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Full Length Research Paper

Screening of thermophilic neutral lipase-producing *Pseudomonas* sp. ZBC1 and improving its enzymatic activity

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From oil-contaminated soil, three lipase-producing microorganisms were selected as good lipase producers using rhodamine B-olive oil plate agar and they were identified as from *Pseudomonas*, *Burkholderia* and *Klebsiella* genera by morphology, biochemical characterization and 16S rRNA gene sequencing. Among the three strains, *Pseudomonas* sp. ZBC1 was found to produce thermophilic neutral lipase which exhibited maximum activity at 80ºC and pH 7.0. Plackett-Burman design (PB design) and response surface methodology (RSM) analysis showed that the optimum values of the most significant factors are $MgSO_4$ (0.85 g/L), inoculum size (65.9 ml/L) and volume of medium (51.57 ml). Under the optimal conditions, lipase production by *Pseudomonas* sp. ZBC1 was enhanced by approximately 2.30 fold (from 8.50 to 19.50 U/ml).

Key words: *Pseudomonas*, lipase, thermophilic, response surface methodology.

INTRODUCTION

Lipases (triacylglycerol acylhydrolases, EC.3.1.1.3) catalyze the hydrolysis of triacylglycerols to glycerol and free fatty acids, while the reverse reaction is also driven by lipases in non-aqueous conditions (Jaeger et al., 1999; Jaeger and Reetz, 1998). Due to their stability, selectivity and broad substrate specificity, microbial lipases are broadly used in industry (Dutra et al., 2008). Besides their widespread use in cosmetic and organic synthesis industries, lipases have become increasingly prevalent in food industry, especially in inter-esterification of oils and fats to produce baker's margarine, cake shortening and vanaspati fat. Moreover, inter-esterification was one of the best methods for trans-free fats production, while trans-fatty acids were reported to be risk factors involved in coronary heart disease (Farmani et al., 2009; Grbavcic et al., 2007; Gupta et al., 2007).

It has been reported that commercial lipases are mainly produced from bacteria (Gao et al., 2000), fungi (Orlando Beys Silva et al., 2005) and yeast (Dalmau et al., 2000). *Pseudomonas* sp. lipases belong to bacterial lipases and are widely used for a variety of biotechnological applications, particularly in food (Gupta et al., 2004). However, the lipases of *Pseudomonas* sp. have relatively low yield and few applicable characteristics so that the enhancement of the production and enlargement of suitability of the lipases become more important. Statistical experimental design is the methodology of conducting and planning experiments to obtain the maximum lipase production in the fewest number of runs.

The main objective of this research was to isolate and screen new strains with potential to produce lipases and optimize the lipase production conditions in the biological lab-scale which would be used for inter-esterification process at high temperature and neutral surroundings. Up to date, there is no thoroughly detailed description

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about how to optimize lipases production conditions with *Pseudomonas* sp. Therefore, different medium compositions, cultivation conditions and combined interactions were analyzed to enhance lipase production from *Pseudomonas* sp. ZBC1. The predicted highest lipase yield was confirmed under the most optimal conditions deduced by the PB design and RSM.

MATERIALS AND METHODS

Rhodamine B, olive oil and polyvinyl alcohol were purchased from Sigma (USA). All other chemicals used in this study were of analytical grade and obtained from Sangon Biotech (Shanghai) Co., Ltd.

Isolating and screening of lipase-producing strains

The lipase-producing strains were isolated from oil-contaminated soil in the production area of Anhui Kangermei Margarine Co., Ltd. They were pre-enriched in enriched medium (0.5% (w/v) yeast extract, 0.1% (w/v) Na_2HPO_4, 0.15% (w/v) KH_2PO_4, 0.05% (w/v) $MgSO_4$, 0.5% (w/v) $(NH_4)_2SO_4$, 0.05% (w/v) NaCl and 1% (v/v) olive oil) and incubated in a rotary shaker at 180 rpm, 37ºC for 48 h. Then appropriate dilutions of the enriched cultures were spread onto Rhodamine B-olive oil agar plates which consisted of 1.8% (w/v) agar, 0.1% (w/v) K_2HPO_4, 0.05% (w/v) $MgSO_4$, 0.1% (w/v) $(NH_4)_2SO_4$, 0.05% (w/v) KCl, 0.001% (w/v) Rhodamine B, 3% (v/v) olive oil and 0.18% (w/v) polyvinyl alcohol, and incubated at 37ºC for 96 h. Lipase-producing strains were identified by orange fluorescence under UV light (350 nm) (Kouker and Jaeger, 1987).

