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Two Step Purification of *Acinetobacter* sp. Lipase and Its Evaluation as a Detergent Additive at Low Temperatures

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Abstract *Acinetobacter* sp. lipase was purified to homogeneity by a two-step process. The crude enzyme (along with biomass) was subjected to partial purification by aqueous two phase system (ATPS), avoiding centrifugation and filtration steps. Conditions for lipase partitioning by ATPS were optimized by response surface methodology (RSM) and a combination of 29.45% polyethylene glycol 8000, 15.5% phosphate, and a pH of 7.0 resulted in an optimal partition coefficient. Partially pure lipase was further purified by a modified batch process using Octyl Sepharose CL-4B in a vacuum filtration apparatus. This two-step process resulted in a purified lipase with a yield of 74.6% having a specific activity of 88.8 U/mg of protein and a purification fold of 14.92. The homogeneity of the lipase preparation obtained by the purification process was confirmed by reversed phase high performance liquid chromatography profile. The molecular weight of the purified lipase was found to be around 32 kDa as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The purified lipase exhibited pH and temperature optima of 8.5 and 37°C, respectively. The lipase was active at low temperatures and it retained 86.8% activity at 10°C. It also displayed other features such as stability over a broad range of pH (3.0–9.0) as well as stability in the presence of hydrogen peroxide and commercial detergents. Based on these characteristics, the potential of this lipase as an additive in laundry detergent formulation was evaluated under low temperature wash conditions. The results indicated that *Acinetobacter* sp. lipase increased the washing efficiency of the detergent Nirma by 21–24% at 15°C–20°C, respectively.

Keywords ATPS · Lipase purification · Response surface methodology · Oxidant stable lipase · Detergent additive

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Introduction

Lipases are triacylglycerol hydrolases (E.C.3.1.1.3), which catalyze the hydrolysis of triglycerides to glycerol and free fatty acids. Purification of lipase is a prerequisite for characterization and also for identifying a suitable range of industrial applications. Among the chromatography methods adopted, ion exchange chromatography (IEC) is the most frequently employed for the purification of different lipases [1, 2]. Chromatographic techniques often result in poor yield [3] and hence, to increase the yield and also to reduce the number of steps, alternative purification strategies are employed viz aqueous two phase system (ATPS), reverse micelles and purification employing immobilized lipases [4]. Purification of various lipases using detergent-based aqueous two-phase system [5] as well as by polyethylene glycol (PEG)–salt ATPS has already been reported [6]. But, in this study, we report the purification of crude lipase (along with biomass) using PEG–salt ATPS.

ATPS has the advantage of handling crude broth directly and apart from purification, it also aids in the concentration of the partially purified lipase. Since ATPS by itself is not sufficient to purify proteins to homogeneity, usually, it is followed by chromatography to obtain homogeneous proteins [7].

The classical method of optimization involves varying one factor at a time and keeping the others constant. But, the method is inefficient as it fails to substantiate the interaction between the variables, while response surface methodology (RSM) is an effective statistical technique for the investigation of complex processes [8]. The main advantage of RSM is the ability to discern the interaction among the factors along with reduced number of experimental runs. Purification of proteins other than lipase by ATPS using RSM as the optimization tool is reported [9].

Hence, the objective of this study is to purify *Acinetobacter* sp. crude lipase by a simplified two-step process. In the first step, crude lipase (along with biomass) is partially purified by PEG–potassium phosphate ATPS using statistical design methods. In the second step, further purification to homogeneity is attempted by a modified batch mode of hydrophobic interaction chromatography (HIC) to increase the yield of purified lipase. In addition, the purified lipase is characterized to find out its suitability as a detergent additive under low temperature wash conditions.

Materials and Methods

Microorganism

Polyethylene glycol (PEG) 8000 and Octyl sepharose CL-4B were obtained from Sigma-Aldrich, CA, USA. All other chemicals were obtained from Merck India, Pvt Ltd and they were of analytical grade. *Acinetobacter* sp. MTCC 6816 used in this study was isolated from forest soil by enrichment technique followed by a novel soil lipase assay developed in our laboratory [10]. The culture was identified at Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. The strain was maintained in nutrient agar slants and it was subcultured at regular intervals. The seed inoculum was prepared by inoculating a loop full of culture from nutrient agar slant into Luria–Bertani broth and after 16 h of growth, 2.5% of this seed inoculum was introduced to the production medium.

Preparation of the Crude Enzyme Extract

The production medium contained 0.5% maltose, 1.5% cornsteep liquor, 1.5% sunflower oil, 1% disodium hydrogen phosphate, 0.1% potassium dihydrogen orthophosphate, 0.1% sodium chloride, 0.05% calcium chloride, 0.001% ferrous sulfate, and 0.002% zinc sulfate. The initial pH of the medium was adjusted to 6.5 with 0.1 M phosphate buffer. After inoculation, the production medium was incubated at 25°C and it was kept for shaking at 150 rpm in an orbital shaker. The enzyme was extracted after 48 h of incubation. After the incubation period, the culture broth was harvested by centrifuging at 8,000 rpm for 10 min and the supernatant, after filtration through 0.45 μ membrane filter, was used for determination of lipase activity and protein content. For ATPS studies, the culture broth was used directly without centrifugation.

Enzyme Assay and Protein Estimation

For the enzyme assay by titrimetry, the method of Ota and Yamada [11] was followed with slight modifications. The substrate consisted of olive oil emulsified with 2% gum arabic in a 1:3 ratio. The substrate (2.5 ml) was mixed with 2 ml of 0.1 M Tris buffer, pH 8.5 and after 10 min of preincubation at 37°C, 0.5 ml of the enzyme was added and the reaction was allowed to proceed for 20 min. The reaction was stopped using 10 ml of acetone. A control was maintained wherein, heat inactivated enzyme (0.5 ml) was added. After stopping the reaction, the liberated free fatty acids in both test and control were titrated against 0.05 N NaOH. Lipase activity is defined as micromole of free fatty acids liberated per milliliter per minute at 37°C. Protein content of the appropriately diluted samples was estimated by Bradford's method [12]. For protein estimation in samples containing PEG, blank was prepared using PEG and the absorbance was subsequently nullified.

