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PURIFICATION AND CHARACTERIZATION OF EXTRACELLULAR LIPASES

TEHREEMA IFTIKHAR[2, 1*], MUBASHIR NIAZ[2], RUKHSANA JABEEN[3] AND IKRAM UL HAQ[1]

[1]*Institute of Industrial Biotechnology, GC University, Lahore*
[2]*Laboratory of Biotechnology, Department of Botany, GC University, Faisalabad*
[3]*Department of Botany, SBKW University, Quetta, Pakistan*

Abstract

The present study describes the enzyme produced after optimization of the cultural conditions subjected to ammonium sulfate precipitation for salting out the proteins. 60% ammonium sulfate showed the enzyme activity of 11.45 U mL^{-1} by wild and 28.2 U mL^{-1} by mutant strain of *R. oligosporus* var. *microsporus* while in 80% ammonium sulfate the enzyme activity by both the wild (13.14 U mL^{-1}) and mutant (29.5 U mL^{-1}) strains increased which indicates the partial purification of enzyme. Desalted enzyme was subjected to DEAE-cellulose column for ion exchange chromatography. Wild strain showed 206.73 fold purification and 82.56% recovery while the mutant strain showed 407.34 fold purification with 62.83% recovery. Sephadex G-100, is a cross-linked polymer used for gel filtration chromatography, which is used for differentiating the molecular size. There is 446.19 fold (W) and 710.02 fold (M) purification of enzyme which showed that most of the contamination proteins are removed. The effect of pH, temperature and metal ions was also investigated on the activity of purified lipases. It is evident from the results that the maximum residual activity by both strains 81% (W) and 100% (M) was observed in the reaction mixture of pH 8.0. The results showed that lipases retained 80% of its activity at 25°C-30°C by wild and 100% of its activity at 20°C-50°C by mutant strain of *R. oligosporus* var. *microsporus.* Mn^{++} stimulated the activity of lipases by the wild while it has inhibitory effect on lipases activity of mutant strain. Other ions Like Ca^{++}, K^{+}, Mg^{++}, Cu^{++} and Na^{+} stimulated the activity of lipases by both wild and mutant strains. Both wild and mutant strains showed the same response of inhibition of enzyme activity in the presence of Hg^{++} and Fe^{++}.

Introduction

Enzymes are considered as nature's catalysts. Lipase (triacyl glycerol acyl-hydrolases, EC 3.1.1.3) catalyses hydrolysis of long chain acyl glycerol at an oil water interface. These are enzymes belonging to the group of hydrolases that present as main biological function to catalyze the hydrolysis of insoluble triacylglycerols to generate free fatty acids, mono and diacylglycerols and glycerol (Lutz, 2004; Kempka *et al.*, 2008). Microbial enzymes are often more useful than enzymes derived from plants or animals because of the great variety of catalytic activities available, the high yields possible, ease of genetic manipulation, regular supply due to absence of seasonal fluctuations and rapid growth of micro organisms or inexpensive media (Saxena *et al.*, 1999; Sharma *et al.*, 2001; Iftikhar & Hussain, 2002; Iftikhar *et al.*, 2003; Iftikhar *et al.*, 2007; Iftikhar *et al.*, 2010b). Microbial lipases are also more stable than their corresponding plant and animal enzymes and their production is more convenient, safer and can be obtained in bulk at low cost (Wiseman, 1995, Vakhlu & Kour, 2006). There is growing interest in large scale purification of lipases. The chemo-, regio- and enantio-specific behavior of these enzymes has caused tremendous interest among scientists and industrialists. Lipases from a large number of bacterial, fungal and a few plant and animal sources have been purified to homogeneity. Different strategies are being used for the purification of various fungal lipases (Saxena *et al.*, 2003). Lipase from *Penicillium candidum* was purified 37-fold using Octyl-Sepharose CL-4B and DEAE-Sephadex columns (Ruiz *et al.*, 2001). The most frequently employed ion-exchangers are the diethylaminoethyl (DEAE) group in anion exchange (58%) and the carboxymethyl (CM) in cation exchange (20%). Strong ion exchangers based on triethylaminoethyl groups (Veeraragavan *et al.*, 1990) and Q-Sepharose (Menge *et al.*, 1990) are becoming more popular in lipase purification. The concentrated protein can be purified on ultrogel, Sephadex G-75, Sephadex G-100, Sephadex G-150 columns by various species of *Rhizopus* (Chattopadhyay *et al.*, 1999). Certain kinetic characteristics are very important in the production of lipases. The extracellular enzymes are the most thoroughly characterized and are produced in much higher amounts (Haas & Joerger, 1995). The optimum pH and temperature for activity of the enzyme were 7.0 and 40°C, respectively (Iftikhar *et al.*, 2010b; Iftikhar *et al.*, 2010c). The lipase was stable in the pH range of 4-9 and at 45°C for 15 min as reported by Hiol *et al.*, (2000). At present, this part of the world has negligible share of enzyme production. The production process for lipases has not been commercialized in Pakistan but country is importing large amount of lipases for industrial application.There is a shifting trend of industrial set up in Pakistan and it is likely that building hi-tech industry will be the major focus of Pakistani entrepreneur in coming days. There is currently interest to permit its production locally (Iftikhar *et al.*, 2010c). The present piece of work has been designed by taking this futuristic view and will provide a base for commercial exploitation of enzymes as an alternative to chemicals. For this purpose the extracellular lipases are purified and characterized to find out its usage in appropriate industry.