The screening of lipase-producing strains was carried out by liquid-state fermentation in shake flask. The basal medium consisted of 1% (w/v) sucrose, 1% (w/v) maltose, 0.2% (w/v) NH_4NO_3, 0.1% (w/v) $MgSO_4$, 0.1% (w/v) Na_2HPO_4, 1% (w/v) soybean flour and 1% (v/v) olive oil. 7.5 ml, 16h-old (AD_{600}=0.8) isolated lipase-producing microorganisms were inoculated into 50 ml of basal medium in 250 ml Erlenmeyer flasks. The culture was incubated at 37ºC on a rotary shaker at 180 rpm. The culture aliquots were centrifuged at 4ºC with 4800 g for 15 min and the supernatants were collected for lipase activity analysis.

Enzymatic activity assay

Lipase activity was measured partially according to the method of Abramic et al. (1999). The reaction mixture contains 4.0 ml olive oil which was emulsified in polyvinyl alcohol, 5.0 ml 0.05 M phosphate buffer saline (PBS) (pH 7.0) and 1.0 ml lipase solution. Incubation was performed at 80ºC for 10 min and terminated by adding 15 ml ethanol. Free fatty acids were titrated against 0.05 M NaOH with phenolphthalein as indicator. One unit of lipase activity (U) was defined as the release of 1.0 µmol of fatty acid per min at 80ºC.

Identification of the selected lipase-producing strains

The strains were identified on the basis of morphological and biochemical characteristics according to the procedures described in Bergey's Manual of Systematic Bacteriology (Bergey and Boone, 2009), and then they were further confirmed by 16S rRNA gene sequencing method. Homology was analyzed in GenBank with the BLAST program.

Effect of pH and temperature on lipase activity and stability

The impact of temperature on lipase activity was measured at various temperatures (30 to 100ºC) under the standard assay method. Lipase thermostability was tested by pre-incubating at 80ºC for various time intervals (0 to 100 min) and then assayed for residual enzyme activity. The optimum of pH was evaluated at different levels (pH 6 to 9) under the standard assay method. For the pH stability, it was pre-incubated at 4ºC in various pH buffers for 24 h.

Medium optimization of lipase production by statistical approach

The impact of 11 factors, including soybean flour, NH_4NO_3, $MgSO_4$, pH, inoculum size, volume of medium, Na_2HPO_4, sucrose, maltose, olive oil and dummy variable were studied by the PB design. The dummy variable effects were used to estimate the experimental error in a PB design. No changes were made to this variable and thus the difference between the high and low levels should be zero. Design Expert 8.05b (Stat Ease Inc., Minneapolis, Minn., USA) was subsequently used to generate a set of 12 experimental designs. The minimum and maximum ranges selected for the variables were given in Table 1. Table 2 shows distribution of factors according to the Design Expert software and the response in the study.

Based on the above results, Design Expert 8.05b was then used to generate a set of experimental designs for medium optimization using RSM. Each factor was studied at three different levels 1, 0, -1 (Table 4). The second order model used for simulating the experimental data was as follows:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3$$

Where, Y was the predicted response; β_0 was the intercept; β_1, β_2 and β_3 were linear coefficients; β_{11}, β_{22} and β_{33} were squared coefficients; and β_{12}, β_{13} and β_{23}, were interaction coefficients.

RESULTS AND DISCUSSION

Isolating and screening of lipase-producing strains

Lipase-producers were screened by the formation of orange fluorescent halos around colonies under UV light (350 nm). Incubation period has a profound influence on extracellular lipase production (Dheeman et al., 2010). Three strains of ZBC1, ZBC2 and ZBC3 with higher lipase activity were isolated and therefore selected for further studies. They reached the maximal lipase activity of 8.5, 8 and 8.25 U/ml at the time scale for 2, 3 and 5 days, respectively.

