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Overproduction of thermoalkalophilic lipase secreted by *Bacillus atrophaeus* FSHM2 using UV-induced mutagenesis and statistical optimization of medium components

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ABSTRACT

Microbial enzymes of extremophilic origin serve as a vital source of stable industrial enzymes. The present study focused on overproduction of a thermoalkalophilic lipase produced by *Bacillus atrophaeus* FSHM2 through UV-induced random mutagenesis (5–45 min exposure to UV light) and factorial experimental design augmented to response surface methodology. Firstly, a UV-induced mutant (designated as UV-45) was developed after the exposure of wild strain to UV irradiation for 45 min which was able to secrete 3484.8 U/L lipase. Afterward, Plackett–Burman experimental approach augmented to central composite design was employed to optimize medium components (olive oil, maltose, glucose, sucrose, yeast extract, tryptone, urea, (NH₄)₂SO₄, NaCl, CaCl₂, and ZnSO₄) for lipase production by the UV-45 mutant strain. The maximum lipase production of 5505.3 U/L were predicted in medium containing 5% of olive oil, 0.69% of glucose, 0.69% of sucrose, 2.5% of maltose, yeast extract (0.7 g/L), urea (0.44 g/L), (NH₄)₂SO₄ (2.44 g/L), tryptone (1.19 g/L), NaCl (1.61 g/L), CaCl₂ (3.81 g/L), and ZnSO₄ (1.42 g/L). A mean value of 5161.3 ± 83.3 U/L of lipolytic activity was acquired from real experiments. To sum up, the lipolytic activity of wild type strain (1720.4 U/L) increased by 3-fold after UV-induced mutagenesis and medium components optimization (5161.3 U/L).

KEYWORDS

Bacillus atrophaeus; Lipase; Medium optimization; UV-induced mutagenesis

Introduction

As a valuable enzyme, the hydrolytic activity of microbial-derived lipases (E.C. 3.1.1.3, triacylglycerol acyl-hydrolases) has been widely applied in different biotechnological and industrial processes.^[1–3] For example, cheese ripening in dairy manufacturing and improving paper quality in paper industries, as well as regio-selective acylation of fatty acids and alcohols and manufacturing of biodiesel, flavoring agents, and biopolymers, have been mediated by the catalytic activity of this unique biocatalyst.^[4,5] Lipases have been also used for the synthesis of chiral pharmaceuticals such as anti-inflammatory analgesics, antidepressants, and antineoplastic agents as well as some food supplements.^[6,7] One of the most serious problems of the lipase application for synthesis of pharmaceuticals is the poor resistance of the majority of enzymes in organic solvents which can be overcome by the employment of solvent tolerant lipases.^[7,8] So, such high demands for lipases especially those with pH, salt, thermal, and organic solvent tolerance encouraged investigators to screen and introduce lipolytic enzymes of the extremophile origin.^[9–13] For instance, Samaei-Nouroozi et al.^[14]

described the isolation of a haloalkaliphilic bacterial isolate, *Alkalibacillus salilacus*, able to produce lipase and applied it for esterification of different alcohols with oleic acid in organic solvents. The produced lipase of *Acinetobacter baylyi* was resistant to organic solvent and could esterify palm oil to fatty acid methyl esters (FAMES) for biodiesel production.^[15] The next steps for overproduction of microbial derived metabolites especially the biotechnologically important enzymes are mutagenesis and statistical optimization approaches to attain the most appropriate strain and cultural conditions led to higher level of enzymes.^[16–19] UV-induced mutagenesis is among the simplest and most commonly applied mutagenesis methods used for developing more suitable microbial mutants.^[20–22] Such a technique has been previously described as a successful approach for production of lipases,^[21,23] proteases,^[24] and kojic acid.^[20]

The optimization of the culture medium components plays a crucial role to enhance enzyme production yields.^[25] Optimization of process parameters using the design of experiments (DOE) is an efficient strategy for minimizing experimental errors and enhancing the production yields.^[26–28] These techniques abundantly used for the

optimization of cultural medium components for the production of lipolytic enzymes by various microorganisms^[27,29–31] and for production of lipases with specific features (pH, salt, thermal, and organic solvent tolerance).^[1,30,31]

Recently, a thermoalkalophilic lipase (optimally work at 70 °C and pH 9) producer bacterial strain, *Bacillus atrophaeus*, was isolated from salty soil.^[32] Characterization of the enzymes revealed that its relative activity increased to $334.2 \pm 0.6\%$ after 1 h of incubation in the presence of xylene (25%). So, the current study was designed to assess the effect of UV-induced mutagenesis on the lipase production potency of the mentioned strain. Thereafter, the influences of some variables on lipase productivity of the selected mutant were determined using statistical experimental design and the related model was applied for the optimization of culture medium components.

