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Enhancement of Halophilic Lipase Production by *Virgibacillus alimentarius* LBU20907 using a Statistical Approach and Scale-Up in a Fermenter

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Abstract

Virgibacillus alimentarius LBU20907 is an efficient extracellular halophilic lipase producer. Isolate LBU20907 was identified by 16S rRNA gene sequence analysis and phenotypic identification before determining the optimal nutritional and cultural conditions for its halophilic lipase activity. Two statistical designs were used to predict the responses to varying factors: Plackett-Burman experimental design (PBD) and central composite design (CCD). The important factors affecting the halophilic lipase production were identified by 2-level PBD of 11 physicochemical parameters screened, 3 factors produced significantly positive effects. These were olive oil, $CaCl_2$, and temperature. The response surface methodology (RSM) was applied to the CCD to predict the most productive levels of these 3 factors. The results predicted that a maximum halophilic lipase activity of 65.96 U mL^{-1} should be achieved with an olive oil concentration of 1.68 %, a $CaCl_2$ concentration of 0.14 g L^{-1} and a temperature of 39.5 °C. The predicted halophilic lipase activity in the optimized medium represented an increase of nearly 42 % compared with the predicted result of 46.50 U mL^{-1} in the non-optimized medium. The model was validated by subsequent experimentation at the optimized conditions. The predicted values were in agreement with the experimental values with a coefficient of determination (R^2) of 0.9995. After scale-up using a 3 L laboratory fermenter, a halophilic lipase yield of 82.50 U mL^{-1} was effectively achieved: 77 % more than predicted to occur in the non-optimized medium. Therefore, halophilic lipase production by *V. alimentarius* LBU20907 can be regarded as promising attractive catalysis for several industrial applications.

Keywords: Central composite design, halophilic lipase, Plackett-Burman design, response surface, methodology, *Virgibacillus alimentarius*

Introduction

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are extensive enzymes that belong to the class of hydrolases and are involved in catalyzing the hydrolysis of triacylglycerols to glycerol, diacylglycerols, monoacylglycerols and fatty acids [1,2]. Lipolytic reactions occur at the oil-water interface and do not induce hydrolysis of substrates dissolved in the bulk fluids [2]. Some lipases are also able to catalyze esterification and transesterification reactions [3]. They are produced and isolated in various plants, animals, and microorganisms. Several microorganisms, such as *Candida rugosa*, *Candida antarctica*, *Burkholderia cepacia*, *Pseudomonas alcaligenes* and *Halomonas* sp. can produce lipase efficiently and their lipases are commercially available [4]. Nowadays there is an increasing interest in the study of lipases, mainly due to their potential applications in detergents, food processing, paper production, dairy

products, agrochemicals, cosmetics, pharmaceuticals, chemical processing and the biodegradation of fatty acid-containing waste [2]. These enzymes also catalyze the synthesis and transesterification of glycerides. Free fatty acids, particularly low molecular weight volatile fatty acids (VFA), are associated with aroma and flavor in food products, and, therefore, lipase has been applied to develop flavor and aroma in bakery products, beverages, cheese ripening, sausages, yogurt, meat and fish products [2,5]. However, since most industrial processes are typically performed under extreme operating conditions, it is of great importance to identify the types of enzymes which retain their optimal activities at various pH values, temperatures and salt concentrations [6]. As researchers search for additional enzymes to improve existing biotechnological applications or design entirely new ones, halophilic organisms and the enzymes they produce are gaining attention. Interestingly, researchers are turning to Archaea as sources of halophilic enzymes [7]. Since halophilic enzymes adapt well to harsh conditions and are unusually tolerant, they could serve as suitable candidates for different industrial processes. Among the various enzymes of halophilic origin, the lipolytic enzymes have potential applications in a variety of biotechnological fields [8]. Halophilic enzymes are active and stable in hypersaline environments or in media with low water activity because, even in these conditions, enough water is present for them to retain a suitable charge distribution at the active site, maintaining the conformation of the enzyme [9,10]. Therefore, their usefulness for biotechnological applications stems from their ability to remain active in the presence of low water conditions. These adaptations would prove useful in harsh industrial process environments where enzymatic reactions may be carried out in an organic solvent [11].

The classical technique used to determine the optimization of biotechnological processes is one factor at a time (OFAT), but it is not as scientific as the response surface methodology (RSM) [12]. The OFAT technique used for optimizing a multivariable system is not only time consuming but also often misses possible alternative effects between components [13]. Although this strategy is simple and easy to apply without the need for statistical analysis, it involves a relatively large number of experiments and the interaction among factors is often ignored [14]. Therefore, the OFAT method is less effective than a statistical screening design and can provide the wrong results where there are strong interactions among the factors. Hence, researchers seek the aid of statistical tools such as RSM for effective optimization of their production media [15].

RSM is a powerful compilation of statistical approaches that is extensively determined and applied to the empirical modeling system, to develop, improve, and optimize complex processes, by examining the relationships between one or more response parameters and a set of experimental input parameters [16]. By varying several different parameters simultaneously, this widely-used technique requires only a limited number of experiments to determine the factors that influence the enzyme activity [17]. Statistical experimental designs such as the Plackett-Burman design (PBD) and central composite design (CCD) have been successfully applied to optimize many biological systems [18,19]. PBD is usually used as the first step in identifying the important factors among many potential factors [20]. It is a screening technique used to examine the effects of several variables in one experiment and avoid multiple runs of the same basic test. This method allows checking of the main effect of various compounds. The CCD is widely used as the second step and determines the optimal levels for the most significant factors and the interactions of variables. Therefore, using RSM resulted in higher production yields and is recently used for optimization studies in several industrial and biotechnological processes. In this study, a halotolerant strain showing halophilic lipase activity was isolated and identified *via* morphological, physiological, and genetic tests as *Virgibacillus alimentarius* LBU20907. Statistical approaches were used to optimize the key nutritional and physicochemical variables that enhance halophilic lipase production of the isolate. Hence, this study is the first report on the optimization of halophilic lipase production by *V. alimentarius* LBU20907. Finally, the predicted optimal conditions were then applied to scale up the halophilic lipase production in the laboratory fermenter.

Materials and methods

Chemicals

All chemicals, solvents, and medium components used in this study were of analytical grade. Olive oil and tween 80 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Casamino acid was purchased from Difco Laboratories (Becton Dickinson, Sparks, MD USA). Yeast extract and potassium chloride (KCl) were procured from Labscan (Bangkok, Thailand). Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) and iron(II) chloride 4-hydrate ($FeCl_2 \cdot 4H_2O$) were obtained from Ajax Finechem (Taren Point, NSW, Australia). Sodium chloride (NaCl), sodium hydroxide (NaOH) and hydrochloric acid (HCl) were purchased from Merck (Darmstadt, Germany). The primers used to identify bacteria were purchased from Pacific Science Co., Ltd. (Bangkok, Thailand).

