PURIFICATION AND CHARACTERIZATION OF LIPASE FROM BACTERIA

Akshatha K.N.[1], Dr. S. Mahadeva Murthy[2], Dr. N. Lakshmidevi[3]

[1]Research Scholar Department of Microbiology, Yuvaraja's College, Mysore
Email: akshata.kn24@gmail.com
Associate Professor, Department of Microbiology, Yuvaraja's College, Mysore
Email: [2]Smmurthy2025@gmail.com

ABSTRACT

Lipases are industrially important enzymes and are produced by a variety of microorganisms. The purpose of this study was purification and characterization of lipase enzyme from lipase producing strains, LBCK and LBPC1. Both the strains showed positive results for lipase activity in different substrates i.e, Olive oil, Mustard oil and Tributryne. Quantitative assay was done using P-nitro phenyl acetic acid. The optimum pH of the enzyme was investigated at neutral pH. Although the bacterial optimum growth temperature is 30^0C, lipase enzyme showed activity even at above temperatures also. Purification of lipase was done by column chromatography and purified protein bands were observed through PAGE electrophoresis. Further purification process can be allowed to get better enzyme activity. Also mass spectrometry can be used to identify amino acid sequence of enzyme after further purification.

***Keywords*:** Lipase, SDS-PAGE, LBCK and LBPC1, Column chromatography

INTRODUCTION

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) catalyze the hydrolysis and the synthesis of esters formed from glycerol and long-chain fatty acids. Lipases occur widely in nature, but only microbial lipases are commercially significant. Microbial lipases are more widely applied in industries due to their shorter generation time, ease of bulk production, which is further enhanced with advancement in fermentation technologies and ease of manipulation, either genetically or environmentally. Lipase producing microorganisms have been found in diverse habitats such as industrial wastes, vegetable oil processing factories, dairies, soil contaminated with oil, etc **(**Bapiraju *et al*., 2004**)**. Each lipase has a number of unique characteristics such as substrate specificity, regio-specificity, and chiral selectivity and some enzymes are important for the industrial production of free fatty acids, synthesis of useful esters and peptides.(Hansan *et.al*.,2006: Dordick, J.S.1989).Many microorganisms such as bacteria, yeast and fungi are known to secret lipases. Of all these, bacterial lipases are more economical and stable (Achamman *et al*., 2003). Several lipases from *Bacillus*

thermoleoverans (Lee et al., 2001;Markossian *et al.,* 2000), *B. stearothermophilus* (Sinchaikul *et al*., 2001),thermoacidophilic bacteria, *B. acidocaldarius* (Auria *et al*., 2000) and alkaliphilic bacteria, *Bacillus* sp. strain A 30-1(Wang *et al*., 1995) have been isolated and characterized. Bacterial lipases are used extensively in food and dairy industry for the hydrolysis of milk fat, cheese ripening, flavour enhancement and lipolysis of butter fat. Bacterial lipases are mostly extracellular and are greatly influenced by nutritional and physico-chemical factors, such as temperature, pH, nitrogen and carbon sources, inorganic salts, agitation and dissolved oxygen concentration.

The present study is planned to isolate and purify bacterial lipases from two strains i.e, LBCK and LBPC1 with two objectives. Screening of lipase from bacterial strains, isolation and identification of lipase by column chromatography.

MATERIALS AND METHODS

Lipase producing LBCK and LBPC1 strains were obtained from departmental culture collection, Dept of biotech, Naintal used for whole experiments.All chemical and glasswares used were procured from Hi-Media, Mercks Bangalore, Genei, SRL Fine chemicals.

Lipase activity

Lipase activity was found with three different substrates Olive oil, Mustard oil and Tributryne.Three different substrates were added at the concentration of 2% of sterilized Luria Broth.Cultures were inoculated into culture tubes containing substrates and incubated at 37 ºC for 24 hrs.

Total protein content

The amount of protein was estimated by Bradford method (Bradford M.M.1976) using Bovine Serum Albumin (BSA) as a standard.

Biomass determination

Bacteria biomass was determined by measuring the absorbance at 600nm **(**Henroette *et al*., 1993**).**

Effect of incubation time

The effect of incubation time on lipase production was determined by incubating the production medium and the enzyme activity was assayed at different time intervals (0-120 h) with an interval of 12 h.