Experimental

Chemicals

ρ -nitrophenyl palmitate (ρ -NPP) and ρ -nitrophenol (ρ -NP) were provided by Sigma-Aldrich (St. Louis, MO, USA). Peptone, Rhodamine B, nutrient broth (NB), nutrient agar, yeast extract, and brain heart infusion broth (BHIB), as well as glucose and sucrose, were bought from Merck Chemicals (Darmstadt, Germany).

Microorganism and culture condition

B. atrophaeus FSHM2, a wild strain previously isolated from a hypersaline soil sample,^[32] was used in this study. Characterization of the secreted lipase of this bacterial isolate (maximum activity at 70 °C and pH 9) categorized it as thermoalkalophilic group. The culture of *B. atrophaeus* FSHM2 was grown on BHI agar plate containing NaCl (15% w/v) for 24 h and then stored at 4 °C for 1 month. This isolate was also kept in glycerol (20% v/v) supplemented culture media at –80 °C for a long duration.

UV irradiation

UV irradiation method was done based on the previously described method.^[20] Briefly, *B. atrophaeus* FSHM2 was cultured in BHIB and incubated for 24 h at 60 °C. The produced biomass was then washed three times by successive centrifugation (8000×g, 15 min) with sterile phosphate buffer (pH 7). Several test tubes, containing 4 mL of the bacterial cell suspensions in sterile water (OD_{600} :0.1), were consequently illuminated by UV ray in a cabinet equipped with a UV lamp (255 nm) at the distance of 20 cm from the surface of the test tube. UV exposure continued for 5 min, 10 min, 15 min, 30 min, and 45 min, where each UV treated test tube was remained in darkness for 24 h, followed by serial dilution in phosphate buffer (pH 7), streaking on BHIA medium, and incubating at 37 °C for 24 h. Viable colony numbers of each plate were then counted and plates having less than 1% survival rate were selected for the isolation of

mutants. Several bacterial colonies were isolated and evaluated for the production of thermoalkalophilic lipase.

Mutants' selection

After UV irradiation, the obtained bacterial colonies were streaked on the BHIA plates supplemented with Rhodamine B (0.001%, w/v), NaCl (15% w/v), and olive oil (1%, v/v) and incubated at 37 °C for 24 h. The plates were consequently checked for lipase production by the intensity of appeared orange fluorescent halo around the colonies by exposure to UV light (350 nm). The potent mutants with wider fluorescent were chosen for the next steps of studies.

Lipase activity assay

The selected mutants of the previous step were aerobically cultivated (60 °C, 120 rpm) in 150 mL of basal medium composed of nutrient broth supplemented with olive oil (1%, v/v). After 16 h, the culture broth was centrifuged (10,000×g, 15 min) and the supernatant was used for measuring the lipase activity. The lipolytic activity of the culture broth was measured assisted by ρ -NPP as substrate based on a modified method explained by Hasan-Beikdashti et al.^[29]

At first, the fresh solution of lipase substrate (ρ -NPP, 0.01 M) was prepared and 0.1 mL of this solution was mixed with 0.8 mL of carbonate buffer (0.1 M, pH 9) containing Triton X-100 (0.4%). Thereafter, the collected supernatant sample (0.1 mL) was added to the prepared mixture and incubated for 30 min at 60 °C under mild shaking followed by recording the related absorbance at 410 nm in order to measure the amount of liberated ρ -NP assisted by spectrophotometer (UV-1800, Shimadzu Corporation, USA). Enzyme activity (U) was then defined as the amount of the enzyme leading to the release of 1 μ mol of ρ -NP per min from the ρ -NPP substrate.

Experimental design

The statistical optimization of medium components to increase thermoalkalophilic lipase production of the selected *Bacillus* mutant was performed through Plackett–Burman design (PBD) and response surface methodology (RSM) approaches.^[20,29,33,34] Eleven variables consisting different carbon sources (olive oil, glucose, sucrose, and maltose), diverse nitrogen supplements (yeast extract, urea, $(NH_4)_2SO_4$, and tryptone), and ions (NaCl, $CaCl_2$, and $ZnSO_4$) (Table 1) were selected.^[27,29] These factors were chosen according to the literature review in order to specify the most important factor influencing bacterial lipase production. PBD was then applied to screen medium components exhibited the main effects on lipase production by the selected mutant. The levels of the selected components were consequently optimized for maximum lipase secretion using central composite design (CCD) of RSM. Variables were coded according to the following formula:

$$x_i = (X_i - X_{i0})/\Delta X_i \quad i = 1, 2, 3, \dots, k \quad (1)$$

where x_i is the coded value of an independent variable, X_i is the real value of the independent variable, X_{i0} is the real

Table 1. The applied levels of independent variables of Plackett–Burman design for lipase production of UV-45 mutant.