Microorganisms and cultural conditions

The isolate LBU20907 used in the present study was screened from samples of laboratory prepared Budu (a traditional salt-fermented fish sauce popular in Southern Thailand and Malaysia) and identified through phenotypic characteristics and 16S rRNA sequencing. The culture was grown on Sehgal and Gibbons complex (SGC) agar comprising the following constituents (g/L): casamino acid 7.5, yeast extract 10, KCl 2, tri-sodium citrate 3, $MgSO_4 \cdot 7H_2O$ 20, $FeCl_2 \cdot 4H_2O$ 0.01, NaCl 200, agar 15 in 1000 mL distilled water pH 6.8 - 7.0 [21].

Screening of halophilic lipase production

In the primary screening process, the culture was tested on SGC agar supplemented with 2 % (v/v) tween 80 using different salt concentrations in the agar on the basis of the typical values reported for classifying halophiles: 0, 5, 10, 15, 20, 25 and 30 % (w/v). The pH was adjusted to 6.8 - 7.0 and the plates were incubated at 37 °C for 72 h. We defined a positive reaction for the lipase test as an opaque lipolytic zone around the colony: the width of the opaque zone was considered to be directly related to the amount of extracellular lipase produced. Selection of the isolate was based on the ratio of enzyme production on SGC-tween 80 agar plates and we selected the isolates that showed the highest lipase activity on SGC-tween 80 agar containing high salt concentrations (15 - 25 %, w/v). The cultures were maintained at 20 % (v/v) glycerol at –20 °C for further investigation.

Secondary screening included the study of growth and enzyme in 250 mL Erlenmeyer flasks containing 50 mL SGC liquid medium supplemented with 1 % (v/v) olive oil. We prepared an inoculum by transferring one loop of the selected strain into 9 mL SGC seed medium (pH 6.8 - 7.0) containing an optimal NaCl concentration and it was incubated on a rotary shaker (200 rpm) at 37 °C for 5 - 7 days. The inoculation was performed with 1 % (v/v) seed culture that had been grown for 3 days in the above medium. The inoculated medium was also incubated on a rotary shaker (200 rpm) at 37 °C for 5 - 7 days. Culture samples (5 mL) were aseptically withdrawn at 12 h intervals and centrifuged at 8,000×g and 4 °C for 20 min. We used the cell-free supernatants as crude enzyme solutions for lipase activity assays [22]. The harvested cells were washed twice with a NaCl solution and were used for measuring growth. Growth was measured turbidically at 600 nm using a spectrophotometer (UV-1800 Shimadzu, Japan). The strain which showed the most promising result was selected for further experiments.

Bacterial strain identification

The morphological and physiological characterization of the selected isolate was performed according to the standard method [23,24]. Acid production from carbohydrates was determined using the method described by Leifson [25]. For 16s rRNA analysis, we isolated the bacterial genomic DNA using the method of Hosek *et al*. [26], then PCR amplified the highly purified DNA in a thermocycler under the following conditions: 35 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min. We used the universal primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGYTACCTTGTTACG ACTT-3′) [27,28]. The 16S rRNA sequence analysis of the selected isolate was performed using an automatic DNA sequencer and the output was compared, using the BLAST program, with other bacterial 16S rRNA sequences deposited in the GenBank database provided by the National Center for

Biotechnology Information (NCBI). All sequences were aligned using the Clustal W program [29]. A phylogenetic tree and a UPGMA phylogeny were constructed using the MEGA software package version 4.0, and bootstrapping was used to estimate the reliability of the phylogenetic reconstructions (1000 replications).

Halophilic lipase production in shaken flasks

An inoculum was prepared as described above. Twelve experimental runs were performed in 250 mL Erlenmeyer flasks, where the medium components were added to the flasks at various concentrations based on the PBD. Inoculation was performed using various concentrations of 1 - 5 % (v/v) seed culture, which had been grown for 72 h in the medium described above. The inoculated media were incubated on a rotary shaker (200 rpm) at various initial pH values (6.0 - 8.0) and temperature (37 - 42 °C) levels for 72 h. Next, 5 mL culture samples were removed aseptically after 72 h and centrifuged at 8,000×g and 4 °C for 20 min. The culture supernatants were used as crude enzyme solutions in the lipase activity assays.

Determination of halophilic lipase activity

The lipase activity was measured using a modified version of the method described by Yamada *et al*. [22]. Olive oil was used as a substrate, treated with 2 % polyvinyl alcohol and distilled water. The resulting olive oil emulsion (5 mL) was hydrolyzed with a solution that contained 2 mL of enzyme solution and 3 mL of 0.2 M phosphate buffer (pH 7.0). Hydrolysis occurred for 1 h at 37 °C and at 200 rpm and we stopped the reaction by adding 20 mL of a mixed solution that contained acetone and 95 % ethanol at a ratio of 1:1. The volume of liberated oleic acid was calculated by titration with 0.05 N NaOH. One unit of lipase activity was calculated as the amount of enzyme required to release 1 μmol of oleic acid per mL per h of reaction.

Experimental design and data analysis

Identification of important variables by Plackett-Burman Experimental Design

We assessed the effects of eleven factors on halophilic lipase production. These factors were olive oil, tween 80, yeast extract, casamino acid, KCl, $MgSO_4$, NaCl, $CaCl_2$, temperature, pH, and inoculum size. The PBD was applied for screening and evaluating these significant variables. These components were studied and varied by concentration in the modified SGC medium at 2 different levels. The minimum (−1) and maximum (+1) ranges of variables were designated as the parameters (**Table 1**). The concentration levels were based on previous reports in the literature of lipase production using different substrates [20]. The statistical software package Design-Expert 10.0 (Stat-Ease Corporation, USA) was used to construct and analyze a set of 12 experimental runs in 250 mL Erlenmeyer flasks according to the design matrix shown in **Table 2**. The analysis of the data obtained from PBD was performed by following the first-order polynomial model equation;

$$Y = \beta_0 + \sum \beta_i X_i \quad (1)$$

where Y represents the predicted response (halophilic lipase activity), X_i is the level of the independent variable, and β_0 and β_i are the model intercept and the linear coefficient, respectively. The model does not explain the interactions among factors. It is used to identify and estimate the major factors affecting the response.

All the experimental runs were carried out in triplicate and the averages were considered to be the responses at 95 % confidence level. For each experimental run, the halophilic lipase activity was calculated in terms of U mL^{-1}. From the regression analysis, the factors showing confidence levels of more than 95 % ($P < 0.05$) were considered to have a significant effect on halophilic lipase production. Three factors met this criterion and were therefore selected for further optimization by CCD.

Table 1 Range of various variables studied in Plackett-Burman design.

