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Purification of a Novel Thermophilic Lipase from *B. licheniformis* MTCC-10498

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Abstract

Lipases have emerged as one of the leading biocatalysts with proven potential for contributing to the multibillion dollar underexploited lipid technology bio-industry and have been used in situ lipid metabolism and ex situ multifaceted industrial applications. For certain applications, such as synthetic reactions in pharmaceutical industry, further purification is needed. Since lipases are known to be hydrophobic in nature, having large hydrophobic surfaces around the active site, the purification of lipases may best be achieved by opting for affinity chromatography, such as hydrophobic interaction chromatography. The purification of bacterial lipase was performed using techniques of ammonium sulphate salting out, dialysis and hydrophobic interaction chromatography (Octyl sepharose) respectively. The analysis of bacterial lipase under reducing and denaturing SDS- PAGE (polyacrylamide gel electrophoresis) revealed that the purified lipase possessed a single band of MW 19kDa as visualized with Coomasie Brilliant Blue R-250.

Keywords: *Bacillus licheniformis, lipase, purification, dialysis, octyl sepharose.*

Introduction

Lipases have emerged as one of the leading biocatalysts with proven potential for contributing to the multibillion dollar underexploited lipid technology bio-industry and have been used in *in situ* lipid metabolism and *ex situ* multifaceted industrial applications[1-6]. Purification of the enzyme is essential in industries such as fine chemicals, pharmaceuticals and cosmetics and also for understanding the 3-D structures and the structure function relationships of proteins[7-8]. Various purification strategies used for lipase have been reviewed several times highlighting clearly the importance of designing optimal purification scheme for various microbial lipases[8-9]. For certain applications, such as synthetic reactions in pharmaceutical industry, further purification is needed. Since lipases are known to be hydrophobic in nature, having large hydrophobic surfaces around the active site, the purification of lipases may best be achieved by opting for affinity chromatography, such as hydrophobic interaction chromatography[10].

Material and Methods

Chemicals: $NaNO_3$, K_2HPO_4, KCl, $MgSO_4$, $FeSO_4.7H_2O$, $(NH_4)_2SO_4$ Celite-545 (S.D. Fine –Chem. Ltd., Hyderabad, India); yeast extract and gum acacia (Hi-Media Laboratory, Ltd., Mumbai, India); cinnamic acid, methylcinnamate, butyl butyrate, butyric acid, methanol, ethanol, butanol, sucrose, KCl, KI, KNO_3, isopropanol, ammonium persulphate, 2-mercaptoethanol, HCl, bromophenol blue and molecular sieves (3Å X 1.5 mm; Merck-Ltd., Mumbai, India); p-nitrophenyl formate (p-NPF), p-nitrophenyl acetate (p-NPA), p-nitrophenyl benzoate (p-NPB) p-nitrophenyl caprylate (p-NPC), p-nitrophenyl laurate(p-NPL), p-nitrophenyl palmitate (p-NPP) from Alpha-aesar, Heysham, England. n-Hexane, silica gel (0.040-0.063 mm, 230-400 mesh) acetic acid and triton–X100, tween-20, 40 and 80 (Qualigens Chemicals, Mumbai, India); phenyl methylsulphonyl fluoride (PMSF), sodium dodecyl sulphate (SDS), sodioum lauryl sarcosine (SLS), acrylamide, bisacryl amide (N,N-methylenebisacrylamide) glycerol, glycine and tris (2-hyroxymethyl-2-methyl-1,3-propanediol) (Sigma Chemicals Co., USA). All chemicals were of analytical grade and were used as received.

