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STATISTICAL OPTIMIZATION OF ALKALINE LIPASE PRODUCTION BY EXTREME HALOPHILIC ARCHEAN *NATRIALBA ASIATICA*

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ABSTRACT

In this study, extreme halophilic archean *Natrialba asiatica* was utilized as a new source for lipase production. Lipases from halophilic archaea are appealing for utilization in assorted industrial and biotechnological applications. The optimum temperature and pH of *N. asiatica* lipase in the crude mixture were 50 °C and 10, respectively. The growth conditions influencing lipase production were determined using a two-level fractional factorial Plackett–Burman design. Among the 9 factors screened, $MgCl_2$ concentration, temperature, and shaking were found to be effective. The optimum levels of these factors for the production process were determined by employing the central composite design of response surface methodology. The 27 g L^{-1} of $MgCl_2$, 50 °C, and 133 rpm were determined as optimized conditions for lipase production. The enzyme activity increased from 3.39 to 6.1 U mL^{-1} using predicted optimum levels. These findings help understanding factors affecting the production of lipase by halo-archean *N. asiatica*. Moreover, using the optimized level of temperature, shaking, and MgCl2, it is possible to increase the production of valuable alkaline lipase by *N. asiatica*.

Keywords: Lipase, *Natrialba asiatica*, Plackett–Burman Design, Response Surface Methodology, Extreme halophilic archaea

INTRODUCTION

Lipases (triacylglycerol acyl-hydrolases, E.C. 3.1.1.3) catalyze the hydrolase reaction of triacylglycerol into fatty acids and glycerol. Lipases are also accomplished to catalyze the synthesis of esters from fatty acids and glycerol. Both reactions were performed at the water-insoluble substrate interface **(C. H. Tan *et al.*, 2015b)**. Moreover, lipases can catalyze interesterification, acidolysis, alcoholysis, and aminolysis reactions. They usually exhibit good chemoselectivity, regioselectivity, and enantioselectivity besides broad substrate specificity **(Joseph *et al.*, 2008)**. These broad specifications made lipases one of the powerful essentials in several biotechnological aspects including synthesis of biopolymer, biodiesel, pharmaceuticals, agro-chemicals, and flavor compounds **(Jaeger & Eggert, 2002)**. These daily growing applications lead to the demand for the lipases with new specificities and therefore the isolation of new lipases from new natural sources is increasing potential value **(Hasan *et al.*, 2009)**. Lipases are produced by many microorganisms and eukaryotes. Among microorganisms, bacteria, fungi, yeasts and actinomycetes are the most important producers of lipase. Microbial lipases are very useful commercially and are broadly used in several industries **(Gupta *et al.*, 2004; Sharma *et al.*, 2014).**
Natrialba asiatica is a gram-negative, strongly lipolytic, and extreme halophilic coccobacillus isolated from beach sand in Japan. Optimum temperature and pH for growth are 50 °C and 6.6-7, respectively. NaCl range for growth is from 2 M to saturation, with an optimum at 4 M **(Hezayen *et al.*, 2001)**. Extreme halophilic organisms are bacteria or archaea that requiring high salt conditions for growth (3.5–5 M NaCl) **(Margesin & Schinner, 2001)**. With some adaptations, the proteins of these organisms can remain active in high salt concentrations (Karan *et al.*, 2012). These adaptations besides the existing robust nature of lipases make halophilic lipases especially attractive for industrial and biotechnological applications. Moreover, halophilic archaea use simple carbon sources and high salt concentrations favored for industrial simple production systems in which enclosed sterile conditions omitted **(Hezayen *et al.*, 2001)**. However, studies on halophilic lipase-producing organisms are limited. Currently, halophilic microorganisms, especially halo-archaea, have received a lot of attention for lipase production **(Delgado-García *et al.*, 2012; Litchfield, 2011; Schreck & Grunden, 2013)**. The most common technique for the production of lipases is submerged fermentation (SmF) but solid-state fermentation (SSF) methods can be used also **(Gupta et al., 2004; Sharma *et al.*, 2014)**. Carbon and nitrogen sources, stimulators, activators, inhibitors, surfactants, the temperature of incubation, pH of production medium, inoculum source and level can affect the lipase production in both SmF and SSF. **(Hasan *et al.*, 2009)**. Also, the production of lipase by every microorganism has different dependencies. Some of the factors influencing the optimal growth of a microorganism not only do not play a role in lipase production but in some cases have a negative effect. In optimization processes, these factors must be replaced by inducers of lipase production to become an economic bioprocess **(Chennupati *et al.*, 2009)**. Nowadays, Plackett–Burman design (PBD) is employed for recognizing effective factors among numerous growth conditions **(Cai *et al.*, 2008; Sadeghi-Dastjerdi *et al.*, 2019; Yele & Desai, 2014)**. To achieve an optimum condition for production, combinatorial interactions of effectors are investigated using response surface methodology (RSM) **(Chennupati *et al.*, 2009).** In this study, PDB was used to determine the effectors of lipase production from nutrition and culture parameters of N. asiatica. The lipase production process was optimized using a model that had been introduced by RSM to gain higher lipase production and was compared with unoptimized process.