Symbol coded	Variables	Experimental values	
		Low actual (-)	High actual (+)
A	Olive oil (%, v/v)	1.0	2.0
B	Tween 80 (%, v/v)	1.0	2.0
C	Yeast extract (g L^{-1})	5.0	10.0
D	Casamino acid (g L^{-1})	3.25	7.5
E	KCl (g/L)	1.0	2.0
F	$MgSO_4.7H_2O$ (g L^{-1})	10.0	20.0
G	NaCl (%, w/v)	10.0	20.0
H	$CaCl_2$ (g/L)	0.05	0.2
I	Temperature (°C)	37.0	42.0
J	pH	6.0	8.0
K	Inoculum size (%, v/v)	1.0	5.0

Table 2 The Plackett-Burman design matrix set up for the screening of 11 variables with coded values for halophilic lipase production by *V. alimentarius* LBU20907 and the response of the design.

Trials	Coded variable label											Halophilic lipase activity (U mL^{-1})	
	A	B	C	D	E	F	G	H	I	J	K	Experimental[a]	Predicted
1	−	−	+	+	+	−	+	+	−	+	−	35.00	33.92
2	−	+	+	+	−	+	+	−	+	−	−	33.00	33.08
3	−	−	−	−	−	−	−	−	−	−	−	35.00	37.50
4	+	−	−	−	+	+	+	−	+	+	−	43.00	43.50
5	−	+	+	−	+	−	−	−	+	+	+	34.00	33.08
6	−	−	−	+	+	+	−	+	+	−	+	30.00	29.50
7	+	−	+	−	−	−	+	+	+	−	+	40.00	39.92
8	+	+	−	+	−	−	−	+	+	+	−	39.00	39.92
9	+	−	+	+	−	+	−	−	−	+	+	50.00	47.92
10	−	+	−	−	−	+	+	+	−	+	+	43.00	43.50
11	+	+	−	+	+	−	+	−	−	−	+	48.00	47.92
12	+	+	+	−	+	+	−	+	−	−	−	43.50	44.33

A: olive oil (%, v/v); B: tween 80 (%, v/v); C: yeast extract (g L^{-1}); D: casamino acid (g L^{-1}); E: KCl (g L^{-1}); F: $MgSO_4$ (g L^{-1}); G: NaCl (%, w/v); H: $CaCl_2$ (g L^{-1}); I: temperature (°C); J: pH; K: inoculum size (%, v/v)

[a] Data are means of triplicate measurements, when halophilic lipase activity was maximum.

Optimization of identified variables by Central Composite Design

After selection of these 3 significant factors, RSM was employed to estimate their significant effects and the interactions between them which would positively influence halophilic lipase production; and to identify the optimal value of each factor that would produce the maximum amount of halophilic lipase. The optimal levels of the significant factors and the interactions of these variables on halophilic lipase production were then analyzed by CCD. In this study, the 3 selected factors were the initial concentration of olive oil (X_1), the initial concentration of $CaCl_2$ (X_2), and temperature (X_3). Each factor was studied at 5 different levels: combining factorial points (−1, +1); axial points (−α, +α), and a central point (0) which was selected from the preliminary work (**Table 3**). CCD developed by the Stat-Ease software (Design-

Expert 10.0, Stat-Ease Corporation, USA) was adopted to optimize the above 3 significant factors, yielding a set of 20 experimental runs which were generated using a 2^3 full-factorial CCD with 6 star points ($\alpha = 1.682$) and 6 replicates at the center point to derive a statistical model for obtaining maximum halophilic lipase production. The levels of the factors in each experiment were as specified by the design software. The experiments were carried out in shake flasks as specified above. The less significant variables were kept constant during the optimization of significant variables by RSM. The following values were fixed at the levels shown: yeast extract 10 g L^{-1}, casamino acid 7.5 g L^{-1}, KCl 3 g L^{-1}, $MgSO_4 \cdot 7H_2O$ 20 g L^{-1}, NaCl 200 g L^{-1}, pH 6.8 - 7.0, and the inoculum size 5 % (v/v). All of the flasks were incubated on a rotary shaker (200 rpm) for 72 h.

In accordance with the design applied, the trials were executed in triplicate and their observations of the average of halophilic lipase yield were considered the response. Regression analysis was performed on the data obtained. The CCD results, were analyzed by a multiple regression procedure to estimate the data to a quadratic model equation. This resulted in a model that empirically related the response measured to all the independent variables of the experiment, and determined the interrelationships of these variables. Predicted relationships between the response (Y) representing the produced halophilic lipase and the independent variables X_1, X_2, and X_3 were calculated using the following second-order polynomial model;

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

where Y is the response (halophilic lipase activity); X_1, X_2, and X_3 are significant independent variables; β_1, β_2, and β_3 are linear regression coefficients; β_{11}, β_{22}, and β_{33} are quadratic regression coefficients; β_{12}, β_{13}, and β_{23} are interactive regression coefficients; β_0 is the intercept term.

Analysis of variance (ANOVA) was determined by Fisher's test (F-test) and counter and 3-dimensional response surface graphs were designed to represent the interactions between different variables. A P value below 0.05 was considered to be statistically significant. The accuracy and general reliability of the above polynomial model should be indicated by the coefficient of determination R^2.

Table 3 The coded and the actual values of the 3 selected factors for parameter optimization using central composite design.

Variable codes	Variables	Units	Coded variable levels				
			–α	**–1**	**0**	**+1**	**+α**
X_1	Olive oil	%, (v/v)	0.32	1	2	3	3.68
X_2	$CaCl_2$	g L^{-1}	0	0.05	0.13	0.2	0.25
X_3	Temperature	°C	35.3	37.0	39.5	42.0	43.7

Validation of the model

To validate the response surface model and evaluate the precision of the quadratic model, a random set of optimized checkpoint experiments was conducted in triplicate under the conditions predicted by the model. The experimental results of halophilic lipase activity were measured and compared with the predicted values.

Statistical analysis

Design-Expert 10.0 software (Stat-Ease Corporation, USA) was used to analyze the experimental data, and also to plot the response surface graphs. All experiments were performed in triplicate. Statistical analysis of the model was performed to evaluate the ANOVA using the same software. The quality of the polynomial model equation was judged statistically by the coefficient of determination (R^2) and its statistical significance was determined by an F-test. Comparison of the mean values was carried out by

the Tukey's multiple range test. A *T*-test was used for comparison of pairs and analysis was performed using the SPSS package (SPSS 16.0 for windows, SPSS Inc., Chicago, IL, USA).

