Biotechnology Reports 8 (2015) 64–71

Contents lists available at ScienceDirect

Biotechnology Reports

journal homepage: www.elsevier.com/locate/btre

A statistical approach for optimization of alkaline lipase production by ascidian associated—*Halobacillus trueperi* RSK CAS9

Ramamoorthy Sathishkumar, Gnanakkan Ananthan*, Kathirvel Iyappan, Chinnathambi Stalin

Centre of Advanced Study in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai 608 502, Tamil Nadu, India

ARTICLE INFO

Article history:
Received 8 July 2015
Received in revised form 2 September 2015
Accepted 14 September 2015
Available online 16 September 2015

Keywords:
Haloalkaline lipase
Didemnum candidum
Marine wastes
Response surface methodology

ABSTRACT

A marine ascidian-associated bacterium, *Halobacillus trueperi* RSK CAS9, was optimized for lipase production by response surface methodology using marine waste as substrate. The central composite design was employed, and the optimal medium constituents for maximum lipase production (1355.81 U/ml) were determined to be tuna powder (14.58 g/l), olive oil (5.05 ml/l); NaCl (72.42 g/l), temperature (45 °C) and pH 9.0. An alkaline lipase was purified to 8.46 fold with 1193.59 U mg^{-1} specific activities with the molecular weight of 44 kDa. The activity was substantially inhibited by EDTA and PMSF, indicating that it was a metalloenzyme serine residue which was essential for catalytic activity. Thus, lipase production by microbial conversion of marine fish wastes in this study suggested its potential utilization for the production of high value products.

1. Introduction

Lipases (triacylglycerol acylglycerol EC 3.1.1.3) belong to the group of serine hydrolases which catalyze the hydrolysis of triglycerides to glycerol and free fatty acids over the oil–water interface [1]. Microbial lipases catalyze the hydrolysis and transesterification of other esters as well as the synthesis of esters and exhibit enantioselective properties [2]. These unique properties of lipases make many relevant industrial applications like chemical processing, dairy industries for improvement of flavor, paper and oleochemical industries, pharmaceuticals, synthesis of surfactants, detergent industries, leather industries and polymer synthesis [3].

The marine industry generates nearly 50–60% of the total weight of fish as waste contains enormous amount of protein (35–50%) and chitin (15–25% of dry weight) which are considered as major environmental pollutants due to its uncontrolled dumping [4]. Production cost of lipase is strongly influenced by expensive medium components like nitrogen, carbon sources such as fatty acids, triglycerides and sugars or complex polysaccharides like glycogen and surfactants. Bioconversion of these disposed fish waste materials has been proposed to be a safer waste treatment option that will solve environmental problems, but also decrease to a large extent the production costs of using microbial enzymes [5]. The majority of lipase producing bacteria was isolated from soil. Recently, marine ascidians are remarkable array of microorganisms and the marine ascidians-associated bacteria have been recognized as rich source for marine enzymes [6]. However many studies were made on production and purification of lipase, no attempt has been made on lipase from *Halobacillus trueperi* through bioconversion of marine wastes. Hence in the present study, an attempt was made on production, purification and characterization of lipase from *H. trueperi* RSK CAS9 by utilization of marine wastes through response surface methodology.

2. Materials and methods

2.1. Fish wastes

Fish wastes include inedible parts eliminated during the industrial process (tuna: heads, viscera, skin, and some muscle tissues, sardinella: heads and viscera; shrimp: heads, shells and tails; cuttlefish: ink sacs and viscera). Fish wastes were collected from local fish process unit and were cooked by boiling and dried at 80 °C for 24–48 h. The dried materials were minced and sieved (100 μM) to get a uniform fine powder and stored in glass bottles at room temperature.

2.2. Microorganism

Marine ascidians (*Didemnum candidum*) were collected from the Campbell Bay (06°59.749′N 93°56.718′E), Great Nicobar

* Corresponding author. Fax: +91 4144 243555, +91 9894414889.
E-mail address: casananthan@yahoo.co.in (G. Ananthan).

http://dx.doi.org/10.1016/j.btre.2015.09.002

Biosphere Reserve, India by SCUBA diving at a depth of 10–15 m and were brought to the laboratory in a sterile container with pure seawater. The ascidian was gently washed with sterile seawater and small piece of ascidian tissue were homogenized using sterile mortar and pestle. Serially diluted homogenates were plated on modified halophilic agar medium containing (peptone, 1.0%; NaCl, 5.0%; glucose, 0.5%; yeast extract, 0.5%; olive oil, 0.1%; KH_2PO_4, 0.1% and $MgSO_4$, 0.05%) and incubated at 40 °C, pH 9 for 72 h. The isolated colonies were screened for extracellular lipase activity by cross streaking on a spirit blue agar plate. Further, potential strain was identified by morphological and biochemical schemes according to Bergey's manual of determinative bacteriology [7] and confirmed through molecular characterization by 16S rRNA gene sequence. Briefly, bacterial genomic DNA was extracted by the phenol chloroform method [8] and the 16S rRNA gene was amplified by using primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGGCGGTGTGTACAAGGC-3′). PCR conditions were as follows; 35 cycles consisting of an initial denaturation at 95 °C for 5 min, denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min and followed by a final extension of 5 min. The 16S rRNA gene was sequenced by an automated DNA sequencer (Megabace, GE) and homology was analyzed with the sequences in GenBank using CLUSTAL X software. The phylogenetic tree was constructed using the neighbor-joining method [9].