Materials and Methods

Microorganisms and culture conditions: A potent identified lipase producing wild strain of *R. oligosporous* var. *microsporous* was obtained from Laboratory of Biotechnology, Department of Botany, GC University Faisalabad. The mutant strain was obtained after treating the wild strain with various physical and chemical mutagens as reported by Iftikhar *et al.*, (2010a). Microorganism was cultured in the medium as reported by Iftikhar *et al.*, (2010a).

Spectrophotometric assay of lipases: Extracellular lipase activity was assayed spectrophotometrically using *p*-nitrophenyl palmitate (*p*-NPP) as substrate according to the method of Krieger *et al*., (1999). "One unit of enzyme activity is defined as the amount of enzyme that released 1 µmole *p*-nitrophenol per minute. Calculations of lipases units was done by the using the standard curve of *p*-nitrophenol".

Protein determination: Protein assay was done by using BCA protein assay kit (PIERCE, Product # 2161297A) as reported by Iftikhar *et al.*, (2008).

*Corresponding author: pakaim2001@yahoo.com/tehreema@gmail.com, 92-42-36854508 (Res.), 92-41-9201488 (Off.)

Glucose estimation: Glucose was estimated by glucose kit (BMI 30092, Korea) as reported by Iftikhar *et al.,* (2008).

Purification of lipase: Purification of lipases was carried out by ammonium sulfate precipitation, ion exchange and gel filtration chromatography (Zia, 2006).

Ammonium sulfate precipitation technique: Crude enzyme was subjected to ammonium sulfate precipitation by the method of Saxena *et al*., (2003).

Salting out of other proteins: Solid ammonium sulfate was added to the crude extract until it was 60% saturated by adding 42 g 100 mL^{-1}. It was kept for 4 h at 4°C, then centrifuged at 10,000 rpm and 4°C for 15 min. The supernatant was separated from the sediment.

Salting out of lipases: The supernatant of above step was adjusted to 85% Ammonium sulfate saturation by adding 17.5 g more salt. It was centrifuged at 10,000 rpm for 15 min. After 4 hours the sediment was separated from the supernatant, the sediments were re-dissolved in minimum distilled water.

Desalting of lipases: The re-dissolved sample was dialyzed in the dialysis bag against continuous stirring distilled water for some hours. All the fractions i.e., supernatant, sediment and desalted samples were subjected to enzyme assay and protein estimation.

Purification by ion exchange chromatography: A column of DEAE-(Diethyl amino ethyl) cellulose was prepared by the method of Kelley & Reddy (1986) and Sukhacheva *et al*., (2004).

Gel filtration chromatography: A column of sephadex G-100 (Pharmacia) was prepared by the method of Jakoby (1971) and Sukhacheva *et al*., (2004).

Characterization of purified lipases: The enzyme purified from the culture supernatant through various steps was then characterized.

Effect of pH on the activity of purified lipases: The effect of pH on the activity of purified enzyme was studied by measuring the enzyme activity at various pH in the range of 4-11, using *p*-nitophenyl palmitate as substrate. Buffer solutions (20mM) of different pH values were used which includes acetate buffer (4, 5, 6), phosphate buffer (7, 8, 9, 10). The purified enzyme was incubated for 1 h in buffers with varying pH values at 30°C and then remaining enzyme activity was measured under standard conditions.