Scale-up of halophilic lipase production in the laboratory fermenter

Halophilic lipase production from *V. alimentarius* strain LBU20907 was scaled up from 250 mL flasks to a 3 L laboratory fermenter equipped with a 6-blade turbine for agitation (Bioflo 3000; New Brunswick Scientific Company, Edison, NJ, USA). A working volume of 2 L of the optimized production medium was prepared by batch fermentation. Prior to fermentation, the optimized production medium was autoclaved at 121 °C for 15 min, and inoculated with a 5 % (v/v) inoculum. The fermentation was carried out under controlled conditions of temperature (39.5 °C), agitation (200 rpm), and aeration rate (1 vvm). A pH value of 8.0 was maintained using automatic additions of 1.0 N NaOH and 1.0 N HCl. Cultivation continued for 5 days, and samples (10 mL) were withdrawn every 24 h, and then centrifuged at 8,000×g and 4 °C for 15 min. The culture supernatants were used as an enzyme solution for determination of halophilic lipase activity.

Results and discussion

Isolation, identification and phylogenetic analysis of bacterial isolate

The bacterial isolate LBU20907, which is a good lipase producer, was isolated from samples of laboratory prepared Budu, a fermented fish product. In order to obtain the most efficient isolate, we used SGC agar supplemented with 2 % (v/v) tween 80 and SGC broth medium containing 1 % (v/v) olive oil for primary screening and secondary screening of halophilic lipase-producing microorganisms, respectively. The isolate LBU20907 exhibited the highest ratio of colony size to opaque zone size, indicating that it exhibited the highest lipase producing ability. Following 5 - 7 days incubation (data not shown), this isolate had lipase activity ratios of 6.73, 5.04, and 4.77 at 20, 25, and 30 % (w/v) NaCl, respectively. This isolate also showed the best halophilic lipase activity in SGC liquid medium containing 20 % (w/v) NaCl. Therefore, the results indicated that this strain can be used as a potential bacterial source of lipase in the presence of high salt concentrations. The morphological and physiological characteristics of isolate LBU20907 were determined by standard methods [24-26], as shown in **Table 4**. LBU20907 was characterized morphologically as a Gram-positive rod with cream pigmentation. Growth occurred at temperatures ranging from 25 - 50 °C and at NaCl concentrations of 0 – 30 % (w/v), with 20 % determined to be optimal. Isolate LBU20907 was positive for oxidase, catalase, urease, and nitrate reduction abilities, but negative for H_2S production. Furthermore, this isolate was capable of hydrolyzing gelatin, casein, and tween 80, but was unable to produce acid from L-arabinose, D-fructose, D-galactose, D-glucose, lactose, D-mannitol, D-mannose, D-sorbitol, raffinose, ribose, or sucrose. From its morphological and physiological characteristics, we determined that LBU20907 was closely related to *Virgibacillus* sp., which is similar to *V. alimentarius* J18 [24,25]. Further identification was performed using comparative sequence analysis of the 16S rRNA gene of the selected isolate and other bacteria in the database. We found that the LBU20907 16S rRNA gene had 99 % similarity to *Virgibacillus alimentarius* (GenBank accession number GU202420) as shown in **Figure 1**.

Evaluation of important variables by Plackett-Burman Experimental Design

The Plackett-Burman experimental design (PBD) is an efficient and rapid screening approach for the identification of the important factors among a large number of variables that influence a process [31]. PBD was used to screen and evaluate the nutritional and cultural conditions for halophilic lipase production by *V. alimentarius* strain LBU20907. A total of eleven parameters were studied with respect to their effect on halophilic lipase production. They were: olive oil, tween 80, yeast extract, casamino acid, KCl, $MgSO_4$, NaCl, $CaCl_2$, temperature, pH, and inoculum size. The concentration range for each variable is shown in **Table 1**. The amount of halophilic lipase enzyme synthesized by *V. alimentarius* was found to vary from 30.0 - 50.0 U mL^{-1} in the 12 experimental runs conducted based on PBD (**Table 2**). This variation may be due to the strong influence of cultural parameters on halophilic lipase production. The highest and lowest halophilic lipase activities were 65.5 U mL^{-1} and 40.0 U mL^{-1} produced in trials 9

and 8, respectively (**Table 5**). This indicates that various factors had major effects on halophilic lipase production. The relative effects of the factors on the halophilic lipase activity are summarized in the Pareto chart in **Figure 2**. The bars in **Figure 2** show the magnitude of the influence of each factor on the response.

Table 4 Morphological and physiological properties of isolate LBU20907.

Characteristics	1	2	3
Gram stain	Positive	Positive	Positive
Morphology	Rods	Rods	Rods
Pigment	Cream	Cream	Cream
Temperature range for growth (°C)	25 - 50	4 - 40	15 - 42
NaCl range for growth (%)	0 - 30	0 - 30	0 - 14
Optimum NaCl for growth (%)	20	9 - 10	1 - 3
Nitrate reduction	+	ND	+
H_2S production	−	−	ND
Oxidase activity	+	+	+
Catalase activity	+	−	+
Urease activity	+	−	−
Hydrolysis of:			
Gelatin	+	−	+
Casein	+	−	+
Tween 80	+	−	−
Acid production from:			
L-Arabinose	−	−	−
D-Fructose	−	−	+
D-Galactose	−	−	−
D-Glucose	−	−	+
Glycerol	+	+	W
Lactose	−	−	+
D-mannitol	−	−	+
D-mannose	−	−	+
D-sorbitol	−	−	ND
Raffinose	−	−	ND
Ribose	−	−	ND
Sucrose	−	−	ND

Texa: 1, *Virgibacillus* strain LBU20907; 2, *Virgibacillus alimentarius* J18 (data from Kim *et al.* [24]); 3, *Oceanobacillus profundus* CL-MP28 (data from Kim *et al.* [30]). +, Positive; −, Negative; ND, no data available; W, weakly positive

The 3 most influential factors affecting the response were the concentrations of olive oil, temperature, and $CaCl_2$ (**Figure 2**). All the other factors within the ranges tested, had no significant impact on the activity. Statistical analysis of the responses was carried out and the results are shown in **Table 6**. The magnitudes of the effects indicate the level of the significance of each variable during lipase production. Among the screened variables, olive oil (A), $CaCl_2$ (H), and temperature (I) were identified as the variables which most enhanced halophilic lipase production (**Table 6**). The statistical analysis showed that lipase production was affected by these 3 variables based on the *F*-values and *P*-values shown in **Table 6**. The model *F*-value of 76.83 indicates that this model is significant because there was only a 0.01 % chance that a model *F*-value this large could be attributable to noise. The model terms were significant ($P < 0.05$). In addition, the coefficient of determination (R^2) for the model was 0.9665. Thus,

the modeled factors explained 96.65 % of the variability in the data, which confirms a good agreement between the experimental data and the model-predicted values. The coefficient of variation (CV) is the ratio of the standard error of the estimate relative to the mean value of the observed response (as a percentage). Thus, CV is a measure of the reproducibility of the model, and a model is generally considered to be reasonably reproducible if CV ≤ 10 % [32,33]. The low CV value (3.50 %) obtained demonstrates that the experimental values had a high degree of precision and reliability.