2.3. Plackett–Burman designs

For the suitable nutritional sources for lipase production, the bacterium was cultivated in a modified halophilic medium at pH 9, 40 °C for 48 h with 1% (w/v) inoculum. The culture was incubated in a shaker (125 rpm) and the cells were collected by centrifugation at 10,000 × *g* for 15 min and the supernatant was further used for lipase assay.

The Plackett–Burman (PB) design is a preliminary screening method for the main physico-chemical parameters among a large number of process variables which are required for lipase production. In the present study, ten different medium components (tuna powder (TP), sardinella powder (SP), cuttlefish powder (CP), shrimp powder (SP), K_2HPO_4, NaCl, $MgSO_4$ and olive oil) and cultivation parameters (incubation temperature and pH) were investigated using PB design to identify the variables that significantly affected the lipase production. The PB design allows for the evaluation of N variables in N + 1 experiments; each variable was examined at two levels: −1 for a low level and +1 for a high level (Table 1). Table 2 represents the 10 variables were evaluated in 12 experimental trials. The design was run in a single block and the order of the experiments was fully randomized.

2.4. Central composite design (CCD)

Based on the choice of selection of significant variables for lipase production by Plackett–Burman design experiment, the significant variables were chosen for further CCD analysis (tuna powder, olive oil, NaCl and temperature). A total of 31 experiments were formulated using the MINITAB, version 16 (PA, USA) and the central values of all variables were coded as zero. The minimum and maximum ranges of the variables were used and the detailed experimental plan with regard to their values in the actual and coded form is presented in Table 3. Data obtained from the RSM on lipase production were subjected to analysis of variance (ANOVA). The experimental results of RSM were fitted with the response surface regression procedure using the following second order polynomial equation:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j$$

where Y is the predicted response, X_i and X_j are independent factors, β_0 is the intercept, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, and β_{ij} is the interaction coefficient. Statistical analysis of the data and surface plots were performed using MINITAB, version 16 (PA, USA). The fitted polynomial equation was then expressed as three dimensional surface plots to illustrate the relationship between the responses and interaction effects of the variables.

2.5. Production and purification of lipase

The lipase production was carried out in 100 ml of optimized liquid medium in an Erlenmeyer flask (500 ml) containing tuna powder 14.58 g/l, olive oil 5.05 ml/l, NaCl 72.42 g/l, temperature 45.29 °C and pH 9 were seeded with 1% inoculum and incubated in shaking incubator (125 rpm) for 42 h. After incubation, the culture broth was centrifuged (4 °C at 12,000 × *g* for 20 min) and the crude enzyme was used for further purification.

2.6. Purification of enzyme

For purification of lipase, ammonium sulfate was added to the culture supernatant to obtain 60% saturation (w/v) and allowed to stand overnight at 4 °C. The precipitate was collected through centrifugation at 6000 × *g* for 15 min and dissolved in 50 mM Tris–HCl buffer (pH 9) and dialyzed against the same buffer (4 °C). The dialysate was loaded on a DEAE-cellulose column (5 cm × 25 cm) and eluted with a linear gradient of NaCl (0–1 M) at a flow rate of 0.5 ml/min. Fractions were collected and assayed for enzyme activity and fractions which exhibited enzyme activity were pooled together and concentrated by ammonium sulfate

Table 1
Experimental variables at different levels used for the production of alkaline lipase by *H. trueperi* RSK CAS9 using Plackett–Burman design.

Symbol code	Variables	Levels		Effect	Coefficient	*t*-value	*p*-value
		+	−				
Constant					329.24	191.05	0.003
X_1	Tuna powder (g/l)	10	1	203.36	101.68	59.00	0.011
X_2	Sardine powder (g/l)	10	1	15.18	7.59	4.40	0.142
X_3	Cuttlefish powder (g/l)	10	1	−13.84	−6.92	−4.02	0.155
X_4	Shrimp powder (g/l)	10	1	−31.54	−15.77	−9.15	0.069
X_5	Olive oil (ml/l)	1	0.1	48.24	24.12	14.00	0.045
X_6	NaCl (g/l)	30	50	185.11	92.55	53.71	0.012
X_7	K_2PHO_4 (g/l)	5	0.1	−16.18	−8.09	−4.69	0.134
X_8	$MgSO_4$ (g/l)	1	0.01	13.23	6.61	3.84	0.162
X_9	Temperature	6	10	67.06	33.53	19.46	0.033
X_{10}	pH	30	40	−1.19	−0.59	−0.34	0.789

Table 2
Plackett–Burman design matrix for ten variables with coded values along with observed and predicted lipase production.

Run order	X_1 (g/l)	X_2 (g/l)	X_3 (g/l)	X_4 (g/l)	X_5 (ml/l)	X_6 (g/l)	X_7 (g/l)	X_8 (g/l)	X_9 (g/l)	X_{10} (g/l)	Lipase activity (U/ml)	
											Observed	Predicted
1	+1	−1	+1	−1	−1	−1	+1	+1	+1	−1	332.02	333.743
2	+1	+1	−1	+1	−1	−1	−1	+1	+1	+1	279.12	277.397
3	−1	+1	+1	−1	+1	−1	−1	−1	+1	+1	154.42	156.143
4	+1	−1	+1	+1	−1	+1	−1	−1	−1	+1	449.13	450.853
5	+1	+1	−1	+1	+1	−1	+1	−1	−1	−1	395.10	396.823
6	+1	+1	+1	−1	+1	+1	−1	+1	−1	−1	518.12	516.397
7	−1	+1	+1	+1	−1	+1	+1	−1	+1	−1	317.03	315.307
8	−1	−1	+1	+1	+1	−1	+1	+1	−1	+1	163.22	161.497
9	−1	−1	−1	+1	+1	+1	−1	+1	+1	−1	277.25	278.973
10	+1	−1	−1	−1	+1	+1	+1	−1	+1	+1	612.06	610.337
11	−1	+1	−1	−1	−1	+1	+1	+1	−1	+1	357.20	358.923
12	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	119.25	114.527

