

RESEARCH ARTICLE

Purification and characterization of *Aspergillus japonicus* lipase from a pig fat production medium

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Abstract

Aspergillus japonicus lipase was purified to homogeneity by Q-Sepharose and Sephadex G-100 column chromatography. The enzyme had a molecular weight of 43 KDa in SDS-PAGE. Purified *A. japonicus* lipase had a specific activity of 480 U/mg protein. The purification magnitude was 3.44 and the total yield was 4.53%. Purified *A. japonicus* lipase had an optimum pH of 7.5 while the optimum temperature was 40°C. Metal ions such as MnCl₂ and CoCl₂ exhibited an inhibitory effect on the enzyme. The enzyme displayed substrate specificity for p-nitrophenyl palmitate. The enzyme was stable in two organic solvents-methanol and acetone. An-ionic detergent, SDS inhibited its activity.

Keywords: *Aspergillus japonicus*, Q-Sepharose, Sephadex G-100, p-nitrophenyl palmitate, methanol.

Introduction

Lipases (Triacylglycerol acyl hydrolase, E.C.3.1.1.3) are lipolytic serine esterases that are secreted by many fungi, yeasts and bacteria. Lipases are active at their interface between their hydrophobic lipid substrate and the hydrophilic space medium (oil-water interface) cleaving water insoluble glycerides into molecules that can readily be assimilated by cells (Jaeger and Eggert, 2002). Microbial lipases have assumed a great deal of importance as industrial enzymes in view of their potential for use in various biotechnological processes. Fungi are important enzyme producers since they produce enzymes extracellularly (Costa and Peralta, 1999). Lipases are exploited owing to their low cost of extraction, thermal and pH stability, substrate specificity, and activity in organic solvents. Lipases are the most widely used enzymes in organic syntheses and more than 20% biotransformations are performed with lipases (Gittlesen *et al.*, 1997). Besides their role in synthetic organic chemistry, these enzymes also find extensive applications in chemical, pharmaceutical, food and leather industries (Gulati *et al.*, 2005).

In view of the variety in applications, there has been a renewed interest in the identification of sources of lipases. Numerous species of bacteria, yeasts and moulds produce lipases with different enzymological properties and specificities but moulds are considered to be more potent lipase producers (Choo *et al.*, 1998). These microorganisms produce lipases both by solid substrate and submerged fermentations (He *et al.*, 2004). Because of a huge variation in applications, the identification of lipases with specific characteristics

remained elusive. Thus, a search for lipases with new attributes and to improve their production emerged as new goals for researchers. Most commercially viable lipases are synthesized by fungi and yeasts. Commercial lipolytic enzymes are produced from *Rhizopus delemar* (Espinosa *et al.*, 1990; Cruz *et al.*, 1993; Shimada *et al.*, 1996); *Humicola lanuginosa* (Morinaga *et al.*, 1986, Ivanova *et al.*, 2002) *Penicillium chrysogenum* (Ferrer *et al.*, 2000), *Fusarium heterosporum* (Nagao *et al.*, 1998), *Rhizopus chinensis* (Nakashima *et al.*, 1988) and *Candida rugosa* (Valero *et al.*, 1988; Obradors, 1993).

A number of extracellular fungal lipases have been purified and their physico-chemical properties ascertained. Many lipases have been thoroughly purified and characterized in terms of their activity and stability profiles relative to pH, temperature, and effects of metal ions and chelating agents. In many cases, lipases have been purified to homogeneity and crystallized. Purification methods used have generally depended on nonspecific techniques such as precipitation, hydrophobic interaction chromatography, gel filtration, and ion exchange chromatography. The present day industrial demand for lipolytic enzymes continues to stimulate the search for new enzyme sources. In view of the diversity in applications, renewed interest is evinced in the development of new sources of lipases.

Against these backdrops, this study was aimed to purify extracellular lipase from *Aspergillus japonicus* isolated from the paper nest of *Ropalidia marginata*. Purified lipase was further characterized and checked for its pH, temperature and solvent tolerability.