Microorganism: For the present study, water samples were collected from hot-spring of Tattapani (Mandi, Himachal Pradesh, India) in sterile containers. For the isolation of lipolytic microorganisms, 100 µl of water samples were plated on triolien and tributyrene agar plates. The formation of clear zone around the colony on the plate was considered as lipolytic microorganisms. In total, 101 isolates were found. Microorganisms which formed large clear zone around the colony were assayed for lipase activity. The microorganism which shows highest lipase activity was designated as BTS-20. The microbial culture was maintained by repeated sub-culturing at 55°C on a mineral based (MB) medium, supplemented with 0.5% (w/v) sucrose and 1.0% (v/v) of cottonseed oil as a sole carbon source (pH 7.5). Further, identification and biochemical characterization was done by Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh. It was identified as *Bacillus licheniformis* MTCC 10498. Glycerol stocks were prepared (25%; v/v) and stored at -20°C till further use.

Assay of Lipase activity and unit of Lipase activity: Lipase assay were performed by a colorimetric method[11]. The absorbance of *p*-nitrophenol released were measured at 410_{nm} (Lab India, UV/ Visible spectrophotometer, India). The enzyme activity was defined as µmole (s) of *p*-nitrophenol released per min by one ml of free enzyme or per g of immobilized enzyme (weight of matrix included) under standard assay conditions. Specific activity was expressed as µmole (s) of the *p*-nitrophenol released per min per mg of protein.

Production of Lipase through Culture conditions: The effects of various physico-chemical and nutritional requirements affecting lipase production by *Bacillus licheniformis* MTCC-10498 were studied systematically. The effect of carbon-source, nitrogen source, amino-acids, fatty acids, surfactants, pH and temperature on bacterial lipase productions was individually evaluated at pre-defined time intervals. All experiments were performed in triplicates unless otherwise stated and mean values were presented. The MB broth 50 ml taken into 250 ml Erlenmeyer flasks in different experiments was inoculated with 10 % (v/v) of 36 h old seed culture. The broth was incubated at 55°C under shaking (150 rpm). After 72 h of incubation, the lipase activity was assayed in cell free supernatant obtained after centrifugation at 10,000 x g. The optimized broth containing 0.5 (%; w/v) yeast extract , 0.3% sodium nitrate and Tween 80 (0.5%, w/v) was calibrated to a final pH of 7.5 to determine cumulative effect of all the selected components on lipase production by *B. licheniformis* MTCC 10498. The MB broth was autoclaved at 1.1 bar for 20 min. at 121°C. This broth was inoculated with 10% (v/v) of 36 h old seed culture and incubated under shaking at 55 ºC for 72 h. The inoculated MB broth was harvested at 72 h by centrifugation (10, 000 X g for 20 min. at 4ºC; Sigma 3K30, Germany). The supernatant was filtered through Whatman filter paper no. 1. This enzyme preparation was termed as crude lipase. The lipase produced by *B. licheniformis* MTCC-10498 in various batches was recorded. The pH was adjusted to 7.5 ± 0.2 and the final volume was made to 1000 ml with sterile distilled water. The culture broth was further processed to obtain purified lipase.

Protein Estimation: Following two methods were used for the estimation of protein in the sample:

Spectroscopic Method: Absorbance of protein sample was measured at A_{280} against the experimental buffer as blank using a spectrophotometer during column chromatography experiments[12] to determine protein concentration in the fractions.

Lowry's Method: A standard quantitative assay for determining the protein content in a solution was used[13]. BSA was used as a reference for protein assay.

Purification of *B. licheniformis* MTCC-10498 lipase: The purification of bacterial lipase was performed using techniques of ammonium sulphate salting out, dialysis and hydrophobic interaction chromatography (Octyl sepharose) respectively.

Precipitation of crude lipase: The cell-free supernatant obtained after 36 h of lipase production was used for purification of bacterial lipase. Required amount of ammonium sulphate was added to the supernatant to achieve10 to100% saturation[14]. The contents were thoroughly mixed and kept at 4 ºC over night to achieve maximum precipitation. There after the precipitate were sedimented by centrifugation at 12,000 X g for 30 minutes at 4 ºC. The precipitates were reconstituted in minimal buffer (tris 0.05 M; pH 8.0), precipitated fractions were analyzed separately for lipase activity and protein content.

