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Statistical optimization of cultural medium composition of thermoalkalophilic lipase produced by a chemically induced mutant strain of *Bacillus atrophaeus* FSHM2

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Abstract

Extremophilic microbial derived lipases have been widely applied in different biotechnological processes due to their resistance to harsh conditions such as high salt concentration, elevated temperature, and extreme acidic or alkaline pH. The present study was designed to overproduce the halophilic, thermoalkalophilic lipase of *Bacillus atrophaeus* FSHM2 through chemically induced random mutagenesis and optimization of cultural medium components assisted by statistical experimental design. At first, improvement of lipase production ability of *B. atrophaeus* FSHM2 was performed through exposure of the wild bacterial strain to ethidium bromide for 5–90 min to obtain a suitable mutant of lipase producer (designated as EB-5, 4301.1 U/l). Afterwards, Plackett–Burman experimental design augmented to D-optimal design was employed to optimize medium components (olive oil, maltose, glucose, sucrose, tryptone, urea, $(\text{NH}_4)_2\text{SO}_4$, NaCl, CaCl_2 , and ZnSO_4) for lipase production by the EB-5 mutant. A maximum lipase production of 14,824.3 U/l was predicted in the optimum medium containing 5% of olive oil, 0.5% of glucose, 0.5% of sucrose, 2% of maltose, 2.5 g/l of yeast extract, 1.75 g/l of urea, 1.75 g/l of $(\text{NH}_4)_2\text{SO}_4$, 2.5 g/l of tryptone, 2 g/l of NaCl, 1 g/l of CaCl_2 , and 1 g/l of ZnSO_4 . A mean value of $14,773 \pm 576.9$ U/l of lipase was acquired from real experiments.

Keywords Lipase · *Bacillus atrophaeus* · Ethidium bromide · Mutagenesis · Medium optimization

Introduction

Microbial derived enzymes have been widely applied as catalysts in biotechnological procedures due to their high activity under mild conditions, suitable stability, and high substrate specificity (Barbosa et al. 2015; Riyadi et al. 2017). Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are among the most useful and most studied enzymes in the biotechnological processes. Microbial lipases catalyze the hydrolysis of triacylglycerols to diacylglycerols, monoacylglycerols, free fatty acids (FFA), and glycerol (Das et al. 2016; Khoobi et al. 2014, 2016; Yao et al. 2013; Jaeger and Eggert 2002). Lipases possess a high level of enantio- and regioselectivity and substrate specificity which make them suitable candidates to catalyze the processes of esterification, interesterification, transesterification, acidolysis, and aminolysis (Adlercreutz 2013; Aravindan et al. 2007; Zhang et al. 2014). From the mechanistic point of view, most of lipases have a ‘lid’ domain, a polypeptide sequence nearby the active site, able to change lipase conformation from a ‘close’ to an ‘open’ (more active) form (Arana-Peña et al.

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2018; Manoel et al. 2015; Rios et al. 2018). It has been shown that immobilization of lipases on hydrophobic surfaces (designated as interfacial activation) lets the 'open' form of lipases to be more stable (Cantone et al. 2013; Hane-feld et al. 2013; Sheldon and Pelt 2013).

This valuable biocatalyst has been widely employed in food industries (improving the quality of dairy products and meat processing), detergent, pharmaceuticals, and cosmetic industries, as well as in the synthesis of fine chemicals, agrochemicals, biodiesel, and new polymeric materials (Darvishi et al. 2011; Khoobi et al. 2014; Li et al. 2018). In pharmaceutical industries, lipases have been also used for kinetic resolution of drug intermediates. Lipases have been applied as co-catalysts in the preparation of enantiomer compounds from racemates (Pinheiro et al. 2018; Zhang et al. 2019; Galvão et al. 2018).

Particularly, the specific lipases produce by extremophiles have been used in harsh industrial conditions, because most of them remain active at alkaline pH and in elevated temperature as well as in the presence of high salt and organic solvent concentrations (Samaei-Nouroozi et al. 2015; Yao et al. 2013). There are many reports on bacterial derived lipases categorized as extremophilic lipases. For example, the secreted solvent-tolerant lipase of *Burkholderia ambifaria* YCJ01 was applied for resolution of (*S*)-mandelic acid in the presence of diisopropyl ether (Yao et al. 2013). Yele and Desai (2015) isolated a thermoalkalophilic lipase from *Staphylococcus warneri* which maximally worked at pH of 8 and temperature of 55 °C. Maximum esterification of ethyl butyrate assisted by lipase of *Burkholderia multivorans* V2 occurred at *n*-hexane (Dandavate et al. 2009).

In the last few decades, the ever-increasing usage of lipases required the expansion in both qualitative improvement and quantitative enhancement (Bisht et al. 2012; Toscano et al. 2011). Quantitative enhancement could be achieved by strain improvement and medium optimization (Toscano et al. 2011). Various methods have been used to improve the lipase producing strains including site-directed mutagenesis and random mutagenesis (Bisht et al. 2012; Chang and Shaw 2009; Iftikhar et al. 2010). The classical random mutagenesis approach mainly involves exposing the desired microbe to the physical mutagens (such as X-rays, gamma rays, and UV irradiation), or chemical mutagens [like *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG), ethyl methanesulfonate (EMS), and ethidium bromide (EtBr)]. Devi et al. (2015) used chemical mutagens of EMS, EtBr, sodium azide, and NTG to enhance the production and lipolytic potential of *Pseudomonas* sp. This mutant represented a total increase in activity (4960 U/l) over its wild strain (3100 U/l) for the production of extracellular lipase (Devi et al. 2015). In another study, the strain improvement of *P. aeruginosa* MTCC 10055 was performed by chemical mutagenesis using the mutagen 4-nitroquinoline 1-oxide for

alkaline lipase production (Bisht et al. 2012). The improved strain was capable to secrete a high titer of alkaline lipase (1609 U/l) into the medium (Bisht et al. 2012). Darvishi et al. (2011) also applied the EMS to improve the productivity of lipase from *Yarrowia lipolytica*.