MATERIAL AND METHODS

Microorganism and Preparation of inoculum

Natrialba asiatica IBRC-M 10341 was obtained from the Iranian Biotechnology research center as an active culture. a loop full of the bacterial active colony was added aseptically to 25 mL of MGM 23% broth (pH 7.5) to prepare pre-culture. Pre-culture was incubated at 37 °C for 48 h with shaking (150 rpm). A volume of 50 ml of broth culture medium was inoculated with 1 ml of 48-hour culture in a 250 ml Erlenmeyer flask and incubated under similar conditions. The optical density of the culture was recorded every 24 hours at 600 nm and was used to plot the growth curve of *N.asiatica*. One optical density unit of bacterial culture was prepared with the appropriate amount of fresh culture after 36 h of incubation and was used for inoculation of the experimental flasks **(Prajapati *et al.*, 2014)**.

Lipase assay

Lipase activity was measured using p-NPP as substrate **(Vorderwülbecke *et al.*, 1992)**. Substrate solution was prepared by addition of 10 mL *p*-NPP (0.1 M) to a mixture of natrium deoxycholate (207 mg) and Arabic gum (100 mg) in 90 mL

phosphate buffer (0.05 M, pH 6.5). The reaction was started by mixing crude enzyme extract (0.1 mL) into substrate solution (2.4 mL). A solution of phosphate buffer (50 mM, pH 6.5) was used instead of crude enzyme extract to prepare the blank solution. The reaction mixture was incubated at 50 °C for 15 min and absorbance was recorded at 410 nm. One enzyme unit (U) was defined as the lipase activity that liberates 1 µmol of p-NP per mL per minute under the standard assay conditions **(Demir & Tükel, 2010)**.

Effect of temperature and pH on the lipase activity

N. asiatica lipase activity at temperatures from 30 to 60 °C (with 5 °C intervals) was measured to determine the optimum temperature. The reaction mixture was incubated for 15 minutes at each temperature and absorbance was recorded at 410 nm. To determine the pH profile of *N. asiatica* lipase, substrate solution was prepared in sodium phosphate buffer (0.5 M, pH 7-8.5) and Britton-Robinson buffer (0.5 M, pH 9-11) with 0.5 intervals.

Plackett–Burman design

To determine the factors affecting production of N. asiatica lipase, a two factorial Plackett–Burman (PB) method was used to design experiments. PB method design n+1 experiments for n factors and statistically explain interactions between the factors (Plackett & Burman, 1946). In this study, 9 parameters including olive oil (g L^{-1}), lactose (g L^{-1}), peptone (g L^{-1}), $MgCl_2$ (M), NaCl (M), shaking (rpm), pH and incubation temperature (°C), and time (h) were used to design experiments. For each factor, 2 levels, high (+) and low (–), with one central point was determined. A collection of 15 experiments were designed for 9 factors. All experiments were replicated three times and the average was used for statistical analysis. Minitab 17 (Stat-Ease Inc., USA) statistical software was used to design experiments and analysis of results in the PB method. Variables and their levels in PB design were represented in Table 1. The PB design is based on the following first-order polynomial equation:

$$Y = ßo + \Sigma ßi\ Xi$$

Where, Y, is the lipase activity; ßo, is the model intercept; ßi, is the linear coefficient; Xi, is the level of the independent variable.

Response surface methodology

Significant factors obtained from PB design ($MgCl_2$, shaking, and temperature) were used for the optimization of lipase production based on the response surface methodology (RSM). In this methodology, a central composite design (CCD) was performed to explore the effect of these factors on lipase production. Each variable was considered at three levels. In central composite design, 8 cube points, 6 center points in the cube, and 6 axial point runs (20 different experiments) were designed (Table 2). Moreover, to evaluate the pure error, 5 replications at the centeral point were performed. The lipase activity (U mL^{-1}) in each experiment was taken as a response. The relationship among variables was determined according to the following second-order polynomial equation:

$$Y = ßo + \Sigma ßi\ xi + \Sigma ßii\ xi^2 + \Sigma ßij\ xixj,\ \ i = 1, 2, 3... k$$

Where, Y, is the predicted response; k, is the number of factor variables; ßo, is the model constant; ßi, is the linear coefficient; ßii, is the quadratic coefficient; ßij, is the interaction coefficient.

Validation of the model

Statistical significance of the polynomial model was evaluated using Fischer's F-test. The coefficient of determination (R^2) was used for the evaluation of the quality of the represented model **(Chennupati *et al.*, 2009; Sumrin *et al.*, 2011)**.