Identification of lipase-producing strains

The morphological and biochemical characteristics of the three strains were illustrated in Table 5. The 16S rRNA gene sequence of ZBC1, ZBC2 and ZBC3 were

Table 1. Minimum (-1) and maximum (+1) ranges of the eleven parameters used in Plackett-Burman run.

Variable	Parameter	Range coding	
		-1	+1
A	Soybean flour (g/L)	10	20
B	NH_4NO_3 (g/L)	1	3
C	$MgSO_4$ (g/L)	0.7	1
D	pH	6	9
E	Inoculum size (ml/L)	100	150
F	Volume of medium (ml)	50	80
G	Na_2HPO_4 (g/L)	0.5	2
H	Sucrose (g/L)	5	10
I	Maltose (g/L)	5	10
J	Olive oil (ml/L)	10	20
K	dummy variable	-1	+1

Table 2. Plackett–Burman experimental design and results.

Run	A	B	C	D	E	F	G	H	I	J	K	Lipase activity (U/mL)	
												Experimental	Predicted
1	-1	-1	1	-1	1	1	-1	1	1	1	-1	2.60	4.47
2	-1	-1	-1	1	-1	1	1	-1	1	1	1	9.80	10.13
3	1	-1	1	1	1	-1	-1	-1	1	-1	1	7.80	6.97
4	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	9.80	9.03
5	1	1	1	-1	-1	-1	1	-1	1	1	-1	9.70	10.00
6	-1	1	1	1	-1	-1	-1	1	-1	1	1	8.50	8.60
7	1	1	-1	-1	-1	1	-1	1	1	-1	1	8.80	9.03
8	-1	1	-1	1	1	-1	1	1	1	-1	-1	9.30	8.50
9	1	-1	1	1	-1	1	1	1	-1	-1	-1	6.30	6.10
10	-1	1	1	-1	1	1	1	-1	-1	-1	1	4.30	3.07
11	1	-1	-1	-1	1	-1	1	1	-1	1	1	6.20	7.10
12	1	1	-1	1	1	1	-1	-1	-1	1	-1	4.50	4.60

Table 3. Screening of variables and their ranking for influence on the response based on the Plackett-Burman run.

Parameter	Lipase production	
	***E* value**	**Rank p-value**
Inoculum size (ml/L)	-3.03	1 0.0018
Volume of medium (ml)	-2.50	2 0.005
$MgSO_4$ (g/L)	-1.53	3 0.0425
Olive oil (ml/L)	-0.83	5 >0.05
Sucrose (g/L)	-0.70	7 >0.05
Soybean flour (g/L)	-0.17	11 >0.05
NH_4NO_3 (g/L)	0.43	10 >0.05
Virtual variables	0.53	9 >0.05
Na_2HPO_4 (g/L)	0.60	8 >0.05
pH	0.80	6 >0.05
Maltose (g/L)	1.40	4 >0.05

Table 4. Central (0), minimum (-1) and maximum (+1) ranges of the three parameters used in RSM studies for final media optimization.

Variable	Parameter	Range coding		
		-1	0	+1
X_1	$MgSO_4$ (g/L)	0.4	0.7	1
X_2	Inoculum size (ml/L)	50	100	150
X_3	Volume of medium (ml)	30	50	70

Table 5. The morphological and biochemical characteristics on strains of ZBC1, ZBC2 and ZBC3.

Characteristic	ZBC1	ZBC2	ZBC3
Shape	Rod	Rod	Rod
Gram	-	-	-
Spore	-	-	-
Nitrate reduction	-	+	+
Oxidase	+	-	-
Aerobic	+	-	-
Catalase	+	+	+
Motile	+	+	-
VP test	-	-	+

+, Positive result; -, Negative result.

submitted to GenBank (http://www.ncbi.nlm.nih.gov/genbank/) with accession numbers JQ638271, JQ638272 and JQ638270, respectively. Based on biochemical characterization and 16S rRNA gene sequence, ZBC1, ZBC2 and ZBC3 maybe from the genus *Pseudomonas*, *Burkholderia* and *Klebsiella*, respectively, but we could not affirm their specific species. A phylogenetic tree was constructed by 16S rRNA sequence of numerous strains using MEGA 2.0 software (Figure 1), which demonstrated that the closest neighbors of the three strains were the same as the results through morphological and biochemical characteristics.