Variable	Component	Unit	Low level (−1)	High level (+1)
X ₁	Olive oil	%	1	5
X ₂	Glucose	%	0.5	2.5
X ₃	Sucrose	%	0.5	2.5
X ₄	Maltose	%	0.5	2.5
X ₅	Yeast extract	g/L	0.5	3
X ₆	Urea	g/L	0.5	3
X ₇	(NH ₄) ₂ SO ₄	g/L	0.5	3
X ₈	Tryptone	g/L	0.5	3
X ₉	NaCl	g/L	1	5
X ₁₀	CaCl ₂	g/L	1	5
X ₁₁	ZnSO ₄	g/L	1	5

value of the independent variable at the center point, and ΔX_i is the value of step change. The experimental runs were performed randomly to negate the effect of nuisance and unwanted variables. The obtained results were then analyzed and augmentation of the designed PBD to a CCD was performed by addition of further 24 new experiments (Table 2, Run 16–39) to the previous design. Furthermore, an additional 10 new experiments (Table 2, Run 40–49) were also added to improve the leverage. The selected variables can be related to the response by the model as exhibited in the following quadratic polynomial equation:

$$Y = b_0 + \sum_{i=1}^n b_i x_i + \sum_{i=1}^n b_{ii} x_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^n b_{ij} x_i x_j + e \quad (2)$$

In this equation, Y is the predicted response (here is lipase production), x_i ($i = 1-11$) are the variables of the experiment, b_0 is the model constant; b_i ($i = 1-11$) are the linear coefficients, b_{ij} ($i \neq j$) are the second order coefficients, and b_{ii} ($i = 1-10$) are the second order interaction coefficients and e is the error of the experiment, x are the independent variables.

Data analysis

The statistical software package Design Expert version 6.0.4 (Stat-Ease Inc., Minneapolis, MN) was applied for analysis of the results.^[20,33,34] The characterizations of the fitted quadratic polynomial models were inspected by the coefficient of determination R^2 . The lipase activity was analyzed using the analysis of variance (ANOVA) along with the F-test to assess whether a stated term had a significant effect ($p \leq .05$). The position of the optimum was specified by resolving the set of equations derived by the variation of the final quadratic model.

Results and discussion

Mutagenesis and screening of a high-level lipase producer mutant

UV-induced mutagenesis is a common and easy approach to achieve more efficient microorganisms able to produce valuable metabolites especially those of enzyme producers. For instance, Zhao et al.^[35] described the development of a variant strain (UV-01) of *Serratia marcescens* ECU1010 exhibited 2.3-fold higher lipase activity than the wild type.

Toscano et al.^[21] reported about a 2-fold increase of lipase production of *Aspergillus niger* after UV irradiation. Shakibaie et al.^[20] investigated on the impact of UV mutagenesis for kojic acid production of *A. terreus*.

The attained results of the current study revealed that five mutant isolates showed the intense orange fluorescent halos under UV light exposure (350 nm) on the BHIA plate supplemented by Rhodamine B (0.001%, w/v), NaCl (15% w/v), and olive oil (1%, v/v) (Figure 1A). These five mutants were tested for thermoalkalophilic lipase production in shake flask mode among which three UV mutants including UV-10, UV-30, and UV-45 (2896.7 U/L, 3186.3 U/L, and 3484.8 U/L, respectively) showed significantly higher lipase activity compared to the wild type (1720.4 U/L) (Figure 1B). The maximum lipase production by UV-45 mutant was also considerably higher than other mutants ($p < .05$) (Figure 1B). Hence, the mutant strain UV-45 was selected for further studies. Similar results were reported by Bisht et al.^[23] who observed a 3.6-fold increase in the lipase secretion by a mutant strain of *Pseudomonas aeruginosa* MTCC 10,055 compared with cells before exposure to UV. The exact mechanism(s) by which the mutagenesis alter the metabolite biosynthesis is not clear but it seems that they cause some damage to the DNA through deletion, addition, transversion or substitution of bases or breakage of DNA.^[22] Consequently, random mutagenesis could affect numerous pathways of the cell machinery.^[19,22]

Medium optimization

Eleven variables were screened using PBD to demonstrate their influences on lipase productivity of UV-45 mutant (Table 1). For medium optimization, the PBD augmented to CCD exhibited variation in lipolytic activity from 1927.6 U/L to 3354.8 U/L (Table 2). This obtained variation assessed the significance of media optimization to achieve higher productivity of lipase. Statistical analysis of the lipase production (U/L) was then carried out and summarized in Table 3. Amongst all selected variables, maltose, olive oil, urea, (NH₄)₂SO₄, and NaCl were displayed significant positive effects on lipase production ($p < .05$), whereas glucose, sucrose, yeast extract, tryptone, CaCl₂, and ZnSO₄ had a significant negative effect ($p < .05$). The attained CCD results were well described by a second-order quadratic polynomial phrase. The well coordinative quadratic model for assessing lipase productivity by the UV-45 mutant was:

$$\begin{aligned} \text{Lipase production} = & +3036.22 + 138.82X_1 - 83.75X_2 - 42.24X_3 \\ & + 67.67X_4 - 60.93X_5 + 77.88X_6 + 155.19X_7 \\ & + 56.83X_9 - 215.44X_{10} - 210.85X_{11} - 491.65X_1X_3 \\ & - 191.80X_1X_6 + 635.15X_1X_7 - 764.24X_1X_8 - 580.53X_1X_9 \\ & + 353.88X_1X_{10} + 301.04X_2X_3 - 358.16X_2X_4 + 568.53X_2X_8 \\ & + 322.69X_2X_9 - 481.26X_2X_{10} + 54.32X_2^2 - 44.64X_3^2 - 27.60X_4^2 \\ & - 100.75X_5^2 - 93.35X_6^2 - 64.56X_7^2 - 62.53X_8^2 - 102.50X_9^2 \\ & - 53.08X_{10}^2 - 81.63X_{11}^2 - 739.88X_1X_2X_3 \end{aligned} \quad (3)$$

The non-significant lack of fitness (0.5307) and significant p -values ($< .05$) of the model proposed that the

Table 2. Experimental design and results of the Plackett–Burman design augmented to central composite design for optimization of culture conditions for maximum lipase production of UV-45 mutant.

Run	Actual levels											Lipase activity (U/L)	
	X ₁	X ₂	X ₃	X ₄	X ₅	X ₆	X ₇	X ₈	X ₉	X ₁₀	X ₁₁	Observed	Predicted
1	3	1.5	1.5	1.5	1.75	1.75	1.75	1.75	3	3	3	3023.1	3036.2
2	5	2.5	2.5	0.5	0.5	0.5	3	0.5	5	5	1	2670.2	2660.6
3	3	1.5	1.5	1.5	1.75	1.75	1.75	1.75	3	3	3	3044.1	3036.2
4	5	2.5	0.5	0.5	0.5	3	0.5	3	5	1	5	2680.7	2670.1
5	3	1.5	1.5	1.5	1.75	1.75	1.75	1.75	3	3	3	3049.4	3036.2
6	1	0.5	2.5	0.5	3	3	0.5	3	5	5	1	2722.9	2709.3
7	1	0.5	0.5	2.5	0.5	3	3	0.5	5	5	5	3102.1	3092.9
8	1	2.5	2.5	0.5	3	3	3	0.5	1	1	5	2301.5	2293.8
9	1	2.5	2.5	2.5	0.5	0.5	0.5	3	1	5	5	2822.9	2819.7
10	1	2.5	0.5	2.5	3	0.5	3	3	5	1	1	3207.4	3200.2
11	5	0.5	0.5	0.5	3	0.5	3	3	1	5	5	3112.6	3117.5
12	5	0.5	2.5	2.5	0.5	3	3	3	1	1	1	2859.8	2855.4
13	5	0.5	2.5	2.5	3	0.5	0.5	0.5	5	1	5	1927.6	1931.7
14	5	2.5	0.5	2.5	3	3	0.5	0.5	1	5	1	2506.9	2495.4
15	1	0.5	0.5	0.5	0.5	0.5	0.5	0.5	1	1	1	2638.6	2628.9
16	3	1.5	1.5	1.5	1.75	1.75	1.75	4.03	3	3	3	2833.5	2828.2
17	3	1.5	1.5	1.5	1.75	1.75	4.03	1.75	3	3	3	3081	3104.5
18	3	1.5	0	1.5	1.75	1.75	1.75	1.75	3	3	3	2938.8	2999.1
19	3	1.5	1.5	1.5	1.75	1.75	1.75	1.75	3	3	6.64	2375.2	2382.1
20	3	1.5	1.5	1.5	1.75	1.75	1.75	1.75	3	6.64	3	2443.7	2468.3
21	3	1.5	1.5	3.32	1.75	1.75	1.75	1.75	3	3	3	3049.4	3068
22	3	3.32	1.5	1.5	1.75	1.75	1.75	1.75	3	3	3	3028.3	3063.7
23	3	1.5	1.5	0	1.75	1.75	1.75	1.75	3	3	3	2791.3	2872.6
24	6.64	1.5	1.5	1.5	1.75	1.75	1.75	1.75	3	3	3	3244.3	3288.9
25	3	1.5	3.32	1.5	1.75	1.75	1.75	1.75	3	3	3	2786.1	2811.5
26	3	1.5	1.5	1.5	0	1.75	1.75	1.75	3	3	3	2780.8	2812.2
27	3	1.5	1.5	1.5	1.75	1.75	0	1.75	3	3	3	2512.2	2538.4
28	3	1.5	1.5	1.5	1.75	1.75	1.75	1.75	0	3	3	2575.4	2593.3
29	3	1.5	1.5	1.5	1.75	1.75	1.75	0	3	3	3	2775.5	2828.2
30	3	1.5	1.5	1.5	1.75	1.75	1.75	1.75	3	3	0	3112.6	3149.6
31	0	1.5	1.5	1.5	1.75	1.75	1.75	1.75	3	3	3	2728.1	2783.6
32	3	0	1.5	1.5	1.75	1.75	1.75	1.75	3	3	3	3354.9	3368.6
33	3	1.5	1.5	1.5	1.75	1.75	1.75	1.75	3	3	3	3049.4	3036.2
34	3	1.5	1.5	1.5	4.03	1.75	1.75	1.75	3	3	3	2575.4	2589.9
35	3	1.5	1.5	1.5	1.75	1.75	1.75	1.75	3	3	3	3059.9	3036.2
36	3	1.5	1.5	1.5	1.75	0	1.75	1.75	3	3	3	2564.9	2583.6
37	3	1.5	1.5	1.5	1.75	4.03	1.75	1.75	3	3	3	2833.5	2867.7
38	3	1.5	1.5	1.5	1.75	1.75	1.75	1.75	6.64	3	3	2765	2800.1
39	3	1.5	1.5	1.5	1.75	1.75	1.75	1.75	3	0	3	3228.5	3252.5
40	3.45	1.98	1.36	2.08	1.47	1.83	1.45	2.25	3.35	3.21	2.11	3002	2996.7
41	2.63	1.11	1.05	1.51	1.1	2.62	2.02	2	3.84	4.07	1.88	3160	3149.5
42	2.13	2.05	1.59	1.5	1.05	2.67	1.3	1.76	3.62	4.25	2.59	2896.7	2867
43	3.7	1.57	1.96	1.58	1.91	0.78	2.31	2.03	4.21	3.25	2.68	2949.3	2906.5
44	4.47	2.13	2.09	1.97	1.59	1.01	1.55	2.83	3.24	3.95	2.69	2264.7	2244.3
45	2.96	1.11	0.57	1.08	1.74	1.55	1.51	1.46	3.42	3.47	2.79	3107.3	3081.8
46	3.09	1.24	1.83	1.54	2.28	1.35	1.9	2.23	3.18	4	4.38	2633.3	2651.1
47	3	1.5	1.5	1.5	1.75	1.75	1.75	1.75	3	3	3	3002	3036.2
48	3	1.5	1.5	1.5	1.75	1.75	1.75	1.75	3	3	3	3107.3	3036.2
49	3	1.5	1.5	1.5	1.75	1.75	1.75	1.75	3	3	3	3054.7	3036.2