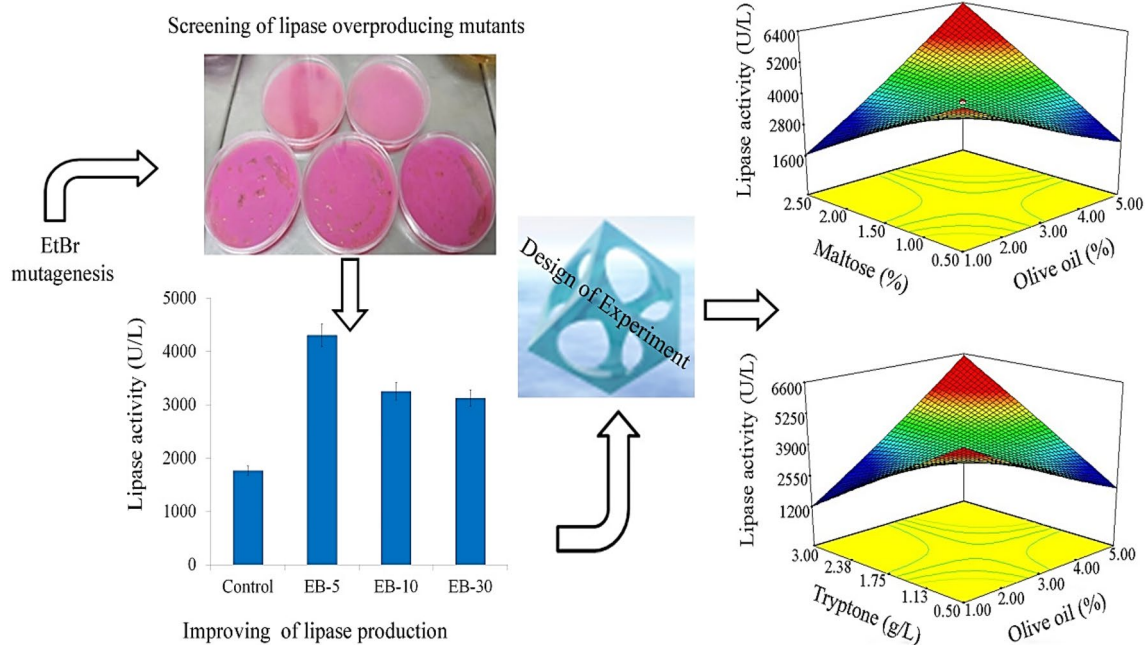
Investigation on the factors influencing the production of biomolecules is essential in many bioprocess development procedures (Forootanfar et al. 2015; Moshafi et al. 2011). Generally, a higher productivity could be achieved by culture medium optimization (Haider Ali et al. 2015; Hasan-Beikdashti et al. 2012; Riyadi et al. 2017). The statistical optimization of processes has advantages compared to the traditional method of varying one factor at a time (Colla et al. 2016; Faisal et al. 2014) such as the lower number of experiments and the possibility of evaluating the interaction effects between factors. Statistical experimental techniques such as two-level factorial experiments, Plackett–Burman design (PBD), and response surface methodology (RSM) are effective tools applied for optimization of lipase production by different microorganisms such as *P. aeruginosa*, *Candida rugosa*, *C. antarctica*, *B. cepacia*, *P. alcaligenes*, *Rhizopus* sp. and *Bacillus pumillus* (Liu and Zhang 2011; Lo et al. 2012; Riyadi et al. 2017; Vasiee et al. 2016; Verma et al. 2012).

The present study was aimed to develop a *B. atrophaeus* mutant strain with high ability for thermoalkalophilic lipase production using chemically induced mutagenesis. Furthermore, the main factors affecting lipase biosynthesis by the selected mutant were then screened and the related model was evaluated. On the other hand, this research tried to increase lipase production by a simple mutation method combined with medium optimization using a statistical experimental design (Scheme 1).

Materials and methods

Chemicals and bacterial strain

Brain heart infusion broth (BHIB), nutrient broth, tryptone, yeast extract, sucrose, glucose, rhodamine B, and agar were supplied by Merck Chemicals (Darmstadt, Germany). ρ -Nitrophenylpalmitate (ρ NPP), ρ -nitrophenol (ρ NP), and ethidium bromide (EtBr) were provided by Sigma-Aldrich (St. Louis, MO, USA). All applied chemicals were of analytical grade. The lipase-producing bacterial strain (*B. atrophaeus* FSHM2, NCBI accession number of KF682367) employed in the present study was isolated in a previous study from a hypersaline location (30°36'18"N, 59°04'04"E) in Kerman, Iran. The secreted lipase of this strain maximally worked at 70 °C, pH 9, and in the presence of 4 M NaCl (Ameri et al. 2017).