precipitation. The resultant precipitate was collected by centrifugation and dissolved in 50 mM Tris–HCl buffer (pH 9). Concentrated fractions were loaded onto a Sephadex G-50 column (2.5 cm × 25 cm) equilibrated with 50 mM Tris–HCl buffer (pH 9) and eluted with same buffer at a flow rate of 15 ml/h. The fractions exhibiting lipase activity were pooled together and used as purified enzyme for further characterization study.

2.7. Protein estimation

Protein contents were determined by the method of Lowry et al. [10] using bovine serum albumin (BSA) as standard.

2.8. Lipase assay

Estimation of lipase activity was carried out by a modified method of Kilcawley et al. [11]. The lipase assay methods were made with the following ingredients: 1 ml Tris buffer (pH 9), 1 ml *p*-nitro phenyl palmitate (*p*-NPP) (10 mM) and 0.5 ml culture supernatant. Approximate controls were further maintained. The mixture was incubated for 15 min on a rotary shaker at 150 rpm at 37 °C. Then the absorbance was measured at 400 nm using UV–vis Spectrophotometer. The amount of lipase was established with the help of *p*-nitro phenol standard graph. One unit of lipase activity is equivalent to one microgram of *p*-nitro phenol released under standard assay condition.

Table 3
Experimental conditions in variables of the CCD design and the corresponding experimental responses.

Run order	TP (X_1) (g/l)	Olive oil (X_5) (ml/l)	NaCl (X_6) (g/l)	Temperature (X_9) (°C)	Lipase activity (U/ml)	
					Observed	Predicted
1	12.5	3.25	60	42	750.10	751.46
2	17.5	3.25	60	42	627.27	627.83
3	12.5	7.75	60	42	681.07	682.27
4	17.5	7.75	60	42	767.20	766.23
5	12.5	3.25	80	42	1072.16	1072.04
6	17.5	3.25	80	42	810.20	809.44
7	12.5	7.75	80	42	640.17	638.60
8	17.5	7.75	80	42	582.10	583.58
9	12.5	3.25	60	47	806.12	804.37
10	17.5	3.25	60	47	711.46	713.97
11	12.5	7.75	60	47	771.17	772.88
12	17.5	7.75	60	47	890.22	890.07
13	12.5	3.25	80	47	1035.10	1037.01
14	17.5	3.25	80	47	809.12	807.65
15	12.5	7.75	80	47	642.10	641.27
16	17.5	7.75	80	47	619.91	619.49
17	10.0	5.50	70	45	787.21	786.59
18	20.0	5.50	70	45	641.23	641.18
19	15.0	1.00	70	45	719.40	718.62
20	15.0	10.00	70	45	461.17	461.28
21	15.0	5.50	50	45	705.31	703.41
22	15.0	5.50	90	45	752.19	753.41
23	15.0	5.50	70	40	978.15	977.89
24	15.0	5.50	70	50	1067.12	1066.70
25	15.0	5.50	70	45	1346.27	1346.98
26	15.0	5.50	70	45	1345.71	1346.98
27	15.0	5.50	70	45	1351.02	1346.98
28	15.0	5.50	70	45	1345.20	1346.98
29	15.0	5.50	70	45	1350.32	1346.98
30	15.0	5.50	70	45	1345.10	1346.98
31	15.0	5.50	70	45	1345.22	1346.98

2.9. Time course of lipase production

To study the relation between lipase production and the growth profile of the bacterium, 50 ml of the optimized production medium was inoculated in 250 ml flasks and the growth was measured at regular intervals by viable count (spread plate method) determination. Lipase production at different time intervals (0–72 h) was determined using the standard lipase assay.

2.10. Molecular weight determination

Molecular weight of the purified lipase was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method of Laemmli [12] with 5% stacking gel and 10% resolving gel. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 in methanol–acetic acid–water (5:1:5, w/v) and detained in 7% acetic acid.

2.11. Substrate specificity to various p-nitrophenyl esters

The substrates, *p*-nitrophenyl fatty acid esters such as *p*-palmitate, *p*NP-laurate, *p*NP-stearate, *p*NP-octanoate, *p*NP-decanoate, *p*NP-acetate, *p*NP-butyrate and *p*NP-valerate at 1 mM concentration.

2.12. Effect of metal ions, inhibitors, detergents and organic solvents on lipase activity

Effect of metal ions on lipase activity was investigated using Mg^{2+}, Ca^{2+}, Fe^{2+}, Mn^{2+}, Co^{2+} and Hg^{2+} at 0.1 and 1 mM concentration. The effect of enzyme inhibitors such as phenylmethylsulfonyl fluoride (PMSF), ethylene diaminetetraaceticacid (EDTA) and β-mercaptoethanol (1 and 5 mM) were investigated. Effect of ionic and non-ionic surfactants such as Tween 80, Triton X-100, SDS and sodium deoxycholate (1% and 10%, w/v) and 25% various organic solvents (*iso*-octane, methanol, benzene, toluene, hexane and chloroform) on the stability of the enzyme was also checked. The purified lipase (10 μL enzyme + 190 μl 50 mM Tris–HCl buffer) was pre-incubated with the aforementioned metal ions, inhibitors and surfactants at 50 °C for 1 h and then assayed for residual activity.