Optimization of the variable's level

The aim of the optimization process in this study was an increment in the production of lipase by *N. asiatica*. To achieve this, we set the temperature level between 30-50 °C that is the minimum and maximum growth temperature of *N. asiatica*. The best temperature in this range is 50 °C that was predicted by the model. Shaking and concentration of $MgCl_2$ were also determined by the model as 133 rpm and 27 g L^{-1}, respectively. The predicted lipase activity by the model in 95% of confidence was 6.74 U.mL^{-1}.

RESULTS AND DISCUSSION

Preparation of inoculum

Factors such as inoculum size and growth profile can affect the amount of enzyme production by bacteria. In addition, the number of bacterial cells in the culture medium affects nutrient accessibility. As the time of bacterial culture increases, the concentration of growth inhibitory compounds also increases **(Thakur *et al.*, 2014)**. To overcome these limiting factors of lipase production and also to use a constant number of active bacteria in all experiments, a *N. asiatica* growth pattern was determined. According to Figure 1, absorbance in 600 nm increases up to 1.4 during 48 h and then remains nearly constant. After 144 h, absorbance decreases due to the death of bacteria. We used 1ml of a 36 h bacterial culture with absorbance 1 in 600 nm to inoculate culture medium in all experiments **(Prajapati *et al.*, 2014)**.

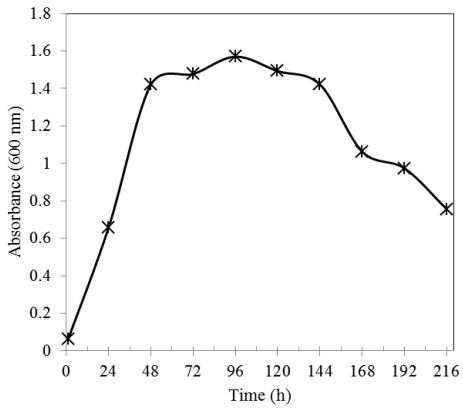

Figure 1 Growth profile of *N. Asiatica* during the different incubation times.

Optimization of enzyme assay

Changes in temperature and pH affect the activity of enzymes and therefore the measurement of enzyme activity should be done under optimal temperature and pH conditions. Therefore, to determine the optimum temperature and pH, the activity of *N. asiatica* lipase was measured at different temperatures and pH (Figure 2). According to the pH profile, the maximum activity of *N. asiatica* lipase was obtained at pH 10 and so this enzyme could be considered as an alkaline lipase. Lipases with similar optimum pH range were reported from other sources such as *Staphylococcus sp*. strain ESW (pH 9-13), *Pseudomonas aeruginosa* (pH 9), *Bacillus sonorensis* 4R (pH 9), and some others **(Bhosale *et al.*, 2016; Cherif *et al.*, 2011; Karadzic *et al.*, 2006)**. Maximum activity of *N. asiatica* lipase (50 °C) is like to the other moderate thermophile lipases from *Spirulina platensis* (45 °C), *Penicillium camemberti* Thom PG-3 (48 °C), and *Aspergillus niger* F044 (45 °C) **(Demir & Tükel, 2010; Shu *et al.*, 2007; T. Tan *et al.*, 2004)**.

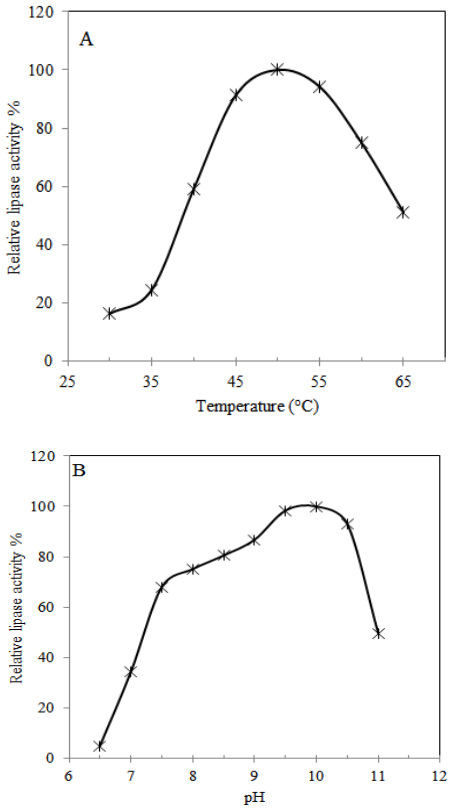

Figure 2 Activity of *N. asiatica* lipase in different temperatures (A) and pH (B).

Detection of significant factors using PB design

The submerged culture method has been widely used to produce lipase by various bacteria **(Sharma *et al.*, 2014)**. Optimal culture conditions and the nutritional needs of each microorganism should be considered to increase lipase production. The most important parameters that affect the amount of lipase production are the type and concentration of carbon and nitrogen sources, temperature and pH of bacterial growth and the concentration of dissolved oxygen **(Elibol & Ozer, 2000)**. In conventional methods for determining the effective parameters in the production of a product by a microorganism, it was only possible to evaluate one parameter in each experiment. In other words, only one parameter is changed in each evaluation and the other parameters are constant. This method is very time-consuming and expensive in cases where the number of factors to be evaluated is large. Today, the Plackett–Burman (PB) method is widely used as a powerful method to evaluate and determine the effective parameters in a process **(Chennupati *et al.*, 2009; Heydari *et al.*, 2012; Liu *et al.*, 2011; Pareek *et al.*, 2011; Prajapati *et al.*, 2014; Yele & Desai, 2014)**. One of the advantages of this method is the reduction of the number of tests required to determine the effective parameters.