Salt removal by Dialysis: The precipitates transferred into a dialysis apparatus were extensively dialyzed against tris buffer (0.05M, pH 8.5) at a regular interval of 2 h so as to completely remove ammonium sulphate. Finally the lipase was assayed in the dialyzate and was further concentrated using freeze-drying technique. The concentrated lipase was stored at -20 ºC until further used.

Hydrophobic Interaction (Octyl-Sepharose) Chromatography: A column of pre-swollen Octyl sepharose (Sigma Chemical Co. MU, Saint Louis, USA) was packed (V_t = 25 $cm^{3)}$ in a sintered glass column. Equilibration was done using 20 ml of start buffer (50 mM) sodium phosphate, 1.0 M ammonium sulphate, pH 7.2) at a flow rate of 1.0 ml/ min. The dialyzed lipase was loaded on the column. The elution was performed with 20 ml of elution buffer (50 mM Sodium phosphate, pH 7.2). All fractions (3 ml) were assayed for both lipase activity and protein content. The fraction showing lipase activities under a peak were pooled (14 ml) and quantified. Fold purification as well as yield of lipase was determined at each stage of purification procedure. Purified lipase was stored at -20 ºC until subsequent use.

Determination of Molecular Mass: The SDS-PAGE were performed to determine the purity and its subunit molecular mass.

Polyacryl amide Gel Electrophoresis (PAGE): The polyacryamide gel electrophoresis was based on a standard method[15].

Preparation of gel for SDS-PAGE: The electrophoresis system from ATTO Corporation, Japan was used to perform the electrophoresis. To prepare one gel of 1.0 mm thickness for and SDS-PAGE, 6 ml of separating gel mixture was poured between the two glasses plates sandwiched together with 1.0 mm spacer. The gel solution was overlaid with n-butanol water and allowed to polymerize for 30 min. The water layer at top was removed using filter paper and 1 ml of stacking (4%) gel mixture was poured over it. The comb was inserted carefully to avoid air bubbles and kept for 1 h for polymerization. The composition used were separating gel (12%) and stacking gel (4%).

Protein Molecular Weight Markers: SDS- Protein molecular weight markers: The protein molecular weight markers (medium range) for SDS-PAGE from Bangalore Genei Pvt. Ltd., Bangalore were used for molecular mass analysis of bacterial isolate of *B. licheniformis* MTCC-10498. The molecular weight of the marker proteins ranged from 14.3 kDa to 97.4 kDa.

Sample loading and Electrophoresis: The protein samples for SDS-PAGE were prepared by mixing protein samples with 5x sample loading buffer in the ratio of 4:1 (v/v) in an eppendorf tube and heated to 100°C in a water bath for 5 min. In case of native-PAGE the samples were mixed with the 5x sample loading buffer (without SDS). The prepared samples were loaded on to the gel and electophoresed at constant voltage of 100 V in case of SDS-PAGE until the dye front reached 0.5 cm above the bottom of the gel.

Procedure: After electrophoresis, the gel was carefully transferred to a clean gel staining box containing Coomassie Brilliant Blue R-250 gel stain (50 ml), and was kept over rocker for agitation for 10-20 min at room temperature. The staining solution was poured off and washed twice with water. The de-staining solution (about 50 ml) was added and gently shook over rocker platform for 1 h. Further, changes of de-staining solution were made and finally the gel was left to de-stain overnight.

Documentation of Gel: The images of gel were recorded in a gel documentation system (Alpha Innotech Corporation, USA). The molecular weight analysis was done using Alpha Digi Doc RT and Alpha Ease FC software.

Results and Discussion

Purification of *B. licheniformis* MTCC-l0498 crude lipase: The cell free broth when saturated with varying concentration of ammonium-sulphates resulted in a maximal lipase activity in the pellet obtained using 80% (w/v) of ammonium sulphates. The sedimented precipitates were reconstituted in 10 ml of Tris buffer (0.05 M, pH 8.0). This suspension transferred into a dialysis bag was subjected to extensive dialysis against Tris buffer (0.05 M, pH 8.0). The dialyzate showed lipase activity of 116.57 U/ml (protein content 3.71 mg/ml, and specific activity of 31.38 U/mg) that indicated an approximate 31.06 fold concentration of lipase.