Effect of temperature on the activity of purified lipases: Lipolytic activity of the purified enzyme was determined after 1 h of incubation at temperature 0, 5, 15, 30, 40, 50, 60, 70, 80, 90, 100°C and then remaining activity was measured under standard conditions.

Effect of metal ions on the activity of purified lipases: The effect of various metal ions on the activity of enzyme was examined by assaying the residual activities after incubation of the enzyme with 1mM metal ions for 1 h at 30°C. The chloride salts of the metal ions tested were Co^{++}, Ca^{++}, K^{+}, Mg^{++}, Cu^{++}, Hg^{++}, Fe^{++}, Mn^{++}, Zn^{++} and Na^{+}).

Statistical analysis: The experiments was statistically analyzed by the method of Snedecor & Cochran (1980) using a computer software Co Stat 3.03 CoHort Software, Berkley, CA 94701. Duncan multiple range test was applied under one way ANOVA. Significance has been presented in the form of probability (p 0.05) values.

Results and Discussion

The enzyme produced after optimization of the cultural conditions was subjected to ammonium sulfate precipitation for salting out the proteins. It is evident from the results that 60% supernatant ammonium sulfate showed the enzyme activity of 11.45 U mL^{-1} by wild and 28.2 U mL^{-1} by mutant strain of *R. oligosporus*. While in 80% sediment ammonium sulfate the enzyme activity by both the wild (13.14 U mL^{-1}) and mutant (29.5 U mL^{-1}) strains increased which indicates the partial purification of enzyme. After desalting process the enzyme attained 1.73 fold (W) and 2.34 fold (M) purification and 90.24% and 77.01% recovery by wild and mutant strains respectively (Table 1). Desalting is carried out for removing the traces of salt in sediments that would result in increased enzyme activity by both wild (22.56 U mL^{-1}) and mutant (44.22 U mL^{-1}) strains. Chahinian *et al.,* (2000) reported 25.4 U mL^{-1} extracellular lipases after 80% ammonium sulfate precipitation. Pabai *et al.,* (1995) also reported that an increased lipase activity depends on the concentration of ammonium sulfate solution used.

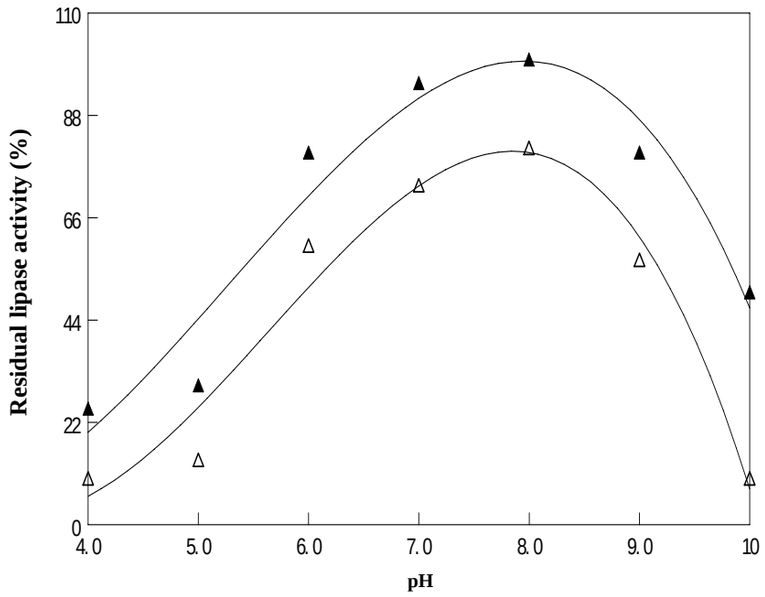
Desalted enzyme was subjected to DEAE-cellulose column for ion exchange chromatography. Out of total fractions taken 13th fraction in elution mechanism contained the maximum lipase activity [20.64 U mL^{-1} (W) and 36.08 U mL^{-1} (M)]. Wild strain showed 206.73 fold purification and 82.56% recovery while the mutant strain showed 407.34 fold purification with 62.83% recovery. Kashmiri *et al.,* (2006) reported 134 fold purification and 46% recovery of lipases.