3. Results and discussion

3.1. Microorganism

In the present study, lipase producing bacteria was isolated from marine ascidian and designated as RSK CAS9. Based on the morphological, physiological and biochemical characteristics, the strain was a gram-positive and endospore-forming bacillus, with catalase which grows in both aerobic and anaerobic environments. The 16S rRNA gene sequencing analysis evidenced that this strain RSK CAS9 exhibited the highest homology (98%) with *H. trueperi* RO7. Based on the evolutionary distance and the phylogenetic tree analysis (Fig. 1), this strain was identified as *H. trueperi* and designated as *H. trueperi* RSK CAS9 (GenBank ID: KJ862542).

3.2. Identification of significant variables using Plackett–Burman design

The Plackett–Burman design, a total of ten variables was analyzed to determine their effects on lipase production (Table 1). The main effect of each variable upon the lipase activity was calculated as the difference between both averages of measurements made at the highest level (+1) and at the low level (−1) of that factor. The obtained results of the Plackett–Burman design experiment showed variation in lipase activity from 119.25 to 612.06 U/ml (Table 2). This variation takes into account the importance of media optimization to attain higher productivity. Statistical analysis of the responses were performed and represented in Table 1. Among the total variables, tuna powder, olive oil, NaCl and temperature were displayed positive effects and significant *p*-value (<0.05) in lipase production, whereas sardine powder, cuttlefish powder, shrimp powder, K_2HPO_4, $MgSO_4$ and pH had a negative effect. The optimal levels of the four selected variables (tuna powder, olive oil, NaCl and temperature) and their interactions were then examined by a central composite design.

3.3. Optimization of significant variables using RSM

A total of 31 experiments with different combinations of these four selected variables were performed; the experimental design is presented in Table 3. The central composite design was employed

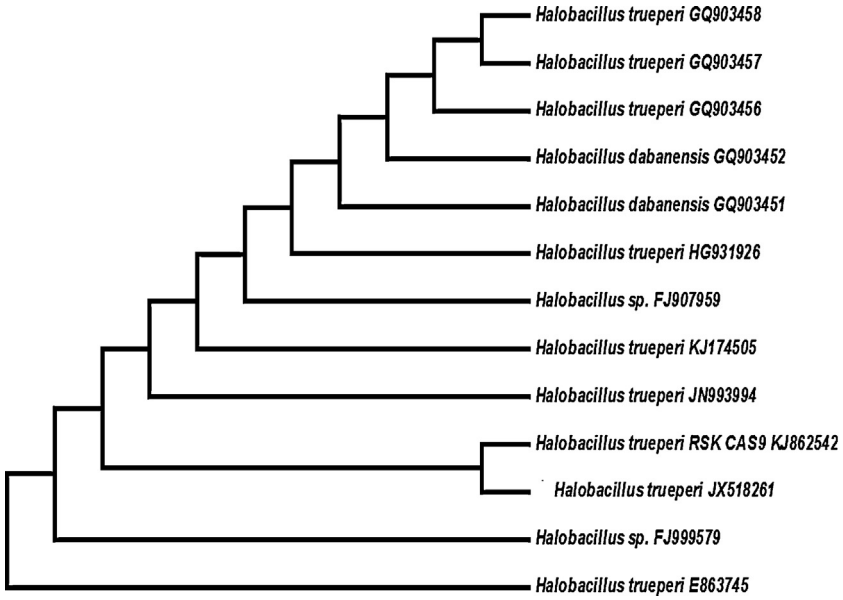

Fig. 1. Phylogenetic analysis of *H. trueperi* RSK CAS9 strain 16S rRNA gene sequence with other *Halobacillus* species/strains.

Table 4
Analysis of variance (ANOVA) for the quadratic model.

Source	DF	Seq SS	Adj SS	Adj MS	*f*–value	*p*-value
Regression	14	2397697	2397697	171264	36742.97	0.000
Linear	4	146635	462737	115684	24818.86	0.000
Square	4	2045718	2045718	511430	109722.00	0.000
Interaction	6	205344	205344	34224	7342.40	0.000
Residual Error	16	75	75	5		
Lack-of-Fit	10	35	35	4	0.54	0.817
Total	30	2397772				

to determine the optimum levels of the four screened factors, and the results of the second-order response surface model fitted to ANOVA are presented in Table 4. Lipase production (Y) by *H. trueperi* RSK CAS9 can be expressed in terms of the following regression equation:

$$Y = -43184.0 + 731.9\ X_1 + 452.2\ X_5 + 300.4\ X_6 + 1210.1\ X_9 - 25.3\ X_1^2 - 37.4\ X_5^2 - 1.5\ X_6^2 - 13.0\ X_9^2 + 9.2\ X_1X_5 - 1.4\ X_1X_6 + 1.3\ X_1X_9 - 4.0\ X_5X_6 + 1.7\ X_5X_9 - 0.9\ X_6X_9$$

X_1,X_5, X_6 and X_9 are tuna powder, olive oil, NaCl and temperature, respectively. The statistical significance was evaluated by performing *F*-test and ANOVA with the MINITAB, version 16 (PA, USA). The significant *p*-values (<0.05) and non-significant lack of fit (0.817) were suggested that the experimental data were the best fit with the model. In addition, larger magnitudes of *t*-test value and