experimental data were well fitted with the linear-quadratic model (Table 3). The squared correlation coefficient (R^2) was considered to be 0.9958, demonstrating that the proposed model can describe 99.58% of the variation in the response. When the predicted R -squared (0.9107) and adjusted R -squared (0.9861) values are closer to one, a better fit was achieved. The above-mentioned values supported that the quadratic model was adequate to explain the response of the experimental observations considering lipase production. The value of the coefficient of variation (C.V.%) was 1.22. In addition, the enhanced precision of the model (48.187) is an indicator of a suitable signal to noise ratio.

To compare the predicted and actual data, predicted values plotted versus observed values as represented in Figure 2A. The obtained results verified the validity of the model and the concurrence between predicted and actual values.

The associated residual versus predicted response obtained from the final model is shown in Figure 2B. An approximately constant variance was clearly observed in all over of the response range.

The actual values and the predicted values by the final model for lipase production represented in Table 2. Evaluation of these values demonstrated that there was great accordance between the model and the experimental results.

The optimum levels of the tested variables, by the selected model for lipase activity was predicted to be olive oil (5%), glucose (0.69%), sucrose (0.69%), maltose (2.5%), yeast extract (0.7 g/L), urea (0.44 g/L), $(\text{NH}_4)_2\text{SO}_4$ (2.44 g/L), tryptone (1.19 g/L), NaCl (1.61 g/L), CaCl_2 (3.81 g/L), and ZnSO_4 (1.42 g/L). The maximum lipase production in the optimum levels was achieved by the mutant UV-45. Compared to that of the wild bacterial strain (lipase activity

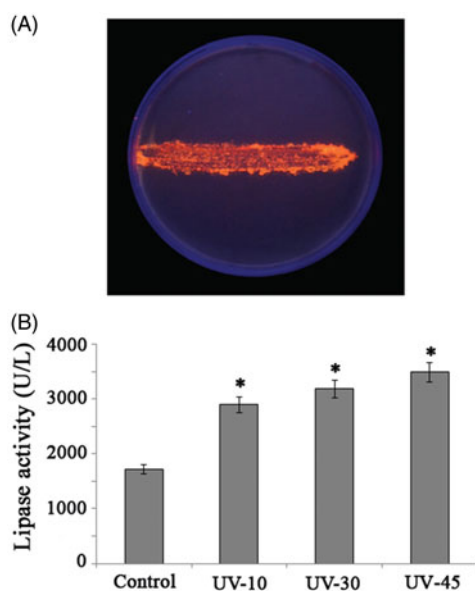


Figure 1. (A) Positive colony of lipase producing mutant of *B. atrophaeus* on BHI plate containing Rhodamine B (0.001%) after exposure to UV light. (B) Screening the best mutants of *B. atrophaeus* after different exposure time to UV light irradiation compared with the wild strain (as a positive control). Significance (*) was evaluated after ANOVA analysis of the obtained results (p -value $< .05$).

of 1720.4 U/L) the lipase productivity of the UV-45 mutant was increased by 5161.3 U/L (3-fold, $p < .05$) after optimization procedures.