In the present study, olive oil, lactose, peptone, $MgCl_2$, NaCl, pH, shaking, and incubation time and temperature as independent variables were investigated in the PB design. The results of experiments designed by PB method and analysis of variance (ANOVA) are shown in Table 1 and Table 2, respectively.

The effect of variables in the lipase production by *N. asiatica* was represented as the following mathematical model:

Lipase activity (U mL^{-1}) = 2.62 - 0.0442 temperature - 0.135 pH - 0.01436 Shaking- 0.01074 Incubation time + 0.00158 NaCl + 0.2114 $MgCl_2$ + 0.0575 Lactose- 0.042 Peptone + 0.0367 Olive oil + 0.575 Ct Pt

The F- and P-value of the assumed model are 6.35 and 0.045, respectively. These values mean that the model is significant. The confidence of determination (R^2) of the PB design (94.08%) represented that the mathematical model can fit 94.08% of total variables in the range of studied values.

The p-value and F-value show the significance of variables at the confidence level. The significant variables (with p-value less than 0.05 and high F-value) **(Pareek *et al.*, 2011)** are represented in Table 2. According to Table 2, three parameters including temperature, shaking, and $MgCl_2$ were known as significant. This means that olive oil, lactose, and peptone used in the culture of *N. asiatica* as carbon and nitrogen sources have not effect on lipase production and could be replaced by other components. Since *N. asiatica* is a halophilic bacterium, high NaCl concentration is essential for bacterial growth but not for lipase production. The dependency of lipase production on carbon sources different between species. Olive oil was known as a lipase production inducer in different bacterial sources (Mobarak-Qamsari *et al.*, 2011; Stergiou *et al.*, 2012; Yele & Desai, 2014). In *N. asiatica*, olive oil has not a significant effect on lipase production like in some other reports **(Burkert, 2004; Chennupati *et al.*, 2009; Ito *et al.*, 2001)**. Lactose is another carbon source that was not determined as an effective factor. This result is similar to the study on the production of cold-active lipase production by marine bacterium *Wangia* sp. C52 **(Liu *et al.*, 2011)**. In the study reported by Tianway et al., lactose was the optimal carbohydrate for lipase production by *P. camemberti* Thom **(C. H. Tan *et al.*, 2015a)**. Rajendran et al. used PBD to investigate the effect of 12 medium components in lipase production by *Bacillus sphaericus* and report glucose, olive oil, peptone, NaCl and $MgSO_4.H_2O$ as effective factors **(Rajendran & Thangavelu, 2007)**. Comparison of the results of these studies with our finding shows that there is a different dependency of medium components for the production of lipase among different bacterial species and so, determining the significant parameters is the most important step in designing a production process.

Table 1 The Placket-Burman design for detection of significant factors affecting lipase production in *N. asiatica*.

Run Order	Temp. (°C)		pH		Shaking (rpm)		Inc. time (h)		NaCl (g L^{-1})		$MgCl_2$ (g L^{-1})		Lactose (g L^{-1})		Peptone (g L^{-1})		Olive oil (mL L^{-1})		Lipase activity (U mL^{-1})	
	EV	level	EV	level	EV	level	EV	level	EV	level	EV	level	EV	level	EV	level	EV	level	Observed	Predicted
1	50	+	8.5	+	190	+	24	-	204	+	26	+	15	-	6.25	+	15	-	2.97	3.18
2	50	+	6.5	-	190	+	72	+	164	-	26	+	15	-	3.75	-	15	-	3.31	3.09
3	30	-	8.5	+	190	+	24	-	204	+	20	-	15	-	3.75	-	25	+	3.60	3.38
4	50	+	8.5	+	110	-	72	+	204	+	20	-	25	+	3.75	-	15	-	3.11	3.26
5	30	-	6.5	-	110	-	72	+	204	+	26	+	15	-	6.25	+	25	+	5.22	5.42
6	30	-	8.5	+	190	+	72	+	164	-	26	+	25	+	3.75	-	25	+	4.32	4.58
7	40	0	7.5	0	150	0	48	0	184	0	23	0	20	0	5	0	20	0	4.73	4.57
8	50	+	8.5	+	110	-	72	+	164	-	20	-	15	-	6.25	+	25	+	3.09	2.94
9	50	+	6.5	-	190	+	24	-	164	-	20	-	25	+	6.25	+	25	+	2.95	3.09
10	50	+	6.5	-	110	-	24	-	204	+	26	+	25	+	3.75	-	25	+	5.89	5.68
11	40	0	7.5	0	150	0	48	0	184	0	23	0	20	0	5	0	20	-	4.84	4.57
12	40	0	7.5	0	150	0	48	0	184	0	23	0	20	0	5	0	20	-	4.15	4.57
13	30	-	6.5	-	110	-	24	-	164	-	20	-	15	-	3.75	-	15	0	4.05	4.30
14	30	-	8.5	+	110	-	24	-	164	-	26	+	25	+	6.25	+	15	0	6.06	5.77
15	30	-	6.5	-	190	+	72	+	204	+	20	-	25	+	6.25	+	15	0	3.36	3.21