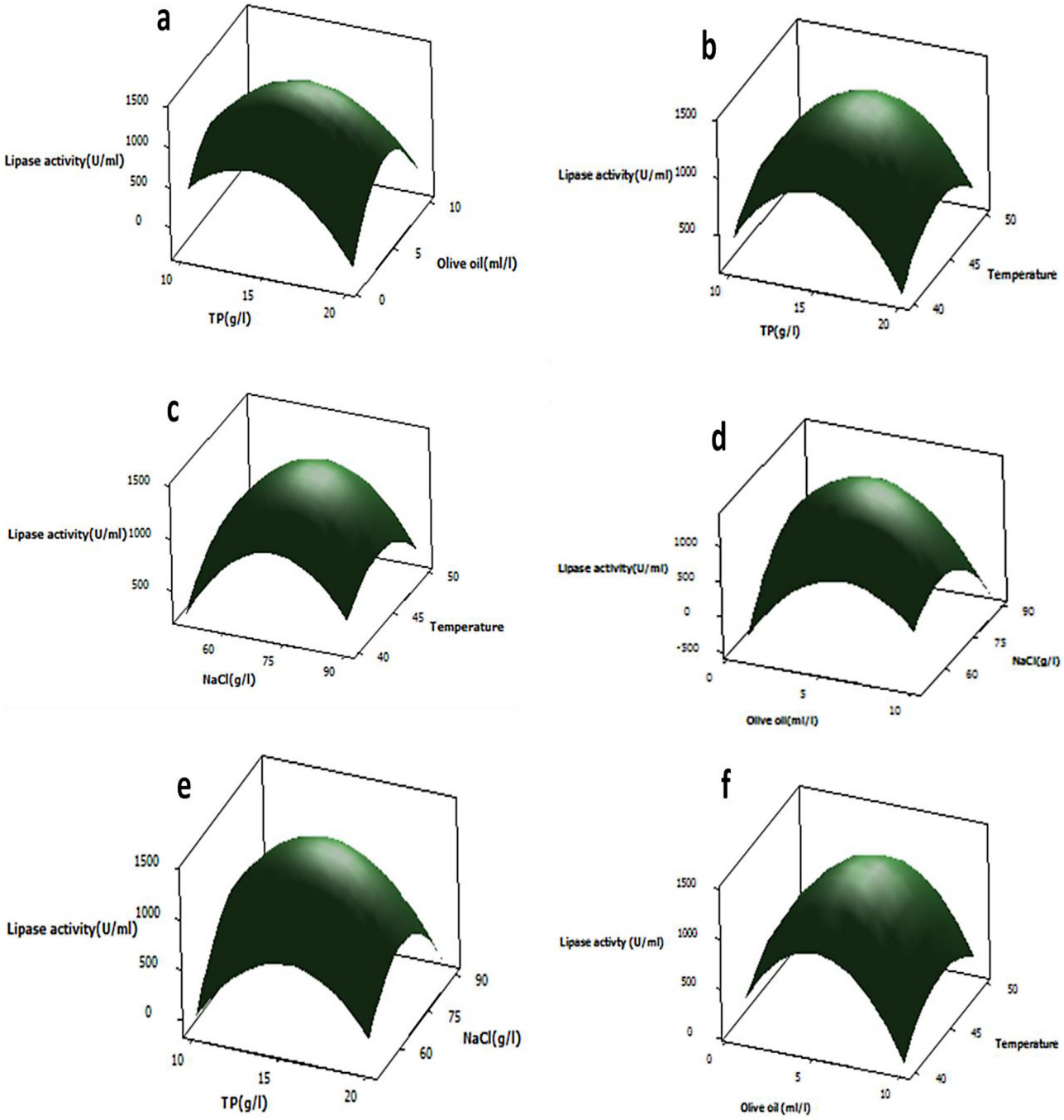

Fig. 2. Three dimensional response surface plot for lipase production showing the interactive effects of the tuna powder and olive oil (A), tuna powder and temperature (B), NaCl and temperature (C), olive oil and NaCl (D), tuna powder and NaCl (E) and olive oil and temperature (F).

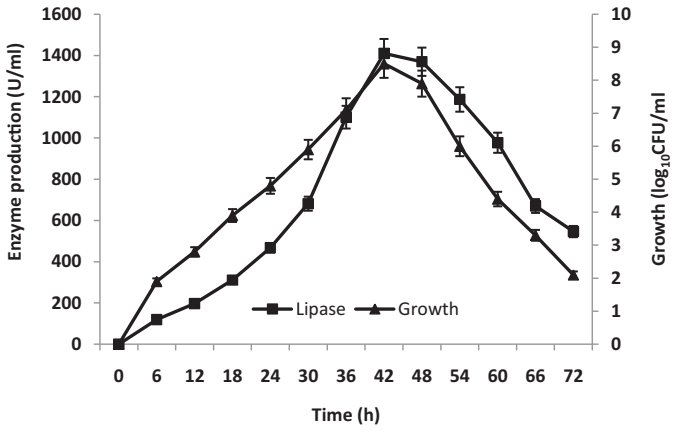

Fig. 3. Kinetics of bacterial cell growth and alkaline lipase production (CFU/ml) by optimized medium.