Partitioning by ATPS

Optimization of parameters for the partitioning of lipase by ATPS was carried out by RSM. Before optimization by RSM, selection of factors influencing the partitioning of *Acinetobacter* sp. lipase was based on preliminary experimentation in which, three critical factors viz, concentration of PEG 8000 as well as phosphate and pH were selected. A five-level three factorial central composite rotatable design (CCRD) was employed in this study with an alpha value of ±1.689 requiring 20 experiments for the selected factors. The design consisted of eight factorial points, six axial points, and six center points. The variables and their levels selected for partial purification of lipase by ATPS were: (A) PEG concentration (8–24%), (B) phosphate concentration (11–20%), and (C) buffer pH (6.0–8.0). The relationship between the variation of the response, Y_c (partition coefficient of lipase{devoid of biomass}) and the variation of factors A, B, and C was represented by a second order mathematical model using the following equation:

$$
\begin{aligned}
Y_c = {} & \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + && \text{(Intercept and main effects)} \\
& \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + && \text{(Interactions)} \\
& \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 && \text{(Quadratic effects)}
\end{aligned}
$$

where, Y_c was the response calculated by the model and X_1, X_2, and X_3 were the coded variables corresponding to factors A, B, and C, respectively. The response was the partition

coefficient of enzyme and the value of the dependent response was the mean of three replications. The results were analyzed by analysis of variance (ANOVA) and multiple regression analysis to calculate the equation coefficients. Statistical analysis of the model was performed using the 'Design Expert' software package (Version 5.0, Stat-Ease Inc., and Minneapolis, USA).

Preparation of Two-phase System

PEG/potassium phosphate systems were investigated to study the partition behavior of lipase from *Acinetobacter* sp. For the RSM experiments, 10 g systems were prepared as per the design matrix, using stock solutions of PEG dissolved in deionised water and the phase forming salt dissolved in buffer of appropriate pH. All the experiments were performed in triplicates and the responses were mean of three experiments. All the systems had sodium chloride at a constant weight (0.2 g). Crude enzyme (along with biomass) was added to the system at a constant weight of 1.7 g. The remaining weight of the system was made up using deionised water. The systems were thoroughly mixed first and then centrifuged at 2,000×*g* at room temperature (28°C–32°C) for 10 min to expedite the phase separation. The centrifuged samples were allowed to stand for 30 min at room temperature (28°C–32°C). Then, the bottom phase was carefully pipetted out and weighed. Lipase activity in each phase was estimated by titrimetry and protein content was determined by the method of Bradford [12].

Under the optimized conditions, a scale up of the process was attempted using 30 g of the crude enzyme (along with biomass) in a separating funnel. To obtain homogeneous lipase with increased yield, a modified batch process using membrane filter holder apparatus (Tarsons product Pvt Ltd, India) was employed. A 0.45 μ membrane filter (Sartorius make) was placed in the membrane filter holder apparatus. Octyl sepharose CL-4B matrix pre-equilibrated with 1.5 M ammonium sulfate in 0.1 M Tris–HCl, pH 8.5 was poured on to the upper chamber of the membrane filtration apparatus. The upper PEG-rich phase containing the partially pure lipase obtained after ATPS was added to the upper chamber and it was allowed to bind to the matrix. Washing was performed by vacuum suction. Elution was performed by using step gradient of ammonium sulfate from 1.5 to 0 M in steps of 0.5 M and a second gradient of isopropyl alcohol from 10% to 80% *v*/*v*. Elution by vacuum suction was employed for complete recovery. Isopropyl alcohol was removed by dialysis against 0.05 M Tris buffer, pH 8.5. The dialyzed fractions were analyzed for enzyme activity and protein content. Purified protein was tested for homogeneity using reversed phase high performance liquid chromatography (RP-HPLC).

Analytical RP-HPLC

The culture filtrate (10 μl each; crude enzyme devoid of biomass), partially pure lipase obtained after ATPS and purified lipase obtained after batch process were separately diluted with 90 μl of 0.1% trifluoroacetic acid (HPLC grade). The diluted sample (15 μl) was then loaded onto a C18 column (HiQ Sil reversed phase C18 column, 10 μm particle size, 2.2 mm i.d., 25 cm length) and the elution was carried out as described by Rugani et al. [13].

The electrophoretic analysis of the purified lipase was carried out by sodium dodecyl sulfate -polyacrylamide gel electrophoresis (SDS-PAGE) [14] followed by silver staining [15].

Effect of pH on Enzyme Activity and Stability

To determine the optimum pH for the purified lipase, 2.5 ml aliquots of olive oil emulsion was suitably buffered with 2 ml of 0.1 M buffers [Glycine–HCl buffer (pH 2.0–3.0), citrate buffer (pH 3.0–6.0), phosphate buffer (pH 6.0–7.5), Tris–HCl buffer (pH 7.5–9.0), and carbonate–bicarbonate buffer (pH 9.0–10.0)]. Purified enzyme (0.5 ml) was then added and the mixture was incubated at 37°C for 20 min. Lipase activity was estimated by titrimetry.

For stability studies, purified lipase was incubated with equal volume of 0.1 M buffers of different pH (3.0–10.0) for 1 h, after which aliquots were withdrawn and the residual activity was determined by titrimetry.

Effect of Temperature on Enzyme Activity and Stability

To determine the optimum temperature of the purified lipase, 2.5 ml aliquots of olive oil emulsion and 2 ml of 0.1 M Tris–HCl buffer, pH 8.5 were mixed with 0.5 ml of purified enzyme and incubated at different temperatures (10°C–70°C) for 20 min. After the incubation period, lipase activity was determined by titrimetry.

The effect of temperature on lipase stability was determined by incubating the enzyme at various temperatures (10°C–70°C) with 0.1 M Tris–HCl buffer, pH 8.5 for 1 h after which, aliquots were withdrawn and the residual activity was determined by titrimetry.

Residual Enzyme Activities of Detergents

To select a suitable detergent for use with *Acinetobacter* sp. lipase, the residual enzyme activities viz, lipase, protease, and amylase, if any, present in commercially available detergents were tested to avoid interference of these enzymes with the added lipase [11, 16, 17].

Effect of Hydrogen Peroxide on Lipase Stability

To determine the ability of *Acinetobacter* sp. lipase to withstand strong oxidizing agents present in commercial detergents, purified lipase was incubated with equal volume of hydrogen peroxide (30% *w*/*v*) in 0.1 M Tris–HCl buffer, pH 8.5 such that the final concentration of hydrogen peroxide varied between 0.5–2.0%. After incubation at room temperature (28°C–32°C) for 1 h, aliquots were removed and the residual activity in each sample was estimated by the standard assay procedure and for control, enzyme sample was incubated with buffer at room temperature without hydrogen peroxide.

