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

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A B S T R A C T

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.

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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 trans-esterification 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
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%; KH₂PO₄, 0.1% and MgSO₄, 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'-GGGCGGTTGTGTTACAAGGC-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), K₂HPO₄, NaCl, MgSO₄ 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 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:

\[ Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i^2 + \sum \beta_{ij} X_i X_j \]

where Y is the predicted response, X_i and X_j are independent factors, \( \beta_0 \) is the intercept, \( \beta_i \) is the linear coefficient, \( \beta_{ij} \) is the quadratic coefficient, and \( \beta_{ij} \) 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.

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**Table 1**

Experimental variables at different levels used for the production of alkaline lipase by *H. truaperi* RSK CA59 using Plackett–Burman design.

| Symbol code | Variables                  | Levels | Effect | Coefficient | t-value | p-value |
|-------------|----------------------------|--------|--------|-------------|---------|---------|
|             |                            | +      |        |             |         |         |
| Constant    |                            | ×      |        |             |         |         |
| X₁          | Tuna powder (g/l)          | 10     | 1      | 203.36      | 329.24  | 0.003   |
| X₂          | Sardine powder (g/l)       | 10     | 1      | 15.18       | 101.68  | 0.117   |
| X₃          | Cuttlefish powder (g/l)    | 10     | 1      | –13.84      | 7.59    | 0.142   |
| X₄          | Shrimp powder (g/l)        | 10     | 1      | –31.54      | –6.92   | 0.015   |
| X₅          | Olive oil (ml/l)           | 1      | 0.1    | 48.24       | 24.12   | 0.045   |
| X₆          | NaCl (g/l)                 | 30     | 50     | 185.11      | 92.55   | 0.012   |
| X₇          | K₂HPO₄ (g/l)               | 5      | 0.1    | –16.18      | –8.09   | 0.134   |
| X₈          | MgSO₄ (g/l)                | 1      | 0.01   | 13.23       | 6.61    | 0.162   |
| X₉          | Temperature (°C)           | 6      | 10     | 67.06       | 33.53   | 0.033   |
| X₁₀         | pH                         | 30     | 40     | –1.19       | –0.59   | 0.34    |
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 2

| Run order | $X_1$ (g/l) | $X_2$ (g/l) | $X_3$ (g/l) | $X_4$ (g/l) | $X_5$ (g/l) | $X_6$ (g/l) | $X_7$ (g/l) | $X_8$ (g/l) | $X_{10}$ (g/l) | Lipase activity (U/ml) |
|-----------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|----------------|------------------------|
| 1         | +1          | −1          | +1          | −1          | −1          | +1          | +1          | +1          | −1          | 332.02                |
| 2         | +1          | +1          | −1          | +1          | −1          | +1          | +1          | +1          | −1          | 279.12                |
| 3         | −1          | +1          | +1          | +1          | −1          | −1          | −1          | −1          | +1          | 154.42                |
| 4         | +1          | −1          | −1          | +1          | −1          | −1          | −1          | −1          | +1          | 449.13                |
| 5         | +1          | +1          | −1          | +1          | −1          | −1          | −1          | −1          | +1          | 395.30                |
| 6         | +1          | +1          | +1          | +1          | +1          | +1          | +1          | +1          | +1          | 518.12                |
| 7         | −1          | +1          | +1          | +1          | +1          | +1          | +1          | +1          | −1          | 317.03                |
| 8         | −1          | −1          | −1          | +1          | −1          | −1          | −1          | −1          | +1          | 161.22                |
| 9         | −1          | −1          | −1          | −1          | +1          | −1          | −1          | −1          | +1          | 277.25                |
| 10        | +1          | −1          | −1          | −1          | +1          | −1          | −1          | −1          | +1          | 612.06                |
| 11        | −1          | +1          | −1          | −1          | −1          | −1          | −1          | −1          | +1          | 357.20                |
| 12        | −1          | −1          | −1          | −1          | −1          | −1          | −1          | −1          | −1          | 119.25                |