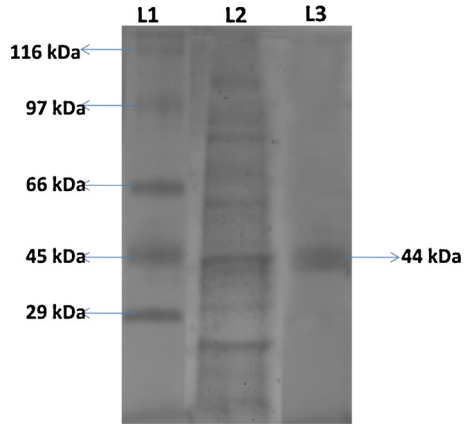

Fig. 4. SDS-PAGE analysis of lipase from *H. trueperi* RSK CAS9. Lane 1: 116 kDa, β-galactosidase (*E. coli*); 97 kDa, phosphorylase b (rabbit muscle); 66 kDa, albumin (bovine serum); 45 kDa, ova albumin (chicken egg); sigma protein 29 kDa, and carbonic anhydrase (bovine erythrocytes). Lane 2: crude lipase. Lane 3: purified lipase.

smaller the magnitudes of *p*-value indicate a high significance of the corresponding coefficient [13]. The high R^2 value (99.99%) suggested that the experimental results and theoretical values predicted by the model agreed well [14]. The values of the adjusted determination coefficient (adjusted R^2, 99.90%) and predicted determination coefficient (predicted R^2 99.98) were also high, supporting for high significance of this model [15].

The regression coefficients of all linear, quadratic terms and two cross products are important at 1% level. The 3D response surface plots are graphical representations of the regression equation and are used to visualize the relationship between the response and experimental levels of each variable and the type of interaction between the variables to realize the optimum conditions (Fig. 2). Each figure displays the effect of two variables while the other variable was held at zero level. As present in the surface plots, there was interaction between each pair of variables and all the interaction between the selected three variables was important. The model predicted that maximum lipase production of 1355.81 U/ml appeared at (g/l): tuna powder, 14.58 g/l; olive oil, 5.05 ml/l; NaCl, 72.42 g/l; temperature, 45 °C and pH 9.0. The time course of lipase production and cell growth of *H. trueperi* RSK CAS9 for the optimized conditions are shown in Fig. 3. These results show that the enzyme production in relation with incubation time and cell growth revealed that both bacterial cell growth and lipase production reached a maximum (1410.17 U/ml) at 42 h and started to decrease gradually after 48 h. The optimization strategy led to the enhancement of lipase from un-optimized conditions (467.33 U/ml) to optimize conditions (1410.17 U/ml) and also lipase production from the experiment was similar with predicted value that reveals about the higher accuracy of the model. The use of fish processing wastes for lipase production was a very useful one and it would significantly reduce the production cost. This was proved earlier by the studies of Esakkiraj et al. [16] who utilized the tuna wastes for maximum lipase production in *Staphylococcus epidermidis* CMST Pi 2. Esakkiraj et al. [16] also reported that esterase production by *Bacillus altitudinis* was greatly enhanced by tuna wastes. Bapiraju et al. [17] reported that lipase production from *Rhizopus* sp. BTNT-2 was induced positively by olive oils and esterase production by *Trichodermareesei* was high in olive oil [18].

3.4. Purification and characterization of lipase from H. trueperi RSK CAS9

The summary of the purification profile was presented in Table 5. The overall purification fold of lipase was 8.46 with specific activity of 1193.59 U/mg and 14.31% of yield. The homogeneity of the purified enzyme was tested and confirmed through single band obtained in SDS-PAGE analysis. The molecular weight of the purified lipase was estimated as 44 kDa (Fig. 4) and it is different from other reported lipases *Staphylococcus* sp. (28.95 kDa) [19], *B. licheniformis* (35 kDa) [20], *Pseudomonas* sp. DMVR46 (32 kDa) [21] and *Burkholderiacepacia* ATCC 25416 (33 kDa) [22].

3.5. Substrate specificity

The lipase activity was higher when *p*NP-palmitate (119.21%) was used as substrate, followed by *p*NP-laurate (97.12%), *p*NP-stearate (90.31%), *p*NP-octanoate (83.2%), *p*NP-decanoate (74.03%), *p*NP-acetate (26.13), *p*NP-butyrate (17.4%) and *p*NP-valerate (7.4%) respectively. Similarly, lipase of *P. aeruginosa* AAU2 [23] and *P. aeruginosa* PseA [24] were also reported producing higher activity when *p*NP-palmitate used as substrate.

3.6. Effect of metal ions, inhibitors, surfactants and organic solvents on lipase activity

The effect of various metal ions on lipase activity was studied and the results were depicted in Table 6. The enzyme activity was much enhanced by Mg^{2+}, Ca^{2+} and Mn^{2+} and suppressed with Co^{2+},

Table 5
Summary of purification factors of lipase by *H. trueperi* RSK CAS9.

Purification step	Total lipase activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Culture filtrate	28,532.5	202.45	140.93	100	1.0
Ammonium sulfate precipitation	15,216.12	57.33	265.41	53.32	1.88
DEAE-cellulose	6,483.05	8.10	771.79	22.72	5.47
Sephadex G-75	4,082.10	3.42	1193.59	14.31	8.46

Table 6
Effects of metal ions, surfactants and organic solvents on stability of the purified lipase of *H. trueperi* RSK CAS9.