Regression analysis was performed on the results and a first-order polynomial Eq. (3) was derived that represented halophilic lipase production as a function of the independent variables.

$$Y = 38.71 + 5.21A - 1.79H - 2.21I \quad (3)$$

The Plackett-Burman evaluation result shows that the variables olive oil, $CaCl_2$, and temperature have great significance in halophilic lipase production. The most important factor associated with the expression of halophilic lipase activity is usually the carbon source because lipases are inducible enzymes and are generally produced only in the presence of lipid sources such as oil. We found olive oil the most suitable carbon source for inducing lipase production. This agrees with the results reported by Rajendran and Thangavelu [33]. The results of a previous study [34] reported that olive oil had a significant effect on lipase production, which was in accordance with Suhigura *et al*. [35], who reported that lipase production from *Bacillus* sp. increased in the presence of 10 mL L^{-1} olive oil in the culture medium and observed very low lipase activity in the absence of olive oil even after prolonged cultivation. In their study [35], the *Bacillus* strain A30-1 produced the maximum level of thermostable alkaline lipase when olive oil and corn oil (10 mL L^{-1}) were used as carbon sources. Therefore, it is evident that olive oil is an essential component for the secretion of lipase. In addition, olive oil induces lipase production best when it is the sole carbon source [36,37]. In contrast, Essamri *et al.* [38] found that higher concentrations of oils decreased lipase production due to the lower oxygen transfer caused by the excessive viscosity of the culture medium.

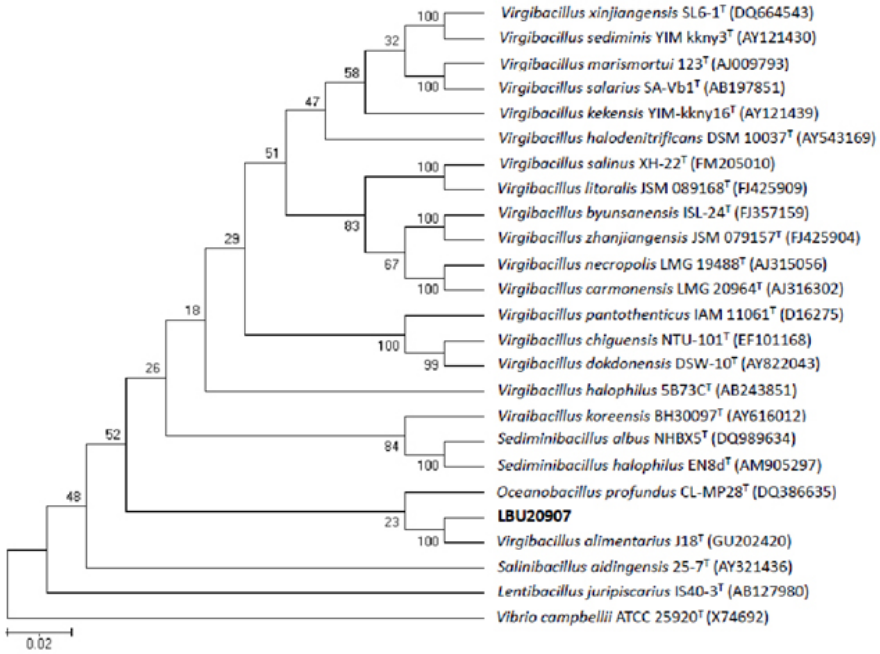


Figure 1 Phylogenetic tree based on 16S rRNA gene sequences showing the relationships between strain LBU20907 and related halophilic bacterial species. The branching pattern was generated using the UPGMA method.

PBD revealed a significant positive effect of $CaCl_2$ on halophilic lipase production. Therefore, increasing $CaCl_2$ from a low level (0.05 g L^{-1}) to a high level (0.13 g L^{-1}), should increase lipase production. Similar results were reported by Sabat *et al*. [39] for *Bacillus subtilis* 168, *B. thermoleovorans* ID-1, and *Pseudomonas aeruginosa* EF2. This result suggested that $CaCl_2$ stimulates the activity of enzymes. The stimulatory effect of calcium is measured in terms of reduced surface tension and increased cell membrane permeability [39]. In other studies, metal ions such as Ca^{2+} and Mg^{2+} are required for halophilic protein stability and activity in some halophilic enzymes [40]. In this study, increasing the temperature from 37 - 42 °C, increased the halophilic lipase production. Temperature is a crucial factor that needs to be controlled differently from organism to the organism because it enhances enzyme production by changing the physical properties of the cell membrane thereby influencing the secretion of extracellular enzymes [41]. According to previous results, Kumar *et al*. [20] found that temperature had a significant effect on the production of lipase. For *Bacillus licheniformis*, the maximum lipase production was observed at 50 °C [42]. It was reported that the maximum lipase production by *Pseudomonas xinjiangensis* was at 37 °C [43], and also that the maximum growth and lipase enzyme production of *Pseudomonas fluorescens* occurred at 36 °C [44]. In this study, the 3 significant variables identified by Plackett-Burman design were evaluated for the next stage in the medium optimization process by using RSM techniques and studies in the fermenter. Thus, olive oil, $CaCl_2$, and temperature were selected for further optimization using the CCD.

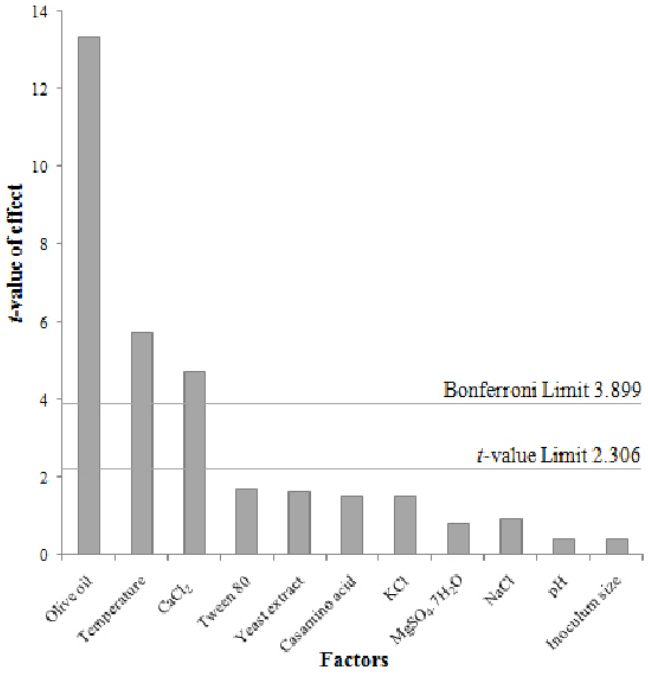


Figure 2 The Pareto chart for the relative effect of the significant factors which influenced the halophilic lipase activity.