Legend: EV: experimental value; Temp: temperature; Inc: incubation

Table 2 Analysis of Variance for experimental results respects to lipase activity of Placket-Burman design.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	10.000	14.387	1.439	6.350	0.045
Linear	9.000	13.592	1.510	6.670	0.042
Temperature	1.000	2.345	2.345	10.350	0.032
pH	1.000	0.219	0.219	0.970	0.382
Shaking	1.000	3.961	3.961	17.490	0.014
Incubation time	1.000	0.797	0.797	3.520	0.134
NaCl	1.000	0.012	0.012	0.050	0.830
$MgCl_2$	1.000	4.829	4.829	21.320	0.010
Lactose	1.000	0.993	0.993	4.390	0.104
Peptone	1.000	0.033	0.033	0.150	0.722
Olive oil	1.000	0.404	0.404	1.780	0.253
Curvature	1.000	0.795	0.794	3.510	0.134
Error	4.000	0.906	0.226		
Lack-of-Fit	2.000	0.629	0.314	2.270	0.306
Pure Error	2.000	0.277	0.139		
Total	14.000	15.293			

Legend: DF: Degree of Freedom, Adj SS: the adjusted sum of squares, Adj MS: adjusted mean of squares.

Standardized effects of variables were shown in the Pareto chart (Figure 3). According to the Pareto chart, significant factors have effects upper than the t-value (2.776). Among the three significant factors, $MgCl_2$ and temperature have the highest and lowest effects, respectively.

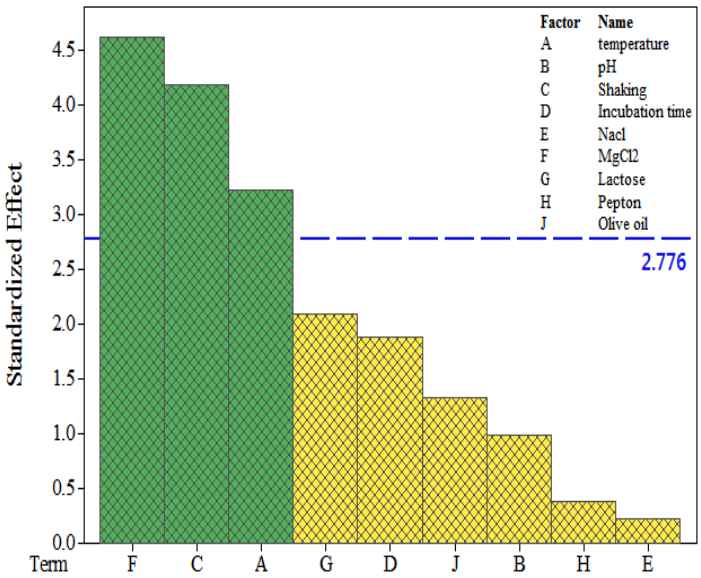

Figure 3 Pareto chart of the standardized effect of variables for the detection of significant factors for lipase production from *N. asiatica*.

Optimization of lipase production using RSM

The response surface methodology (RSM) was used to investigate the interaction of effective parameters in the production of lipase by *N. asiatica* (temperature, vibration and magnesium). The predicted and observed values of the experiments designed based on CCD are shown in Table 3. The minimum and maximum lipase activity obtained in these experiments were 4.112 to 6.701 U mL^{-1}, respectively.

Table 3 Data for the central composite design of significant factors in the production of lipase from *N. asiatica.*

Run Order	Temperature (°C)		Shaking (rpm)		$MgCl_2$ (g L^{-1})		Observed	Predicted
	actual	Coded	actual	Coded	actual	Coded		
1	30	-1	110	-1	20	-1	6.541	6.264
2	50	1	110	-1	20	-1	6.065	5.809
3	30	-1	190	1	20	-1	4.897	4.816
4	50	1	190	1	20	-1	4.582	4.361
5	30	-1	110	-1	26	1	5.668	5.783
6	50	1	110	-1	26	1	6.701	6.317
7	30	-1	190	1	26	1	6.114	5.906
8	50	1	190	1	26	1	6.269	6.440
9	23	-1.682	150	0	23	0	6.725	6.855
10	57	1.682	150	0	23	0	6.647	6.921
11	40	0	83	-1.682	23	0	5.338	5.678
12	40	0	217	1.682	23	0	4.499	4.564
13	40	0	150	0	18	-1.682	4.112	4.471
14	40	0	150	0	28	1.682	5.770	5.814
-15	40	0	150	0	23	0	5.794	5.804
16	40	0	150	0	23	0	5.867	5.804
17	40	0	150	0	23	0	5.828	5.804
18	40	0	150	0	23	0	5.847	5.804
19	40	0	150	0	23	0	5.760	5.804
20	40	0	150	0	23	0	5.799	5.804

The ANOVA was used to investigate the effect of each variable on lipase production by linear, square, and 2-way interaction (Table 4). Considering F- and P-value (P =< 0.05), just temperature has not a significant effect in the linear model. While all other combinations of square and 2-way interactions of three variables are significant. The following second-order polynomial equation represents these effects in the production of lipase.