Effect of Detergents on Lipase Stability

To determine the stability of lipase in presence of different commercial and laboratory detergents, lipase was incubated with equal volume of detergent solution containing 7 mg/ml of respective detergent in 0.1 M Tris–HCl buffer, pH 8.5 for l h. Aliquots were removed after the incubation period and the residual activity in each sample was estimated by the standard assay procedure. For control, enzyme sample was incubated with buffer at room temperature without any detergent.

Evaluation of Lipase as a Detergent Additive

Preparation of soiled fabric, washing solutions viz, buffer (B), buffer–lipase (BL), buffer–detergent (BD), and buffer–detergent–lipase (BDL) extraction of oil and its determination were carried out as reported earlier [18]. To state briefly, ten pieces of the soiled fabric, each 5×10 cm^2 were put into the flask containing the washing solutions. The soiled fabrics were washed for 20 min at 20°C with shaking at 100 rpm using B/BL/BD/BDL. At the end of 20 min, the fabrics from different washes were removed and rinsed three times with 100 ml of water, each for a period of 2 min and then air-dried. The residual oil present in the fabric after washing was extracted using soxhlet apparatus and it was quantified as follows

$$\% \text{ Oil removal } = W_b - W_a / W_b \times 100$$

$$W_b = \text{Weight of olive oil before washing}$$

$$W_a = \text{Weight of olive oil after washing}$$

Effect of Detergent Concentration on the Removal of Olive Oil

For studying the efficacy of detergent formulation on the removal of olive oil from the soiled fabric, different concentrations of detergent Nirma were introduced into the washing solution from 0.25–1.0% with and without 100 U of *Acinetobacter* sp. lipase. Washing was done by the standard procedure and oil removal was quantified by the procedure mentioned earlier.

Effect of Lipase Concentration on the Removal of Olive Oil

To study the effect of lipase concentration on the removal of olive oil, *Acinetobacter* sp. lipase of various concentrations from 25 to 100 U were included in the formulation with Nirma (0.5%) and the oil removal was determined by the above mentioned procedure**.**

Results

The compositions of the various runs of the CCRD design matrix and the response viz. partition coefficient of *Acinetobacter* sp. lipase to the upper phase were shown in Table 1. The results implied that the partition coefficient varied from 0.5–21.4 indicating that the ATPS experiments resulted in almost negligible partitioning to complete partitioning of lipase to the upper phase. Optimal partition coefficient of 21.4 was obtained with the combination of PEG 29.45%, phosphate 15.5%, and pH 7.0.

Model Fitting

The independent and dependent variables were fitted to the second order model equation and examined in terms of goodness of fit. ANOVA was used to examine the adequacy of the fitted model. On the basis of ANOVA (Table 2), a second order model equation was established describing the partition coefficient of lipase as a function of PEG 8000, phosphate concentration, and pH. The ANOVA of quadratic regression model showed that the model was highly significant, as shown by Fisher's F test with a very low probability value ($P_{model} > F = 0.0001$). The pure error was also very low indicating good reproducibility

Table 1 Design matrix of CCRD in terms of actual and coded factors and responses.

Std. number	BLOCK	PEG (% *w*/*v*)	Phosphate (% *w*/*v*)	pH	Partition coefficient	
					Observed	Predicted
1	Block 1	8.00 (−1)	11.00 (−1)	6.00 (−1)	3.5	3.77
2	Block 1	24.00 (+1)	11.00 (−1)	8.00 (+1)	8	9.03
3	Block 1	8.00 (−1)	20.00 (+1)	8.00 (+1)	2.6	1.61
4	Block 1	24.00 (+1)	20.00 (+1)	6.00 (−1)	20	19.82
5	Block 1	16.00 (0)	15.50 (0)	7.00 (0)	5	3.61
6	Block 1	16.00 (0)	15.50 (0)	7.00 (0)	2.35	3.61
7	Block 2	8.00 (−1)	11.00 (−1)	8.00 (+1)	1.85	2.23
8	Block 2	24.00 (+1)	11.00 (−1)	6.00 (−1)	0.5	1.7
9	Block 2	8.00 (−1)	20.00 (+1)	6.00 (−1)	5	4.18
10	Block 2	24.00 (+1)	20.00 (+1)	8.00 (+1)	20	19.94
11	Block 2	16.00 (0)	15.50 (0)	7.00 (0)	3	2.07
12	Block 2	16.00 (0)	15.50 (0)	7.00 (0)	1.85	2.07
13	Block 3	2.55 (−α)	15.50 (0)	7.00 (0)	3.25	4.04
14	Block 3	29.45(+α)	15.50 (0)	7.00 (0)	21.4	20.31
15	Block 3	16.00 (0)	7.93 (−α)	7.00 (0)	1.8	0.19
16	Block 3	16.00 (0)	23.07 (+α)	7.00 (0)	11	12.31
17	Block 3	16.00 (0)	15.50 (0)	5.32 (−α)	1.16	0.98
18	Block 3	16.00 (0)	15.50 (0)	8.68 (+α)	2.5	2.38
19	Block 3	16.00 (0)	15.50 (0)	7.00 (0)	1.4	2.04
20	Block 3	16.00 (0)	15.50 (0)	7.00 (0)	1.8	2.04

of the experimental data. Based on the F test, the model was predictive since calculated F value was greater than the critical F value and in addition, the regression coefficient R^2 for the model was high (0.98) which showed that almost 98% of the variation could be accounted by the model. Hence, the model was suitable to represent the real relationship among the selected factors.

The coded model was used to generate response surfaces for the analysis of variable effects on selective partitioning of lipase. The final predictive equation in terms of coded factors was as follows:

$$\text{Partition coefficient} = 2.58 + 4.84 * \text{A} + 3.60 * \text{B} + 0.42 * \text{C} + 3.58 * \text{A}^2 + 1.49 * \text{B}^2 + 3.66 * \text{A} * \text{B} + 1.44 * \text{A} * \text{C}$$

Three-dimensional (3D) response surface plots were constructed according to the above-mentioned equation to illustrate the main and interactive effects of the independent

Table 2 ANOVA for the quadratic model for partition coefficient.