Optimization of significant variables using Central Composite Design

The important variables, olive oil, $CaCl_2$, and temperature, for halophilic lipase production using *V. alimentarius* strain LBU20907 were identified through PBD. The model of the effects of their interactions to enhance halophilic lipase production was built by CCD. The experimental range selected for CCD is shown in **Table 3**. The experimental and predicted results along with the design matrix were presented in **Table 5** and they were in good agreement.

By applying multiple regression analysis on the experimentally determined data in Eq. (2), the regression coefficients were estimated and the following second-order polynomial equation was obtained using Stat-Ease software (Eq. (4));

$$Y = 65.06 - 4.21X_1 + 1.48X_2 - 0.81X_3 - 1.50X_1X_2 - 1.75X_1X_3 - 1.00X_2X_3 - 6.37X_1^2 - 4.41X_2^2 - 6.55X_3^2 \quad (4)$$

where Y is the predicted halophilic lipase activity (U mL^{-1}), and X_1, X_2, and X_3 are the coded values of the independent variables olive oil (%, v/v), $CaCl_2$ (g/L), and temperature (°C), respectively.

The adequacy of the model was checked using ANOVA, as shown in **Table 7**. The F-value, calculated as the ratio between the lack-of-fit mean square and pure error mean square, is a statistically valid measure of how well the factors describe the variation in the data about its mean. The further the F-value from unity, the more certain it is that the factors adequately explain the variation in the data about its mean and that the estimated values are real [20]. From **Table 7**, the ANOVA for the CCD, it was found that the ''F-value" of the model was 2249.03. The value of ''$Prob > F$" < 0.0001, suggesting that the model terms were highly significant. Linear terms: X_1, X_2, and X_3, Quadratic terms: X_1^2, X_2^2, and X_3^2, Interactive terms: X_1X_2, X_1X_3, and X_2X_3 were significant for halophilic lipase activity.

The correlation measures for testing the validity of the model are the determination coefficient R^2, which was 0.9995, indicating that 99.95 % of the variability of the response could be described by the model. The adjusted R^2 in this study was 0.9991, which is very close to the R^2 value. Similarly, Doddapaneni *et al*. [45] suggested that the closer the value of R^2 is to 1.0, the stronger the model is and the better its prediction efficiency of the responses. Thus, its value's proximity to 1.0 suggested that the model represents better correlation between experimental and predicted values. In addition, the non-significant value 0.9552 for lack-of-fit showed that the quadratic model was valid for the present study.

Adequate precision determines the signal to noise ratio. A ratio greater than 4 is desirable. The adequate precision of 122.913 indicated low adequate signal to noise ratio. The coefficient of variation (CV) is the ratio of the standard error of estimate to the mean value of the observed response, and as a general rule a model can be considered reasonably reproducible if the CV is not greater than 10 % [33]. In this model, the low values of the CV (0.54 %) indicate good reliabilities of the experiments performed. Thus, this model could be applied to navigate the design space.

The predicted optimum levels of X_1, X_2, and X_3 were obtained by applying regression analysis of Eq. (2), and they were 2 % (v/v) of olive oil, 0.13 g L^{-1} of $CaCl_2$, and 39 °C for temperature. As shown in **Table 5**, the predicted value of halophilic lipase was 65.06 U mL^{-1} which is very close to our experimental result (65.50 U mL^{-1}).

Table 5 The central composite design matrix of independent variables used in response surface methodology (RSM) with corresponding experimental and predicted values of halophilic lipase activity (U mL^{-1}).

Standard order	X_1: Olive oil	X_2: $CaCl_2$	X_3: Temperature	Halophilic lipase activity (U mL^{-1})	
				Experimental[a]	Predicted
1	- 1	- 1	- 1	47.00	47.00
2	+1	- 1	- 1	45.00	45.18
3	- 1	+1	- 1	55.00	54.98
4	+1	+1	- 1	47.00	47.05
5	- 1	- 1	+1	51.00	50.89
6	+1	- 1	+1	42.00	41.96
7	- 1	+1	+1	55.00	54.86
8	+1	+1	+1	40.00	39.90
9	0	0	0	65.50	65.06
10	0	0	0	65.00	65.06
11	0	0	0	65.00	65.13
12	0	0	0	65.00	65.13
13	- α	0	0	54.00	54.12
14	+α	0	0	40.00	39.94
15	0	- α	0	65.00	65.06
16	0	+α	0	55.00	55.07
17	0	0	- α	48.00	47.89
18	0	0	+α	45.00	45.08
19	0	0	0	64.50	65.06
20	0	0	0	65.00	65.06

[a] Data are means of triplicate measurements, when halophilic lipase activity was maximum.

Table 6 Statistical analysis of the model.

Source	Sum of squares	Degrees of freedom	Mean square	*F*-value	*Prob* > *F*
Model	422.56	3	140.85	76.83	< 0.0001
A	325.52	1	325.52	177.56	< 0.0001
H	38.52	1	38.52	21.01	0.0018
I	58.52	1	58.52	31.92	0.0005
Residual	14.67	8	1.83		
Corr. Total	437.23	11			

R^2 = 0.9665; Adj R^2 = 0.9539; CV = 3.50 %

Table 7 Analysis of variance (ANOVA) for response surface quadratic polynomial model of halophilic lipase activity.

Source	df	Sum of squares	Mean square	*F*-value	*Prob > F*
Model	9	1701.90	189.10	2249.03	< 0.0001[a]
X_1	1	242.47	242.47	2883.83	< 0.0001[a]
X_2	1	19.69	19.69	234.18	< 0.0001[a]
X_3	1	8.93	8.93	106.25	< 0.0001[a]
X_1^2	1	578.46	578.46	6879.84	< 0.0001[a]
X_2^2	1	167.71	167.71	1994.69	< 0.0001[a]
X_3^2	1	611.00	611.00	7266.87	< 0.0001[a]
X_1X_2	1	18.00	18.00	214.08	< 0.0001[a]
X_1X_3	1	24.50	24.50	291.39	< 0.0001[a]
X_2X_3	1	8.00	8.00	95.15	< 0.0001[a]
Residual	10	0.84	0.084		
Lack of fit	5	0.13	0.026	0.19	0.9552
Pure error	5	0.71	0.14		
Cor total	19	1702.74			

R^2 = 0.9995; Adj. R^2 = 0.9991; CV = 0.54 %; Adequate precision = 122.913
[a] Model terms are significant

The optimum level of each variable and the effect of their interaction on halophilic lipase production were studied by constructing 3 dimensional (3D) response surfaces and contour plots (**Figures 3 - 5**). The figures were generated using Eq. (2), keeping one variable constant at its optimum value and varying the other 2 within the experimental range.