Effect of temperature and pH on lipase activity and stability

The temperature tolerance curve of ZBC1 lipase indicated that the optimum lipase activity temperature was 80ºC (Figure 2A). As displayed in Figure 2B, the lipase was stable at 80ºC, with over 90% residual activity for 25 min and 65% for 75 min. Although the optimum temperature of the lipase from *Bacillus stearothermophilus* MC 7 (Kambourova et al., 2003) was similar to *Pseudomonas* sp. ZBC1, the lipase from *Pseudomonas* sp. ZBC1 was more thermostable. To the best of our knowledge, the lipase of *Pseudomonas* sp. ZBC1 seemed to be more thermostable than the other *Pseudomonas* lipases, such as *P. aeruginosa* lipases (Karadzic et al., 2006), *P. gessardii* (Ramani et al., 2010), *P. fluorescens* RB02-3 (Boran and Ugur, 2010) and *P. aeruginosa* LX1 (Ji et al., 2010) whose optimal temperature were found at 70, 37, 50 and 40ºC, respectively. The pH tolerance curve of the ZBC1 lipase indicated the maximum lipase activity at pH 7.0 (Figure 3A), in agreement with most other lipases from the *Pseudomonas* sp. (Baharum et al., 2003; Boran and Ugur, 2010; Cadirci and Yasa, 2010; Ji et al., 2010; Li et al., 2011; Ogino et al., 2004; Wang et al., 2009). The optimal pH of lipase from ZBC1 was significantly different from that of other *Pseudomonas* reported, such as *P. fluorescens* HU380 (Kojima and Shimizu, 2003), *P. aeruginosa* CS-2 (Peng et al., 2010) and *P. gessardii* (Ramani et al., 2010) whose optimal pH were 8.5, 8.0 and 5.0, respectively. Morever, the lipase of ZBC1 showed stability at pH 6.5 to 9.0, and the residual activity retained above 70% after pre-incubation for 24 h at 4ºC (Figure 3B). According to previous reports, *Pseudomonas* lipases do have some special characteristics, such as thermophile, thermostability and wide pH range (Cadirci and Yasa, 2010; Gao et al., 2000; Ji et al., 2010). The strain ZBC1 exhibited higher lipase activity and shorter fermentation period for the production of extracellular lipase than ZBC2 and ZBC3, as shown. Thus, further study