Metal ions	Concentrations (mM)	Relative activity (%)
$MgSO_4$	0.1	105.3 ± 2.2
		120.4 ± 1.8
$CaCl_2$	0.1	$121 \pm 2.2 \pm 1.6$
	1	142.3 ± 2.5
$FeSO_4$	0.1	75.3 ± 2.5
	1	63.6 ± 3.3
$MnSO_4$	0.1	116 ± 1.5
	1	130 ± 1.5
$CoCl_2$	0.1	70 ± 2.0
	1	54.3 ± 1.5
$HgCl_2$	0.1	43.2 ± 1.5
	1	24.1 ± 3.0
Detergents (%,v/v)		
Tween 80	1	111.03 ± 2.0
	10	100.13 ± 2.0
Triton X 100	1	116.02 ± 1.5
	10	102.10 ± 2.2
SDS	1	41.16 ± 2.5
	10	29.21 ± 3.0
Sodium deoxycholate	1	50.33 ± 3.5
	10	27.67 ± 2.1
Inhibitors		
PMSF	1	95.10 ± 2.2
	5	82.03 ± 2.5
EDTA	1	25 ± 1.8
	5	10.21 ± 3.0
β-Mercaptoethanol	1	88.16 ± 2.0
	5	76.12 ± 2.5
Organic solvents (25%, v/v)		
Iso-octane		107.12 ± 1.0
Methanol		71.22 ± 1.5
Benzene		52.04 ± 2.0
Toluene		13.03 ± 2.5
Hexane		90.16 ± 3.0
Butanol		4.20 ± 2.0
Chloroform		10.20 ± 1.8

$Hg^{2+}$and Fe^{2+} at both 0.1 and 1 mM concentrations. Earlier, stimulated lipase activity was reported with the effects of metal ions such as Ca^{2+}, Mg^{2+} and Ba^{2+} [24]. Rahman et al. [25] reported that the presence of Ca^{2+}, Ba^{2+} and Mg^{2+} increases lipase activity, this may be due to binding of the metal complex to the active site of the enzyme which leads to the conformational changes in protein.

The enzyme inhibitors such as PMSF and β-mercaptoethanol exhibited no significant effect on enzyme activity. But, the inhibition effect by metalloenzyme inhibitors EDTA was clearly evidenced that the purified enzyme of *H. trueperi* RSK CAS9 belongs to the family metallolipases. But, PMSF, a serine inhibitor has a marginal effect on lipase at 1 mM (95.10%) and 5 mM (82.03%) concentration exhibiting on relative lipase activity (Table 6). This may be revealed to the fact that the catalytic serine residue is inaccessible owing to the presence of the lid covering the active site which is a characteristic feature for most of the lipases [24]. The stability of the lipase with surfactants, the enzyme retained 116.02% and 111.03% of its original activity in the presence of 1% Triton X-100 and Tween 80 (non-ionic surfactant) whereas 41.16% and 50.33% with SDS and sodium deoxycholate (anionic surfactant), respectively (Table 6). Several lipases had enhanced lipolytic activity in the presence of Triton X-100 and Tween 80 [20]. Accordingly, non-ionic surfactants with low hydrophile–lipophile Balance value (TritonX-100:HLB13.5; Tween20: HLB 16.7 and Tween 80:HLB15) were less inimical on activity of lipase in comparison to SDS with a higher HLB of 40. Its unfavorable electrostatic interaction with the enzyme may cause unfolding and/or disrupt substrate binding, thus resulting in diminished enzyme activity [26] and so the stability of the purified lipase towards surfactants and commercial detergents could be certainly useful in detergent industry dealing with lipase.

The effects of different organic solvents on the stability of *H. trueperi* RSK CAS9 lipase are shown in Table 6. The enzyme was found to be quite stable and active in most of the organic solvents. The highest stability was present in *iso*-octane (107.12%), hexane (90.16%) and methanol (71.22%) whereas, other organic solvents were inhibiting the enzyme stability. Similarly, organic solvents such as *iso*-octane and hexane had stimulated effects on lipase activity have been reported previously [20]. When organic solvents such as toluene, butanol and chloroform were in addition to the purified lipase solution and the enzyme got drastically inactivated. The possible reason for this may be the incubation of enzyme in polar solvents (Log *p*-values < 3.0), remove water molecules necessary for its catalytic function which leads to decrease in activity of the enzyme. Reasonably high stability of *H. trueperi* RSK CAS9 lipase in organic solvents makes it potentially useful for practical application in many synthetic reactions in non-conventional media.

4. Conclusion

Based on the statistical optimization, maximum lipase production (1410.17 U/ml) was obtained when the medium supplemented with tuna powder, 14.58 g/l; olive oil, 5.05 ml/l; NaCl, 72.42 g/l; temperature, 45 °C and pH 9.0. Considering the production cost and reutilization of bioresources, lipase production by microbial reclamation of marine wastes seems to be promising approach. Moreover, the interesting properties of the purified lipase compatible with ionic, non-ionic and commercial detergents and organic solvents will make the strain as a potential candidate for applications in biotechnological processes and industrial applications.

Acknowledgments

The authors thank to the Dean and Director, CAS in Marine Biology and authorities of Annamalai University for providing the facilities. The authors are also grateful to Ministry of Environment, Forests and Climate Change (MoEn&F, Grant No.: 22-48/2010-CS-1 dt: 28/02/2013), Government of India, New Delhi for providing fund.