The proposed optimum culture medium components were utilized five times to determine the model validation. The lipase production in these five independent experimental trials was 5161.3 ± 83.3 U/L that presented appropriate accordance with the predicted results (5505.3 U/L).

The response surface graphs exhibited in Figure 3 were drawn according to the selected model, holding other factors constant (in center point), while the other two factors were varying within their experimental range. Figure 3A exhibited the response surface for olive oil (-1 : 1 , $+1$: 5%) and maltose (-1 : 0.5 , $+1$: 2.5%), while other factors kept at their center point levels. Maximum response was seen with the high level of olive oil (5%) and high level of maltose (2.5%). When olive oil and maltose were at high levels, the production of lipase (3556 U/L) was significantly different compared to those of low level ($p < .05$). Also, the results suggested the considerable interaction between olive oil and maltose at the constant level of the other factors. Bora and Bora^[36] reported that the carbon substrates as maltose significantly increased the lipase activity of *Bacillus* sp. LBN. Abdel-Fattah^[37] evaluated the influences of variables on thermostable lipase production by *Geobacillus thermoleovorans* using RSM. They introduced olive oil (60 g/L) as an important factor in maximum enzyme activity.^[37] The attained results of the current investigation were also similar to the study of Rajendran et al.^[38] who found that maximum lipase production was achieved when a high concentration of olive oil (3620 U/L) was applied. Liu et al.^[39] evaluated lipase secretion by stepwise adding of olive oil; thereby the lipolytic activity maximally happened until the olive oil was inserted stepwise at 1 mM/day for 72 h. In

Table 3. Analysis of variance of central composite design for medium compositions in relation to lipase activity (U/L) by UV-45 mutant.

Source	Sum of Squares	df	Mean Square	F Value	p-value	Prob > F
Block	122000	2	60975.72	—	—	—
Model	3953000	32	123500	103.13	<.0001	significant
X ₁	161100	1	161100	134.45	<.0001	—
X ₂	56372.17	1	56372.17	47.06	<.0001	—
X ₃	14086.48	1	14086.48	11.76	.0041	—
X ₄	35826.81	1	35826.81	29.91	<.0001	—
X ₅	27838.28	1	27838.28	23.24	.0003	—
X ₆	49153.97	1	49153.97	41.03	<.0001	—
X ₇	180300	1	180300	150.53	<.0001	—
X ₉	24097.74	1	24097.74	20.12	.0005	—
X ₁₀	366800	1	366800	306.2	<.0001	—
X ₁₁	353400	1	353400	294.99	<.0001	—
X ₁ X ₃	47483.36	1	47483.36	39.64	<.0001	—
X ₁ X ₆	31876.9	1	31876.9	26.61	.0001	—
X ₁ X ₇	22874.78	1	22874.78	19.1	.0006	—
X ₁ X ₈	459500	1	459500	383.58	<.0001	—
X ₁ X ₉	67074.21	1	67074.21	55.99	<.0001	—
X ₁ X ₁₀	7423.51	1	7423.51	6.2	.026	—
X ₂ X ₃	21457.95	1	21457.95	17.91	.0008	—
X ₂ X ₄	7360.62	1	7360.62	6.14	.0265	—
X ₂ X ₈	18803.36	1	18803.36	15.7	.0014	—
X ₂ X ₉	21483.76	1	21483.76	17.93	.0008	—
X ₂ X ₁₀	182300	1	182300	152.15	<.0001	—
X ₂ ²	63385.25	1	63385.25	52.91	<.0001	—
X ₃ ²	43903.41	1	43903.41	36.65	<.0001	—
X ₄ ²	16313.4	1	16313.4	13.62	.0024	—
X ₅ ²	212500	1	212500	177.38	<.0001	—
X ₆ ²	185300	1	185300	154.67	<.0001	—
X ₇ ²	87167.05	1	87167.05	72.77	<.0001	—
X ₈ ²	86333.35	1	86333.35	72.07	<.0001	—
X ₉ ²	219400	1	219400	183.14	<.0001	—
X ₁₀ ²	59877.1	1	59877.1	49.98	<.0001	—
X ₁₁ ²	140600	1	140600	117.4	<.0001	—
X ₁ X ₂ X ₃	155800	1	155800	130.1	<.0001	—
Residual	16770.82	14	1197.92	—	—	—
Lack of Fit	10779.81	9	1197.76	1	.5307	not significant
Pure Error	5991.01	5	1198.2	—	—	—
Cor Total	4092000	48	—	—	—	—

contrast, Tommaso et al.^[40] reported that the medium consisted of olive oil (55.6%) and Tween 80 (44.4%) had a negative effect on the lipolytic activity of *Candida rugosa*. Hasan-Beikdashti et al.^[29] also reported that olive oil negatively affected the lipolytic activity of *Stenotrophomonas maltophilia*. It seems that adding oils to culture media has a different influence on lipase production that is mainly inferred by the type of microorganism.^[30,31,38]

