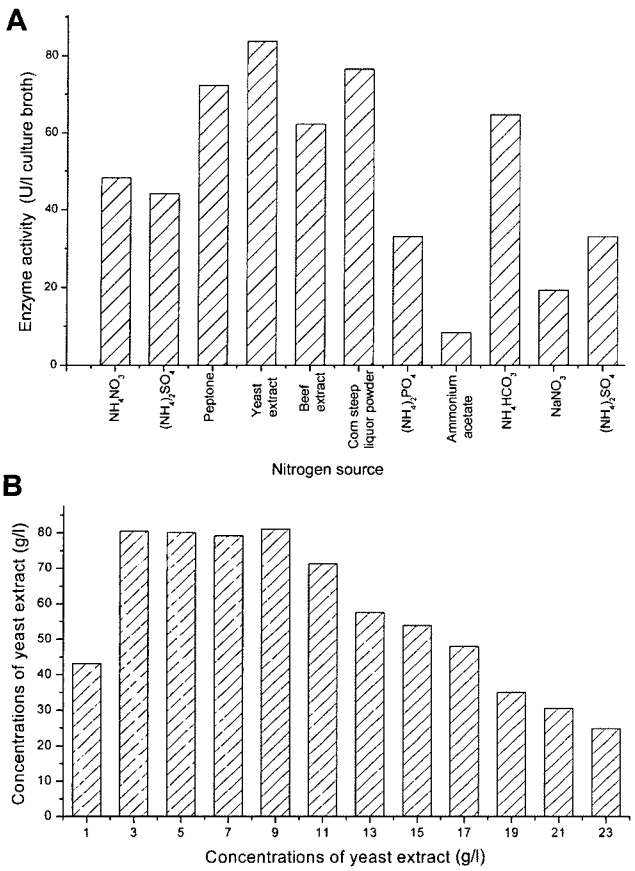

Fig. 2. Effects of different nitrogen sources (**A**) and different yeast extract concentrations (**B**) on amidase production of *D. tsuruhatensis* ZJB-05174.

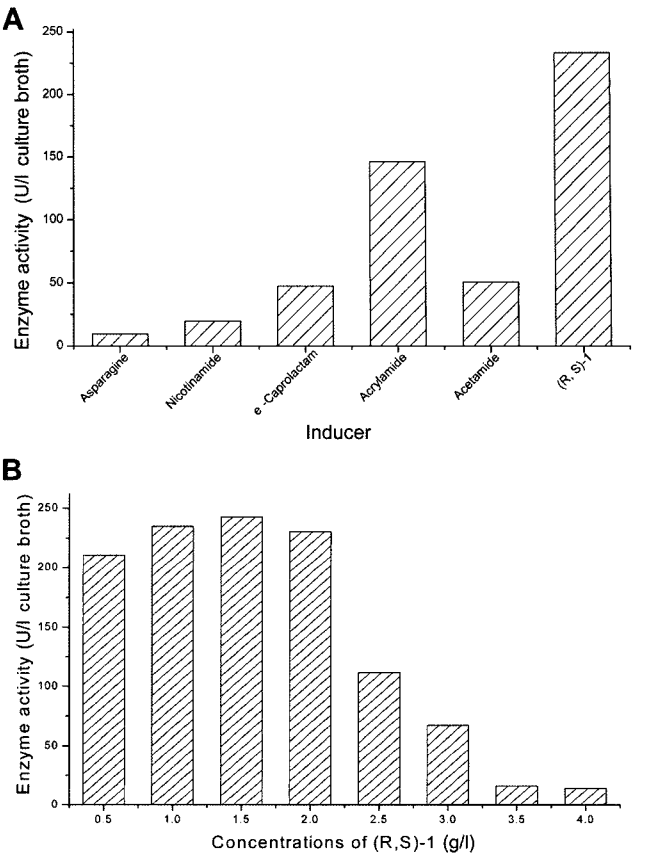

Fig. 3. Effects of different inducers (**A**) and different (*R,S*)-1 concentrations (**B**) on amidase production of *D. tsuruhatensis* ZJB-05174.