Materials and methods

Test isolate

The paper nest of *Ropalidia marginata* was collected from a glass house at CAS in Botany, University of Madras. The paper nest was surface sterilized with 0.1% mercuric chloride for 2 min and washed thoroughly in sterile distilled water for 2 min and the bits plated on PDA and incubated at 25°C for a week. A fungus, identified as *A. japonicus* (Deuteromycete: Hyphomycetes), was isolated from the paper nest of *R. marginata*. After isolation, the fungus was subcultured and the pure culture was stored in PDA at 4°C.

Lipase purification

Aspergillus japonicus was reared in 1 litre of optimized lipase production medium which was previously optimized using variables such as 2.5 g/L sucrose, 7.16 g/L peptone, 5.61 g/L pig fat at pH 7.6, 39°C and at 120 rpm for 7 days. The culture filtrate was used as the enzyme source for the purification process. One litre of culture filtrate was concentrated by adding 70% to 90% ammonium sulphate. The culture filtrate was incubated overnight at 4°C for precipitation. The precipitated protein was removed by centrifugation at 12,000 × g for 15 min. and dialyzed.

The crude enzyme sample was loaded onto a Q-Sepharose FF column (50 mL, 30 cm × 1.6 cm) equilibrated with 0.1 M Tris-HCl buffer (pH 7.5) at a flow rate 5 mL/min. The unbound protein was washed out with 250 mL of the equilibration buffer. Subsequently, a linear gradient of 0–0.5 M sodium chloride (NaCl) in the same buffer was used to elute bound lipase activity and 5 mL fractions were collected. The protein content of the each fraction was determined and the lipase positive fractions were pooled together and concentrated and stored for further use.

The enzyme fractions collected from Q-Sepharose column were concentrated and loaded on to a Sephadex G-100 containing 0.1 M Tris-HCl buffer (pH 7.5). Elution was made with the same buffer at a flow rate of 15 mL/h and the fractions were collected. The active fractions were combined, dialyzed and lyophilized. The protein content of each fraction was determined and the lipase positive fractions were pooled together and concentrated for further studies.

Lipase assay

Lipase activity was assayed quantitatively with p-nitrophenyl palmitate as the substrate (Winkler and Stuckmann, 1979). 10 mL isopropanol containing 30 mg p-nitrophenyl palmitate (Sigma) was mixed with 90 mL 0.05 M sodium phosphate buffer (pH 8.0) containing 207 mg sodium deoxycholate and 100 mg gum arabic. A total volume of 2.4 mL freshly prepared substrate solution was prewarmed at 37°C and mixed with 0.1 mL enzyme solution. After 15 min. incubation at 37°C, absorbance at 410 nm was measured against a blank. One enzyme unit

was defined as 1 μM p-nitrophenol enzymatically released from the substrate in mL/min.

Protein estimation

The total protein at different stages of purification was estimated at 595 nm according to the method of Bradford (1976) by using Bovine Serum Albumin (BSA) as a standard.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed in polyacrylamide slab gel (12% W/V) Separating and 5% (W/V) stacking according to the method of Laemmli (1970).

Zymogram

The purified *A. japonicus* lipase was run on native PAGE (12%, without SDS) for zymography. The gels were transferred to a 2% agar plate containing 1% tributyrin emulsion in 0.1 M Tris-HCl, pH 7.5 for lipase activity detection. After incubation for 3 h at 40°C lipase activity was visualized as a band of clearing on the tributyrin plate.

Effect of pH on lipase activity

Fifty μg of purified lipase was incubated in 0.1 M sodium citrate buffer (pH 3.5-5.5), 0.1 M sodium phosphate buffer (pH 5.6-7.4) 0.1 M Tris-HCl buffer (pH 7.5-8.0) and 0.01 M carbonate buffer (pH 9-12). Further, 0.05 M sodium carbonate solution was used for adjusting the pH of the substrate to 12. About 2.4 mL of the appropriate buffer containing p-nitrophenyl palmitate as substrate and 50 μg purified enzyme were incubated at 37°C for 15 min. and the release of p-nitro phenol was read at 410 nm. Since, the enzyme can develop the characteristic colour of p-nitrophenol by the enzymatic hydrolysis of its colourless substrate only at an alkaline pH, the optimum and the stability of the pH was assayed in the pH range 7.5-12. The relative activity was calculated by the following formula.