Hydrophobic Interaction Chromatography (Octyl-sepharose): The purification of dialyzed lipase on Octyl-sepharose column resulted in a single peak (figure-1). The fractions under the single peak correspond to total lipase activity of 588U (2.0 U/ml; table 1). This hydrophobic –column purified lipase was further evaluated for its homogeneity in on electrophoresis.

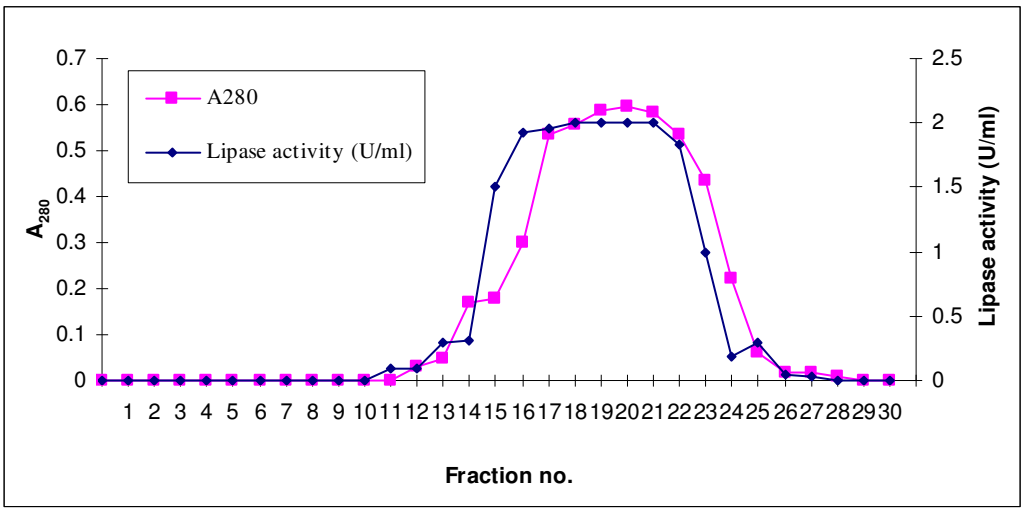

Figure 1

Purification profile of *B.licheniformis* MTCC-l0498 using hydrophobic interaction chromatography

Table-1
Summary of stepwise purification *B.licheniformis* MTCC-l0498

Purification steps	Total lipase Activity (U)/ Volume	Total protein (mg)	Specific activity (U/mg)	Fold purification	Yield (%)
Crude	7648 /(4 L)	7535	1.01	1	100
Dialyzed	2448 /(21ml)	78	31.38	31.06	32
Octyl-sepharose	588 /(294ml)	18	32.66	32.33	24.01

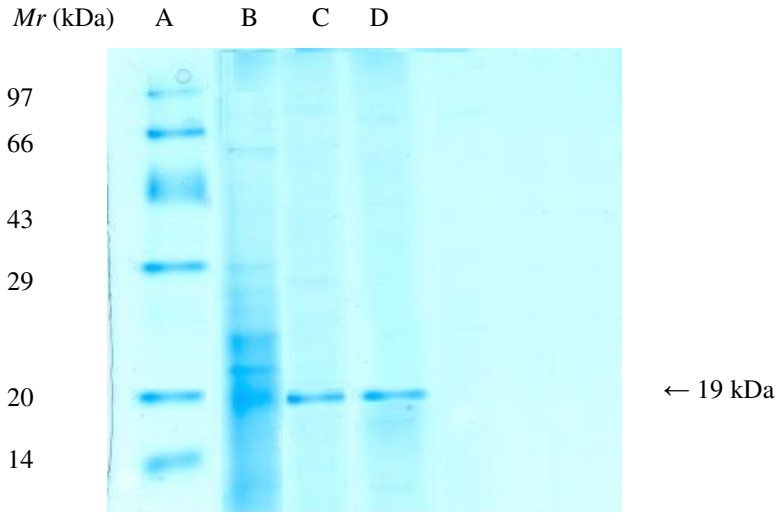

Figure 2
SDS-PAGE (A=Marker, B=crude lipase, C=dialysed lipase and D=purified lipase)