beef extract, and corn steep liquor powder) was higher than that obtained from single nitrogen sources, except NH_4HCO_3. Maximum amidase production (83.67 U/l) was achieved with yeast extract. The data suggest that complex nitrogen sources might be optimum for amidase production. In order to determine the optimum concentration of yeast extract for amidase production, yeast extraction concentrations of 1 to 23 g/l were used in the medium. The amidase production increased quickly from 43.15 to 80.47 U/l when yeast extract concentrations increased from 1 to 3 g/l, then remained stably with the increase of yeast extract concentration up to 9 g/l, and declined thereafter (Fig. 2B).

Effect of Inducer on Amidase Production

As shown in Fig. 3A, the effects of various tested inducers on amidase production were different. Significant enhancement of amidase production was observed when acrylamide and (*R,S*)-1 were tested. Maximum amidase production (233.25 U/l) was achieved with 1 g/l of (*R,S*)-1. The effect of acetamide on amidase production was almost the same as that of ε-caprolactam. Asparagine and nicotinamide tested appeared to be poor inducers for amidase production by *D. tsuruhatensis* ZJB-05174. In order to determine the optimum concentration of (*R,S*)-1 for amidase production, (*R,S*)-1 concentrations of 0.5 to 4.0 g/l were used in the medium. The results are shown in Fig. 3B. The amidase production increased from 210.86 to 230.09 U/l when (*R,S*)-1 concentrations increased from 0.5 to 1.0 g/l. Maximum amidase production (242.80 U/l) was achieved when 1.5 g/l (*R,S*)-1 was used. When the (*R,S*)-1 concentration was further increased to 2.0 g/l, amidase production of 230.32 U/l was obtained, and declined drastically when the (*R,S*)-1 concentration further increased. Therefore, amidase production of *D. tsuruhatensis* ZJB-05174 could be remarkably enhanced by low concentrations of (*R,S*)-1.

Optimization of Medium with Response Surface Methodology

From the above experiments, glucose, yeast extract, and (*R,S*)-1 were found to be key factors for amidase production. They were chosen for RSM optimization. The ranges of the factors are given in Table 1. The results of 20 CCD runs are shown in Table 2. It shows the amidase production corresponding to the combined effect of the three components

Table 1. Factors and their levels (in g/l) for the experimental design.

Factors	Symbols	Actual levels of coded factors				
		-1.52465	-1	0	1	1.52465
Glucose	X_1	2.852	6	12	18	21.148
Yeast extract	X_2	2.377	5	10	15	17.623
(*R*,*S*)-1	X_3	0.2377	0.5	1.0	1.5	1.762

in their specified ranges. The DPS statistic software was used to fit the polynomial Eq. (1) to the experimental data. The mathematic model that represents a second-order polynomial is given by Eq. (2) in coded values of variables:

$$Y=416.5741+51.2605X_1+64.8047X_2+56.5854X_3 -45.3969X_1^2-54.7874X_2^2-24.3020X_3^2+22.8705X_1 +X_2-2.0022X_1*X_3+28.0682X_2*X_3$$

where X_1 is glucose, X_2 is yeast extract, and X_3 is (*R*,*S*)-1.

The results were analyzed using the analysis of variance (ANOVA), and the analysis results are summarized in Table 3. The model *F*-value of 29.00 for amidase production suggests that the second-order model is significant. The quality of fit of the model was examined by the coefficient of determination R^2. The value of R^2 was 0.9631 for amidase production, which indicates 96.31% of the variability in the response could be explained by the model. Therefore, this equation can be used for predicting response at any combination of the three variables in the experimental range. Amidase production at a specified combination of the three variables can be predicted by substituting the corresponding coded values in Eq. (2).

Table 2. Experimental design and results of the central composite design of response surface methodology.