Lipase assay

Lipase activity was assayed quantitatively by using para-nitro phenyl acetate (*p*NPA) as the substrate. One milliliter of methanol containing 0.2 mg *p*NPA and sixty milliliter of acetonitrile was mixed with 50 mM phosphate buffer containing 0.087g of $K_2H_2PO_4$ mg and 0.06 mg $K_2H_2P0_4$. A total volume of 0.9 ml freshly prepared substrate solution in the ratio of 1:4:95 Acetonitrile (1ml) with PNPA, Ethanol (4ml) and Phosphate buffer (95ml) was mixed with 0.3 ml enzyme solution. After 15 min incubation at 60°C in water bath, absorbance at 405 nm was measured against the blank. The amount of enzyme that liberates 1µg *p*NPA per

minute is considered as one unit. Lipase activity was detected by measuring the hydrolysis of p-nitrophenyl esters. (Winkler and Stuckmann 1979).

Effect of pH on lipase activity and stability

The effects of pH on lipase activity and stability were determined at 37 °C over a pH range of 5.0–9.0 using p-nitrophenyl acetate as substrate. For optimum pH determination, reaction mixture was incubated at 37 °C for 15 min. The enzyme was incubated in 50 mM buffer at the specific pH for 6 h at 37 °C and then the residual activity was determined at pH 8.0.Buffer systems were acetate (pH 5.0–6.0), phosphate (pH 6.0–8.0), Tris-HCl (pH 7.0–9.0).

Effect of temperature on lipase activity and stability

LBCK and LBPC1 strains were inoculated with active cultures. One tube from each set was incubated at 20,30,40,50 and 60ºC for 6h in a water bath and then measuring the residual activity using the activity at 37 °C as control. The half-life of the enzyme at 60 °C was monitored periodically up to 72 h.

Purification of lipase

The lipase enzyme produced in the optimized medium with the best pH and temperature was precipitated with 85% ammonium sulphate and the crude enzyme was used for the purification process. Lipase was purified to apparent homogeneity by a single Sephadex G-100 gel filtration step. The crude enzyme for column chromatography was concentrated by ultrafiltration using a stirred cell (Amicon) with a Diaflo® membrane of 10,000 Da cut off. Specifically, 50 ml of cell-free supernatant of culture broth was filtered at 2.76 kPa pressure for approximately 2 h. The concentrated solution of approximately 10 ml was applied to a Sephadex G-100 gel filtration column (2.6×70 cm; Pharmacia Biotech Co., Sweden) equilibrated with 10 mM potassium phosphate buffer (PPB, pH 8.0) and eluted with the same buffer. Fractions of 2.0 ml were collected every 15 min and assayed for protein and lipase activity. Fractions with high lipase activity were pooled and concentrated. The samples were then stored at 4°C until used.

Electrophoresis

PAGE under non-denaturing conditions and SDS-PAGE were carried out as described by Laemmli(1970). For nondenaturing PAGE, a 6% separating gel, a 3% stacking gel and incorporated with tributyrne were used. For SDSPAGE, a 7.5% separating gel and a 3% stacking gel were used, each containing 5 M urea and 0.5% (v/v) Triton X-100. All gels were run at 20 mA for 120 min. Proteins were stained using CBB R-250.

RESULTS AND DISCUSSION

In this study, LBCK & LBPCI strains were selected to investigate lipase activity. Lipase activity was found with three different substrates olive oil, Mustard oil & Tributryne Purification of lipase was done by column chromatography and purity of protein was checked by PAGE electrophoresis. (Mukesh *et al*, 2012).

Effect of Temperature on Lipase activity

The maximum lipase activity was observed within the temperature range 30-40^{0}c, with different substrates. For olive and Mustard oil, maximum activity was at 30^{0}C, whereas for tributryne it was at 40^{0}C. It was reported earlier also that temperature for lipase activity varies with species to species. Toida *et. al* (1998) reported the maximum lipase activity of *Apergillus oryzae* at 40^{0}C using olive oil as a substrate and enzyme essay was done using 4-Nitropheyl palmitate as substrate. Sztejer *et. al* (1992) reported that lipase from *Penicillium simplicissimum* lost its activity completely at 60^{0}C. In another study Kim *et. al* (1998) reported that lipase from thermophilic strain, *Bacillus stearothermophilus* was most active at 65^{0}C. Substrates used for the above studies were C_6-C_{10} P-nitrophenyl esters. Lipase from thermophilic strain *pseudomonas putida* 35K showed a thermal stability upto 75^{0}C with P-Nitrophenyl caproate as substrate. The results are summarized in table 1and figures 1-3.