Source	Sum of squares	Degrees of freedom	Mean square	F value	Prob>F
Model	840.14	9	93.35	48.29	0.0001
Residual	15.47	8	1.93	–	–
Lack of fit	11.21	5	2.24	1.58	0.3746
Pure error	4.25	3	1.42	–	–
Total	864.46	19	–	–	–

R^2 0.98; CV 23.57%

variables on the dependent ones. They were drawn imposing constant values (i.e. the center points of the interval taken into consideration) to two of the independent variables of the CCRD.

Figure 1 depicted the 3D response surface plot showing the interactive effect of PEG 8000 and phosphate concentrations on the partition coefficient of lipase in ATPS at a pH of 7.0. To favor the selective partitioning of lipase to the upper phase, increase in phosphate concentration should be accompanied by a concomitant increase in PEG concentration. The synergetic effect of PEG and phosphate resulted in an optimal partition coefficient of 19.74.

The cumulative effect of pH and PEG 8000 on selective partitioning of lipase to the upper phase at a phosphate concentration of 15.5% was shown in Fig. 2. The response surface plot showed that increase in PEG 8000 concentration had a significant impact on partition coefficient irrespective of the change in pH. Hence, to obtain an increased partition coefficient, PEG 8000 had to be maintained at a concentration of 24%, phosphate at 15.5% concentration. and pH could be maintained at a midpoint value of pH 7.0.

Partitioning of culture broth (crude enzyme along with biomass) was performed under these optimized conditions to check the reliability of the model and the results showed that 21±2% increase in partition coefficient during the partitioning of lipase to the upper PEG rich phase was obtained. The predicted value for partition coefficient under these optimized conditions was 20.31 (Table 1). The predicted value was in reasonable agreement with the observed value (21.4) along with the statistical significance of the model as depicted by ANOVA (Table 2). Hence, the present model could be deemed suitable. Thus, by using RSM with a minimal number of experimental runs, a maximal increase in partition coefficient was observed.

The preliminary RSM optimization studies were carried out with 10 g system in a centrifuge tube, suitable for standardization of a process and the efficacy of the system could be evaluated only when the process was suitable to handle greater enzyme volumes. Hence, under the optimized conditions, ATPS was performed in a separating funnel using

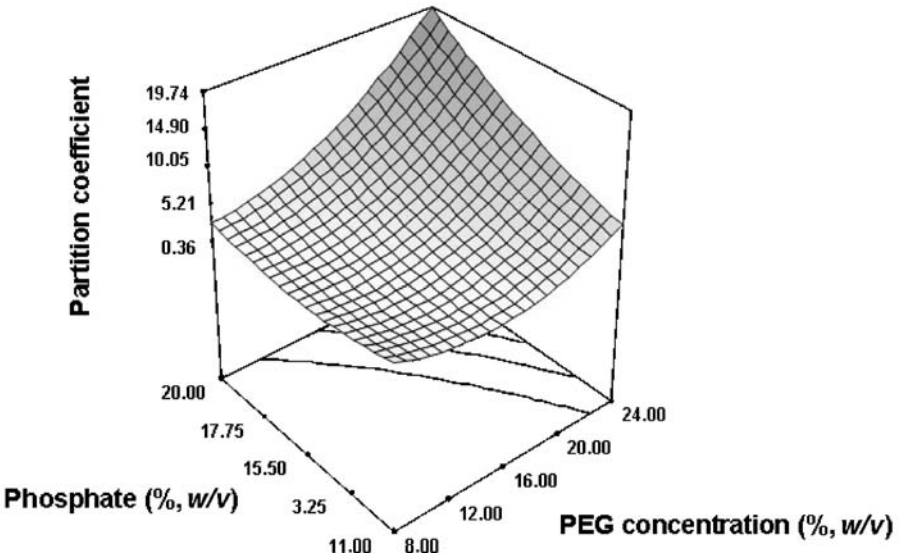

Fig. 1 Response surface plot showing the effect of phosphate and PEG on the partitioning of *Acinetobacter* sp. lipase

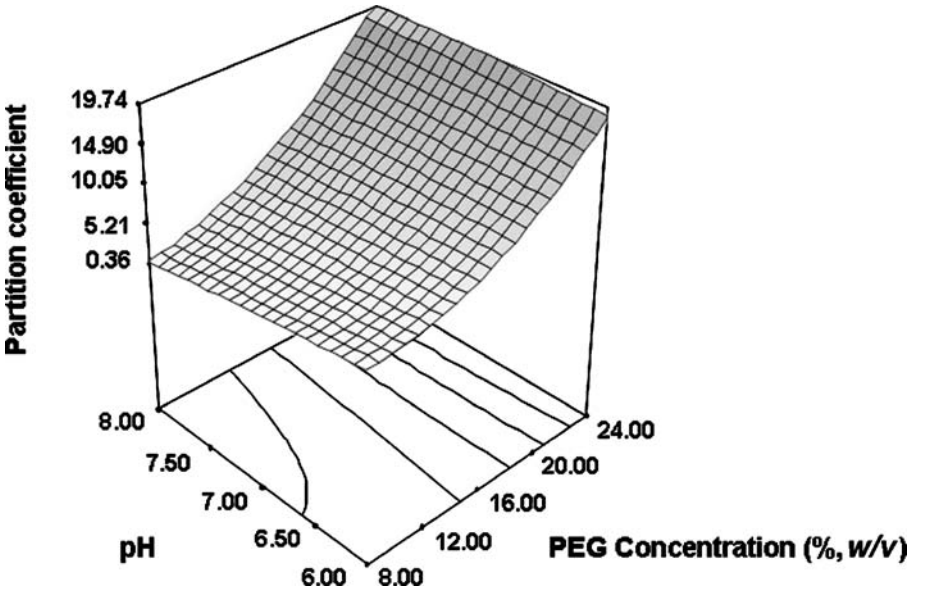

Fig. 2 Response surface plot showing the effect of PEG and pH on the partitioning of *Acinetobacter* sp. lipase

30 g of crude enzyme (along with biomass). The results of this study (Table 3) showed that the system was reproducible even when larger enzyme volumes along with biomass were employed. Along with a yield of 88%, the ATPS system resulted in almost a sevenfold increase in specific activity and an equivalent increase in purification fold.

By the use of ATPS, under these optimized conditions, the biomass partitioned preferentially to the interphase, most of the unwanted proteins partitioned to the lower phase and partially pure lipase got enriched in the upper PEG rich phase as evidenced by the high partition coefficient of 21.4 and by RP-HPLC profile (Fig. 3).

Modified Batch Process by HIC

To increase the yield of purified enzyme, a modified batch process was performed in a membrane filtration apparatus as described in "Materials and Methods". By this mode,

Table 3 Purification profile of lipase from *Acinetobacter* sp.