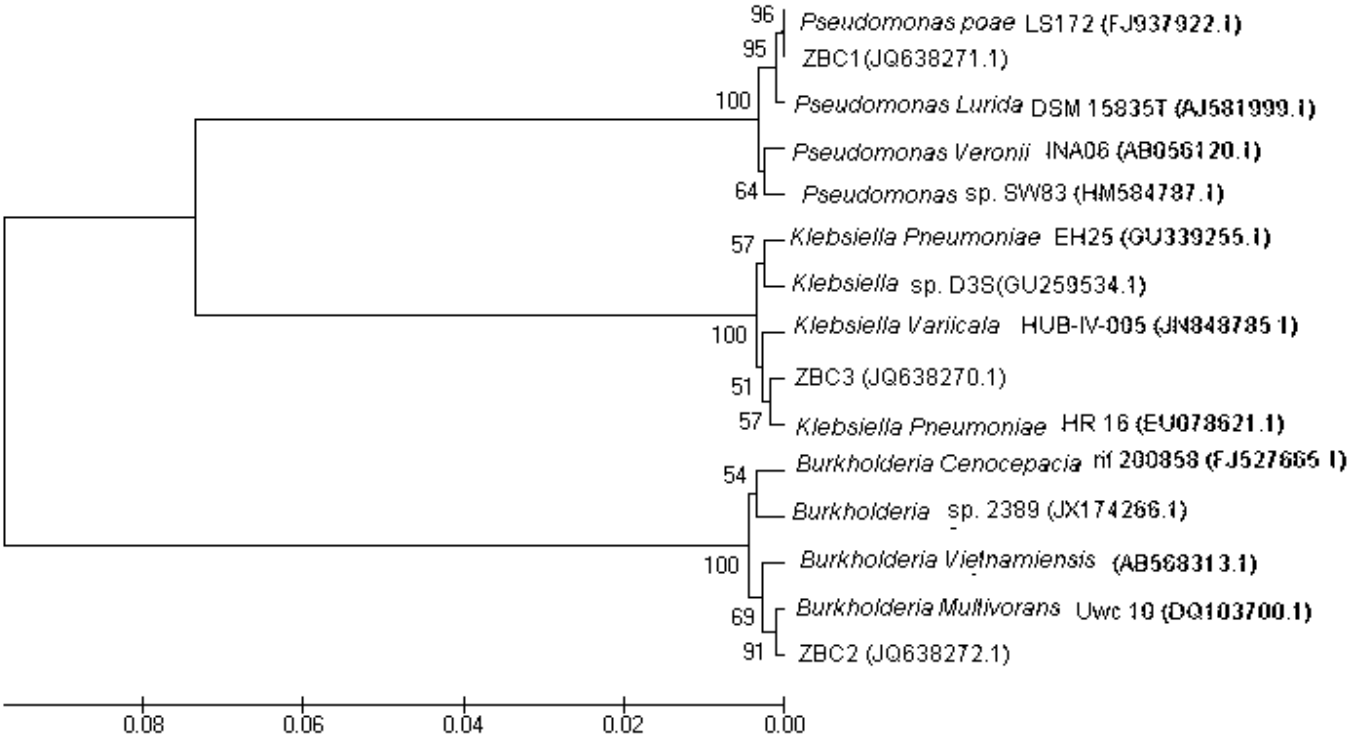

Figure 1. The phylogenetic tree was based on 16S rRNA sequences information of the genomic DNA of ZBC1, ZBC2 and ZBC3 and constructed by MEGA2.0 software. The sequences used in the analysis were obtained from GenBank database.

would be done on ZBC1.

Medium optimization of lipase production by statistical approach

PB design serves as a very useful tool for initial screening of large number of seemingly important factors in a very small number of experiments for reliable short-listing of relevant factors for further optimization (Kalil et al., 2000). The influence of each variable on the production was determined by calculating their respective *E*-values. The statistical analysis of the PB design showed that inoculum size (P = 0.0018) was the most significant factor, followed by medium volume (P = 0.005), $MgSO_4$ (P = 0.0425), as they had high confidence level of more than 95% (Table 3). Therefore, the optimum concentration and interaction amongst the variables were studied using RSM.

Using the Box–Behnken method, a set of 17 experiments with 5 replicates at the center point was conducted. The design matrix of the variables in coded units was given in Table 6 along with the predicted and experimental values of response. The results obtained were analyzed by standard analysis of variance (ANOVA). The predicted response could be expressed by the following second-order polynomial equation in terms of coded values:

The Y was the yield of lipase activity (U/ml), χ_1, χ_2 and χ_3 were the coded variables for $MgSO_4$, inoculum size and volume medium, respectively. The R^2 value of 0.9869 for lipase production pointed to the accuracy of the model. The quality of fit of the polynomial equation was tested by the coefficient of determination R^2 (Sen and Swaminathan, 1997). The R^2 implied that 98.69% for the

$$Y=+19.76-0.16\chi_1-2.18\chi_2+0.69\chi_3+0.85\chi_1\chi_2+0.33\chi_1\chi_3+0.50\chi_2\chi_3-4.67\chi_1^2-1.24\chi_2^2-3.27\chi_3^2$$

lipase activity was attributed to the independent variables. The predicted R^2 of 0.9218 pointed to a good agreement between the predicted and experimental values for lipase production. The predicted R^2 of 0.9218 was also in reasonable agreement with the adjusted R^2 of 0.9700. The adjusted R^2 arranged the R^2 values for the sample size and for the number of variables in the model. The purpose of RSM was to determine which experimental values generated signals, which were large in comparison to do noise. A ratio greater than 4 was desirable. (Haaland, 1989) The ratio of 19.163 indicated an adequate signal. The “Lack of fit F-value” of 3.06 for lipase activity implied the lack of fit was insignificant. Insignificant lack of fit was good and indicated that the model equation was adequate for predicting the lipase activity under any combination of values of the variables.