Table 3

| Run order | TP ($X_1$) (g/l) | Olive oil ($X_2$) (ml/l) | NaCl ($X_3$) (g/l) | Temperature ($X_4$) (°C) | Lipase activity (U/ml) |
|-----------|-----------------|--------------------------|-------------------|--------------------------|------------------------|
| 1         | 12.5            | 3.25                     | 60                | 42                       | 750.10                 |
| 2         | 17.5            | 3.25                     | 60                | 42                       | 627.27                 |
| 3         | 12.5            | 7.75                     | 60                | 42                       | 681.07                 |
| 4         | 12.5            | 7.75                     | 60                | 42                       | 767.20                 |
| 5         | 12.5            | 3.25                     | 80                | 42                       | 1072.16                |
| 6         | 17.5            | 3.25                     | 80                | 42                       | 810.20                 |
| 7         | 12.5            | 7.75                     | 80                | 42                       | 640.17                 |
| 8         | 17.5            | 7.75                     | 80                | 42                       | 582.10                 |
| 9         | 12.5            | 3.25                     | 60                | 47                       | 806.12                 |
| 10        | 17.5            | 3.25                     | 60                | 47                       | 711.46                 |
| 11        | 12.5            | 7.75                     | 60                | 47                       | 771.17                 |
| 12        | 17.5            | 7.75                     | 60                | 47                       | 890.22                 |
| 13        | 12.5            | 3.25                     | 80                | 47                       | 1035.10                |
| 14        | 17.5            | 3.25                     | 80                | 47                       | 809.12                 |
| 15        | 12.5            | 7.75                     | 80                | 47                       | 642.10                 |
| 16        | 17.5            | 7.75                     | 80                | 47                       | 619.91                 |
| 17        | 10.0            | 5.50                     | 70                | 45                       | 787.21                 |
| 18        | 20.0            | 5.50                     | 70                | 45                       | 641.23                 |
| 19        | 15.0            | 1.00                     | 70                | 45                       | 719.40                 |
| 20        | 15.0            | 10.00                    | 70                | 45                       | 461.17                 |
| 21        | 15.0            | 5.50                     | 50                | 45                       | 705.31                 |
| 22        | 15.0            | 5.50                     | 90                | 45                       | 752.19                 |
| 23        | 15.0            | 5.50                     | 70                | 40                       | 797.15                 |
| 24        | 15.0            | 5.50                     | 70                | 50                       | 1067.12                |
| 25        | 15.0            | 5.50                     | 70                | 45                       | 1346.27                |
| 26        | 15.0            | 5.50                     | 70                | 45                       | 1345.71                |
| 27        | 15.0            | 5.50                     | 70                | 45                       | 1351.02                |
| 28        | 15.0            | 5.50                     | 70                | 45                       | 1345.20                |
| 29        | 15.0            | 5.50                     | 70                | 45                       | 1350.32                |
| 30        | 15.0            | 5.50                     | 70                | 45                       | 1345.10                |
| 31        | 15.0            | 5.50                     | 70                | 45                       | 1345.22                |
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 detainted in 7% acetic acid.

2.11. Substrate specificity to various p-nitrophenyl esters

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

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

Effect of metal ions on lipase activity was investigated using Mg²⁺, Ca²⁺, Fe²⁺, Mn²⁺, Co²⁺ and Hg²⁺ at 0.1 and 1 mM concentration. The effect of enzyme inhibitors such as phenylmethysulfonyl fluoride (PMSF), ethylene diaminetetraacetic acid (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 R07. 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, K₂HPO₄, MgSO₄ 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.](image-url)
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

| 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  | 203344  | 203344  | 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 |

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).
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 *P.aea* was greatly enhanced by tuna wastes. Bapiraju et al. [17] reported that lipase production from *Rhizopus* sp. BNT-2 was induced positively by olive oils and esterase production by *Trichoderma reesei* 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. DMVR-46 (32 kDa) [21] and *Burkholderia cenocepacia* ATCC 25416 (33 kDa) [22].

#### 3.5. Substrate specificity

The lipase activity was higher when pNP-palmitate (119.21%) was used as substrate, followed by pNP-laurate (97.12%), pNP-stearate (90.31%), pNP-octanoate (83.2%), pNP-decanoate (74.03%), pNP-acetate (26.13%), pNP-butyrate (17.4%) and pNP-valerate (7.4%) respectively. Similarly, lipase of *P. aeruginosa* AAU2 [23] and *P. aeruginosa* PseA [24] were also reported producing higher activity when pNP-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 Mg²⁺, Ca²⁺ and Mn²⁺ and suppressed with Co²⁺.

### 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              |

![Fig. 3](image-url) Kinetics of bacterial cell growth and alkaline lipase production (CFU/ml) by optimized medium.

![Fig. 4](image-url) SDS-PAGE analysis of lipase from *H. trueperi* RSK CAS9. Lane 1: 116 kDa, β-galactosidase (E. coli); 97 kDa, phosphoryse 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.
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]. Rahaman 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 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 ºC 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.

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