Fraction	Activity U[a]	Vol (ml)	Total activity	Protein mg/ml	Specific activity	Yield %	Purification fold
Crude	25	30	750	4.2	5.9	100	1.0
ATPS[b]	60	11	660	1.4	42.2	88	7.1
Modified batch process by HIC	80	7	560	0.9	88.8	74.6	14.92

[a] Lipase activity U = μmole of free fatty acids released per milliliter per minute

[b] ATPS was performed in a separating funnel with 30 g of crude enzyme (along with biomass)

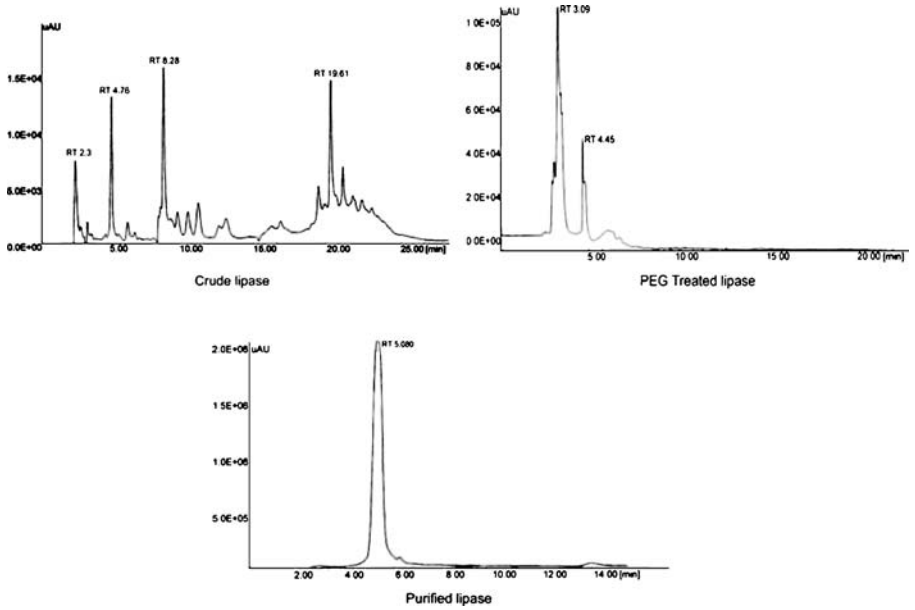

Fig. 3 RP-HPLC profile of culture supernatant, partially pure (ATPS upper phase) and purified lipase

enzyme sample up to 10 ml volume obtained from the upper PEG rich phase after ATPS extraction, was added to the octyl sepharose CL-4B HIC matrix and the bound lipase was found to elute with 60% isopropyl alcohol. The eluted portion was immediately dialyzed against 0.05 M Tris buffer, pH 8.5 to remove the solvent, and after lyophilization, the sample was resuspended in 0.1 M Tris buffer, pH 8.5 and checked for activity. The modified batch process resulted in a yield of 74.6%, purification fold of 14.92 and a specific activity of 88.8 (Table 3).

The purity of this fraction was also confirmed by RP-HPLC (Fig. 3). Upon comparison of the RP-HPLC profile of crude and ATPS-treated sample, it was shown that the majority of peaks found in the crude enzyme were absent in the PEG-rich partially purified enzyme wherein, only two major peaks were present, thereby confirming the ability of ATPS to partially purify the lipase with high selectivity. The chromatogram of the sample obtained after batch process by HIC showed a single peak with a retention time of 5.08 min confirming the homogeneity of the purified lipase. The molecular weight of the purified lipase was approximately 32 kDa as evidenced by SDS-PAGE analysis (Fig. 4).

Effect of pH on Lipase Activity and Stability

Acinetobacter sp. lipase was found to possess alkaline pH optima of 8.5 and the results presented in Fig. 5 showed that the enzyme was found to be highly stable between pH 3.0–9.0 and at pH 10.0, 65% of the activity was retained.

Effect of Temperature on Lipase Activity and Stability

The purified lipase was found to exhibit temperature optima of 37°C. The activity profile of purified lipase at various temperatures (Fig. 6) indicated that the enzyme retained 86.8% of

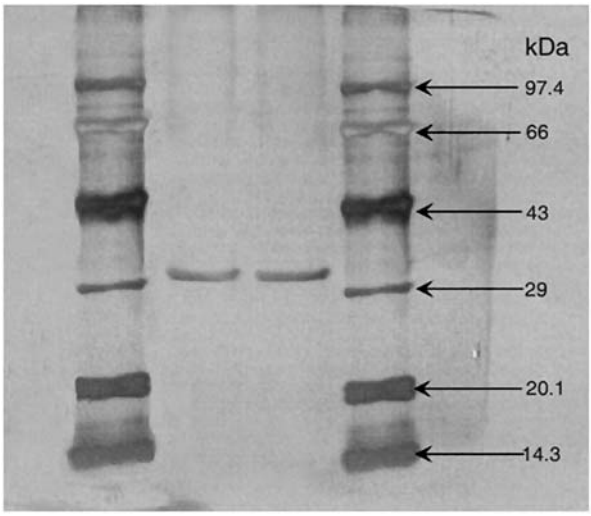

Fig. 4 SDS-PAGE showing *Acinetobacter* sp. lipase purified by modified batch process. *Lanes 1* and *4* molecular weight markers. *Lanes 2* and *3* purified lipase obtained by modified batch process

activity at 10°C, whereas, at 20°C, up to 89.4% of activity was maintained indicating the efficacy of this lipase to function effectively at lower temperatures.

When the enzyme was incubated with 0.1 M Tris buffer, pH 8.5 for 1 h at different temperatures, it was found to be stable up to 50°C. Even at low temperatures (10°C), the enzyme was found to retain the original activity indicating its low temperature stability (Fig. 6).