Lipase activity (U mL^{-1}) = 11.76- 0.4942 Temp- 0.0382 Shaking + 0.508 $MgCl_2$+ 0.003832 Temp*Temp- 0.000151 Shaking*Shaking-0.02599 $MgCl_2$*$MgCl_2$+ 0.00824 Temp*$MgCl_2$+ 0.003273 Shaking*$MgCl_2$

The F- and P-value for the model are 16.80 and 0.00, respectively and therefore the model terms are significant.

Table 4 ANOVA for the central composite design based experiments.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	8	9.554	1.194	16.800	0.000
Linear	3	3.683	1.228	17.270	0.000
A	1	0.005	0.005	0.070	0.791
B	1	1.498	1.498	21.080	0.001
C	1	2.179	2.179	30.670	0.000
Square	3	4.148	1.383	19.460	0.000
A*A	1	2.116	2.116	29.770	0.000
B*B	1	0.842	0.842	11.840	0.006
C*C	1	0.789	0.789	11.100	0.007
2-Way Interaction	2	1.723	0.862	12.120	0.002
A*C	1	0.489	0.489	6.880	0.024
B*C	1	1.234	1.234	17.360	0.002
Error	11	0.782	0.071		
Lack-of-Fit	6	0.774	0.129	84.830	0.000
Pure Error	5	0.008	0.002		
Total	19	10.335			

Legend: A: Temperature, B: shaking, C: $MgCl_2$

The coefficient of variations (R^2) always lies between 0 and 1 and shows the capability of the model to describe the variability in the response.
(Uma & Satyanarayana, 2003). The closer R^2 is to one, the greater the model's ability to predict results (Frank Ph. D, 1992). Considering the R^2 value (0.924), the confirmed model can describe 92.4% of the total variability within the range of values studied. In our study, there is an appropriate agreement between R^2 (0.924) and adjusted R^2 (0.869). The adjusted R^2 corrects the R^2 value for the sample size and the number of terms in the model (Uma & Satyanarayana, 2003). It means that experimental and predicted values for lipase production are enough to close together. The ability of the given model in the prediction of results can be investigated by comparing the actual results and predicted values in the same experiment. Figure 4 shows a good fit for actual and predicted values in the lipase production.

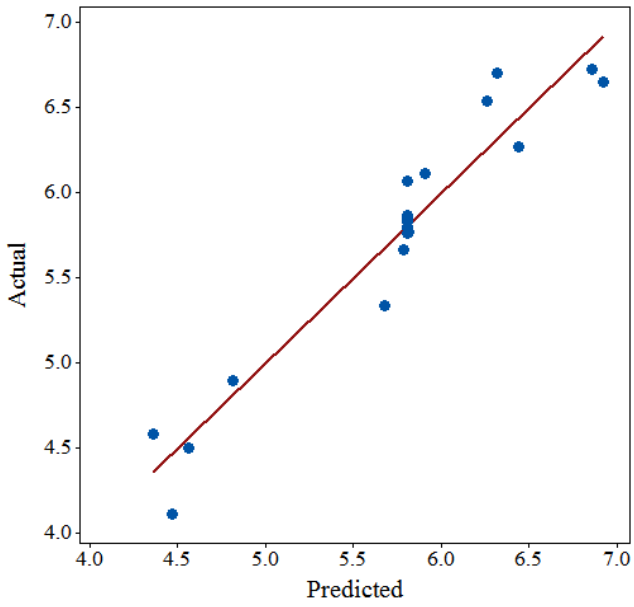

Figure 1 Scattering plot for actual results against predicted values of CCD experiments.