Scheme 1 Steps to get the maximum level of thermoalkalophilic lipase production via EtBr-induced mutagenesis of *Bacillus atrophaeus* FSHM2 followed by screening of overproducing mutant

EtBr mutagenesis

The chemical mutagenesis of *B. atrophaeus* FSHM2 cells was carried out by exposing to EtBr by a modified method of Raju and Divakar (2013). The overnight culture of the bacterial strain in BHIB was harvested by centrifugation at 10,000×g for 10 min, washed twice with phosphate buffer (0.1 M, pH 7.4) and suspended in the same buffer. Five ml of the cell suspension containing 1×10^8 cells/ml was mixed with 10 ml of EtBr solution (5 mg/ml) and incubated at 37 °C. One ml sample from this solution was taken at intervals of 5–90 min and centrifuged immediately at 10,000 rpm for 10 min. The obtained cells were then re-suspended in 2 ml phosphate buffer (0.1 M, pH 7.4). Preliminary screening of lipase-overproducing mutants was carried out on BHI agar medium containing rhodamine B (0.001%, w/v), olive oil (1%, v/v), and NaCl (15% w/v). The EtBr-treated cell suspension was diluted and spread on a series of pre-sterilized plates of the above-mentioned medium and incubated for 24–48 h. The bacterial colonies exhibiting an orange-colored fluorescent halos (under UV light, 350 nm) around their colonies were selected for further studies.

Screening of a potent lipase-overproducing mutant strain

The selected mutants of the previous step were grown in the basal medium composed of nutrient broth fortified by olive oil (1%,

(Et-5) and optimization of cultural medium compositions using statistical experimental design

v/v) to determine their ability for alkaline lipase production in shake flasks. Fermentation experiments were performed at 60 °C and 120 rpm in 500 ml flasks containing 100 ml of medium inoculated by 1% (v/v) of the seed culture. At the end of process, the culture media was centrifuged (10,000×g, 15 min) and the supernatant was used for determination of lipase activity.

Enzyme assay

Extracellular lipase activity was measured spectrophotometrically using p -NPP as substrate by the modified method of Hasan-Beikdashti et al. (2012). First, the related standard curve of p -NP (the product of lipase hydrolysis on p -NPP substrate) was drawn by plotting each absorbance of p -NP (at 410 nm) against its related concentration (performed in triplicates). The reaction mixture consisted of 0.8 ml of 0.1 M carbonate buffer (pH 9) supplemented by Triton X-100 (0.4%), 0.1 ml of the attained culture broth sample, and the freshly prepared p -NPP solution (0.01 M, 0.1 ml) was then prepared and incubated at 60 °C for 30 min under mild shaking. An amount of 0.25 ml Na_2CO_3 (0.1 M) was consequently added to the reaction mixture and the amount of released p -NP was determined by recording the absorbance at 410 nm assisted by a spectrophotometer (UV-1800, Shimadzu Co., Tokyo, Japan) using the line equation obtained from the above-mentioned standard curve. One unit of the lipolytic activity was then specified as the amount of the enzyme that catalyzed the formation of 1 μmol of p -NP per min from p -NPP substrate.

Table 1 Applied levels of independent variables in the experimental design for optimization of lipase production by EB-5 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

Experimental design

To optimize the medium components affecting the production of thermoalkalophilic lipase by the chemically induced mutant of *B. atrophaeus* FSHM2, PBD and RSM was employed. At the first step, the effect of eleven factors (Table 1, selected according to a preliminary literature reviews) on lipase productivity was determined and analyzed using PBD drawn by Design Expert software (6.0.4, Stat-Ease Inc., Minneapolis, MN, USA). Afterwards, the levels of the main components achieved by PBD were optimized by D-optimal approach of RSM. Coding of the factors was performed based on the following equation:

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

where x_i is the coded value of an independent variable, X_i is the real value of each independent variable, X_{i0} is the real value of each independent variable at the center point, and ΔX_i is the value of step change. To neutralize the influence of nuisance variables, the designed runs (49 experiments) were carried out randomly. The obtained results of Plackett–Burman experimental design were then analyzed and 24 new experiments (Table 2, run 16–39) were added to the previous design using augmentation of a central composite design (CCD). To improve the leverage, the applied design was completed by the addition of ten further experiments (Table 2, run 40–49). The following equation was used as the most appropriate model to correlate the chosen variables with the response.

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

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

Data analysis

As said above, the analysis of the obtained results was performed using the statistical software of Design Expert (6.0.4, Stat-Ease Inc., Minneapolis, MN, USA). The qualities of the fitted polynomial models were tested by the determination of coefficient R^2 . Analysis of variance (ANOVA) together with the F test was applied to evaluate the significance of a given term ($p \leq 0.05$). The location of the optimum was determined by solving the set of equations derived by the difference of the final quadratic model.

Results and discussion

Mutagenesis of lipase-producing bacterial strain

The lipase-producing bacterial strain, *B. atrophaeus* FSHM2, was subjected to chemical mutagenesis using EtBr. This method was repeated three times to isolate a lipase-overproducing mutant. The primary selection was performed considering the orange-colored fluorescent halos that appeared by lipolysis on the BHIA plate supplemented by rhodamine B (0.001%, w/v) (Fig. 1a). Among the five selected mutants attained in this step, only three mutants (EB-5, EB-10, and EB-30) represented significantly higher lipase secretion after cultivation in submerged culture compared to that of wild bacterial strain (Fig. 1b). In addition, it was observed that EB-5 (exposed to EtBr for 5 min at 37 °C) was the most efficient mutant able to produce 4301.1 U/l of lipase which was 2.4-fold of untreated control ($p < 0.05$). Devi et al. (2015) reported about the application of chemical mutagens such as EMS, EtBr, sodium azide, and NTG for the improvement of lipase production of *Pseudomonas* sp. VITSDVM1 among which EMS was introduced as the best mutagen that elevated the lipase production to 4960 U/l. The findings of the present study were also similar to the one described by Iftikhar et al. (2010). They applied chemical mutagenic treatments (using NTG, nitrous acid, and EtBr) to improve the lipolytic potential of the fungal strain *Rhizopus oligosporus* (Ifitikhar et al. 2010). The mutant derived from NTG treatment (IIB-63^{NTG-7}) represented the highest lipase activity (10,370 U/l) which was approximately

