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Original Article

Purification and characterization of lipase from *Acinetobacter haemolyticus* TA 106 isolated from human skin

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Abstract

Acinetobacter sp. isolated from healthy human skin of a tribal population was tested for lipase production.Medium optimization was achieved to increase the production. Purification was carried out by a one-step purification process using DEAE Sephadex A-50. The molecular weight of the lipase was approximately 60 kDa by SDS-PAGE. The purified lipase showed not only good stability in the presence of detergents and organic solvents but also an enhancement of activity. The lipase was active at pH 9 and displayed good activity at 0, 30 and 37°C. It was inhibited in EDTA, suggesting that it is a metalloenzyme. The cations like Ca^{2+}, Mg^{2+}, and Cu^{2+} significantly reduced the lipase activity at 5 mM concentration. The lipase converted 67% oleic acid to methyl oleate at 37°C at 72 h. All these features make this lipase an important candidate from an industrial point of view.

Keywords: *Acinetobacter*, human skin, lipase, one step purification, methyl oleate synthesis

1. Introduction

Lipases (triacylglycerol hydrolases E.C. 3.1.1.3) are enzymes widely distributed in nature. Extracellular lipases have been produced from microorganisms such as fungi, yeast and bacteria beside from plants and animals. Lipases catalyze the hydrolysis and formation of lipids. Therefore they are becoming one of the most important enzymes for industrial applications, which include detergents, cosmetics, pharmaceuticals and dairy products (Jaeger and Reetz, 1998). There has been a renewed interest in the development of sources of lipases due to their wide applications. In our laboratory, we have optimized a medium for lipase production and it was shown that lipase activity reached to 55 U/ml (Jagtap *et al.*, 2010). It is important to note that the members of the genus *Acinetobacter* are the only Gram negative bacteria present on the human skin as normal flora (Patil and Chopade, 2001). The present study describes purification and characterization of lipase produced by *A. haemolyticus* TA 106 from human skin of tribal population in India and its role in methyl oleate synthesis.

2. Materials and Methods

2.1 Bacterial culture and identification of the genus *Acinetobacter*

Acinetobacter sp. (118 cultures) previously isolated in our laboratory from healthy human skin of a tribal population were used (Yavankar *et al.*, 2007) *A. haemolyticus* TA 106, which produced 55 U/ml of lipase after the medium optimization, was used for purification of lipase. The culture was maintained in glycerol stocks at -80°C.

2.2 Chemicals

All chemicals were of analytical grade and commercially available in India. The substrate p- nitrophenyl

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palmitate (p-NPP) for lipase assay and a standard lipase from *Rhizomucor miehei* were obtained from Sigma (St. Louis, USA). Refined plant oils were locally available. Organic solvents used in the study were from Merck (Darmstadt, Germany). High purity water was obtained by Millipore Milli Q plus water purification system.

2.3 Inoculum preparation

Acinetobacter haemolyticus TA106 was grown in Luria Bertani (LB) broth at 37°C with shaking (200 rpm) for 18 h. The optical density of the culture was adjusted to 0.5 McFarland units and used as an inoculum.

2.4 Lipase production

Maximum lipase activity was obtained in LB with emulsified oil 1% (v/v). The medium consisted of (% w/v): Tryptone 1, NaCl 1, Yeast extract 0.5, olive oil 1, Manganese sulphate 5 mM, sucrose 1 and Tween 80 1, pH-7. The production medium was taken in a 250 ml Erlenmeyer flask with 3% inoculum of *A. haemolyticus*. These flasks were then incubated at 37°C under shaking conditions (200 rpm) for 72 h.

2.5 Lipase assay

The lipase assay was carried out at 24 h intervals up to 96 h. In brief, the assay was performed as follows : 30 mg p-nitrophenylpalmitate (p-NPP) was dissolved in 10 ml of isopropanol and mixed with 90 ml of 50 mM phosphate buffer, pH 8 containing 207 mg of sodium deoxycholate and 100 mg gum arabic. 2.4 ml of the freshly prepared substrate solution was prewarmed at 37°C and mixed with 0.1 ml of cell free supernatant. After 30 min of incubation at 37°C, absorbance at 410 nm was checked against enzyme-free control using a UV-visible spectrophotometer (UV 1601 Shimadzu Corporation, Japan) (Winkler and Stuckmann, 1979). Protein content was measured using Folin-Lowry method (Lowry *et al.*, 1951).