References

[1] C.H. Liu, W.B. Lu, J.S. Chang, A reviews on microbial lipases production, Process Biochem. 41 (2006) 1940–1944.
[2] H. Treichel, D.D. Oliveira, M.A. Mazutti, M.D. Luccio, J.V. Oliveira, A review on microbial lipases production, Food Bioprocess Tech. 31 (2010) 82–196.
[3] P. Villeneuveaa, J.M. Muderhwab, J. Graillea, M.J. Haese, Customizing lipases for biocatalysts a survey of chemical, physical and molecular biological approaches, J. Mol. Catal. B Enzyme 9 (2000) 113–148.
[4] M.S. Islam, S. Khan, M. Tanaka, Waste loading in shrimp and fish processing effluents: potential source of hazards to the coastal and near shore environments, Mar. Pollut. Bull. 49 (2004) 103–110.
[5] B.L. Liu, P.M. Kao, Y.M. Tzeng, K.C. Feng, A solvent stable metalloprotease produced by *Bacillus* sp. TKU004 and its application in the deproteinization of squid pen for b-chitin preparation, Enzyme Microb. Technol. 33 (2003) 410–415.
[6] R. Sathishkumar, G. Ananthan, C. Raghunathan, Production and characterization of haloalkaline protease from ascidian-associated *Virgibacillus halodenitrificans* RSKCAS1 using marine wastes, Ann. Microbiol. (2014) , doi:http://dx.doi.org/10.1007/s13213-014-0987-8.
[7] G.M. Garrity, D.R. Boone, R.W. Castenholz, Bergey's manual of systematic bacteriology, 2nd ed., Springer Verlag, New York, 2001.
[8] J. Marmur, A procedure for the isolation of deoxyribonucleic acid from microorganisms, Moll. Biol. J. 3 (1961) 208–218.
[9] N. Saitou, M. Nei, The neighbor-joining method: a new method for reconstructing phylogenetic trees, Mol. Biol. Evol. 4 (1987) 406–425.
[10] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 93 (1951) 265–275.

[11] K.N. Kilcawley, M.G. Wilkinson, P.F. Fox, Determination of key enzyme activities in commercial peptidase and lipase preparations from microbial or animal sources, Enzyme Microb. Technol. 31 (2002) 310–320.
[12] U.K. Laemmli, Cleavage of structural proteins during assembly of head of bacteriophage, Nature 22 (1970) 680–685.
[13] M.S. Tanyildizi, D. Ozer, M. Elibol, Optimization of a-amylase production by *Bacillus* sp. using response surface methodology, Process Biochem. 40 (2005) 2291–2996.
[14] M.A. Haider, K. Pakshirajan, Screening and optimization of media constituents for enhancing lipolytic activity by a soil microorganism using statistically designed experiments, Appl. Biochem. Biotechnol. 141 (2007) 377–390.
[15] W. Wang, T. Yuan, K. Wang, B. Cui, Y. Dai, Statistical optimization of cellulase production by the brown rot fungi, *Fomitopsis palustris*, and its application in the enzymatic hydrolysis of LHW pretreated woody biomass, Process Biochem. 47 (2012) 2552–2556.
[16] P. Esakkiraj, R. Usha, A. Palavesam, G. Immanuel, Solid-state production of esterase using fish processing wastes by *Bacillus altitudinis* AP-MSU, Food Bioprocess Technol. 90 (2012) 370–376.
[17] K.V.V.S.N. Bapiraju, P. Sujatha, P. Ellaiahi, T. Ramana, Sequential parametric optimization of lipase production by a mutant strain *Rhizopus* sp. BTNT-2, Basic Microbiol. 45 (2005) 257–273.
[18] H. Kontkanen, M. Saloheimo, J. Pere, A. Miettinen-Oinonen, Characterization of *Melanocarpus albomyces* steryl esterase produced in *Trichoderma reesei* and modification of fibre products with the enzyme, Appl. Microbiol. Biotechnol. 72 (2006) 696–704.
[19] L. Daoud, J. Kamoun, M. Bou Ali, R. Jallouli, R. Bradai, T. Mechichi, Y. Gargouri, Y. Ben Ali, A. Aloulou, Purification and biochemical characterization of a halotolerant *Staphylococcus* sp. extracellular lipase, Int. J. Biol. Macromol. 57 (2013) 232–237.
[20] N. Annamalai, M.V. Rajeswari, S. Vijayalakshmi, T. Balasubramanian, Purification and characterization of chitinase from *Alcaligenes faecalis* AU02 by utilizing marine wastes and its antioxidant activity, Ann. Microbiol. 61 (2011) 801–807.
[21] V. Patel, S. Nambiar, D. Madamwar, An extracellular solvent stable alkaline lipase from *Pseudomonas* sp. DMVR 46: partial purification, characterization and application in non-aqueous environment, Process Biochem. 49 (2014) 1673–1681.
[22] X. Wang, X. Yu, Y. Xu, Homologous expression: purification and characterization of a novel high-alkaline and thermal stable lipase from *Burkholderia cepacia* ATCC 25416, Enzyme Microb. Technol. 45 (2009) 94–102.
[23] A. Bose, H. Keharia, Production: characterization and applications of organic solvent tolerant lipase by *Pseudomonas aeruginosa* AAU2, Biocatal. Agric. Biotechnol. 2 (2013) 255–266.
[24] R. Gaur, A. Guptam, S.K. Khare, Purification and characterization of lipase from solvent tolerant *Pseudomonas aeruginosa* PseA, Process Biochem. 43 (2008) 1040–1046.
[25] R.N.Z.R.A. Rahman, S.N. Baharum, A.B. Salleh, M. Basri, Lipase: an organic solvent tolerant enzyme, J. Microbiol. 44 (2006) 583–590.
[26] D.E. Otzen, Protein unfolding in detergents: effect of micelle structure, ionic strength, pH, and temperature, J. Biophys. 83 (2002) 2219–2230.