Table 1. Enzyme activity of LBCK and LBCK1 strain at different temperature with different substrates

Substrates	**Strains**	**Temperature range**			
		20^{0}C	30^{0}C	40^{0}C	50^{0}C
Olive oil	LBCK	1.402	2.163	1.187	0.587
	LBPC1	1.719	1.996	0.954	0.179
Mustard oil	LBCK	1.367	2.199	2.079	0.069
	LBPC1	1.739	2.288	2.044	1.715
Tributryne	LBCK	1.779	2.235	2.596	0.587
	LBPC1	1.751	2.000	2.288	0.990

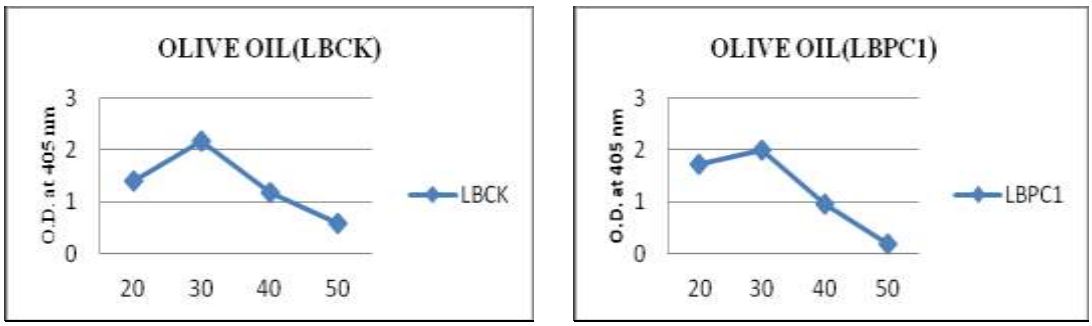

Fig.1. Temperature range for LBCK and LBPC1with olive oil as a substrate

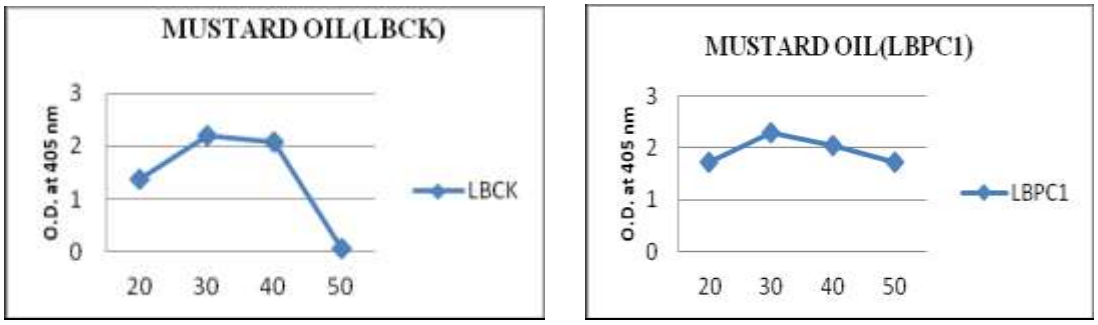

Fig.2. Temperature range for LBCK and LBPC1with mustard oil as a substrate

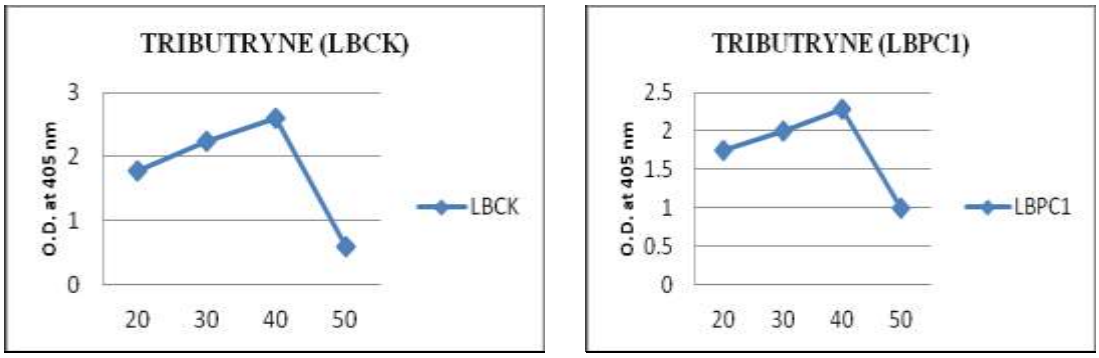

Fig.3. Temperature range for LBCK and LBPC1with tributryne as a substrate

Effect of pH on lipase activity

The maximum lipase activity was within the pH range (7-9) [7 for olive oil & Mustard oil and 9 for Tributryne LBPC1 strain]. The results of pH optimization were shown in table -2and figures 2-6.

Table2. Quantitative essay for lipase activity at different pH

Substrates	**Strains**	**pH range**				
		5	6	7	8	9
Olive oil	LBCK	0.475	0.494	0.501	0.320	0.334
	LBPC1	0.328	0.432	0.518	0.479	0.416
Mustard oil	LBCK	0.349	0.508	0.476	0.439	0.411
	LBPC1	0.349	0.329	0.536	0.372	0.324
Tributryne	LBCK	0.292	0.356	0.469	0.342	0.248
	LBPC1	0.423	0.475	0.508	0.514	0.549