2.6 Purification of lipase

72 h old culture broth of *A. haemolyticus* TA 106 was centrifuged at 1565 g for 20 min at 4°C (Kubota, Japan). The supernatant was removed carefully and addition of 80% ammonium sulphate was done in an ice bath with gentle stirring. Protein was precipitated and it was kept at 4°C overnight. After the complete precipitation of protein, the precipitate was collected by centrifugation at 1565 g for 20 min at 4°C. The precipitate was dissolved in (5 ml) 50 mM phosphate buffer of pH 7. It was then dialyzed for 24 h (Seamless cellulose tubing, width 40 mm, diameter 25 mm, retaining most proteins of molecular weight 12,000 or greater, Sigma Alderich Chemie, Gmbh, Steinheim, Germany) at 4°C against the same buffer. The buffer was changed after 10 h. The dialysate was further concentrated using polyethylene glycol 6000 (Sigma, St. Louis, MO). This concentrated enzyme was loaded on DEAE Sephadex A-50 (Sigma, St. Louis, MO) column (1× 15cm) which was previously equilibrated with 50 mM phosphate buffer, pH-7. The column was washed with 3 bed volumes of the same buffer at a flow rate of 60 ml / h and the bound protein was eluted with a linear gradient of NaCl (0.05 M - 1 M) in the same buffer. 100 fractions of 1 ml each were collected and presence of protein was checked using O.D. at 280 nm. Those fractions which had protein were subjected to lipase assay (Gopinath *et al.*, 2003). The protein content and lipase activity were determined as described above.

2.7 SDS-PAGE of purified lipase

Sodium dodecyl sulphate-polyacrylamide electrophoresis (SDS-PAGE) was carried out according to the Laemmli technique (Laemmli, 1970) on a 10% (w/v) polyacrylamide gel system. Gels were stained using the silver nitrate method for proteins (Blum et al., 1987). The activity was estimated qualitatively using p-NPP as substrate in a micro-titer plate using method described above (Winkler and Stuckmann, 1979). The development of yellow colour indicated the presence of lipase. A blank was run, by adding distilled water, instead of the eluted supernatant.

2.8 Characterization of purified lipase

Effect of pH and temperature on lipase activity: Purified lipase was incubated in the presence of citrate phosphate and glycine –NaOH buffers of pH 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 and 13. Incubation was done at RT for 2 h. Then residual activity was determined at 37°C using lipase assay. Purified lipase was incubated at different temperatures, namely 0, 20, 30, 37, 40 and 45°C, for 2 h. After incubation the residual activity was determined.

2.9 Effect of salts and EDTA

Different metal salts were tested for their effect on lipase. 1 mM and 5 mM of calcium chloride, magnesium chloride, barium chloride, ferric chloride, mercuric iodide, manganese sulphate, sodium chloride and copper sulphate were incubated in the purified lipase. Residual activity was checked at 37°C. EDTA at 1 mM and 5 mM was also tested to check its effect on lipase activity. The lipase activity assayed in the absence of salt and EDTA was defined as control.

2.10 Effect of detergents and surfactants

Purified lipase was incubated in the presence of detergents and surfactants at 1% w/v or v/v at RT for 2 h. The detergents and surfactants used were sodium dodecyl sulphate, Triton X-100, Tween 80, Tween 20, Wheel (Hindustan Lever Ltd.), Rin advanced (Hindustan Lever Ltd.),

Tide (Procter and Gamble Ltd.),Surf Ultra (Hindustan Lever Ltd.), sodium cholate and hydrogen peroxide. Residual activity was determined. The residual activity of lipase from *A. haemolyticus* was compared with that obtained by standard lipase from *Rhizomucor miehei (*Sigma). Control was without the addition of surfactant and detergent.