Figure 3B represented the interactive effect of olive oil and $(\text{NH}_4)_2\text{SO}_4$ on lipase productivity. It revealed that the higher lipase activity (4086.6 U/L) observed at a higher concentration of $(\text{NH}_4)_2\text{SO}_4$ ($p < .05$). Similarly, Rajendran et al.^[38] studied the effect of $(\text{NH}_4)_2\text{SO}_4$ on the production of extracellular lipase by *C. rugosa*. The inorganic nitrogenous compound, $(\text{NH}_4)_2\text{SO}_4$, showed a considerable effect with 93.58% confidence level on lipase production.^[38] Investigations about the influences of other inorganic nitrogen sources such as NH_4NO_3 , $\text{NH}_4\text{CH}_3\text{COO}$, and NH_4Cl revealed their significant effects on lipase production.^[23,27,41,42] It has been proved that the medium containing both inorganic and organic nitrogen sources positively affected the lipase productivity of *Bacillus* sp.^[1,42,43] In most studies,^[29,30,37] the higher productivity of lipase was sufficiently impressed by the existence of different nitrogen sources in the culture medium. Indeed, the nitrogen sources

released the NH_4^+ ions, which stimulated the growth and lipase production of the lipase producer.^[1,38]

As shown in Figure 3C, lipase activity was influenced by olive oil and NaCl. The maximum lipase activity (3928.9 U/L) reached when NaCl was at its lowest level.

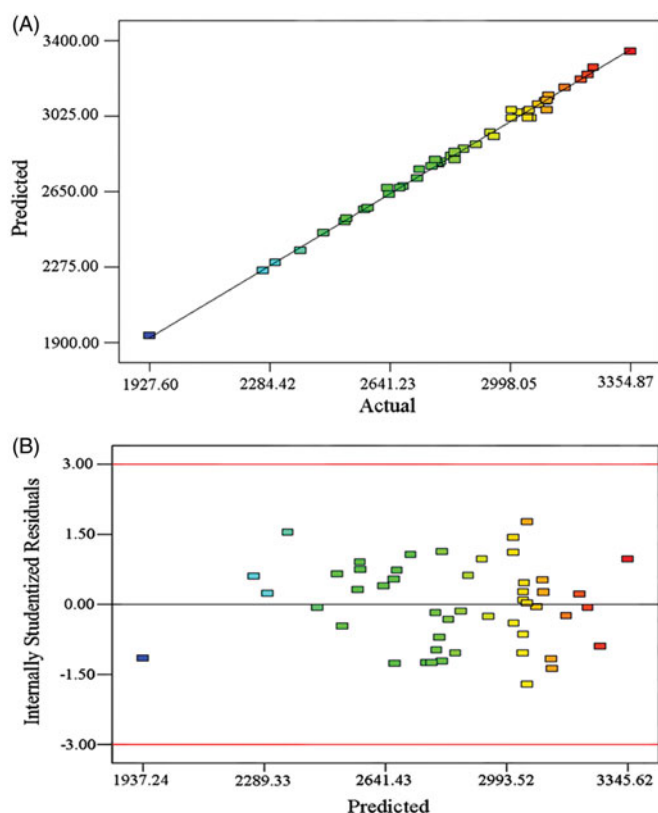


Figure 2. (A) Studentized residuals vs. predicted response by final quadratic model. (B) The normal probability vs. residuals plot by final fitted model.

Therefore, the negative influence of NaCl concentration showed on lipase productivity of mutant UV-45 ($p < .05$). Similar results were stated by Rajendran et al.^[38] where the addition of NaCl decreased the lipolytic activity of *C. rugosa*. Conversely, the positive effect of NaCl on lipase production was reported by Sathishkumar et al.^[44] They observed that lipase production of *Halobacillus trueperi* RSK CAS9 increased (1355.81 U/mL) in the presence of NaCl. Samaei-Nouroozi et al.^[14] also described that olive oil, KH_2PO_4 , NaCl, and glucose positively affected ($p < .05$) the haloalkaliphilic solvent-tolerant lipase production of *Alkalibacillus salilacus*. Several studies have the stimulatory effects of different ions on lipase production by different microorganisms.^[30,45]

Figure 3D illustrated the influence of glucose and maltose concentrations on lipase production. The maximum lipase activity (3764.4 U/L) was achieved when maltose was at the highest level ($p < .05$). These results were in agreement with the finding of Dutta and Ray^[43] where the alkaline thermostable lipase activity of *B. cereus* C7 was enhanced in the presence of maltose ($p < .05$). Nevertheless, the negative influence of maltose on lipase production was reported by Yele and Desai.^[45] They found that lipase secretion of *Staphylococcus warneri* decreased when maltose existed in the culture medium.^[45] As observed in the current study, glucose negatively influenced the production of lipase, which was in agreement with the study of Hasan-Beikdashti et al.^[29] However, Mukesh Kumar et al.^[46] found about the positive influence of glucose on lipase productivity of *Bacillus* sp. MPTK 912 in the presence of glucose. These findings were in contrast to those observed by Papagora et al.^[2] who stated that the enhancing concentrations of glucose and olive oil led to increasing of lipase production.