Electrophoretic characterization of B. *licheniformis* MTCC-10498 lipase: SDS-PAGE analysis for purity and molecular mass determination: The analysis of bacterial lipase under reducing and denaturing SDS-PAGE (polyacrylamide gel electrophoresis) revealed that the purified lipase possessed a single band of MW 19kDa as visualized with Coomasie Brilliant Blue R-250. The characteristics of electrophoretic pattern revealed that *B. licheniformis* MTCC-10498 lipase was a homogeneous protein (figure 2).

Newer diverse microbial sources of lipases have been reported in the recent years[16-18]. In the present study, a thermotolerant bacterial isolate *Bacillus licheniformis* MTCC-10498 that produce a novel lipase was exploited to obtain the this enzyme in its purified form. The purified lipase was subsequently used to perform reaction in both aqueous and organic media, and repetitive use of lipase immobilized onto silica and celite matrices was also studied to economize the catalytic processes in aqueous and organic media. Previously, a moderately thermophillic bacteria *B. coagulans* MTCC 6375 exhibited an extracellular lipase activity[19].

A purified and concentrated lipase was preparation was essential to be obtained before characterization of an enzyme. An impure (crude) lipase preparation could predict undesirable and ambiguous results. In last few years, emphases were given to purification and characterization of bacterial lipases. Previously, different purification procedures were attempted to obtain homogenous lipase preparations[20]. Most of the purification techniques involved fractionation by salting out using ammonium sulphates or use of an organic solvent. However, ammonium sulphate method was relatively inexpensive, efficient, reliable, and reversible and a general storage technique employed in an enzyme system. The potential advantage of the ammonium sulphate precipitation over all other techniques is the increase in stabilization of the protein. A 2-3 M ammonium sulphate suspension of protein precipitates remain stable for years, thus it forms a normal packaging method for many enzymes. Also high salt concentration prevents proteolysis and bacterial activation[16]. Amongst bacteria, till date a few extracellular lipases have been isolated, characterized and studied for their catalytic activities in aqueous or their organic media.

As described for most extracellular lipases, maximum enzyme production appeared when bacterial cells reached the late logarithmic phase[21]. Purification yielded 32 fold with a 31% recovery and molecular weight was estimated as 38 kDa. In general many of the *Bacillus* lipases have molecular weight ranges[22] from 20-112 kDa. The purified enzyme was monomeric and had a molecular mass of 51 kDa by SDS-PAGE. The enzyme had temperature and pH optima of 45^0C and pH 6.5, respectively[23].

The optimum temperature for enzyme activity was 55°C and it is in agreement with most thermotolerant lipases[17]. Lipase from *Bacillus licheniformis* shown to have good thermostability, it retains 50% activity after 3h incubation at 55°C. The thermostability of the lipase was somewhat higher to other reported lipases from Bacillus J 33 and *Pseudomonas fluorescens*[24] NS2 W. Among *Bacillus spp*., *B. coagulans*[25] NCIMB 9365 while a recently reported thermophillic B. coagulans BTS-3 isolate possessed an extracellular (31 kDa) alkalophillic lipase[26]. Thermostable lipases from many *Bacillus* spp. have been found to possess molecular masses in the range[27-28] of 43-45 kDa.

Conclusion

The present study showed that, *B. licheniformis* MTCC-10498 lipase can be purified by the techniques of ammonium sulphate precipitation (80% saturation), dialysis using a membrane and

by octyl-sepharose chromatography and possessed an extra cellular alkaline thermophillic lipase of 19 kDa as analyzed by SDS-PAGE.

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