The effect of olive oil (X_1) and $CaCl_2$ (X_2) on halophilic lipase production while keeping temperature (X_3) constant at zero is depicted in **Figure 3**. The response surface plot (**Figure 3a**) showed that the optimum production of halophilic lipase could be attained while the olive oil concentration was low and $CaCl_2$ concentration was at optimum level.

The effect of olive oil (X_1) and temperature (X_3) on halophilic lipase production while keeping $CaCl_2$ (X_2) constant at zero is depicted in **Figure 4**. The response surface plot (**Figure 4a**) predicted that the halophilic lipase production would increase when the olive oil concentration was low and the temperature at the optimum.

The effect of $CaCl_2$ (X_2) and temperature (X_3) on halophilic lipase production while keeping olive oil (X_1) constant at zero is depicted in **Figure 5**. The response surface plot (**Figure 5a**) of $CaCl_2$ concentration and temperature showed that lipase activity increased when $CaCl_2$ concentration and temperature were at the optimum level.

The shapes of the surfaces, circular, elliptical or saddle, indicated whether the interactions between the different variables were significant or not. An elliptical or saddle pattern indicates that the interactions between the corresponding variables were significant interactions, whereas a circular shape implies a negligible interaction [46]. Here, the 2 dimensional contour plots (**Figures 3 - 5b**) are elliptical, indicating that the interactive effects of the variables on halophilic lipase production were significant.

Lipase activity comparable with that obtained in this study was reported by Rajendran and Thangavelu [47]. They used the Plackett–Burman experimental design to evaluate the medium components for lipase production by *R. arrhizus*. The most significant variables affecting lipase production were reported to be olive oil, peptone, KH_2PO_4, $CaCl_2{\cdot}2H_2O$ and $MgSO_4{\cdot}7H_2O$. A maximum lipase activity of 3980 μmol/L/min and a maximum cell mass concentration of 5.62 g L^{-1} were determined using the optimized medium.

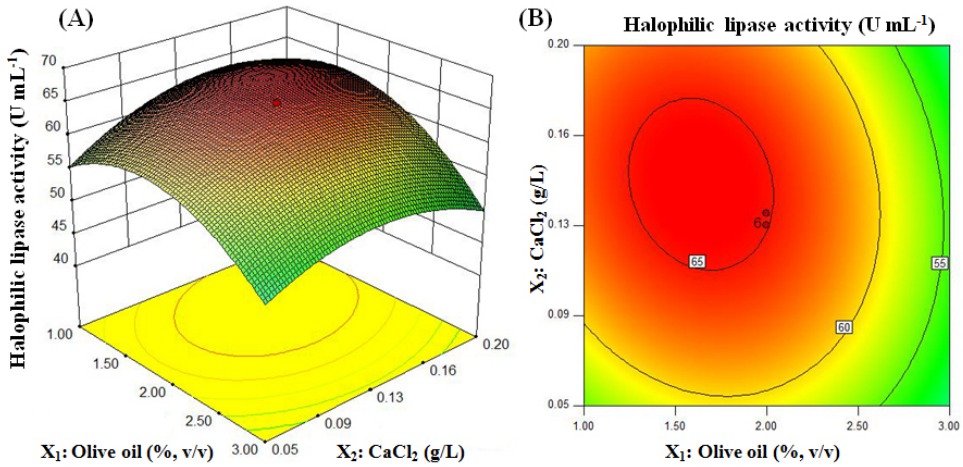


Figure 3 Response surface (a) and contour plots (b) of halophilic lipase activity showing interactive effect of olive oil (X_1) and $CaCl_2$ concentrations (X_2).

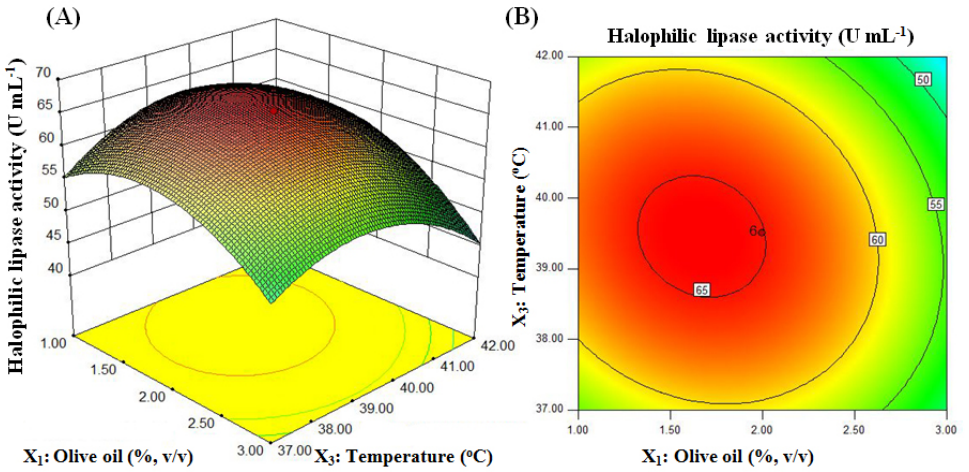


Figure 4 Response surface (a) and contour plots (b) of halophilic lipase activity showing interactive effect of olive oil (X_1) and temperature (X_3).

Validation of the model

Random experimental conditions were evaluated for the validation of the model. The experimental value was found to be very close to the predicted value, and thus, the model was successfully validated. The maximum activity of halophilic lipase was found to be 65.96 U mL^{-1} (olive oil 1.68 % (v/v), $CaCl_2$ 0.14 g L^{-1}, and temperature at 39.5 °C) which was very close to the predicted value, 65.68 U mL^{-1} (**Table 8**). Therefore, the statistical optimization resulted in a 42 % increase of halophilic lipase activity over the non-optimized conditions.

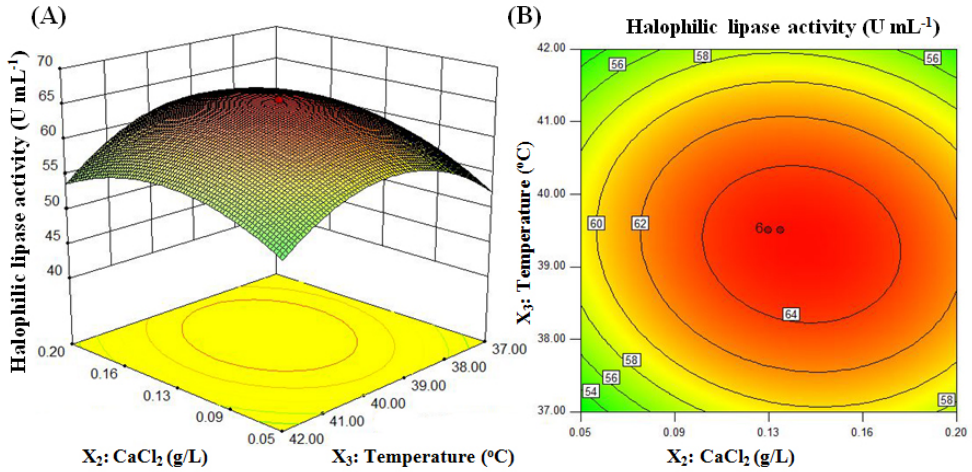


Figure 5 Response surface (a) and contour plots (b) of halophilic lipase activity showing interactive effect of $CaCl_2$ (X_2) and temperature (X_3).