Table 2 Experimental design and results of the Plackett–Burman design augmented to D-optimal design for optimization of culture conditions for maximum lipase production of EB-5 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	3739.3	3727.9
2	5	2.5	2.5	0.5	0.5	0.5	3	0.5	5	5	1	3476	3485.4
3	3	1.5	1.5	1.5	1.75	1.75	1.75	1.75	3	3	3	3686.6	3727.9
4	5	2.5	0.5	0.5	0.5	3	0.5	3	5	1	5	3581.3	3591.1
5	3	1.5	1.5	1.5	1.75	1.75	1.75	1.75	3	3	3	3739.3	3727.9
6	1	0.5	2.5	0.5	3	3	0.5	3	5	5	1	3528.6	3537.3
7	1	0.5	0.5	2.5	0.5	3	3	0.5	5	5	5	3897.3	3906.2
8	1	2.5	2.5	0.5	3	3	3	0.5	1	1	5	3107.3	3116.1
9	1	2.5	2.5	2.5	0.5	0.5	0.5	3	1	5	5	3634	3642.3
10	1	2.5	0.5	2.5	3	0.5	3	3	5	1	1	4002.6	4011.4
11	5	0.5	0.5	0.5	3	0.5	3	3	1	5	5	3897.3	3906.5
12	5	0.5	2.5	2.5	0.5	3	3	3	1	1	1	3844.6	3854.1
13	5	0.5	2.5	2.5	3	0.5	0.5	0.5	5	1	5	3002	3010.9
14	5	2.5	0.5	2.5	3	3	0.5	0.5	1	5	1	3581.3	3590.4
15	1	0.5	0.5	0.5	0.5	0.5	0.5	0.5	1	1	1	3423.3	3432.1
16	3	1.5	1.5	1.5	1.75	1.75	1.75	4.03	3	3	3	3686.6	3669.6
17	3	1.5	1.5	1.5	1.75	1.75	4.03	1.75	3	3	3	3897.3	3882.9
18	3	1.5	0	1.5	1.75	1.75	1.75	1.75	3	3	3	3844.6	3814.8
19	3	1.5	1.5	1.5	1.75	1.75	1.75	1.75	3	3	6.64	2791.3	2777.3
20	3	1.5	1.5	1.5	1.75	1.75	1.75	1.75	3	6.64	3	3107.3	3094.1
21	3	1.5	1.5	3.32	1.75	1.75	1.75	1.75	3	3	3	3950	3933.8
22	3	3.32	1.5	1.5	1.75	1.75	1.75	1.75	3	3	3	3897.3	3880.1
23	3	1.5	1.5	0	1.75	1.75	1.75	1.75	3	3	3	3265.3	3353.7
24	6.64	1.5	1.5	1.5	1.75	1.75	1.75	1.75	3	3	3	5056	5038.6
25	3	1.5	3.32	1.5	1.75	1.75	1.75	1.75	3	3	3	3634	3622.4
26	3	1.5	1.5	1.5	0	1.75	1.75	1.75	3	3	3	3423.3	3405.3
27	3	1.5	1.5	1.5	1.75	1.75	0	1.75	3	3	3	2738.6	2739.1
28	3	1.5	1.5	1.5	1.75	1.75	1.75	1.75	0	3	3	3054.6	3039.4
29	3	1.5	1.5	1.5	1.75	1.75	1.75	0	3	3	3	3581.3	3566.5
30	3	1.5	1.5	1.5	1.75	1.75	1.75	1.75	3	3	0	4055.3	4040.2
31	0	1.5	1.5	1.5	1.75	1.75	1.75	1.75	3	3	3	3423.3	3408.4
32	3	0	1.5	1.5	1.75	1.75	1.75	1.75	3	3	3	5161.3	5146.3
33	3	1.5	1.5	1.5	1.75	1.75	1.75	1.75	3	3	3	3686.6	3727.9
34	3	1.5	1.5	1.5	4.03	1.75	1.75	1.75	3	3	3	3844.6	3830.1
35	3	1.5	1.5	1.5	1.75	1.75	1.75	1.75	3	3	3	3739.3	3727.9
36	3	1.5	1.5	1.5	1.75	0	1.75	1.75	3	3	3	3792	3819.1
37	3	1.5	1.5	1.5	1.75	4.03	1.75	1.75	3	3	3	3886.6	3896.1
38	3	1.5	1.5	1.5	1.75	1.75	1.75	1.75	6.64	3	3	3739.3	3726.2
39	3	1.5	1.5	1.5	1.75	1.75	1.75	1.75	3	0	3	3792	3775.7
40	2.83	0.99	1.69	1.44	1.85	1.74	2.7	1.18	2.83	4.24	3.69	3528.6	3536.6
41	2.11	1.55	2.05	1.63	1.33	1.47	2.22	2.04	2.84	2.92	2.09	3686.6	3706.6
42	2.93	1.83	1.35	2.35	2.34	1.86	1.57	1.26	2.93	1.35	2.57	3581.3	3594.3
43	2.32	1.9	1.12	1.35	1.05	1.77	1.57	2.27	3.28	2.75	3.45	3634	3654.7
44	2.77	2.01	1.04	1.6	1.67	1.88	1.64	1.44	4.28	1.41	3.27	3792	3779.5
45	1.83	1.59	1.36	1.44	1.28	1.7	2.24	1.78	1.73	2.98	3.34	4160.6	4153.8
46	2.61	1.24	1.51	2.26	2.31	1.12	1.83	2.11	3.46	3.07	1.39	3581.3	3586.6
47	3	1.5	1.5	1.5	1.75	1.75	1.75	1.75	3	3	3	3739	3727.9
48	3	1.5	1.5	1.5	1.75	1.75	1.75	1.75	3	3	3	3739.3	3727.9
49	3	1.5	1.5	1.5	1.75	1.75	1.75	1.75	3	3	3	3686.6	3727.9