Effect of pH on the stability of lipase activity

Fifty μg of purified lipase solution was preincubated for 24 h with 1 mL of 0.1 M sodium citrate buffer (pH 3.5-5.5), 0.1 M sodium phosphate buffer (pH 5.6-7.4) 0.1 M Tris-HCl buffer (pH 7.5-8.0) and 0.01 M carbonate buffer (pH 9-12). 0.1 M Tris-HCl buffer (pH 7.5-8.0) at 37°C. The residual activity was calculated after 1 h incubation; the lipase activity was assayed using p-nitrophenyl palmitate as substrate.

Effect of temperature on lipase activity

Purified lipase (50 μg) was incubated at varying temperatures (20-90°C) along with 2.4 mL substrate containing p-nitrophenyl palmitate as substrate. The enzyme activity was measured after incubation of the reaction mixture for 15 min. Reaction mixture without enzyme served as control. The relative activity of the purified lipase was calculated.

Effect of temperature on the stability of lipase activity

The purified enzyme (50 µg) was incubated at varying temperatures (20-90°C) for 1 h. At the end of the incubation period, the enzyme solution was rapidly cooled to the room temperature and the relative activity was calculated. Reaction mixture without enzyme served as control and the test solutions were assayed in triplicate.

Effect of metal ions on lipase activity

The purified enzyme (50 µg) was mixed with metal ions such as barium chloride (BaCl₂), calcium chloride (CaCl₂), cobalt chloride (CoCl₂), copper sulphate (CuSO₄), ferric chloride (FeCl₃), mercuric chloride (HgCl₂), magnesium chloride (MgCl₂), manganese chloride (MnCl₂), sodium chloride (NaCl) or ammonium nitrate (NaN₃) to give a final concentration of 2 mM and was incubated at 30°C for 1 h. A control was run in an identical manner with distilled water instead of metallic salts. The relative activity of the enzyme was calculated with p-nitrophenyl palmitate as substrate.

Substrate specificity of lipase

The substrate specificity of purified lipase toward substrates with different acyl chain lengths was determined under standard conditions using various esters of p-nitrophenyl (p-NP). Substrates and their chain lengths examined were as follows: p-NP acetate (C2); p-NP butyrate (C4); p-NP caproate (C6); p-NP caprylate (C8); p-NP caprate (C10); p-NP laurate (C12); p-NP myristate (C14); p-NP palmitate (C16); and p-NP stearate (C18). The purified lipase (50 µg) was incubated in different substrates to give a final concentration of 2 mM. Relative activity was measured after incubating the reaction mixture for 15 min.

Effect of organic solvents on lipase activity

The stability of the enzyme was determined by incubating 50 µg of purified lipase in 10% and 20% of different organic solvents such as acetone, butanol, chloroform, methanol, ethanol or hexane for 1 h at 40°C. A control was maintained by incubating the enzyme in 0.1 M Tris-HCl buffer at pH 7.5. The relative activity was estimated by the standard assay system.

Effect of inhibitors on lipase activity

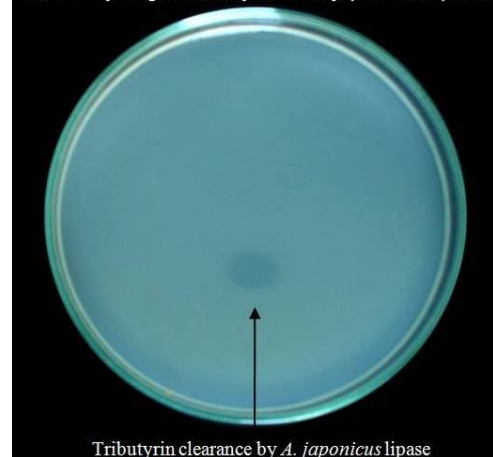
The inhibitory activity of EDTA (Ethylenediamine tetra acetic acid), DTT (Dithiothreitol), IAA (Indole acetic acid), PMSF (Phenyl methyl sulfonyl fluoride) and SDS (Sodium dodecyl sulphate) was determined against purified lipase. About 50 µg of purified lipase was incubated in different reagents to give a final concentration of 2 mM. Relative activity was measured after incubating the reaction mixture for 30 min.