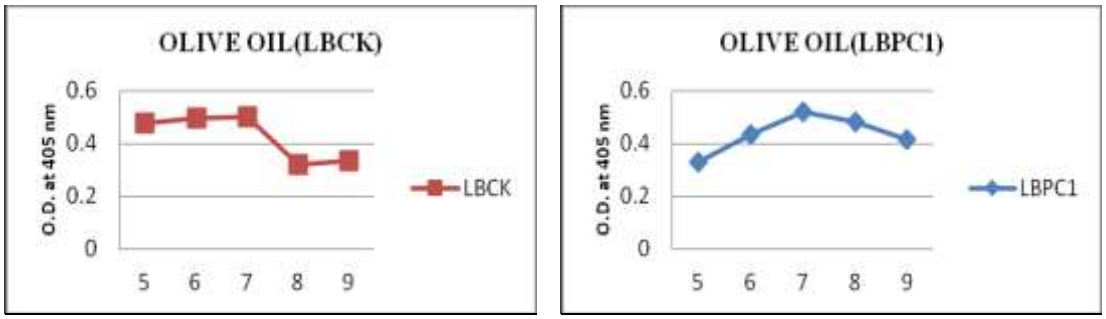

Fig.4. PH range for LBCK and LBPC1with olive oil as a substrate

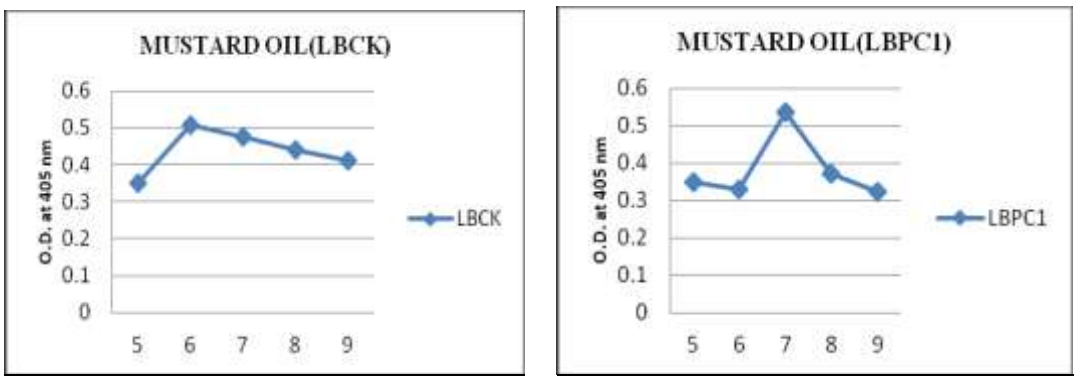

Fig.5. PH range for LBCK and LBPC1with mustard oil as a substrate

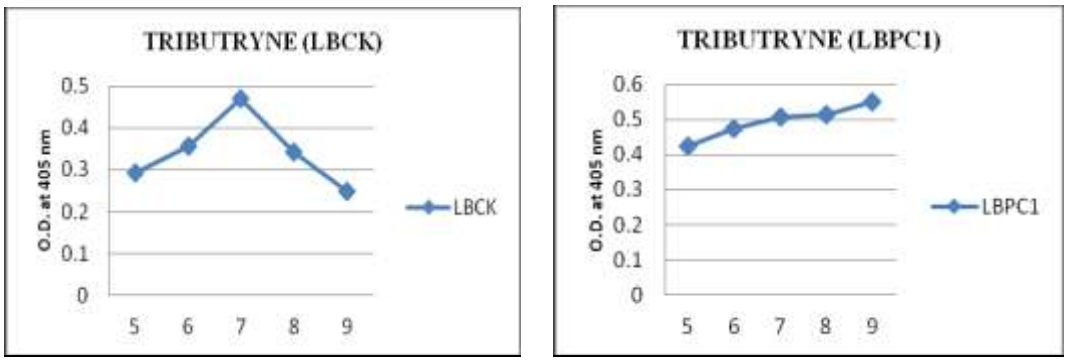

Fig.6. PH range for LBCK and LBPC1with tributryne as a substrate

Motai *et al*. (1966) reported that the optimum pH for *Torulopsis* is 6.5, when Enzyme activity was investigated at 40^0C in buffers of various pH at 50 mM using PNP esters as substrate. In another study, Sztajer *et al*. (1988) reported that optimum pH for *Penicillium Simplicissimum* is 5. Lee *et al.* (1993) reported that optimum pH of lipase from *Pseudomonas putida* 35 K strain is 8.0-9.0. Sugihara *et al* (1995) reported that lipase from *Pichia burgun*i isolated from soil showed a pH of 6.5 P –Nitrophenyl Laurate was used as a substrate. Kim *et al*. (1998) reported that lipase from *Bacillus stearothermophilus* showed a pH range of 9-10. Toida *et al*. (1998) reported that lipase from *Aspergillus Oryzae* showed a

pH range of 9-10. Hardwood *et al*. (1989), Characterized lipase isoenzymes from a technical *Aspergillus niger* enzyme and enzyme activity was determined using 4-Nitrophenyl palmitate and Sodium phosphate buffer, pH was found to be 7.0.

Nambodiri and chattopodhyaya (2000) reported a novel thermostable lipase of *Aspergillus niger* that showed optimum pH range 5.0-6.0. The enzyme was stable upto pH 9.0 and lost its activity when the pH was raised about 12. Table -3: summarized the optimum condition of all the 3 stains for lipase production.

Table 3. Optimum pH & Temperature for Lipase Activity

Substrates	Strains	Temperature	pH
Olive oil	LBCK	30^0C	7
	LBPCI	30^0C	7