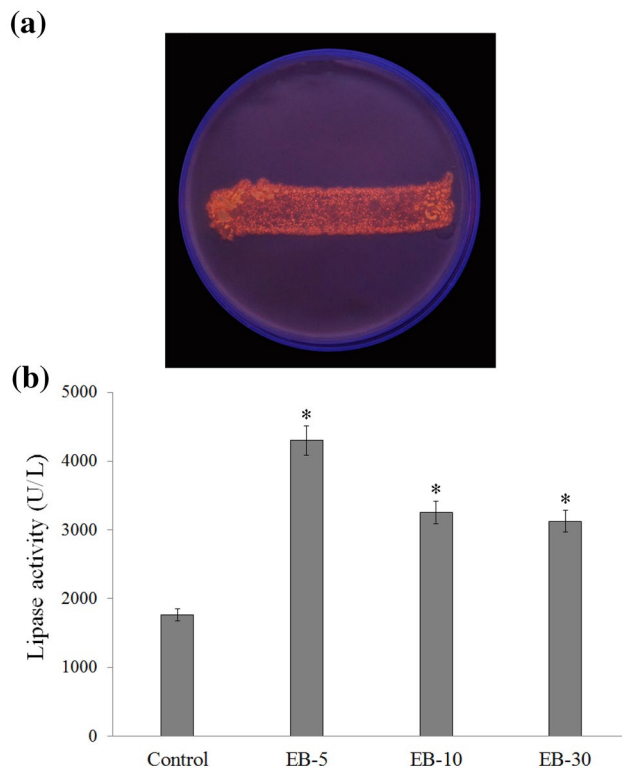


Fig. 1 **a** Positive colony of lipase-producing mutant on BHIA plate containing rhodamine B (0.001%) after exposure to UV light. **b** Screening the best mutant of *B. atrophaeus* after different exposure times to EtBr compared with the wild strain of *B. atrophaeus* (as a positive control). Significance (*) was evaluated after ANOVA analysis of the obtained results (p value < 0.05)

threefold higher compared to that of the wild strain (3200 U/l). IIB-63^{EtBr-7} mutant was also found to be the producer of extracellular lipase with the activity of 6990 U/l which was nearly twofold higher than its untreated strain (Iftikhar et al. 2010).

Medium optimization

The obtained results of the first step of medium components optimization performed by PBD augmented to D-optimal design showed variation of lipase productivity in the range of 2738.7–5161.3 U/l (Table 2). Table 3 represented the statistical analysis of the obtained results among which olive oil, maltose, tryptone, yeast extract, $(\text{NH}_4)_2\text{SO}_4$, and NaCl were found to significantly enhance the lipase productivity of EB-5 mutant ($p < 0.05$), while sucrose, glucose, CaCl_2 , and ZnSO_4 exhibited a significantly negative effect ($p < 0.05$).

According to the attained D-optimal results, a second-order polynomial expression was fitted. The following quadratic model (in the terms of coded values) was then defined by the software for estimating lipase production of the EB-5 mutant.

$$\begin{aligned} \text{Lipase production} = & +3727.90 + 447.87X_1 - 347.86X_2 \\ & - 57.96X_3 + 187.88X_4 + 116.42X_5 \\ & + 318.69X_7 + 28.29X_8 + 188.70X_9 - 187.27X_{10} \\ & - 346.94X_{11} + 81.75X_1X_2 + 726.06X_1X_3 \\ & + 1925.62X_1X_4 + 1928.30X_1X_6 - 3772.39X_1X_7 \\ & + 2207.87X_1X_8 - 1360.31X_1X_9 - 714.41X_1X_{10} \\ & - 2224.58X_1X_{11} + 2142.21X_2X_3 + 2412.88X_1X_4 \\ & - 4560.15X_2X_5 - 1022.32X_1X_6 + 1730.70X_2X_7 \\ & - 332.16X_2X_8 + 1020.46X_2X_9 + 149.63X_1^2 \\ & + 237.09X_2^2 - 41.04X_4^2 - 33.12X_5^2 - 128.13X_7^2 \\ & - 33.00X_8^2 - 104.17X_9^2 - 88.44X_{10}^2 - 96.34X_{11}^2. \end{aligned}$$

According to the significance of the p values (< 0.05) and non-significance of the lack of fitness, it was implied that the experimental data was well fitted with the selected model. In addition, the amount of squared correlation coefficient (R^2 , 0.9989) showed that the model was able to explain 99.89% of the variation in the response. Furthermore, the proximity of the predicted and adjusted R -squared values (0.9494 and 0.9953, respectively) implied that a better fitness could be achieved. The amount of variation coefficient (C.V.%) was 0.81. All of the above-mentioned values together with the sufficient precision of the model (an indicator of the signal to noise ratio) which enhanced to 92.652 suggested that equation of the selected model was adequate to explain the response of the experimental observations related to the lipase production and also navigate the design space.

Figure 2a represented the predicted versus observed data from which the validity of the model and agreement between predicted and observed values was completely obvious. Furthermore, the Box-Cox graph of the natural logarithm (Ln) of the residual SS (sum of square) against lambda (Fig. 2b) showed a minimum value of 0.92 which was in the range of minimum (0.3) and maximum (1.51) confidence interval value (Fig. 2b). The data did not require a transformation, as the current value of confidence interval (lambda) was very close to the optimum value. Comparison of the attained experimental results and the predicted values summarized in Table 2 showed good accordance between the predicted and observed data.