Analysis of response surface methodology (RSM)

The interactions of factors on lipase production by *N. asiatica* are shown in 3D plots. In each plot, one factor is constant and the other two factors change according to the values specified in the CCD. The surface of each curved plate shows the amount of change in lipase production.
Figure 5a shows the effect of shaking and temperature when $MgCl_2$ concentration remaining constant. In each temperature, lipase activity increased with increment in shaking up to 150 rpm. But activity decreases in the shaking upper than 150 rpm. The temperature has different effects. In each shaking value, lipase activity decreases with an increment in the temperature up to 40 °C. In the next step, we can see increments in the activity with the increase in temperature. According to the related contour plot for the mentioned condition (Figure 6a), maximum lipase activity obtains in shaking range from 90 to 150 rpm and temperatures less than 25 °C and more than 55 °C. In Figure 5b simultaneous effects of temperature and $MgCl_2$ in lipase production were investigated. Increments in $MgCl_2$ concentration leads to an increase in lipase activity in all temperatures. Maximum activity of lipase acquires in $MgCl_2$ and temperature upper than 24 g L^{-1} and 55 °C, respectively (Figure 6b). As like as Figure 5a, similar effects of temperature can be seen in different concentrations of $MgCl_2$. Figure 5c shows lipase activity in different levels of $MgCl_2$ and shaking at constant temperature (40 °C). The

concentration of $MgCl_2$ up to 23 g L^{-1} increases lipase activity in the shaking range from 90 to 150 rpm. More $MgCl_2$ concentrations in similar shaking ranges decrease activity. In the shaking range from 150 to 210 rpm, lipase activity increased with increment in the concentration of $MgCl_2$. The maximum activity obtained in broad ranges of $MgCl_2$ concentrations and shaking speed from 21 to 28 g L-1 and 90 to 200 rpm, respectively (Figure 6c).

Predicted values of factors in the model were used in an experiment and the result was compared with non-optimized conditions. The optimized condition was pH 7.5, incubation time 48 h, NaCl 184 g L^{-1}, lactose 20 g L^{-1}, peptone 5 g L^{-1}, olive oil 20 mL L^{-1}, shaking 133 rpm, temperature 50 °C, and $MgCl_2$ 26 g L^{-1}. Lipase activity in the experiment under optimal conditions (6.1 U mL^{-1}) was very close to the value predicted by the model (6.74 U mL^{-1}). In addition, lipase activity increased by 98.8% under optimal conditions compared to non-optimized conditions (3.39 U mL^{-1}). This result confirms that the model appropriately can define optimal levels of factors to increase production of lipase by *N. asiatica*.