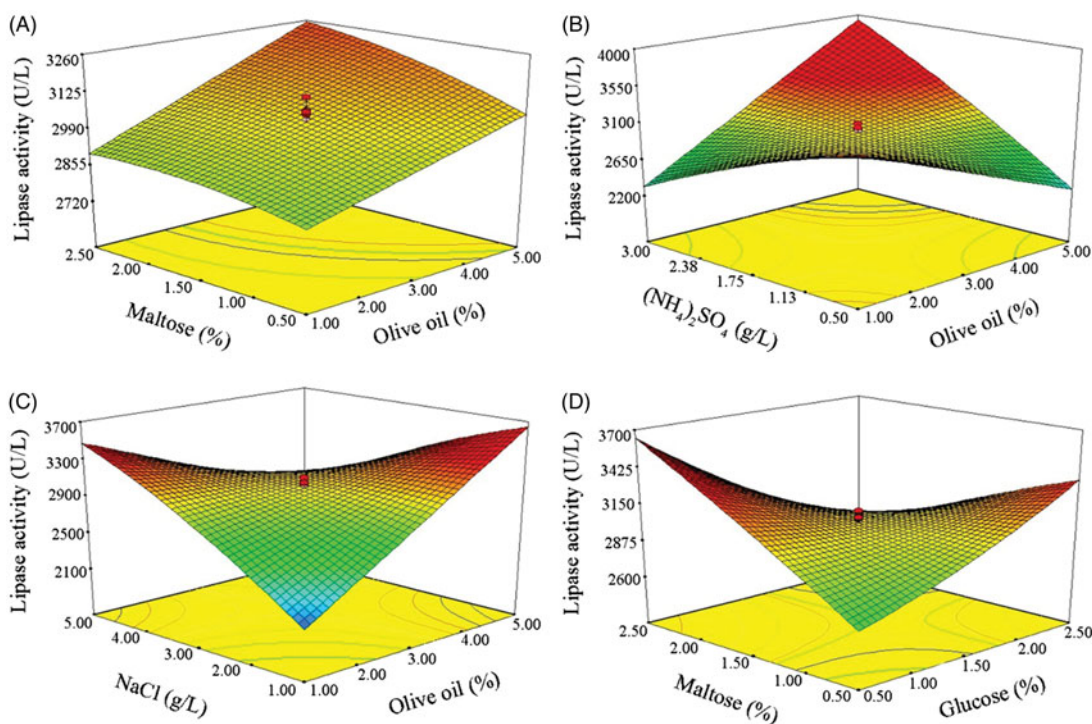


Figure 3. Response surface plots of the impact of various factors on lipase production of the mutant UV-45. (A) olive oil and maltose, (B) olive oil and $(\text{NH}_4)_2\text{SO}_4$, (C) olive oil and NaCl, (D) glucose and maltose. The other factors have constant level (in center point level).

The major factor affecting the production of lipases, usually, is a lipid source such as oil^[30,38] and is generally produced in the presence of carbon sources, such as sugars.^[27,29] However, lipase production is significantly influenced by other factors such as nitrogen sources and different ions.^[27,41,46] Thus, optimized levels could be used successfully for lipase overproduction.

Conclusion

The current study was designed to develop a mutant isolate of *B. atrophaeus* with a high capability for thermophilic lipase biosynthesis assisted by the UV-induced mutagenesis procedure followed by employment of statistical experimental methods to optimize the cultural medium compositions required for lipase production by the most efficient mutant. A 2-fold increase of lipase production achieved after exposure of the wild bacterial strain to UV irradiation for 45 min (UV-45 mutant). Amongst the 11 variables evaluated for optimization of culture medium components, olive oil, maltose, urea, $(\text{NH}_4)_2\text{SO}_4$, and NaCl showed positive and considerable effects ($p < .05$) on lipase production by the UV-45 mutant, while glucose, sucrose, yeast extract, tryptone, CaCl_2 , and ZnSO_4 exhibited negative effects. Eventually, UV-induced mutagenesis together with statistical optimization studies significantly enhanced the thermophilic lipase production of UV-45 mutant by 3-fold (5161.3 U/L) compared to the wild strain (1720.4 U/L) ($p < .05$). Anyhow, further studies must be performed to demonstrate the probable mechanism of mutation and possible application of this strain for overproduction of lipase.

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