Using the selected model, the optimum levels of the applied factors for maximum lipase production of EB-5 mutant were predicted to be 0.5% of glucose, 5% of olive oil, 2% of maltose, 0.5% of sucrose, 2.5 g/l of yeast extract, 2.5 g/l of tryptone, 1.75 g/l of urea, 1.75 g/l of $(\text{NH}_4)_2\text{SO}_4$, 1 g/l of CaCl_2 , 2 g/l of NaCl, and 1 g/l of ZnSO_4 .

To validate the selected model, the suggested optimum medium composition was applied for lipase production

Table 3 Analysis of variance for D-optimal refined model to optimize the medium compositions in relation to lipase activity (U/l) by EB-5 mutant

Source	Sum of squares	df	Mean square	F value	p value Prob > F	
Block	99,247	2	49,623			
Model	8,555,000.0	35	244,400.0	276.6	< 0.0001	Significant
X_1	1,381,000.0	1	1,381,000.0	1563.4	< 0.0001	
X_2	819,000.0	1	819,000.0	927.0	< 0.0001	
X_3	22,317.3	1	22,317.3	25.3	0.0004	
X_4	238,600.0	1	238,600.0	270.1	< 0.0001	
X_5	90,993.3	1	90,993.3	103.0	< 0.0001	
X_7	677,600.0	1	677,600.0	767.0	< 0.0001	
X_8	5344.1	1	5344.1	6.1	0.0317	
X_9	240,500.0	1	240,500.0	272.3	< 0.0001	
X_{10}	241,700.0	1	241,700.0	273.5	< 0.0001	
X_{11}	804,500.0	1	804,500.0	910.6	< 0.0001	
X_1X_2	30,763.4	1	30,763.4	34.8	0.0001	
X_1X_3	91,747.3	1	91,747.3	103.8	< 0.0001	
X_1X_4	285,100.0	1	285,100.0	322.7	< 0.0001	
X_1X_6	368,100.0	1	368,100.0	416.7	< 0.0001	
X_1X_7	598,500.0	1	598,500.0	677.4	< 0.0001	
X_1X_8	340,900.0	1	340,900.0	385.9	< 0.0001	
X_1X_9	150,400.0	1	150,400.0	170.2	< 0.0001	
X_1X_{10}	17,953.3	1	17,953.3	20.3	0.0009	
X_1X_{11}	308,400.0	1	308,400.0	349.1	< 0.0001	
X_2X_3	188,800.0	1	188,800.0	213.7	< 0.0001	
X_2X_4	806,400.0	1	806,400.0	912.8	< 0.0001	
X_2X_5	722,700.0	1	722,700.0	818.0	< 0.0001	
X_2X_6	71,101.2	1	71,101.2	80.5	< 0.0001	
X_2X_7	191,600.0	1	191,600.0	216.9	< 0.0001	
X_2X_8	22,171.5	1	22,171.5	25.1	0.0004	
X_2X_9	36,029.9	1	36,029.9	40.8	< 0.0001	
X_1^2	503,200.0	1	503,200.0	569.6	< 0.0001	
X_2^2	1,264,000.0	1	1,264,000.0	1431.2	< 0.0001	
X_4^2	38,017.1	1	38,017.1	43.0	< 0.0001	
X_5^2	24,724.7	1	24,724.7	28.0	0.0003	
X_7^2	369,100.0	1	369,100.0	417.8	< 0.0001	
X_8^2	24,487.4	1	24,487.4	27.7	0.0003	
X_9^2	244,800.0	1	244,800.0	277.1	< 0.0001	
X_{10}^2	176,600.0	1	176,600.0	199.9	< 0.0001	
X_{11}^2	209,100.0	1	209,100.0	236.7	< 0.0001	
Residual	9718.7	11	883.5			
Lack of fit	4633.4	6	772.2	0.8	0.63	Not significant
Pure error	5085.3	5	1017.1			
Cor total	8,663,000.0	48				

$$R^2=0.9989; R^2_{Adj}=0.9494; R^2_{Pred}=0.9953; \text{adeq precision}=92.652$$

(five times). The mean lipase activity of these five replicates was measured to be $14,773 \pm 576.9$ U/l which was in the confidence interval range indicated by the software and also showed appropriate agreement with the predicted results (14,824.3 U/l). Compared to that of the wild

bacterial strain, the lipase productivity of the EB-5 mutant was increased by 6.5-fold (Fig. 3).

Figure 4 illustrated the 3D plots of lipase activity against two studied variables (in their related range), while others kept constant (in center point). Figure 4a