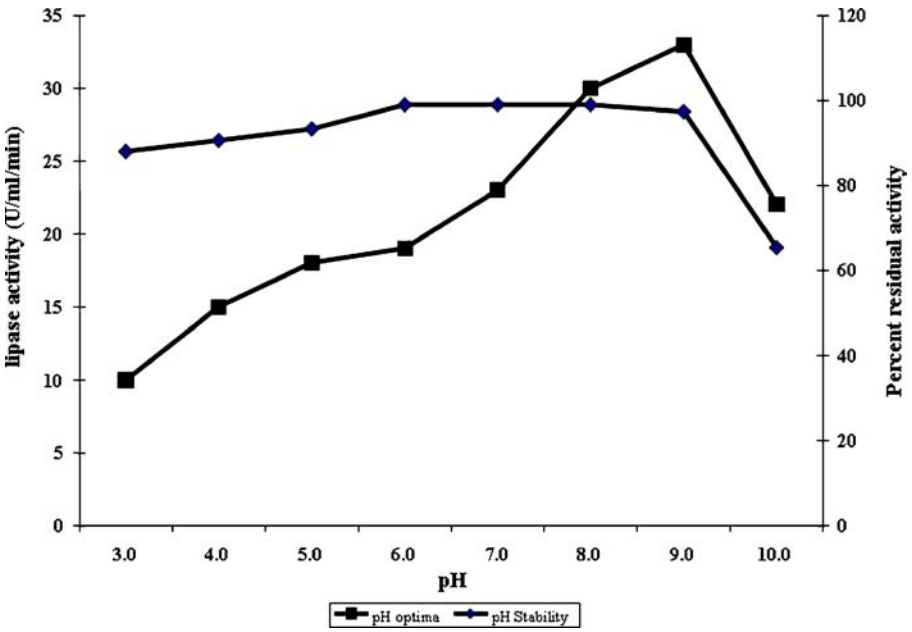

Fig. 5 Effect of pH on the activity and stability of lipase from *Acinetobacter* sp

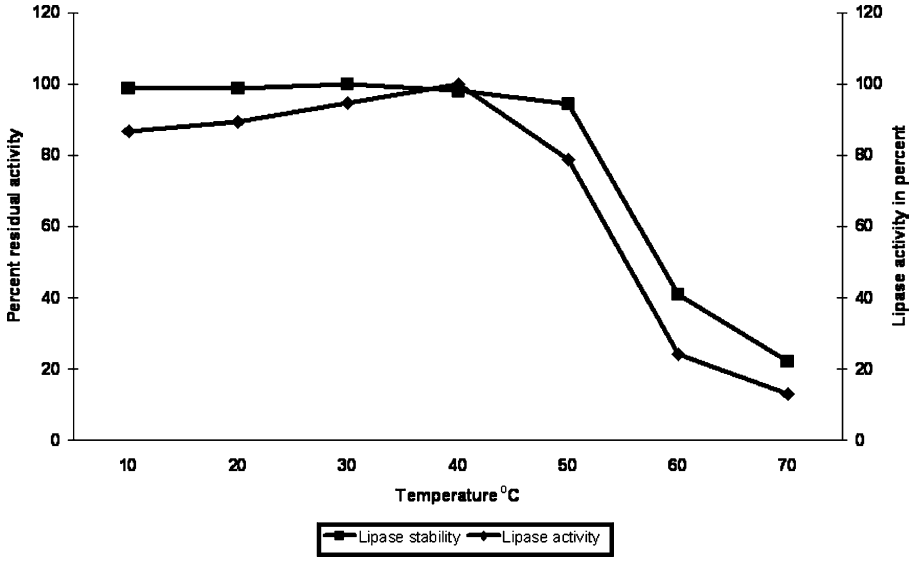

Fig. 6 Effect of temperature on the activity and stability of lipase from *Acinetobacter* sp

Effect of Detergents on Lipase Stability

It could be seen from Fig. 7 that *Acinetobacter* sp. lipase retained its stability when the enzyme was incubated with different commercial detergents for 1 h and maximal stability with enhancement in activity was observed in the presence of detergent Nirma. When the