2.11 Effect of organic solvents and substrates

Organic solvents, namely n-butanol, methanol, chloroform, formaldehyde, absolute ethanol, isopropanol, acetone, and hexane, were checked for the effect on lipase. All the organic solvents at 50% (v/v) were incubated in the presence of lipase at RT for 2 h. Then residual activity was detected and compared with the standard lipase. Different substrates like triolein, tributyrin and plant oils were also tested to know the substrate specificity of lipase. Lipase activity without addition of organic solvents and substrates served as control.

2.12 Synthesis of methyl oleate using purified lipase

Esterification for methyl oleate synthesis was performed with 0.4 M methanol and 0.25 M oleic acid dissolved in 4 ml n-hexane. Purified lipase was used as biocatalyst. It was incubated at 37°C with shaking for 72 h with a control having no enzyme. To 10 ml reaction mixture, 20 ml acetone-methanol (1:1 v/v) was added (Nawani *et al.*, 1998). The ester content was quantified using an alkalimetric method of titrating unreacted acid with 0.1 M NaOH using phenolphthalein as an indicator. The conversion (%) in ester synthesis was based on oleic acid consumed (Wu *et al.*, 1996).

3. Results

3.1 Production of lipase from *A. haemolyticus*:

Lipase production was carried out in an optimized medium containing (% w/v or v/v) tryptone-1, yeast extract-0.5, sodium chloride- 1, olive oil-1, tween 80-1, manganese sulphate-5 mM and sucrose-1, pH 7. Maximum production occurred at late log phase after 72 h at 200 rpm. The maximum activity displayed by the lipase was 55 U/ml (Jagtap *et al.*, 2010).

3.2 Purification and characterization of lipase

Ion exchange chromatography gave a single peak of lipase (Figure 1). The purification steps are shown in Table 1. The molecular weight of the lipase was found to be about 60 kDa (Figure 2). It was found that the purified lipase from *A. haemolyticus* was highly stable at pH 9 for more than 72 h. Significant activities were not observed at all the pH values checked, and therefore are not included in the result (Figure 3). The lipase also showed good stability at different temperatures except at 20°C (Figure 4). Out of all the salts tested, only 1 mM barium chloride could enhance the lipase activity (Table 2). EDTA at 1 mM and 5 mM completely inhibited the

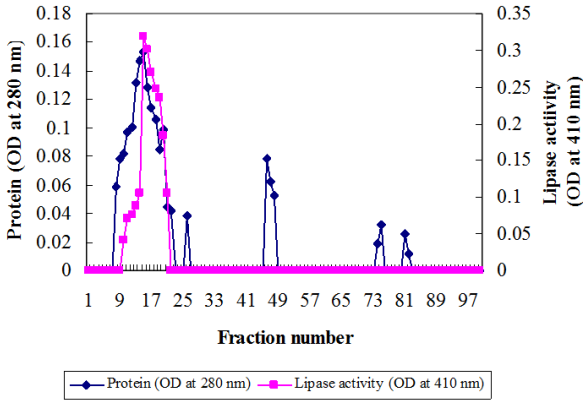

Figure 1. Chromatography profile of lipase from *Acinetobacter haemolyticus* TA 106

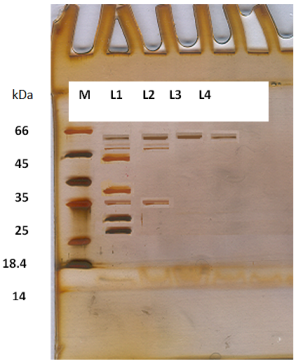

Figure 2. SDS PAGE of lipase from *A. haemolyticus* TA 106

Table 1. Summary of purification of lipase from *A. haemolyticus* TA 106

Purification step	Total protein	Total activity (mg)	Specific activity (U)	Yield (%) (U/mg)	Fold Purification
Supernatant	820	5500	6.7	100	1
80 % ammonium sulphate	360	2500	7.11	45.45	1.06
Dialysis	45	420	9.33	7.63	1.39
Ion-exchange chromatography	5	350	70	6.36	10.44