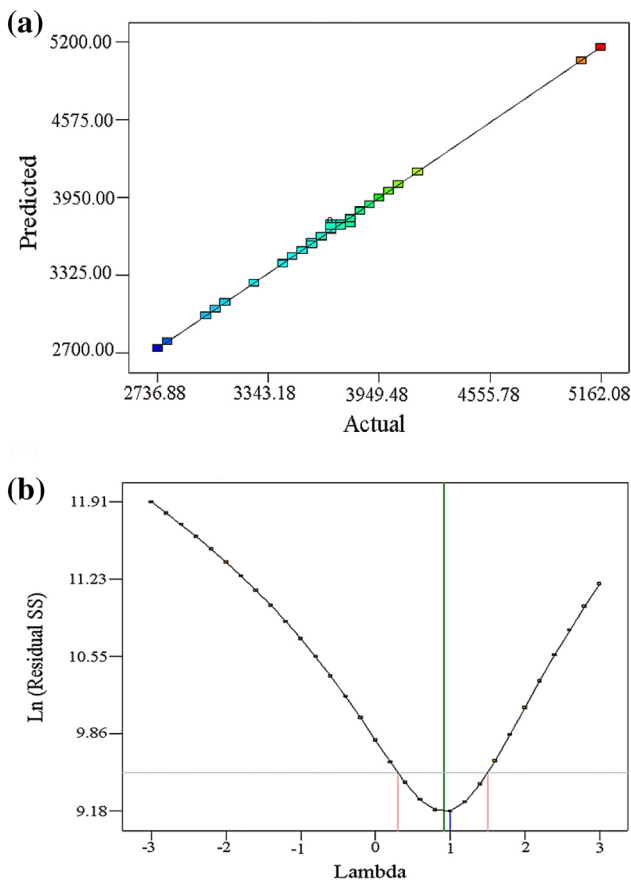


Fig. 2 **a** Studentized residuals vs. predicted response by the final quadratic model, **b** Box-Cox plot for power transformation of lipase production by mutant of *B. atrophaeus* FSHM2

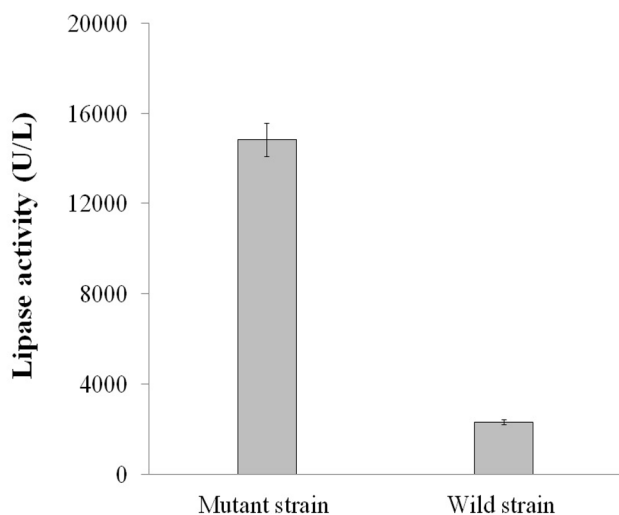


Fig. 3 Overproduction of lipase by the mutant EB-5 under optimized conditions including 5% of olive oil, 0.5% of glucose, 0.5% of sucrose, 2% of maltose, 2.5 g/l of yeast extract, 1.75 g/l of urea, 1.75 g/l of $(\text{NH}_4)_2\text{SO}_4$, 2.5 g/l of tryptone, 2 g/l of NaCl, 1 g/l of CaCl_2 , and 1 g/l of ZnSO_4

represented the response surface for olive oil (-1 : 1%, $+1$: 5; %) and maltose (-1 : 0.5%, $+1$: 2.5; %), while other variables kept constant (their center points). At a high level of olive oil (5%) and a high level of maltose (2.5%), maximum lipase activity (6384.7 U/l) was observed. When olive oil and maltose were at center points, the lipolytic activity considerably decreased to 3727.9 U/l ($p < 0.05$). Liu et al. (2011) reported the significant influence of olive oil (0.5–5.14 mM) as a lipase inducer by *Burkholderia* sp. In addition, Samaei-Nouroozi et al. (2015) found that the maximum lipase production (856.9 U/l) by *Alkalibacillus salilacus* was obtained mainly in the presence of a high concentration of olive oil (2%). Previously, statistical experimental design showed a significant positive effect of olive oil on lipase production by *C. cylindracea* (Salihi et al. 2011). However, Hasan-Beikdashti et al. (2012) reported the negative effect of olive oil on extracellular lipase production by *Stenotrophomonas maltophilia*.

As presented in Fig. 4a, the greatest increase in lipase production by EB-5 mutant (6400 U/l) was observed at a high level of maltose (2.5%). Mahanta et al. (2008) reported higher lipase activity (976 U/g) in the solid-state fermentation (*Jatropha curcas* seed cake as substrate) of *P. aeruginosa* PseA supplemented with maltose as the carbon source. Yele and Desai (2015) who optimized the fermentation media for overproduction of thermostable and organic solvent-tolerant lipase by *Staphylococcus warneri* reported the significant effect of adding maltose (0.054%) on lipase production. In the present study, it seems that a high level (2.5%) of glucose and sucrose exhibited a negative effect on lipase productivity of EB-5 mutant (1545 and 2128 U/l, respectively), while insertion of maltose at a high level (2.5%) lead to higher lipase production (6138 U/l).

The 3D plot of olive oil and urea effects on lipase production by EB-5 mutant was illustrated in Fig. 4b from which it was revealed that a higher lipase activity (6231.4 U/l) was observed at a high level of urea (3 g/l) and olive oil (5%) ($p < 0.05$). Sangeetha et al. (2008) reported about the significant influence of urea on extracellular lipase production of *B. pumilus* SG 2. Furthermore, Bora and Bora (2012) reported that various inorganic nitrogen sources including ammonium sulfate and urea enhanced lipase production by *Bacillus* sp. LBN2 in the range of 10,000–17,000 U/l. On the contrary, Veerapagu et al. (2013) showed that urea had a significantly inhibitory effect on the lipase production by *Pseudomonas gessardii*.