Sephadex G-100, is a cross-linked polymer used for gel filtration chromatography, which is used for differentiating the molecular size. Third fraction of both wild and mutant strain showed an enzyme activity of 13.92 U mL^{-1} (W) and 28.60 U mL^{-1} (M) and specific activity of 464 mg mL^{-1} (W) and 715 mg mL^{-1} (M). There is 446.19 fold (W) and 710.02 fold (M) purification of enzyme which shows that most of the contamination proteins are removed.

The effect of pH on the activity of purified lipases is shown in Fig. 1. The effect of different pH values on the lipolytic activity of purified enzyme was studied by incubating the enzyme in various buffers with pH range of 4.0 to 10.0. After one hour incubation, residual lipolytic activities of both wild and mutant strains were measured using *p*-nitrophenyl palmitate as substrate. It is evident from the results that the maximum residual activity by both strains 81% (W) and 100% (M) was observed in the reaction mixture of pH 8.0. Any change in the pH resulted decrease in the activity of enzyme. It might be due to the fact that changes in the external pH optima may also altere the ionization of the nutrient molecules and thus, reduced their availability to the organism. Our findings are in accordance with Huang *et al.,* 2004; Salah *et al.,* 2006. The enzyme was stable at pH range of 7.5-8.0 and also retained 90% of its activity as reported by Kasana *et al.,* (2008) and Amoozegar *et al.,* (2008). While Cihangir & Sarikaya (2004) reported maximum production of lipases by *Aspergillus* sp. in the acidic range of pH which are not in agreement with our findings.

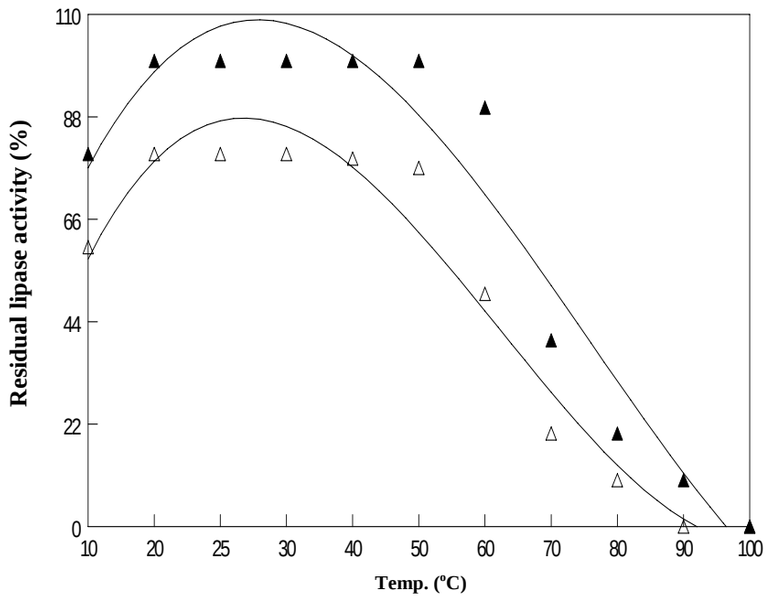
Table 1. Summary of the purification of extracellular lipases by *Rhizopus oligosporus* IIB-63 and its mutant derivative.

Purification stage	Lipase activity (U mL^{-1})		Protein content (mg mL^{-1})		Specific activity of lipase (U/mg protein)		Fold purification		% Age recovery	
	W	M	W	M	W	M	W	M	W	M
Crude	25.0	57.42	24.03	57.02	1.04	1.007	1	1	100	100
Ammonium sulphate	22.56	44.22	12.5	18.74	1.80	2.36	1.73	2.34	90.24	77.01
DEAE-cellulose	20.64	36.08	0.096	0.088	215	410.0	206.73	407.14	82.56	62.83
Sephdex-100	13.92	28.60	0.03	0.04	464	715	446.19	710.02	55.68	49.81



△ Residual Lipase activity % (Wild Type)
▲ Residual Lipase activity % (Mutant)

Fig. 1. Effect of pH on the activity of purified extracellular lipases by *Rhizopus oligosporus* IIB-63 and its mutant derivative.



△ Residual Lipase activity % (Wild Type)
▲ Residual Lipase activity % (Mutant)

Fig. 2. Effect of temperature on the activity of purified extracellular lipases by *Rhizopus oligosporus* IIB-63 and its mutant derivative.