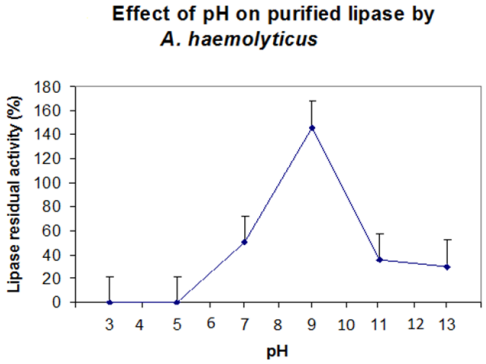

Figure 3. Effect of pH on purified lipase produced by *Acinetobacter haemolyticus*

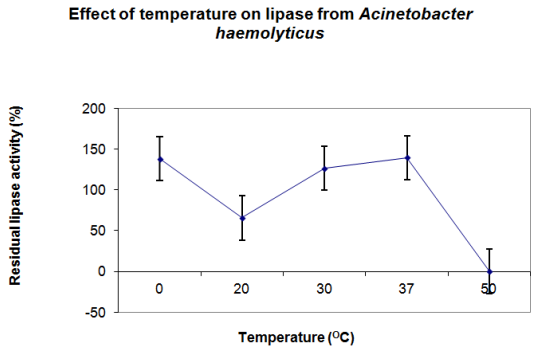

Figure 4. Effect of temperature on purified lipase from *Acinetobacter haemolyticus*

lipase activity suggesting that it is a metalloenzyme.Surfactants like Triton- X-100, Tween 20 and Tween 80 completely inhibited the lipase activity, whereas, it was stable in other surfactants tested (Table 3). It was also observed that lipase from *A. haemolyticus* was better than the commercial lipase. The lipase activity was enhanced in organic solvents like n-butanol, hexane and isopropanol (Table 4). Substrate specificity of lipase indicated that triolein was a better substrate

Table 2. Effect of salts and EDTA on lipase of *Acinetobacter haemolyticus* TA 106.

Salts tested	Residual activity (%)
Control	100
Calcium chloride (1 mM)	84.95 ± 1.42
Calcium chloride (5 mM)	Nil
Magnesium sulphate (1 mM)	86.39 ± 3.17
Magnesium sulphate (5 mM)	56.86 ± 2.26
Ferric chloride (1 mM)	38.97 ± 1.77
Ferric chloride (5 mM)	4.54 ± 0.95
Mercuric iodide (1 mM)	41.11 ± 2.02
Mercuric iodide (5 mM)	36.56 ± 3.39
Manganese sulphate (1 mM)	57.1 ± 2
Manganese sulphate (5 mM)	56.81 ±2.97
Sodium chloride (1 mM)	35.34 ± 1.4
Sodium chloride (5 mM)	99.79 ± 0.94
Copper sulphate (1 mM)	1.63 ± 0.12
Copper sulphate (5 mM)	4.22 ± 1.58
Barium chloride (1 mM)	151.1 ± 1.36
Barium chloride (5 mM)	20.58 ± 0.73
EDTA (1 mM)	Nil
EDTA (5 mM)	Nil

*: The values given are the mean of three independent experiments along with their standard deviation values.

Table 3. Effect of surfactants, detergents and oxidizing agent on lipase from *A. haemolyticus* and its comparison with lipase from *Rhizomucor meihei*

Different agents tested	Standard lipase Residual activity (%) [a]	*Acinetobacter* lipase Residual activity (%) [a]
Control	100	100
Tween 20	106.2 ± 1.05	NA
Tween 80	107.11 ± 1.8	NA
Sodium cholate	104.41 ± 2.14	170.85 ± 1.26
Sodium Dodecyl sulphate	123.5 ± 1.7	232.2 ± 2.35
Tide	103.2 ± 1.36	217.5 ± 3.64
Surf Ultra	107.62 ± 1.49	229.6 ± 6.18
Rin advanced	106.83 ± 2.12	203.7 ± 2.41
Wheel	110.3 ± 1.85	103.4 ± 0.6
Hydrogen peroxide	96.53 ± 1.7	132.26 ± 2.32
Triton X-100	106.1 ± 3.07	NA

NA: No lipase activity

[a] : The values given are the mean of three independent experiments along with their standard deviation.

