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PARTIAL PURIFICATION OF COLD ACTIVE LIPASE FROM *PSEUDOMONAS* SP. VITCLP4 ISOLATED FROM MARINE SAMPLES OF TAMILNADU COAST

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ABSTRACT

An extracellular cold active lipase from *Pseudomonas* sp. VITCLP4 was concentrated by two methods, ammonium sulphate precipitation and ultrafiltration. Ammonium sulphate precipitation with 40% saturation was found to give good results with 125-fold partially purified lipase and yield of 63%. Further purification by DEAE-Sephadex ion exchange chromatography resulted in 4.6-fold purified enzyme with yield of just 0.1%. This may be due to aggregation of protein during elution which is not reversible even after salt exchange. Therefore the enzyme could be partially purified with ammonium sulphate precipitation. Stability studies revealed that ammonium sulphate precipitate was 100% stable at -20°C for 10 days than other fractions. The molecular mass of cold active lipase was estimated to be approximately 52 kDa by SDS-PAGE.

KEYWORDS: **Cold active lipase, *Pseudomonas* sp. Partial Purification and Stability.**

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INTRODUCTION

Lipases (EC 3.1.1.3: Triacyl glycerol acyl hydrolases) are hydrolytic enzymes capable of hydrolyzing and synthesizing fatty acid esters. Cold adapted lipases exhibit high specific activity in the temperature range of 0-30°C[1]. Industrial applications of cold active lipases are based on their high activity at low temperatures and they are used in detergent, leather, food and pharmaceutical industries, fine chemical synthesis and bioremediation[2]. Cold active lipases are mostly obtained from psychrotrophic and psychrophilic microorganisms isolated from Antarctic and Polar Regions or from deep sea environments[3,4&5] and they are unstable at high temperatures. Therefore they may not be used in industrial applications which require high working temperatures[2]. Thermal stability may be improved by immobilization, directed evolution, protein engineering and chemical modification by adding polysaccharides[6, 7&8]. *Geotrichum* sp., a mesophilic yeast isolated from subtropical region was reported to produce two cold active lipases stable at room temperature[9]. In our previous report, we described the isolation of a novel strain, *Pseudomonas* sp. VITCLP4 producing cold active lipase from tropical part of India. In the other studies, the cold active lipase producing strains were isolated from alpine parts of India[10, 11&12]. The enzyme from *Pseudomonas* sp. VITCLP4 was alkaline and exhibited maximum thermal stability at 40°C for 4 h which is higher than cold active lipases from other psychrotrophs and comparable with those from mesophilic yeast, *Geotrichum* sp. SYBC WU-3 [13]. Present paper deals with purification of lipase from *Pseudomonas* sp. VITCLP4 and stability of the enzyme at various stages of purification to ascertain its suitability for industrial applications.

MATERIALS AND METHODS

(1) Materials

p- nitrophenyl palmitate (*p*-NPP), DEAE Sephadex, cellulose tubing were purchased from Sigma Chemicals. Minimate tangential flow filtration (TFF) system was purchased from Pall Corporation. Protein molecular weight marker was obtained from Genei, Bangalore. Olive oil and other culture media ingredients were from Hi-Media, Mumbai. All other chemicals used were of analytical grade.

(2) Organism

Pseudomonas sp. VITCLP4 was isolated from marine samples collected from Tamilnadu coast. The isolation was done by enrichment method in the medium containing olive oil followed by screening for lipolytic activity on tributyrin agar and rhodamine-B-olive oil agar. The strain was identified as *Pseudomonas* sp. closely related to *Pseudomonas taiwenensis* based on biochemical and physiological characteristics by Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India. As revealed by NCBI BLASTn analysis, the 16S rRNA gene sequence of the organism exhibited 97% sequence homology with *Pseudomonas aeruginosa*. Phylogenetic tree displayed the isolate in Pseudomonas cluster of Gammaproteobacteria.

(3) Lipase production

Production medium for lipase contained peptone -5g, yeast extract-2.5g, sodium chloride-0.5g, sodium hydrogen phosphate-3g, disodium hydrogen phosphate-4g, olive oil-10 ml in 1000 ml of distilled water at pH 7.2. 30 ml of the medium was taken in a 100 ml conical flask, inoculated with 10% inoculum and incubated at 25°C in an orbital shaker (180 rpm) for 4 days. The supernatant was obtained by centrifugation at 8000 rpm for 15 min at 4°C and used as crude extract for lipase purification.

(4) Lipase assay

Lipase activity was determined by spectrophotometric assay using *p*-NPP as substrate[14]. The lipase assay conditions were fixed as, pH 8, temperature-25°C and time-10 min. *p*-nitrophenol (*p*-NP) released was quantified spectrophotometrically at 410 nm. One unit of lipase activity was defined as

amount of enzyme extract required to release one μ mol of *p*-NP ml^{-1} min^{-1}.

(5) Protein estimation

Protein was estimated by the method described by Lowry et al[15].