The P-values were used as a tool to check the significance of each of the coefficient, which also indicated

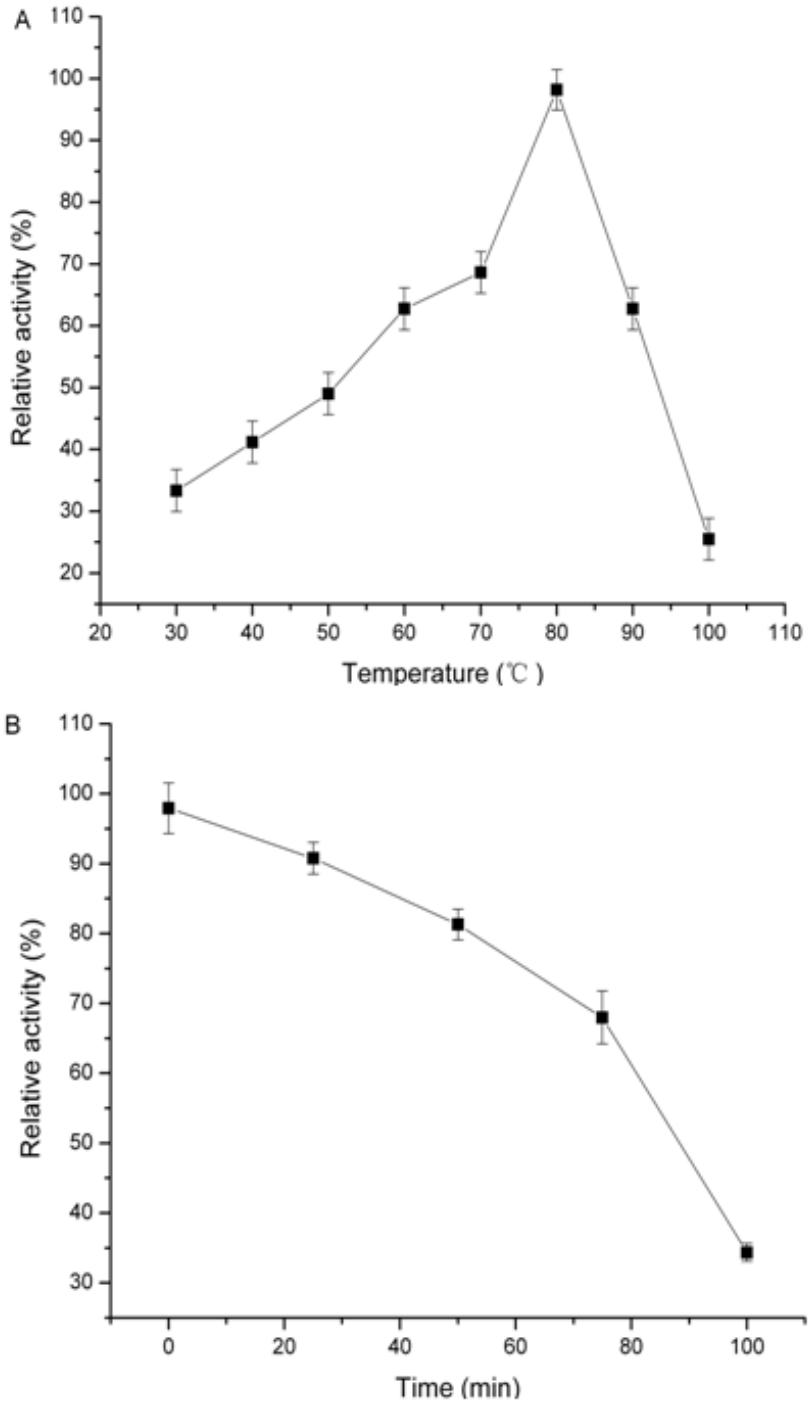

Figure 2. Effect of temperature on ZBC1 lipase activity and stability. (A) It was assayed at various temperatures (from 30 to 100ºC), at pH 7. (B) For the stability, it was evaluated after incubation at 80ºC. Results represent the means of three experiments, and bars indicate ± standard deviation.