Table 4. Effect of organic solvents on lipase from *Acinetobacter haemolyticus* and its comparison with standard lipase from *Rhizomucor miehei*

Organic solvents tested	Standard lipase Residual activity (%) [a]	*Acinetobacter* lipase Residual activity (%) [a]
Control	100	100
Methanol	155.23 ± 1.9	91.5 ± 1.17
Chloroform	121.3 ± 1	84.99 ± 1.36
Formaldehyde	108 ± 1.76	Nil
Absolute ethanol	167.2 ± 2.19	81.4 ± 0.82
Isopropanol	174.1 ± 2.13	180.5 ± 1.5
Acetone	142.5 ± 2.16	13.24 ± 0.74
Hexane	173.3 ± 1.52	98.1 ± 1.54
n-butanol	176.4 ± 2.04	169.36 ± 0.65

NA: No lipase activity;
[a] : The values given are the mean of three independent experiments alongwith their standard deviation.

followed by olive oil (Table 5). The lipase produced by *A. haemolyticus* TA 106 was found to display sn-1, 3 specificity (data not shown).

3.3 Studies on synthesis of methyl oleate using purified lipase

Synthesis of methyl oleate was carried out in hexane as lipase showed good stability in it. It was observed that 62% conversion of oleic acid occurred in 24 h, which reached 65.3% in 48 h and almost 67% in 72 h.

3.4 Confirmation of methyl oleate

Synthesis of methyl oleate was confirmed by using thin layer chromatographic technique. The solvent system used was petroleum ether-ether-acetic acid (60:40:1).

4. Discussion

Production of hydrolytic enzymes by *Acinetobacter* sp. from skin is due to the metabolic requirement of these bacteria. In the present investigation, lipase production occurred extracellularly. Lipases are usually secreted out in the culture medium, though there are a few reports on intracellular lipases (Lee and Lee, 1989) as well as cell-bound lipases (Large *et al.*, 1999). In the present investigation, lipase from *A. haemolyticus* was purified by ion exchange chromatography in single step. Ni^{2+} Nitriloacetic acid affinity chromatography is employed in a one-step purification of cloned *Bacillus licheniformis* lipase (Nthangeni *et al.*, 2001). Lipases are reported to be monomeric proteins having molecular weights in the range of 16-670 kDa. In the present study, the molecular weight of purified lipase from *A. haemolyticus* TA 106 was approximately 60 kDa. Lipase from

Table 5. Activity of purified lipase from *A. haemolyticus* towards various substrates

Substrates	Residual activity (%)*
Triolein	100
Tributyrin	165.5±3.3
Olive oil	106.7 ± 3.1
Sunflower oil	68.4 ± 0.85
Groundnut oil	54.6 ± 1.05
Coconut oil	46.1 ± 3.48
Castor oil	93.9 ± 4.7
Almond oil	80.1 ± 1.9
Soybean oil	42.7±2.33

*: The values given are the mean of three independent experiments along with their standard deviation values

A. sp. is found to be around 32 kDa (Saisubramanian *et al.*, 2008) the same as lipase produced by *A.* sp. ES1 (Lee *et al.*, 2006). *A. radioresistens* CMC-1 lipase is 45 kDa (Hong and Chang, 1998). Lipase from *A. haemolyticus* showed pH optimum of 9. This finding is in agreement with lipase from *Alternaria brassicicola* which also shows pH optima of 9 (Berto *et al.*, 1997). Lipase from *A. radioresistens* was found to be alkaline in nature having pH optima of 10.5 (Hong and Chang, 1998). Lipase from *Streptomyces coelicolor* A3 (2) SCO1725 was reported to be active in a pH range from 7.5 to 9 whereas lipase SCO7513 was found to be active in a pH range of 8.5-10 (Cote and Shareck, 2008). *A. haemolyticus* lipase displayed temperature optima of 37°C and was also active at 0°C. A similar result was shown by lipase from *A.* sp., which showed temperature optima of 37°C and activity at 10°C (Saisubramanian *et al.*, 2008). The cold active lipase from phychrophilic *Acinetobacter* sp. Strain no.6 is reported to retain 40% of its activity at 4°C (Suzuki *et al.*, 2001). EDTA