In the present study the enzyme extract was incubated at different ranges of temperature *i.e.,* from 0-100°C (Fig. 2). The effect of temperature on the activity of purified lipases was observed by incubating the enzyme at different temperatures for one hour. The results showed that lipases retained 80% of its activity at 25-30°C by wild and 100% of its activity at 20-50°C by mutant strain of *R. oligosporus.* This comparison shows that the lipases from mutant strain are stable for a greater range of temperature (20-50°C) as compared to the lipases from wild strain. While Maliszewska & Mastelerz (1992) reported *Penicillium* sp., lipase as unstable above 50°C but most active at 40°C. Further increase in the incubation temperature, the activity of the enzyme was greatly inhibited. It might be due to the denaturation of the enzyme at high temperature. Papapavaraskevas *et al.,* (1992) also optimized 40°C temperature for the optimum activity of lipases. Our findings are in agreement with the work of Hiol *et al.,* (2000).

The residual activities were determined after incubation of the purified lipases with 1mM metal ions (Fig. 3). The results showed that Co^{++} has no effect on the activity of lipase by wild strain of *Rhizopus oligosporus* IIB-63 and has inhibitory effect on lipases by mutant strain (IIB-63NTG7). Mn^{++} stimulated the activity of lipases by the wild strain while it has inhibitory effect on lipases activity of mutant strain. Other ions Like Ca^{++}, K^{+}, Mg^{++}, Cu^{++} and Na^{+1} stimulated the activity of lipases by both wild and mutant strains as previously reported by Huang *et al.,* 2004. Both wild and mutant strains showed the same response of inhibition of enzyme activity in the presence of Hg^{++} and Fe^{++}. Similar kind of work has also been reported by Kumar *et al.,* (2005) and Joshi *et al.,* (2006).

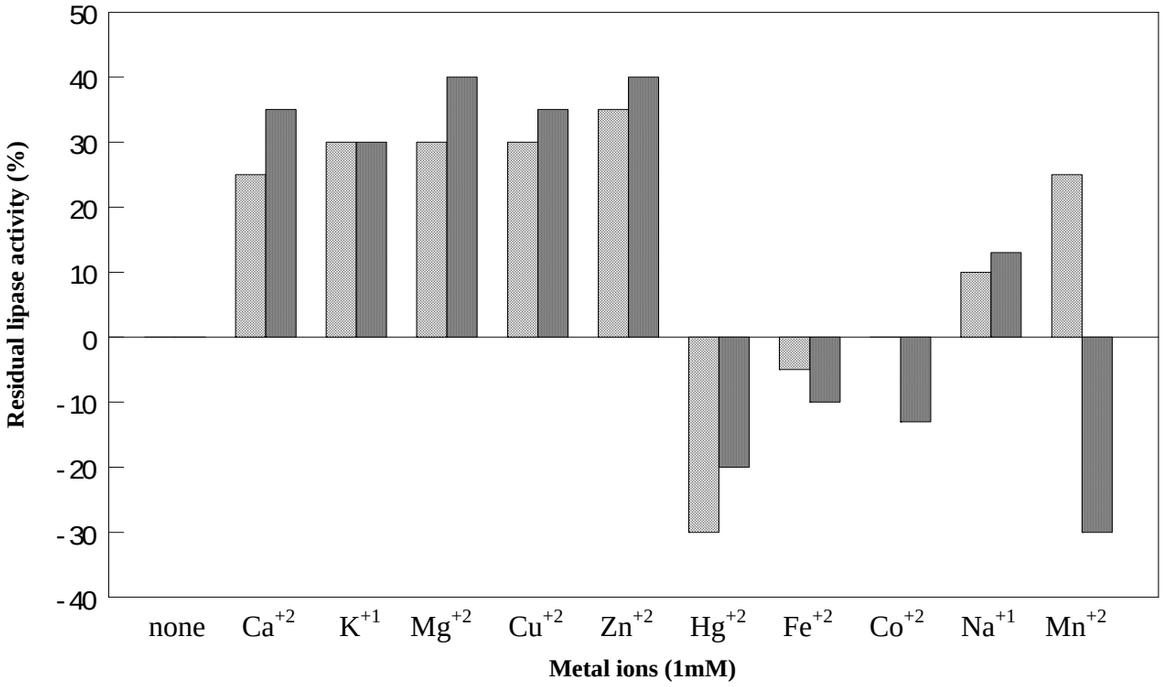


Fig. 3. Effect of metal ions on the activity of purified extracellular lipases by *Rhizopus oligosporus* IIB-63 and its mutant derivative.

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