Results and discussion

In view of their importance in the fields of dairy science, detergent production technology and oleochemistry, lipases have been purified and characterized by various investigators. Several reviews in this area highlighting

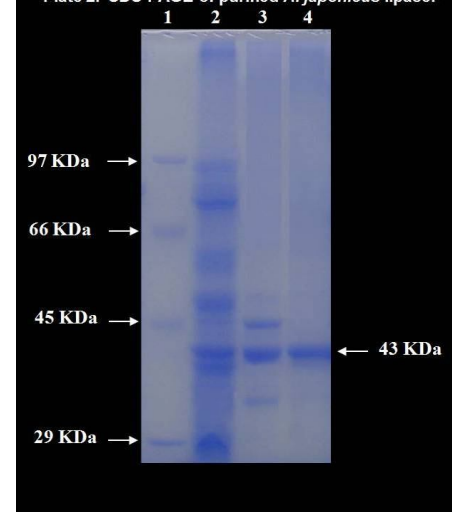
the importance of designing optimal purification schemes for various microbial lipases are available (Antonian, 1988; Taipa *et al.*, 1992; Barros *et al.*, 1994; Palekar *et al.*, 2000). Here an attempt has been made to purify lipase enzyme by conventional biochemical separation techniques. *Aspergillus japonicus* was reared in 1 litre of optimized lipase production medium which was previously optimized using variables such as 2.5 g/L sucrose, 7.16 g/L peptone, 5.61 g/L pig fat at pH 7.6, 39°C and at 120 rpm for 7 days (data not shown). The culture filtrate was used as the enzyme source for the purification process (Plate 1).

Plate 1. Zymogram analysis of *A. japonicus* lipase.



One litre of culture filtrate was concentrated by adding 70% to 90% ammonium sulphate. The culture filtrate was incubated overnight at 4°C for precipitation. The precipitated protein was removed by centrifugation at 12,000 × g for 15 min., dialyzed using 0.1 M Tris-HCl buffer (pH 7.5) for 48 h and then lyophilized. Lipase activity of ammonium sulphate precipitated protein recovered from various concentration of ammonium sulphate was assessed.

Plate 2. SDS-PAGE of purified *A. japonicus* lipase.



Lane 1 – Standard Molecular wt. markers
 Lane 2 – Crude *A. japonicus* lipase (70% ammonium sulphate precipitated)
 Lane 3 – Q-Sepharose column eluted proteins
 Lane 4 – Sephadex G-100 column eluted lipase

Table 1. Purification scheme of *A. japonicus* lipase.

Purification stage	Total Protein(mg)	Total activity (U)	Specific activity (U/mg of protein)	Purification fold	Yield (%)
Culture filtrate	190	26476.5	139.35	1	100
Ammonium sulphate precipitation (70%)	21.0	7710	367.14	2.63	29.12
Q-Sepharose	12.0	4500	375.00	2.69	16.99
Sephadex G-100	2.5	1200	480.00	3.44	4.53

Lipase activity of about 367.14 U/mg protein was realized in 70% precipitation. The total protein yield was 21 mg. The crude lyophilized sample was used as the enzyme source for subsequent purification process. Generally, no single chromatographic method is sufficient enough to obtain the requisite level of purity. Hence, a combination of chromatographic procedures is necessary. Ion exchange chromatography is widely used as the most common separation method accounting for nearly 67% of the purification schemes reported to date (Saxena *et al.*, 2003). Q-Sepharose is gaining importance in most lipase purification exercises (Menge *et al.*, 1990). Gel filtration is the second most frequently employed purification method applied widely in the lipase purification schemes (Saxena *et al.*, 2003).

The crude enzyme was loaded on to a Q-Sepharose FF, an ion exchanger column (50 mL, 30 cm × 1.6 cm) equilibrated with 0.1 M Tris-HCl buffer (pH 7.5) at a flow rate of 5 mL/min which facilitated separation of the active fractions and most other extracellular proteins. The bound proteins were eluted with a linear gradient of sodium chloride (0-0.5 M) at a flow rate of 5 mL/min. The protein content of each of the fractions was determined and the lipase positive fractions were pooled and concentrated for further use. The total protein and specific activity of the enzyme enhanced to 12 mg and 375 U/mg protein respectively at the end of this step of purification (Table 1).