Mustard Oil	LBCK	30^0C	6
	LBPCI	30^0C	7
Tributryne	LBCK	40^0C	7
	LBPCI	40^0C	9

Purification of enzyme

Lipase was purified from culture filtrate by Sephadex G-100 column chromatography and protein bands were observed by SDS-PAGE electrophoresis.Various type of lipases from different microorganisms has been reported by many investigators Hofelmann *et al* (1985) reported that molecular mass of lipase from *Aspergillus niger* is 31 and 19kd.Purification was done by Sephadex G-75 gel filtration chromatography and molecular weight was determined by SDS-PAGE (Vijayaraghavan *et al*., 2011).

In another study Muderhua *et al*.,(1986) reported that molecular mass of lipase from *Rhodotorula pilimanae* is 21.4kDa.Lipase was purified from culture filtrate by ammonium sulphate precipitation, ion exchange chromatography and gel filtration. The isolated enzyme was seen in both native and SDS-PAGE.

Suzuki *et al.,*1986 reported that molecular mass of metal insensitive lipase were isolated from *Rhizopus japonicas* is 30kDa.Purification was carried out by Sephadex G-100 chromatography and stepwise precipitation was carried out using acetone at different saturations. The apparent molecular mass of this purified enzyme was estimated to 30kDa by SDS-PAGE.

Therefore from the above study, it can be concluded that both the bacterial strains i.e, LBCK and LBPC1, produces lipases enzyme at broad range of temperature and pH with different substrates. Lipase from LBPC1 was stable from 20ºC to 40ºC upto pH 9.Whereas LBCK lipase was less stable at 20ºC.Hence further research needs to exploit the industrial application of lipase isolated and purified from both the strains.

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