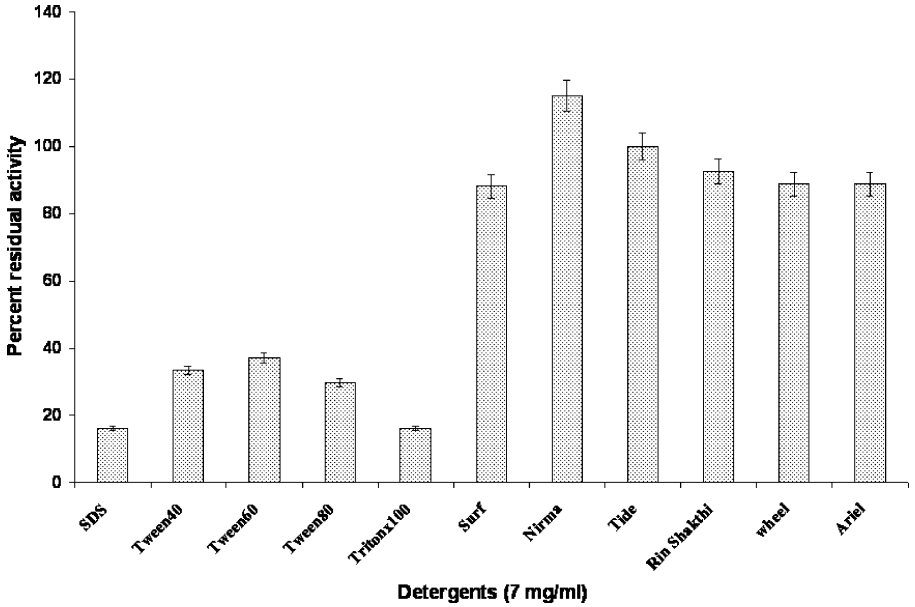

Fig. 7 Effect of detergents on lipase stability

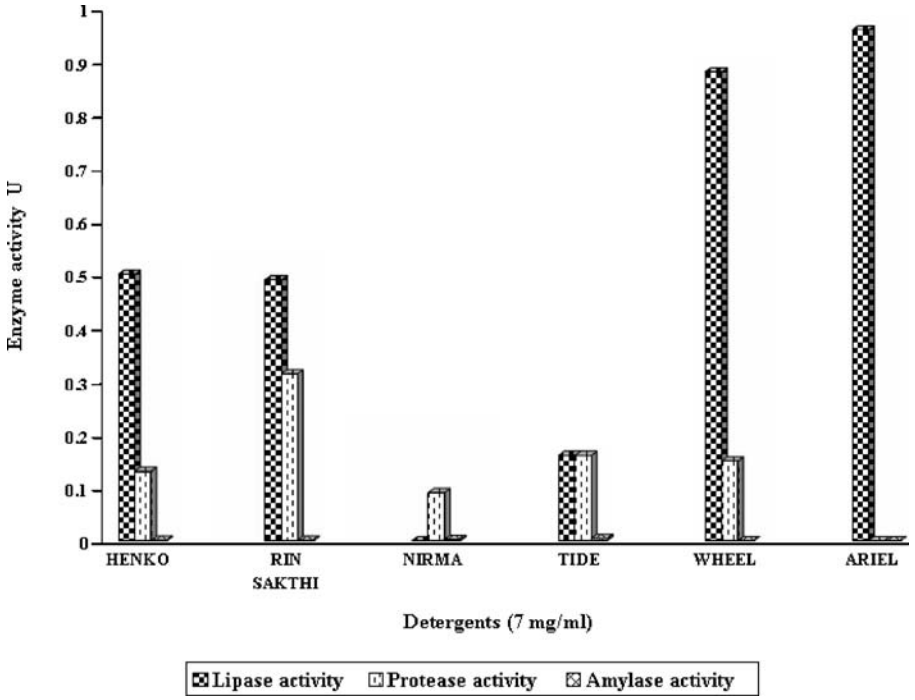

Fig. 8 Residual enzymatic activities of laundry detergent formulations. Lipase activity 1 U = micromole of free fatty acids liberated per milliliter per minute. Protease activity 1 U = milligram of tyrosine liberated per milliliter per 10 min. Amylase activity 1 U = milligram of maltose released per milliliter per minute

enzyme was incubated in presence of laboratory detergents, a significant reduction in enzymatic activity was observed, specifically with SDS and Triton X-100.

Residual Enzymatic Activities of Commercially Available Detergents

The results from Fig. 8 showed that minimal lipase activity was observed with Wheel, Ariel, and Henko. Since Nirma was devoid of lipase activity, it was worthwhile to evaluate *Acinetobacter* sp. lipase as an additive to Nirma.

Effect of Hydrogen Peroxide on Lipase Stability

The results (Table 4) showed that the lipase maintained significant stability over the entire concentration range of hydrogen peroxide tested (0.5–2.0%) and at the highest

Table 4 Stability of lipase in presence of hydrogen peroxide.

S. number	Hydrogen peroxide concentration (%)	Residual lipase activity (%)
1	0.5	96
2	1.0	92.5
3	1.5	90
4	2.0	84

Table 5 Effect of detergent concentration on percent oil removal from the soiled fabric.

S. number	Detergent concentration (Nirma) (%)	Percent oil removal	
		B + N	B + N + L
1	0	5	14
2	0.25	18	34
3	0.5	42	63
4	0.75	43	65
5	1.0	43	67

B + *N* buffer + Nirma, *B* + *N* + *L* buffer + Nirma + lipase

concentration (2%) of hydrogen peroxide tested, 84% stability was observed when compared to control.

Among the commercial detergents evaluated in this study, Nirma, by its ability to activate and stabilize the purified lipase, apart from its lack of residual lipase activity, was chosen for further work.

Effect of Detergent Concentration on the Removal of Oil with Lipase

The effect of different concentrations of detergent Nirma in presence and absence of 100 U of lipase for the removal of oil from the soiled fabric was shown in Table 5. Among the concentration range employed in this study, a detergent concentration of 0.5–1.0% was found to be effective.

Effect of Lipase Concentration on the Removal of Olive Oil

To study the effect of lipase concentration on the removal of olive oil, *Acinetobacter* sp. lipase at concentrations of 25 to 100 U was included along with 0.5% detergent Nirma and a lipase concentration of 100 U was found to be optimal for oil removal (Fig. 9).

The efficacy of *Acinetobacter* sp. lipase for the removal of oil from soiled fabric was evaluated by using it as an additive with detergent Nirma. Table 6 demonstrated that use of lipase increased the washing efficiency of Nirma by 21–24% under low temperature wash conditions (15°C–20°C). At 30°C, an optimal oil removal of 27% from the soiled fabric was observed over the wash by detergent alone.

Discussion

Partitioning of a solute in ATPS was governed by differences between solute–solvent interactions in the coexisting phases. While separating a compound from a mixture, besides interaction of the compound with the phases, there will be interaction between different components of a mixture. In case of proteins, they might form aggregates changing the partition behavior [19].

Bompensieri et al. [20] tried various purification protocols for *Acinetobacter calcoaceticus* lipase including HIC chromatography, PEG–salt ATPS, and Triton X-114 detergent-based ATPS. It was found that Triton X-114 detergent-based ATPS resulted in a maximal purification fold of 68 and a yield of 81%. Terstappen et al. [5] reported partitioning of various prokaryotic and eukaryotic lipases in detergent based aqueous two-

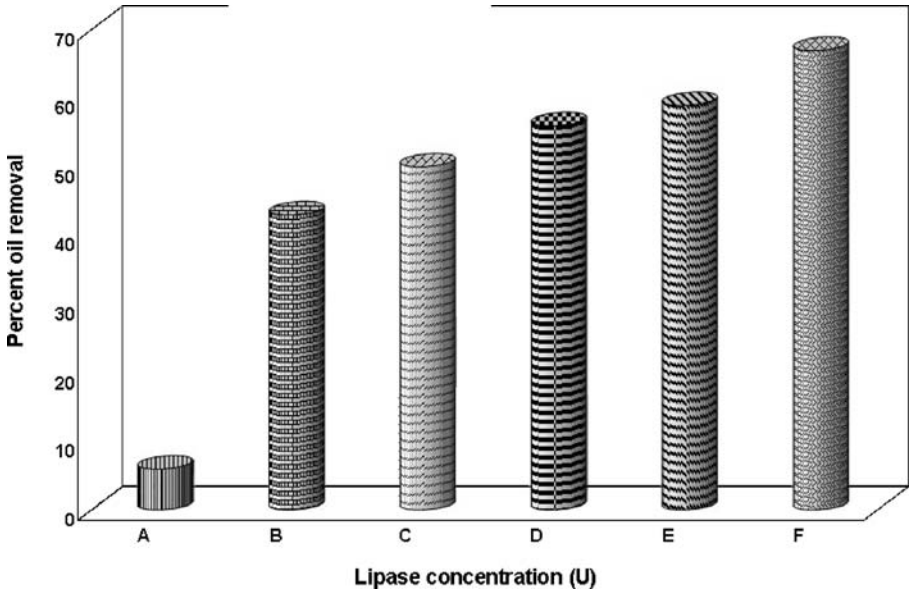

Fig. 9 Effect of lipase concentration on the percent oil removal from the soiled fabric. *A* Buffer wash; *B* buffer + Nirma wash; *C* buffer + Nirma + lipase 25 U wash; *D* buffer + Nirma + lipase 50 U wash; *E* buffer + Nirma + lipase 75 U wash; *F* buffer + Nirma + lipase 100 U wash

phase system and observed that prokaryotic lipases partitioned to the detergent rich phase. In both these reports, the lipase preferred the hydrophobic PEG-rich phase illustrating the hydrophobic nature of various lipases.