the interaction strength between each independent variable. The ANOVA analysis of the optimization study demonstrated that inoculum size ($p < 0.0001$) and volume medium ($P = 0.0164$) had high significance on lipase production. In the fixed period of fermentation, size of inoculum had great influence on the production of lipase (Rahman et al., 2005). ANOVA analysis showed that although $MgSO_4$ was not a significant variable ($p > 0.05$),

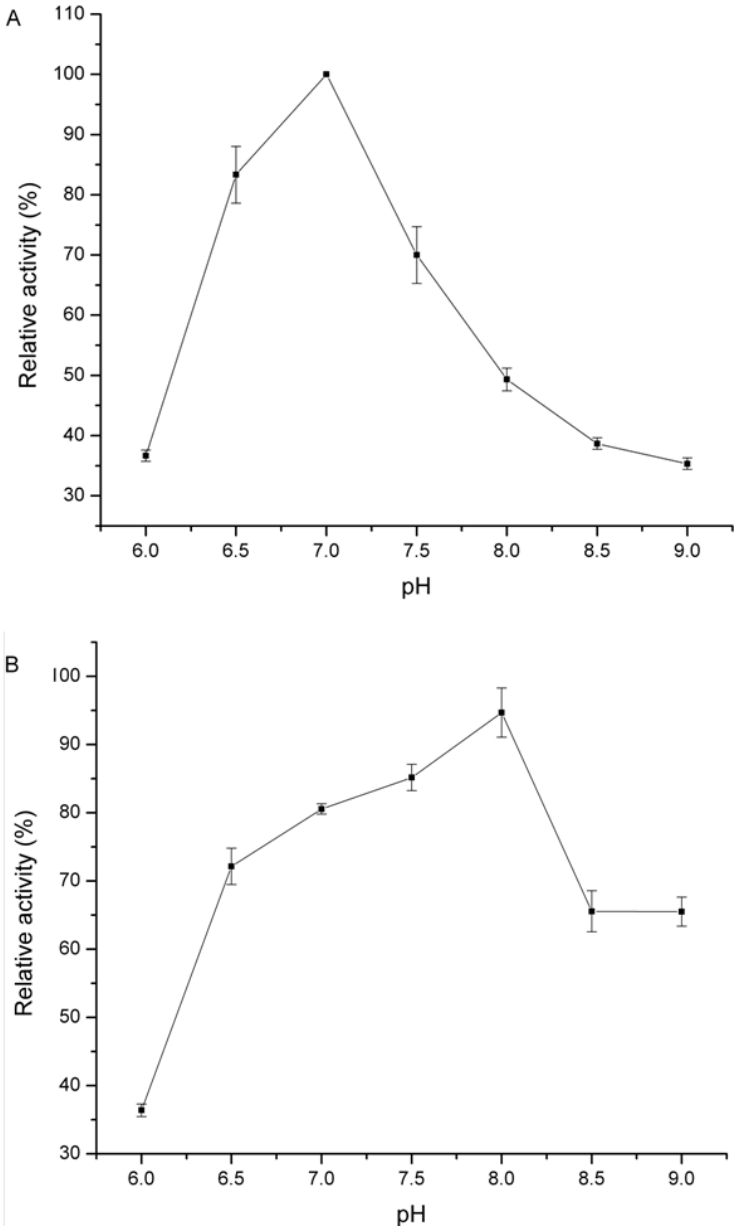

Figure 3. Effect of pH on ZBC1 lipase activity and stability. (A) The optimum pH was assayed after incubation in various pH buffers at 80ºC for 10min. (B) For the pH stability, it was pre-incubated at 4ºC in various pH buffers for 24 h. Results represent the means of three experiments, and bars indicate ± standard deviation.

but it had an important and significant interaction with inoculum size (P = 0.0288), hence been used to develop the model.

The three dimensional response surface curve was plotted by a statistically significant model to understand the interaction of the medium components and the optimum concentration of each component required for optimum lipase production. The interaction of two variables ($MgSO_4$ and inoculum size) at fixed volume of medium (50 ml) was presented in Figure 4. Maximum lipase activity of 19.07 U/ml could be achieved with $MgSO_4$ (0.85 g/L) and inoculum size (65.9 ml/L). Further increase or decrease in $MgSO_4$ led to a slight decline in the enzyme production, however, increase in the inoculum size led to the decrease in the enzyme production.