Run	Factors			Enzyme activity (U/l)	
	X_1	X_2	X_3	Experimental	Predicted
1	1	1	1	526.7051	513.6749
2	1	1	-1	341.4115	348.372
3	1	-1	1	244.564	282.1882
4	1	-1	-1	258.8886	229.1581
5	-1	1	1	345.2443	369.4173
6	-1	1	-1	239.2875	196.1058
7	-1	-1	1	241.9304	229.4124
8	-1	-1	-1	160.901	168.3737
9	-1.52465	0	0	219.6241	232.8921
10	1.52465	0	0	392.9057	389.2007
11	0	-1.52465	0	194.79	190.4131
12	0	1.52465	0	374.0822	388.0221
13	0	0	-1.52465	237.9627	273.8097
14	0	0	1.52465	472.6395	446.3556
15	0	0	0	413.6575	416.5741
16	0	0	0	417.4575	416.5741
17	0	0	0	419.5212	416.5741
18	0	0	0	421.2362	416.5741
19	0	0	0	415.2146	416.5741
20	0	0	0	418.8167	416.5741

R^2, coefficient of determination=0.9631.

Table 3. Analysis of variance (ANOVA) for the response surface quadratic model obtained from experimental designs.

Source	Degree of freedom	Sum of squares	*F*	*P*
Model	9	1.985E+005	29.00	<0.0001
X_1	1	33237.29	43.70	<0.0001
X_2	1	53121.87	69.85	<0.0001
X_3	1	40501.28	53.26	<0.0001
X_1^2	1	22272.12	29.29	0.0003
X_2^2	1	32439.28	42.66	<0.0001
X_3^2	1	6382.54	8.39	0.0159
$X_1* X_2$	1	4184.46	5.50	0.0409
$X_1* X_3$	1	32.07	0.042	0.8414
$X_2* X_3$	1	6302.59	8.29	0.0164
Residual	10	7604.95		
Lack of fit	5	7565.31	190.89	
Pure error	5	39.63		
Total	19	2.061E+005		

P-values were used as a tool to check the significance of each of the coefficients. The results indicated that the model terms X_1, X_2, X_3, X_1^2, X_2^2, X_3^2, X_1X_2, and X_2X_3 were significant ($P<0.05$).

Three-dimensional response surface plots were constructed by plotting the response (amidase production) on the Z-axis against any other two independent variables, while maintaining the other variable at its central level. As is shown in Fig. 4, considerable interaction between glucose and yeast extract could be observed. Fig. 5 also revealed the significant interaction yeast between extract and (*R*,*S*)-1.

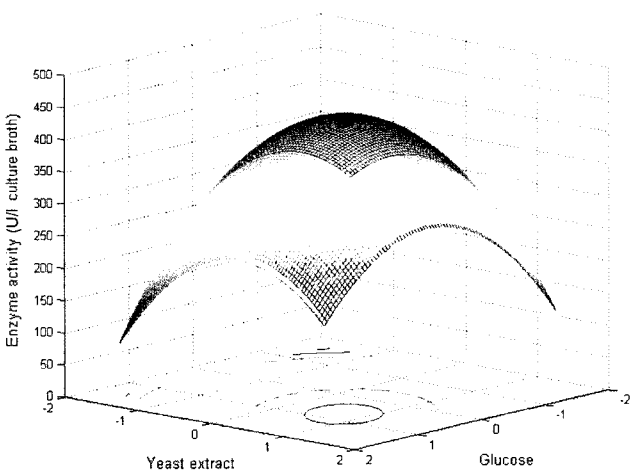

Fig. 4. Three-dimensional contour plots showing the effect of glucose and yeast extract on amidase production at (*R*,*S*)-1 coded level of 0.