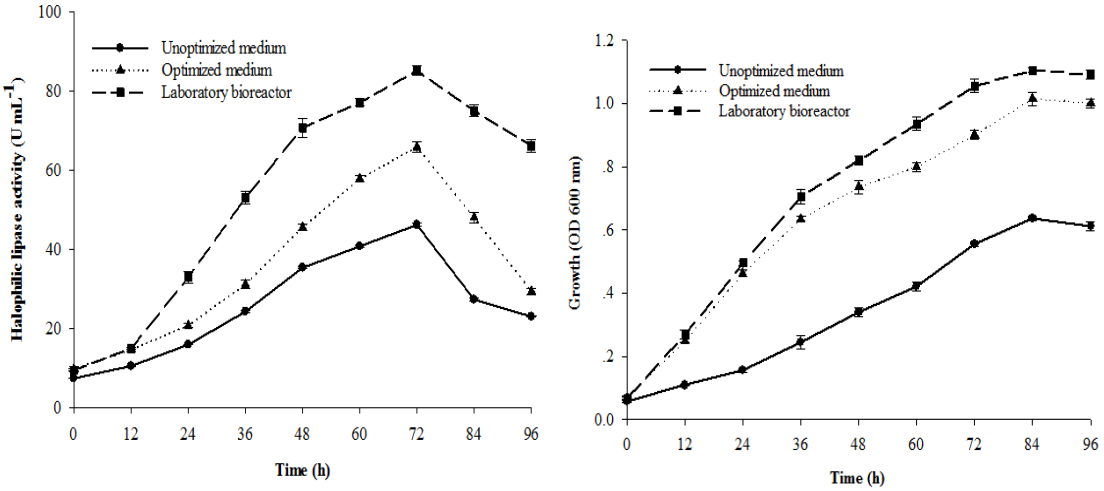
Table 8 Validation of the predicted model showing halophilic lipase production at optimum level of all parameters.

Parameters	Optimal conditions	Halophilic lipase activity (U mL^{-1})	
		Experimental	Predicted
Olive oil (%, v/v)	1.68		
$CaCl_2$ (g/L)	0.14	65.96	65.68
Temperature (°C)	39.5		

Scale-up of halophilic lipase production from V. alimentarius LBU20907

After optimization studies in shake-flasks, the production of halophilic lipase from *V. alimentarius* LBU20907 was scaled up in a laboratory fermenter containing 2 L working volume under controlled conditions of agitation (200 rpm), and aeration (1 vvm). Using the optimal conditions for flask culture, 3 L scaled fermentation reached a maximum halophilic lipase value of 85.20 U mL^{-1} at 72 h of incubation time (**Figure 6a**). By comparison, in optimized and non-optimized media in 250 mL Erlenmeyer flasks, the halophilic lipase production obtained in 72 h was 65.96 U mL^{-1} and 46.50 U mL^{-1}, respectively: a difference of 42 %. Similar results were reported by Kumari *et al*. [48], who found that a 40 % increase in lipase production was observed in *Enrobacter aerogenes* under optimized conditions. Sharma *et al*. [49] reported that a 60 % increase in lipase production was obtained in *Arthrobacter* sp. BGCC#490. Ramakrishnan *et al*. [50] found that an increase of 215 % was obtained in lipase production for *Enterococcus faecium* MTCC5695 by optimization using RSM. An increase in the halophilic lipase production was possible after optimization of nutritional and cultural conditions using RSM. The results indicate that the bacterial strain was induced to produce lipase in the presence of a variety of growth conditions. In this study, the halophilic lipase production progressively increased during the late exponential phase of growth and reached its maximum level when the culture reached the early stationary phase of growth after 72 h (**Figure 6b**). This finding was in agreement with several reports on lipase production from *Pseudozyma aphidis* as reviewed by Dimitrijević *et al.* [51]. This result suggests that when *P. aphidis* entered the stationary phase, lipase production decreased and the protein concentration increased; probably as a result of cell death. Here, the halophilic lipase production observed in the optimized media during scale-up was 77 % higher than observed in the shake flask, because the fermenter

systems provide a more precise control of parameters such as pH, temperature, aeration and agitation speed. This can further enhance the application potential of halophilic lipase for food manufacture especially for fish sauce production. Moreover, the results obtained in this work suggested that RSM is an effective and reliable tool for developing the model, optimizing factors, improving product yield, reducing time, and evaluating the relative significance of several factors affecting halophilic lipase production.



a) b)

Figure 6 Fermentation profiles of halophilic lipase activity (a) and cell growth (b) by *V. alimentarius* strain LBU20907 in 50 mL optimized and non-optimized media in a 250 mL Erlenmeyer flask and 3 L laboratory fermenter with 2 L optimized medium.

Conclusions

RSM was found to be an efficient methodology for the rapid optimization of the parameters influencing the enhancement of halophilic lipase production by *V. alimentarius* LBU20907. Based on the results of our statistical approach, the optimal parameters for production were established as: olive oil 1.68 % (v/v), $CaCl_2$ 0.14 g L^{-1}, and temperature at 39.5 °C. Under such conditions, halophilic lipase production could be increased from 46.50 U mL^{-1} in non-optimized medium to 65.96 U mL^{-1} giving 1.42-fold increase in the yield. The determination coefficient R^2 of 0.9995 for the established model clearly indicated a relatively high correlation between the model and the experimental data. Furthermore, the scale-up study using the optimized medium in a 3 L laboratory fermenter, achieved a 77 % increase in the yield (82.50 U mL^{-1}). Bioreactor fermentations did improve the halophilic lipase activity and biomass concentration relative to the shake flasks clearly because of the ability afforded by bioreactors to carefully control aeration, agitation speed, pH, and temperature. Moreover, the halophilic lipase appeared in the medium when the bacterial cells reached the late logarithmic phase after 72 h of fermentation. The decrease in lipolytic activity in the early stationary phase was also observed. This study may also be helpful and useful to improve the productivity of various industrial applications.

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