To confirm these results, the actual response of 19.50 U/ml was obtained under the optimal conditions, close to the predicted value of 19.07 U/ml.

Table 6. Experimental and predicted values of lipase activity as obtained in the experimental set up of RSM studies for the final medium optimization.

Run	X_1	X_2	X_3	Lipase activity (U/ml)	
				Experimental	Predicted
1	1	0	-1	10.70	10.65
2	1	1	0	12.70	12.36
3	-1	-1	0	16.70	17.04
4	0	0	0	19.70	19.76
5	0	-1	-1	17.20	17.24
6	1	0	1	12.30	12.67
7	-1	0	-1	12.00	11.63
8	0	-1	1	18.00	17.61
9	-1	1	0	11.00	10.99
10	0	0	0	19.70	19.76
11	0	0	0	20.50	19.76
12	-1	0	1	12.30	12.35
13	0	0	0	20.20	19.76
14	1	-1	0	15.00	15.01
15	0	1	1	14.30	14.26
16	0	1	-1	11.50	11.89
17	0	0	0	18.70	19.76

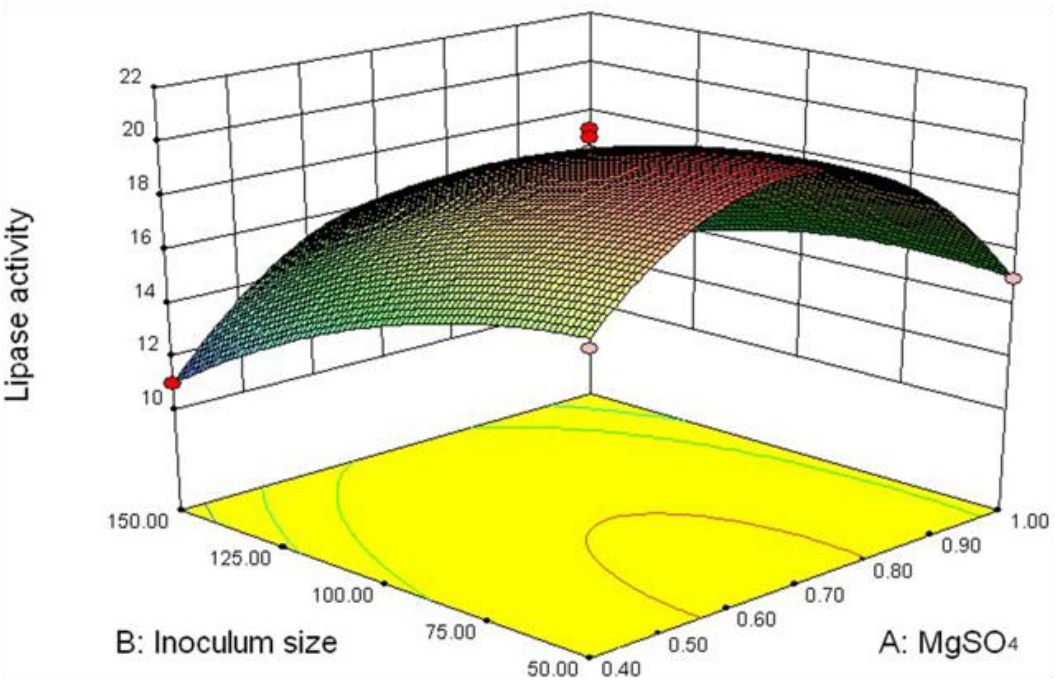

Figure 4. Response surface plot of MgSO4 and inoculum size at an inoculum volume of medium at 50 ml.

Conclusion

At present, *Pseudomonas* sp. lipases are the principal source of lipases used in industry. In this study, we successfully isolated and identified a new *Pseudomonas* sp. ZBC1, which produces thermophilic neutral lipase. In order to further increase the lipase production in the biological laboratory-scale, our work demonstrated the feasibility of using experimental design tools to select significantly influential factors and optimize fermentation

conditions for the lipase production. Under the optimal conditions, the lipase activity increased from 8.50 to 19.50 U/ml. The optimized lipase activity of *Pseudomonas* sp. ZBC1 was significantly higher than the reported (Kaushik et al., 2006; Liu et al., 2006).

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