(6) Ammonium sulphate precipitation

Ammonium sulphate precipitation was performed according to the chart mentioned in Scopes RK, Protein Purification: Principles and Practice[16]. 30 ml of cell free extract was treated with 20, 30, 40, 50, 60, 70 and 80% saturation of ammonium sulphate. Dialysis of the precipitate was carried out using Sigma cellulose tubing (mol. wt. cutoff: 12-14 kDa) for 24 h at 4°C with 3-4 changes of buffer. For each saturation percentage, lipase activity and specific activity were calculated.

(7) Ultrafiltration

One liter of cell free extract was concentrated to 10 ml in Minimate TFF system fitted with cellulose membrane cassette of 30 kDa mol. wt. cutoff.

(8) Ion exchange chromatography

Isoelectric point of lipase produced by *Pseudomonas* sp. VITCLP4 was not known and determining the Isoelectric point by Isoelectric focusing was not possible as the cell free extract was impure. Therefore, a survey of pH conditions was conducted as per the protocol mentioned in Scopes RK, Protein Purification: Principles and Practice[17]. pH 7.5 was found out to be the optimum for the enzyme in DEAE-Sephadex ion exchanger. To perform ion exchange chromatography, the dialyzed sample (5 ml) was applied to a column of DEAE-Sephadex (20 cm X 1 cm) pre equilibrated with 0.01 M Tris-HCl buffer (pH 7.5). The column was washed with same buffer at the flow rate of 60 ml h^{-1}. The bound protein fractions were eluted with a linear gradient of 0.02-1.0 M NaCl at the same flow rate. Lipase active fractions were pooled, dialyzed, concentrated by ultrafitration and stored at -20°C.

(9) Storage stability studies

Storage stability of lipase produced by *Pseudomonas* sp.VITCLP4 at different stages of purification was undertaken. Cell free extract, ammonium sulphate precipitate, retentate and pooled fraction after ion exchange chromatography were stored at 25, 4 and -20°C in 0.05 M sodium phosphate buffer (pH 8) for up to 10 days. Samples were withdrawn at 2 days time intervals and residual lipase activity was measured under standard assay conditions.

(10) SDS-Polyacrylamide gel electrophoresis

To determine the molecular weight of lipase, SDS-PAGE was performed in a 12% polyacrylamide gel slab as described by Laemmli UK[18]. A mixture of Phosphorylase-B (97.4 kDa), Bovine serum albumin (66.0 kDa), Ovalbumin (43.0 kDa), Carbonic anhydrase (29.0 kDa), Soyabean trypsin inhibitor (20.1 kDa) and Lysozyme (14.3 kDa) was used as reference. At the end of the electrophoresis the gel was stained with Coomassie brilliant blue.

(11) Experimental statistics

All the experiments were done twice. The enzyme assay was done in triplicates and repeated twice and best of all was considered for representation. The values in the table and graph represent arithmetic mean and standard deviation. The standard error was within experimental limits. Relative lipase activity was calculated by considering maximum as 100%.

RESULTS AND DISCUSSION

(1) Partial purification of lipase

Cell free extract was concentrated by ammonium sulphate precipitation and ultrafiltration. The results are shown in Table 1. Ammonium sulphate precipitation with 40% saturation and ultrafiltration yielded 125 and1.3 fold partially purified lipase with enzyme yield of 63% and 16% respectively. Therefore ammonium sulphate precipitation with 40% saturation was considered the better option for concentration of the enzyme as the method eliminated more proteins present in cell free extract. DEAE-Sephadex ion exchange chromatography yielded only 4.6-fold purified enzyme with yield of just 0.1% (Table 1).

Table 1
Partial Purification of Cold active lipase from Pseudomonas sp. VITCLP4

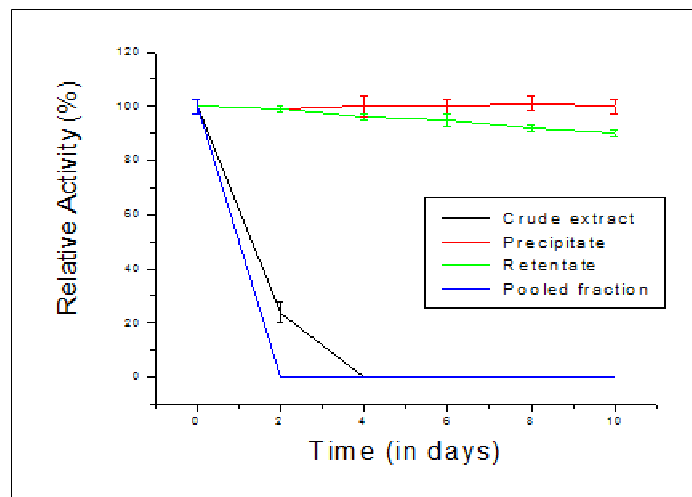
Purification steps	Enzyme Volume (ml)	Lipase Activity (Units ml^{-1} min^{-1})	Protein (mg ml^{-1})	Total activity (Units)	Total Protein (mg)	Specific Activity (Units mg^{-1} of protein)	Yield (%)	Fold Purification
Crude extract	100	171.02	7.5	17102	750	22.80	100	1
Ammonium sulphate precipitate (40%)	5	2146.72	0.75	10733.6	3.75	2862.29	62.76	125
Retentate	10	275.15	8.75	2751.5	87.5	31.44	16.08	1.3
DEAE Sephadex	1	28.43	0.27	28.43	0.27	105.29	0.16	4.6