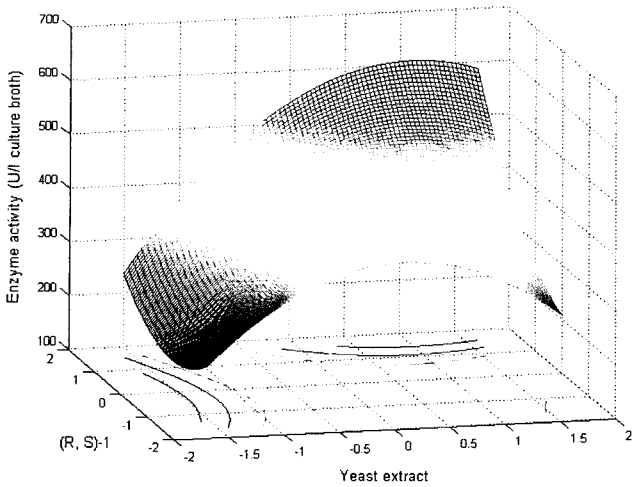

Fig. 5. Three-dimensional contour plots showing the effect of yeast extract and (*R*,*S*)-1 on amidase production at glucose coded level of 0.

Based on the model, the optimum medium compositions for maximum amidase production were as follows: glucose =8.23 g/l, coded as 0.823; yeast extract=11.59 g/l, coded as 1.159; (*R*,*S*)-1=1.76 g/l, coded as 1.525. Under the optimized medium, the predicted amidase production was 528.21 U/l. Validation was performed under conditions predicted by the model. The experimental result obtained was 527.18 U/l, confirming the validity.

Production of amidase by *D. tsuruhatensis* ZJB-05174 with the optimized medium and the initial medium was further carried out using a 50-l bioreactor (FUS-XL, Shanghai Guoqiang Bioengineering Equipment Co. Ltd) with the following operation conditions: medium volume of 30 l, initial pH of 7.5, temperature of 30°C, aeration ratio of 30 l/min, agitation speed of 200 rpm, and inoculum volume of 2.5% (v/v). After 20 h cultivation, amidase production of both media reached 402.36 U/l and 98.80 U/l, respectively.

Discussion

The results of this experiment revealed the role of inducers on amidase production by *D. tsuruhatensis* ZJB-05174. When inducers such as the substrate (*R*,*S*)-1 were supplemented to the medium, amidase production was significantly enhanced. This was consistent with earlier reports on amidase production [11, 13, 25]. However, the induction of inducers on various amidases was different. For example, (*R*,*S*)-1 and acrylamide seemed to be optimal inducers for amidase production by *D. tsuruhatensis* ZJB-05174, whereas 2-azacyclononanone and ε-caprolactam could remarkably enhance the amidase production of *C. acidovorans* KPO-2771-4 and *Rhodococcus butanica*, respectively [12, 25]. This might be caused by the different effect of inducers on the expression of the amidase gene in amidase-producing microorganisms [25].

Process optimization is of great importance for enzyme-based biotechnology production processes. Therefore, optimization of amidase production is essential for its commercial applications. To date, researches on amidases mainly concentrated on the screening of amidase-producing microorganisms, and purification and characterization of amidase, whereas optimization of amidase production is seldom reported [2, 6–9, 11, 14, 18, 22–27, 29]. Far fewer is the optimization of the amidase production process using response surface methodology. Currently, biotechnology process optimization was mainly performed using classical and statistical ways. The "one-factor-at-a-time" conventional method was laborious, time consuming, and sometimes the results obtained might be misleading and inaccurate. Moreover, it does not justify the optimal conditions, and is unable to examine the interactions between two or more factors. On the other hand, RSM, the statistical method, could effectively use quantitative data from appropriate experiments to detect and simultaneously solve multivariate equations [21]. This method has been successfully used in biocatalysis process optimization [1, 5, 15–17, 19–21]. Because of the increasing need for enantioselective amidases, this research was conducted in order to optimize the medium compositions for maximal amidase production. The RSM applied to the optimization of amidase production in this study indicated the importance of various factors at different levels. The central composite design used was a helpful tool for investigating and exploring the culture conditions for maximum amidase production. 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 amidase production.

RSM was successfully applied in the optimization of amidase production by *D. tsuruhatensis* ZJB-05174, and a significant improvement of 370% in amidase production was accomplished compared with the initial medium. Significant improvement in amidase production was also observed with the optimized medium in a 50-l bioreactor level cultivation compared with the initial medium. The results of this work might be helpful for further application of amidase from *D. tsuruhatensis* ZJB-05174.

Acknowledgments

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