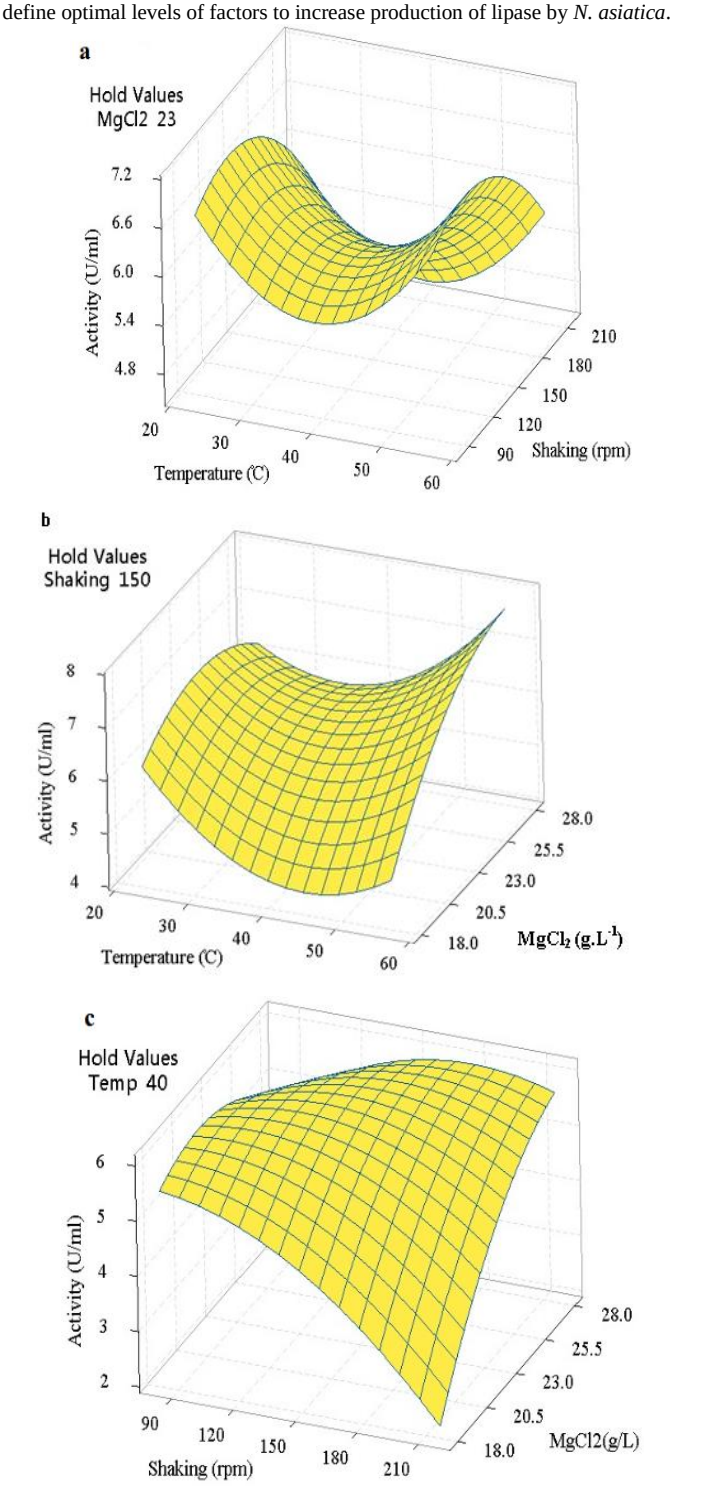

Figure 5 Surface plot for investigation of interactive effects of variables on lipase production. In each plot, one variable remains constant. The constant variables including $MgCl_2$ (23 g.L^{-1}) in (a) and shaking (150rpm) and temperature (40°C) in (b) and (c), respectively.

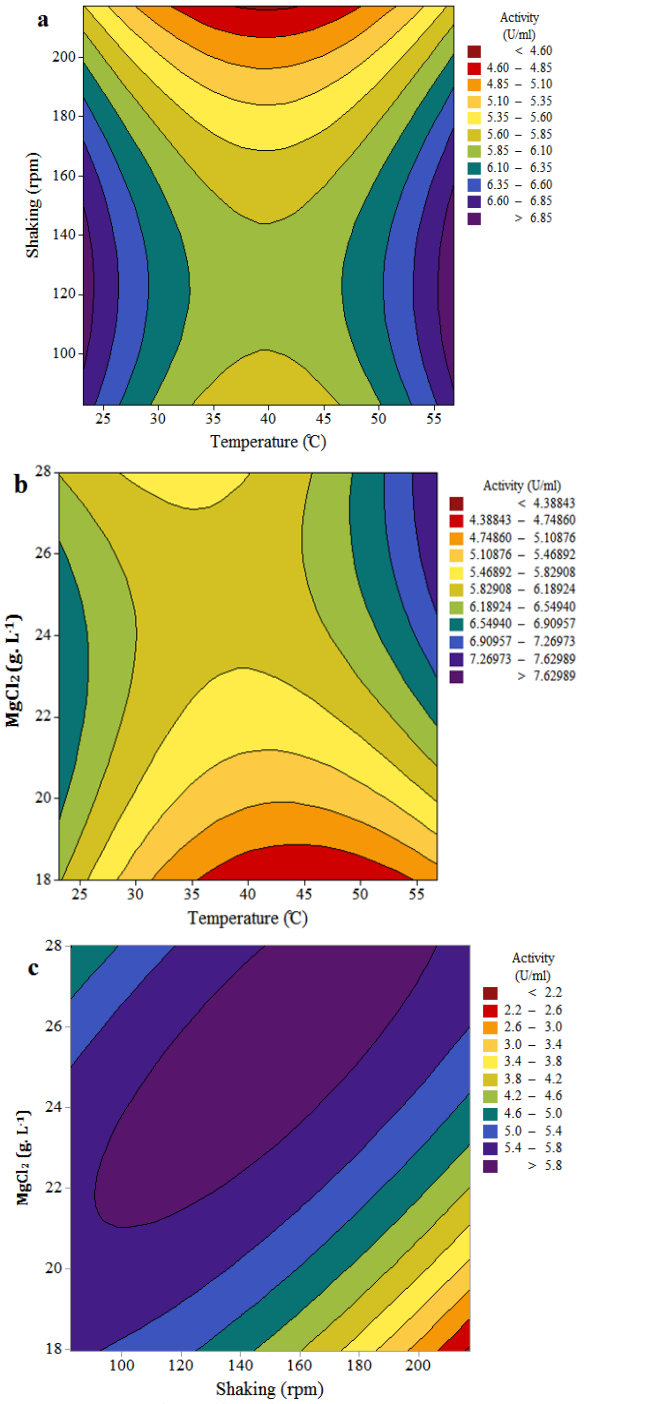

Figure 2 Contour plots of the effect of two variables while the other variable held at 0 level. The $MgCl_2$ concentration, shaking and the temperature held invariant in a, b and c, respectively.

CONCLUSIONS

In the present study, the production of lipase by the halophilic bacterium *N. asiatica* was optimized and modeled. The Placket-Burman design was used for the identification of significant variables affecting lipase production by this bacterium. Among 9 investigated variables, temperature, shaking and $MgCl_2$ concentrations were identified as significant factors. These factors are used as optimization variables using the CCD method in the next step. An increment in lipase production from 3.39 to 6.1 U mL^{-1} (79.9%) was obtained using optimized levels of variables. The maximum lipolysis activity of *N. asiatica* lipase was obtained in pH 10 and so this enzyme was determined as alkaline lipase. Considering the applications of alkaline enzymes and the demands in this field, it seems that *N. asiatica* can be considered and used in the field of biotechnological and industrial processes that are performed under alkaline conditions.

Conflict of Interest: The authors confirm that no part of the manuscript has been plagiarized or self-plagiarized and declare no conflict of interest.

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