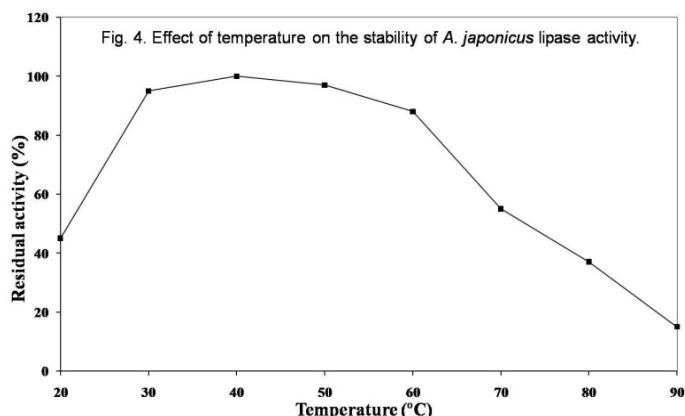
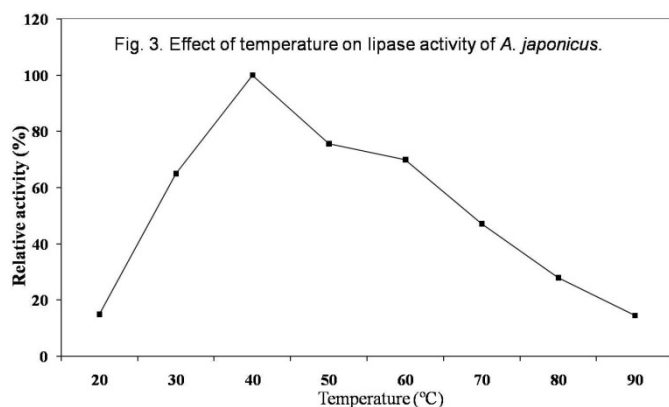
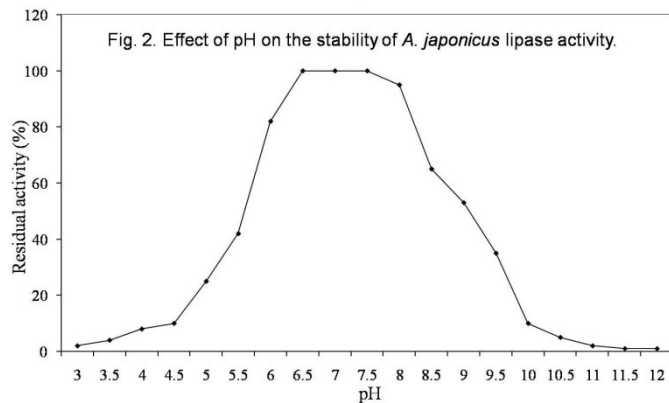
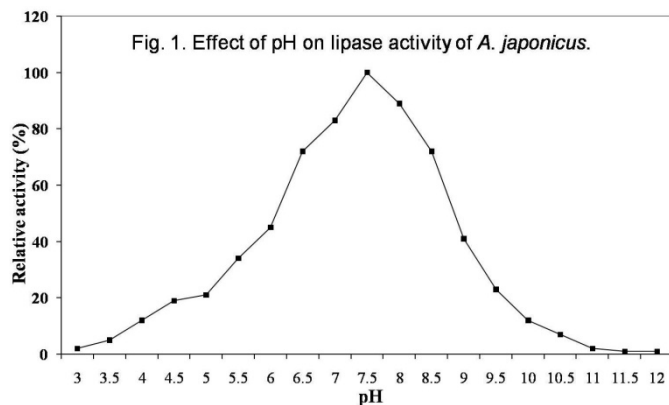
The enzyme realized from the Q-Sepharose column was further concentrated and loaded on to a Sephadex G-100 which was preequilibrated with 0.1 M Tris-HCl buffer (pH 7.5). Elution was carried out with the same buffer at a flow rate of 15 mL/h. The protein fractions were analysed for their lipase activity and protein content. The specific activity rose to 480 U/mg protein. The purification magnitude was 3.44 and the total yield percentage was 4.53 (Table 1). The active enzyme fraction was lyophilized and stored for further use.