In spite of existence of several models for evaluating the partitioning of proteins in PEG–salt ATPS [21, 22], predicting the partition coefficient of proteins remained elusive due to the complex nature of interrelated interactions present in the system. Therefore, development of separation processes using aqueous two-phase systems relied upon extensive empirical experimentation, which could be significantly simplified by using factorial experimental design methods [23]. In this study, a three-factorial central composite design was employed for the purification of *Acinetobacter* sp. lipase by PEG 8000–potassium phosphate ATPS. Zhang et al. [24] reported purification and stabilization of ricin B using sodium sulfate ATPS, using response surface analysis. These studies reiterated the potential importance of statistical design experiments in optimizing the conditions for ATPS.

Table 6 Effect of lipase as a detergent additive at different temperatures on percent oil removal.

Detergent	Wash temperature (°C)	% oil removed			
		B	BL	BD	BDL
Nirma	15	5	18	40	61
	20	6	20	43	67
	25	6	21	43	69
	30	6	23	43	70

Wash conditions 0.1 M Tris–HCl buffer pH 8.5; detergent 0.5%; lipase 100 U; time of wash 20 min

B Buffer, *BL* buffer + lipase, *BD* buffer + detergent, *BDL* buffer + detergent + lipase

By the use of ATPS under these optimized conditions, the biomass partitioned preferentially to the interphase, most of the unwanted proteins partitioned to the lower phase and partially pure lipase got enriched to the upper PEG rich phase as evidenced by RP-HPLC profile and partition coefficient. The greatest advantage of this system lay in its ability to handle the culture broth (crude enzyme along with biomass) directly thereby paving way for reduction in the number of processing steps such as centrifugation, filtration, etc. Hence, while processing the enzyme in a large scale, the avoidance of centrifugation and filtration steps would make this process cost effective and economically viable.

To increase the yield of pure lipase, a modified batch mode of purification was employed using octyl sepharose CL-4B matrix in a filtration apparatus. This design resulted in the optimal purification of lipase devoid of matrix particles by the application of vacuum. Hiol et al. [25] partially purified lipase from *Rhizopus oryzae* in a conventional batch process by mixing the dialyzed solution with Q-sepharose equilibrated in buffer. Batch mode of purification usually resulted in partial purification with limited recovery. But, in this study, this modified batch process resulted in a homogeneous lipase along with the following advantages viz, (1) handling large sample volumes, (2) greater yield with enhanced purity, and (3) lack of matrix contamination during elution due to membrane-based vacuum-aided separation of eluant.

Results from the batch mode of purification (Table 3) showed that this process was highly effective in terms of yield and purification fold. The homogeneity of lipase was confirmed by RP-HPLC chromatogram (Fig. 3) as well as by SDS-PAGE (Fig. 4). The variation in the retention time observed for the PEG treated lipase when compared to crude and purified lipase could be due to the hydrophobic nature of PEG, which interfered with the effective binding of lipase to the C18 matrix thereby altering the retention time.

The cold active lipase from psychrophilic *Acinetobacter* sp. strain no. 6 was shown to retain 40% of its activity at 4°C [26]. Recently, cold active lipase and esterase from metagenomic and bacterial sources were also reported [27, 28] but reports on mesophilic microbes, with enzyme activity at lower temperatures, are relatively scarce. The lipase used in the present study retained 86.8% of activity at 10°C indicating its suitability for use at lower temperatures. It was obtained from mesophilic *Acinetobacter* sp. and hence, it was unique in nature similar to the mycelium bound lipases obtained from *Aspergillus niger* [29].

Laundry detergents consisted of a mixture of granular materials including surfactants, builders, bleaching agents, and enzymes. The surfactants were the main cleansing agents, while the builders provided alkalinity and ionic strength to the wash liquor. Bleaching agents were added to provide a white shine and to remove stains on the fabric upon oxidation. The characteristics of an ideal detergent enzyme were broad substrate specificity, stability at high pH and temperature, ability to withstand oxidizing and chelating agents, and optimal functioning at low concentrations [30].

Commercial detergents contained bleaching agents such as sodium percarbonate (SPC) and it liberated hydrogen peroxide upon solubilization which was shown to oxidize methionine and tyrosine residues of enzymes thereby inactivating them [31]. Hence, it was thought worthwhile to examine the lipase stability in presence of hydrogen peroxide. In this study, the observation of purified lipase being stable in the presence of hydrogen peroxide was in agreement with an earlier report on lipase from *Burkholderia cepacia* [32].

Acinetobacter sp. lipase demonstrated activation in the presence of commercial detergents such as Nirma and Tide. Probably, these detergents tended to disaggregate the lipase, which might lead to a possible enhancement in enzyme activity. Similar results were

observed for activation of cathepsin by detergents [33]. Activation of lipase in the presence of detergents was also observed for lipase from *Ralstonia pickettii* [34].

It was reported that electrostatic interactions between anionic detergents stabilized the open lid conformation of lipases, which could favor the overall activity of the enzyme. However, zwitterionic micelles were shown not to favor the open lid conformation. Hence, a detergent powder needed to balance these two properties to preserve the activity of lipases [35].

SDS, a laboratory anionic detergent, significantly affected the stability of this enzyme (Fig. 7). Inactivation of lipase by SDS could be due to the general phenomenon of denaturing effect of monomeric surfactant above critical micellar concentrations which caused proteins to unfold via electrostatic repulsion and hydrophobic interactions [36].

Earlier, we reported the use of *A. niger* lipase as an additive in laundry detergent formulations which performed well at warm wash conditions [19]. But, from the perspective of energy expenditure especially in colder regions, enzymes active at lower temperatures could be advantageous.

Acinetobacter sp. lipase displayed optimal washing efficiency (21–24%) at low concentrations as well as efficient oil removal at lower temperatures (15°C–20°C). By virtue of these remarkable properties, *Acinetobacter* sp. lipase could be effectively employed as an additive in laundry detergent formulations under low temperature wash conditions.

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