Stability studies (Table 2 and Graph 1) revealed that ammonium sulphate precipitate was 100% stable at -20°C for 10 days. Retentate was 90% stable at -20°C for 10 days. Cell free extract and Pooled fraction obtained after ion exchange chromatography lost their activity completely after 4 and 2 days of incubation respectively at-20°C. Half life of the enzyme at 4°C was found to be 10, 6 and 1 day/s for ammonium sulphate precipitate, retentate and cell free extract respectively. Half life at room temperature was observed as 5days, 2 days and 12 h for the 3 fractions (Data not shown).

Table 2
Storage stability of various fractions at -20°C

	Relative Activity (%)			
Time (days)	Crude extract	Ammonium sulphate precipitate	Retentate	Pooled fraction
0	100±2.5	100±2.5	100±2.5	100±2.5
2	24±3.7	99±1.2	99±1.2	0
4	0	100±3.7	96±1.2	0
6	0	100±2.5	95±2.5	0
8	0	101±2.5	92±1.2	0
10	0	100±2.5	90±1.2	0

Graph 1
Storage stability of various fractions at -20°C

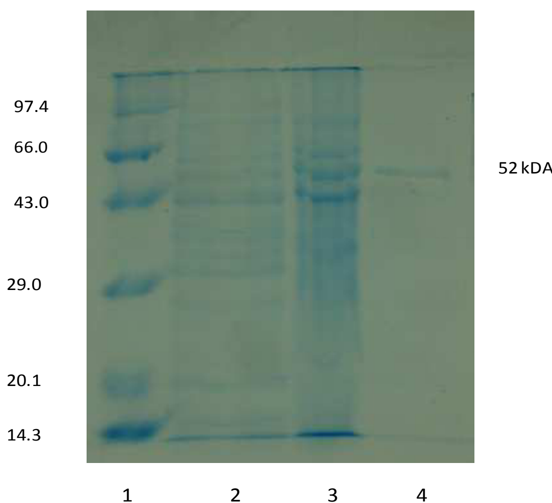


Hence ammonium sulphate precipitate was considered as more stable than other fractions. Pooled fraction tended to lose enzyme activity within 12 hours when stored at -20^oC. This may be due to aggregation of protein during elution with NaCl which is not reversible even after salt exchange. Considering this and the prominent band seen after SDS-PAGE (Figure 1) the yield and fold purification after ion exchange chromatography could have been more. Therefore, alternatively hydrophobic interaction chromatography could be tried as an intermediate purification step which is not available in our laboratory at this time. Unsuccessful chromatographic purification of cold active lipase was reported by Suzuki et al[19]. As observed in our routine studies the ammonium sulphate precipitate retained more than 90% its activity when stored at -20^oC for several months. Therefore it could be used in industrial applications which use crude and stable cold active lipase like detergent, leather processing, oil degradation etc. Partial purification of cold active lipase from *Pseudomonas* sp. MSI057 with ammonium sulphate precipitation and further characterization was reported by Kiran et al[20].

(2) Determination of Molecular mass by SDS-PAGE

SDS-PAGE pattern of cell free extract, ammonium sulphate precipitate and pooled fraction from ion exchange chromatography is shown in Figure 1. The molecular mass of cold active lipase produced by *Pseudomonas* sp. VITCLP4 was estimated to be 52 kDa, which is comparable with cold active lipase from *Pseudomonas* sp. KB700A9 and *Pseudomonas* sp. MSI057[21&20]. Molecular mass of purified cold active lipase varied from 50-85 kDa[2].

Figure 1
SDS-PAGE pattern of partially purified lipase from Pseudomonas sp. VITCLP4



Lane-1: Protein molecular weight marker, Lane-2: Cell free extract, Lane-3: Ammonium sulphate precipitate, Lane-4: Partially purified lipase (after ion exchange chromatography)

CONCLUSION

We previously reported isolation and characterization of cold active lipase producing *Pseudomonas* sp. VITCLP4 from marine samples collected from Tamilnadu coast[13]. The enzyme was alkaline and thermostable making it a prospective candidate for industrial applications. The above study on purification revealed that the enzyme could be partially

purified with conventional purification scheme. The study also suggested ammonium sulphate precipitate is more stable than crude extract, retentate and pooled fraction. Therefore it could be used in detergent, leather processing, oil degradation etc. Further study on characterization of the enzyme and its application as detergent additive is under way in our laboratory.

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