The purified *A. japonicus* lipase was run on native PAGE (12%, without SDS) for zymography. The gels were transferred to a 2% agar plate containing 1% tributyrin emulsion in 0.1 M Tris-HCl, pH 7.5 for lipase activity detection. After incubation for 3 h at 40°C lipase activity was visualized as a band of clearing on the tributyrin plate. The zymogram revealed a single band of hydrolysis of tributyrin by lipase (Plate 1).

Molecular weight of the purified lipase from *A. japonicus* was determined by SDS-PAGE on 12% polyacrylamide gels. The molecular weight was determined by using standard protein molecular markers such as 29 kDa, carbonic anhydrase (bovine erythrocytes); 45 kDa, ova albumin (chicken egg); 66 kDa, albumin (bovine serum) and 97 kDa, phosphorylase b (rabbit muscle). The purified lipase showed a single band on a 12% SDS-PAGE. The molecular weight was found to be 43 kDa (Plate 2). The molecular weight arrived at in this investigation matches with that of lipases from other sources—*Aspergillus niger* 35.5 KDa (Namboodiri and Chattopadhyaya, 2000); *Ophiostoma piceae* 37 KDa (Gao and Breuil, 1998); *Mucor hiemalis* 49 KDa (Hiol *et al.*, 1999). To date, there seemed to be no reports on lipase from *A. japonicus*.

The optimum pH of purified lipase was assayed at pH 3-12. 7.5 was found to be the optimum pH at which the activity of the enzyme was maximum followed by a gradual fall in the enzyme activity with further increase in pH leading to a total loss of the enzyme activity at pH 12 (Fig. 1). The enzyme was stable between pH 6.5-8.0 (Fig. 2). A pH of 5.5 has been reported to be the optimum pH for other fungal lipases such as those of *Aspergillus oryzae*, *Humicola lanuginosa* and *Neurospora* sp. TT-241 (Omar, 1987; Toida, 1995; Lin, 1996). The optimum temperature of the purified lipase was determined by incubating 50 µg of the enzyme at different temperatures (20-90°C) along with 2.4 mL substrate solution containing p-nitrophenyl palmitate as substrate as described in the materials and methods. 40°C was found to be the optimum temperature for the purified lipase. Further increase in temperature proved detriment of the enzyme activity (Fig. 3). The enzyme displayed good stability over temperatures ranging from 30-60°C; the optimum temperature was found to be 40°C. The residual activity of the enzyme showed a declining trend between 60 and 90°C (Fig. 4).

Aspergillus carneus lipase shows optimum activity at 37°C (Saxena *et al.*, 2003). According to Razak *et al.*, (1997), very few fungal lipases exhibit temperature optima above 40°C. According to Kambourova *et al.* (2003), the interest in running bioprocesses at elevated temperatures lies in the favourable changes in most physical properties of fats at elevated temperatures and in the stability of thermostable lipases in organic solvents.



chloride. Manganese chloride and cobalt chloride did not influence the enzyme activity (Table 2). However, Mase *et al.*, (1995) reported that lipase activity is not affected by Ca^{2+} , Mg^{2+} , Mn^{2+} , Na^+ , K^+ , and Cu^{2+} in *Penicillium roqueforti* IAM7268. The substrate specificity of purified lipase toward substrates with different acyl chain lengths was determined under standard conditions using various esters of p-nitrophenyl (p-NP). Substrates and chain lengths examined were examined. The enzyme showed specificity towards p-nitrophenyl ester followed by p-nitrophenyl laurate and p-nitrophenyl caprate (Fig. 5). A similar trend was observed in a *Bacillus* lipase which preferred p-nitrophenyl palmitate to other phenyl esters (Mase *et al.*, 1995).