(Ethylene diamine tetra acetic acid) has been reported to inhibit activity of a few lipases. The inactivation of some of these lipases was overcome by treatment with divalent ions like calcium, magnesium and even barium (Boral and Fox, 1997). Lipase from *A. haemolyticus* was inhibited in the presence of the metal chelator, EDTA and showed activity only with barium chloride. It is in accordance with the lipase from *A. calcoaceticus* LP009 which is also a metalloenzyme (Dharmshtiti *et al.*, 1998). Most of the metal ions except barium inhibited the lipase from *A. haemolyticus*. This result is contrary to the lipase from *A.calcoaceticus* LP009, which is only slightly affected by metal ions retaining more than 80% activity (Dharmshtiti *et al.*, 1998). Similarly lipase from *A. radioresistens* CMC-1 was also neither enhanced nor inhibited in the presence of most of the metal ions except zinc (Hong and Chang, 1998). The lipase from *A.* sp. ES1 also showed 1.5-fold enhanced activity in the presence of calcium (Lee *et al.*, 2008).

Lipase from *A. haemolyticus* showed good enhancement of activity in the presence of detergents and hydrogen peroxide. This is in agreement with the lipase from *A.* sp., which is stable in detergents and hydrogen peroxide (Saisubramanian *et al.*, 2008). Triton X-100, Tween 80 and Tween 20 had negative effect on lipase from *A. haemolyticus* which is contrary to the lipase from *A. calcoaceticus* LP009 (Dharmshtiti *et al.*, 1998). However, this result is in agreement with the lipase from *Bacillus thermoleovorans* CCR11, where Tween 20 and Tween 80 were found to inhibit lipase activity (Castro-Ochoa *et al.*, 2005). In organic media, environment of an enzyme changes dramatically as compared to that in water and therefore enzyme activity and selectivity may be altered (Wescott and Klibanow, 1993). This could be the reason for different activities of lipase in different organic solvents. In the present investigation, organic solvents such as butanol and isopropanol could enhance the lipase activity of *A. haemolyticus* whereas lipase from *A. calcoaceticus* LP009 was unstable in organic solvents (Dharmshtiti *et al.*, 1998). Thus it is observed that lipase from *A. haemolyticus* showed stability at different temperatures and pH optima of 9. The enhancement of activity in the presence of various detergents and organic solvents like butanol and isopropanol as compared to the commercial lipase from *Rhizomucor miehei* makes it an important candidate for application in the detergent industry. In synthetic reactions, choice of adequate solvent is important in which the enzyme remains active. Solvents with log P values >3 are widely used. In the present investigation, we have used hexane for the same purpose. Conversion of 62% oleic acid to methyl oleate occurred using lipase from *A. haemolyticus* after 24 h. This observation is similar to that of thermostable lipase from *Bacillus* sp., which converted 66% of oleic acid to methyl oleate after 16 h (Nawani *et al.*, 1998). The yield is less compared to that obtained by cell bound lipase from *Geotrichum* sp., in which almost 94% conversion is observed within 24 h (Yan and Yan, 2008). Lipase from *A. haemolyticus* TA 106 isolated from healthy human skin of tribal population displayed a good activity of 55 U/ml after medium optimization (Jagtap *et al.*, 2010) and it also showed many industrially important properties.

Thus, we have presented the activity and stability of purified lipase from *A. haemolyticus* from healthy human skin of a tribal population in the presence of various chemicals. The lipase showed stability and enhanced activity at pH 9 and also in different surfactants and commercial detergents, H_2O_2, and therefore can be used as an additive for detergent formulation. The lipase exhibited good stability in organic solvents and was used in the synthesis of methyl oleate which has wide applications in soap making, metal- working fluids, and solvents for inks, paint removal and production of plasticizer alcohols. Lipase from *A. haemolyticus* TA106 was also sn-1, 3 specific and extracellular in nature. It is well known that sn-1, 3 specific lipases can be used in the production of structured lipids with unique functional properties. All these points make it an important candidate from industrial point of view. To the best of our knowledge, it is the first report on lipase production by *A. haemolyticus* from human skin and its role in methyl oleate synthesis. The obtained results allow for assumption that the lipase may have industrial applications.

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