As shown in Fig. 4c, the lipolytic activity of EB-5 mutant culture broth was influenced by olive oil and tryptone. The maximum lipase activity (6503.2 U/l) achieved when tryptone was at its highest level (3 g/l). Therefore, a positive

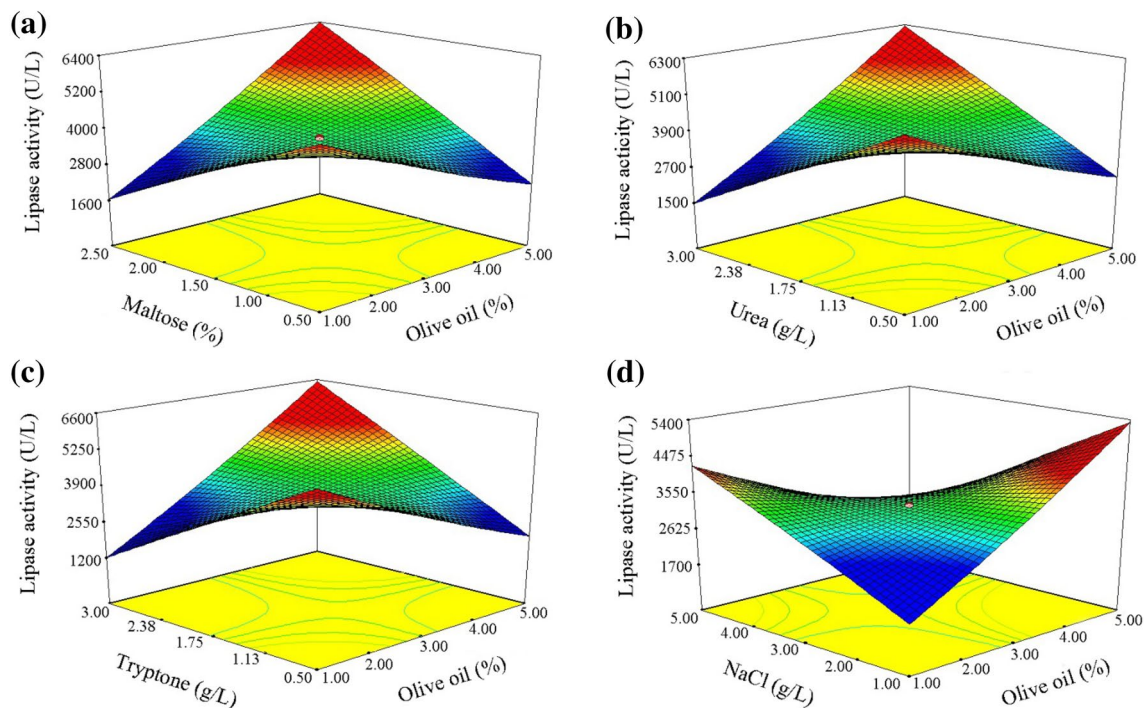


Fig. 4 Response surface plots of the impact of various factors on lipase production of the EB-5 mutant. **a** Olive oil and maltose, **b** olive oil and urea, **c** olive oil and tryptone, and **d** olive oil and NaCl. Other factors are kept at a constant level (their center points)

effect of tryptone concentration influenced the lipase activity of mutant EB-5 ($p < 0.05$). As obtained in the present study, organic nitrogen sources (yeast extract and tryptone) and inorganic nitrogen sources (urea and ammonium sulfate) exhibited a positive significant effect on the lipase production of EB-5 mutant ($p < 0.05$). Kumar et al. (2011) reported the negative effect of yeast extract and peptone on the lipolytic activity of *B. pumilus* RK31 in primary screening using Plackett–Burman statistical design.

Haider Ali et al. (2015) reported the significant negative effect of NaCl on lipase production by *Pseudomonas aeruginosa* FW_SH-1. However, Sathishkumar et al. (2015) reported the positive effect of NaCl on lipase production by *Halobacillus trueperi* RSK CAS9. Several studies reported the stimulatory effects of different ions on lipase production by different microorganisms (Riyadi et al. 2017; Verma et al. 2012). For example, Verma et al. (2012) reported that the Mg^{2+} ions have significant influence on lipase production by *P. aeruginosa* ($p < 0.05$). Yele and Desai (2015) reported the enhancement in the lipase productivity of *S. warneri* in the presence of K_2HPO_4 (0.091%). Figure 4d illustrated the effect of olive oil and NaCl concentrations on lipase production by EB-5 mutant. The maximum lipase activity (5376.4 U/l) was achieved when NaCl was at the lowest level (1 g/l, $p < 0.05$).

Conclusion

The present research aimed to develop a mutant strain of *B. atrophaeus* FSHM2 with high ability for thermoalkalophilic lipase biosynthesis using a chemical mutagenesis method followed by the application of statistical methods to optimize culture medium components required for lipase production by the best mutant. Among the total variables, olive oil, maltose, yeast extract, tryptone, $(NH_4)_2SO_4$, and NaCl displayed positive significant effects ($p < 0.05$) on lipase production by the most efficient mutant (EB-5), while glucose, sucrose, $CaCl_2$, and $ZnSO_4$ had a negative effect ($p < 0.05$). Finally, the best optimum medium composition for maximum production of lipase ($14,773 \pm 576.9$ U/l) by EB-5 mutant was determined as follows: 5% of olive oil, 0.5% of glucose, 0.5% of sucrose, 2% of maltose, 2.5 g/l of yeast extract, 1.75 g/l of urea, 1.75 g/l of $(NH_4)_2SO_4$, 2.5 g/l of tryptone, 2 g/l of NaCl, 1 g/l of $CaCl_2$, and 1 g/l of $ZnSO_4$. Overproduction of thermoalkalophilic lipase by EB-5 mutant of *B. atrophaeus* FSHM2 was achieved using simple mutagenesis and medium optimization methods. However, further studies must be performed to determine the probable mechanism of mutation and potential application of this strain for large-scale production of lipase.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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