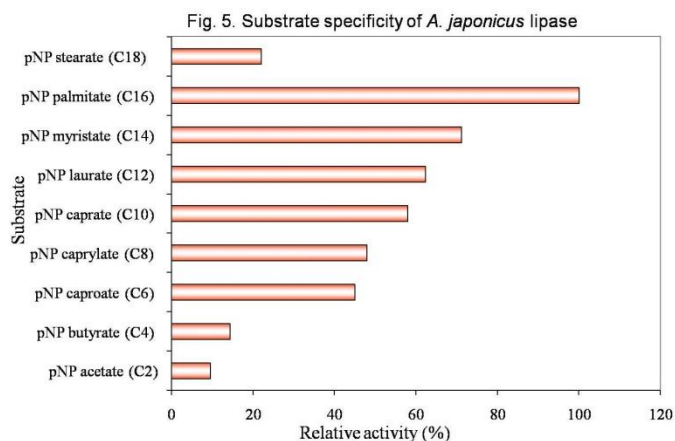


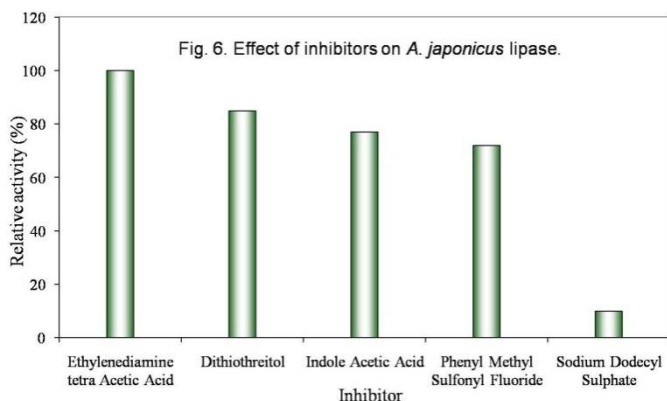
Table 2. Effect of metal ions on purified lipase from *A. japonicus*.

Metal ions (1 mM)	Relative activity (%)
Control	100
CaCl ₂	100
NaCl	56
MnCl ₂	0
CuSO ₄	26
FeCl ₃	12
BaCl ₂	67
CoCl ₂	0
NaH ₃	73
HgCl ₂	82
MgCl ₂	78

The nature of solvent is crucial for enzyme activity. The effects of organic solvents on lipase activity are given in Table 3. The enzyme retained nearly 90% activity in acetone, chloroform, methanol, ethanol and hexane except butanol at 40°C. There was a gradual decrease in the activity of the enzyme with increase in solvent concentration. Activity without the solvent was set as

Metal ions play a key role in enzymology. Therefore, the influence of certain metal ions on the enzyme secretion, activity and stability was investigated in this study. Calcium chloride appeared to be the best inducer of lipase yielding as much as 100% relative activity followed by mercuric chloride, magnesium chloride and barium

100%. It was found that divalent metal chelating agent EDTA, S-S reducing agent DTT, SH carboxy methylation reagent IAA and PMSF had no inhibitory effect on lipase, whereas the anionic surfactant, SDS had an inhibitory effect on the enzyme (Fig. 6). A Similar trend has been reported for *Rhizopus japonicus* lipase by Nishio *et al.*, (1987).



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Table 3. Effect of organic solvents on *A. japonicus* lipase.

Organic solvents	Concentration (%)	Relative activity (%)
Control	-	100
Acetone	10	84
	20	0
Butanol	10	65
	20	45
	30	0
Chloroform	10	38
	20	0
Ethanol	10	23
	20	0
Methanol	10	95
	20	62
	30	0
Hexane	10	5
	20	0

Conclusion

Aspergillus japonicus lipase has been purified to homogeneity by Q-Sepharose and Sephadex G-100 column chromatography. The enzyme has a molecular weight of 43 KDa in SDS-PAGE. Purified *A. japonicus* lipase has a specific activity of 480 U/mg protein. The purification magnitude was 3.44 and the total yield was 4.53%. Purified *A. japonicus* lipase had an optimum pH of 7.5 while the optimum temperature was 40°C. Metal ions such as MnCl₂ and CoCl₂ exhibited an inhibitory effect on the enzyme. The enzyme displayed substrate specificity for p-nitrophenyl palmitate. The enzyme was stable in two organic solvents-methanol and acetone. An-ionic detergent, SDS inhibited its activity.

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