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PURIFICATION AND CHARACTERIZATION OF EXTRACELLULAR LIPASE BY *GEOTRICHUM CANDIDUM* OF DAIRY ORIGIN

ABUBAKAR MUHAMMAD[1], SYED ALI IMRAN BOKHARI[2], NAEEM ALI[1], HAMID MUKHTAR[3*], ALI NAWAZ[3] AND MUHAMMAD IMRAN[1*]

*[*1]Department of Microbiology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, 45320, Pakistan*
[2]Department of Bioinformatics and Biotechnology, International Islamic University, Islamabad, Pakistan
[3]Institute of Industrial Biotechnology, GC University Lahore-54000, Pakistan
[*]Corresponding author's e-mail address: m_imran766@hotmail.com/mmimran@qau.edu.pk; hamidmukhtar@gcu.edu.pk

Abstract

In the present study, thermostable lipase from *Geotrichum candidum* UCMA 91(ATCC 204307) was purified and characterized. Lipase produced after optimization of the various cultural and physico-chemical conditions was purified to homogeneity by two step methods of purification: ammonium sulfate precipitation and column chromatography. The enzyme was purified by 60% ammonium sulfate precipitation and lipase activity of 5.77U mg^{-1} was attained. Then, Sephadex G-75 was used for gel filtration chromatography and 62.36fold purification was achieved. Molecular mass of lipase was estimated to be 59 KDa by using SDS-PAGE. It is also determined from the study that lipase showed stability at varying range of pH (5-12) and thermo stability (15-65ºC). The lipase was completely inhibited by EDTA (3.98%) confirming it as a metalloprotease, whereas the enzyme was found to be stable in various organic solvents. The results demonstrate that lipase hydrolyzes vegetable oils, which validates its technological relevance for use in the dairy, pharmaceuticals and bakery industry.

Key words: Protein purification, *Geotrichum candidum*, Shake flask fermentation, Biocatalysis, Lipase.

Introduction

Enzymes are regarded as nature's catalysts. Almost all enzymes produced nowadays (and perhaps almost all in the future) are produced by the fermentation of bio based materials. Lipase (EC 3.1.1.3), is a subclass of esterases and catalyzes the hydrolysis of triacylglycerol's and convert them to diacylglycerols, monoacyglycerols, fatty acids and glycerol (Salihu *et al*., 2011; Soler *et al*., 2016). During hydrolysis, a lipase cleaves the acyl group from glycerides and transfers it to the –OH (Martinelle & Hult, 1995). There are region specific and non-specific lipases; region-specific as their name suggest they act on specific positions on lipid molecule while non-specific lipases can catalyze reaction at all positions (Sonnet & Gazzilo, 1991).

Though, lipases can be acquired from animal, plant and microbial sources, microbial lipases have been considered most intensively as of their multipurpose properties, for example stability at elevated temperatures, activity at a wide-ranging pH values, selectivity in racemic mixtures as well as ease of mass production (Yang *et al*., 2005). Among microbial lipases, largely bacterial and fungal, represents the most broadly used class of enzymes that produces varied variety of extracellular lipase (Rigo *et al*., 2010; Jaeger *et al*., 1994). Lipases have numerous industrial applications in the food, degreasing formulation, dairy, medicinal, detergent industries and synthesis of fine chemicals (Gupta *et al*., 2004; Louwrier, 1998). In dairy products especially, cheese have *Geotrichum candidum* as dominant microorganism and known to impart significant impact on product organoleptic attributes. Studies on *G. candidum* lipase are scarce but are of particular interest for their widespread industrial demand and with respect to their use in the food industry (Kocabiyik & Ozel, 2007). Therefore, a need was felt to explore native fungal isolates, capable of producing lipase and at the same relatively stable at the operating conditions.

The activity as well as stability of enzymes are significant factors to regulate the monetary viability in manufacturing procedures. High stability is important from the financial perspective due to lessened enzyme turnover (Gohel & Singh, 2012; Li *et al*., 2004). The high temperature resistance and greater specific activities along with physical and chemical characteristics makes enzymes potent candidate for future biotechnological applications (Temiz *et al*., 2008). The current study was aimed at the characterization and purification of lipase from *G. candidum* isolated from yogurt. It includes study of lipase properties, thermo stability, and solvent and pH resistance for its projected industrial applications. To date, there is no report to characterize and purify lipase from a *G. candidum* of dairy fermented product.

Materials and Methods

Yeast strains and maintenance: Twelve *G. candidum* was isolated from indigenous fermented milk product.. *G. candidum* cells were cultured at 25ºC in the presence of oxy-tetracycline glucose agar (OGA) medium (Merck). The medium pH was maintained to 5.0 (with 1N HCL/NaOH).

Shake flask experiment (SFF): Lipase was produced in 1000 ml erlenmeyer flask containing 250 ml of oxy-tetracycline glucose broth supplemented with 1% tween 80 (Merck) and pH was regulated to 5.0. The flask after sterilization was aseptically inoculated with 2 days old slant of *G. candidum*. The inoculated flask was incubated at 30ºC in rotary shaker (VMR, Model No. 1572-2), at 150 rpm, for 48 h. One milliliter of yeast inoculum was transferred to optimized 250 ml of fermentation medium for lipase production (g/l: Peptone 30.0, Yeast extract

10.0, Olive oil 10.0 and NaCl5.0) and was incubated in a shaker at 30ºC for 48 h. at 150 rpm. After 2 days, the contents of the flasks were centrifuged at 4ºC for 10 minutes at 6,000 rpm. The supernatant was stored at 4ºC and used for further study.

Lipase assay and protein concentration. Lipolytic activity was estimated by *p*-nitrophenylpalmitate (p-NPP) at 410nm by spectrophotometric assay. The method of Lesuisse *et al*. (1993) was used to determine lipolytic activity of lipase. One unit of lipase activity was defined as "amount of enzyme that liberates 1 µg of p-Nitrophenol per minute". Bovine serum albumin (BSA) was used as standard and concentration of protein was calculated according to (Lowry *et al*., 1951).

Purification: After 48 h cultivation, the yeast culture medium was subjected to centrifugation at 4ºC for 30 min at 8,000 rpm (Kokusan Model H-251 centrifuge) and was screened through a filter paper (Whatman No. 1, Sigma Aldrich) to remove *G. candidum* biomass. The yeast cells and other debris was discarded. An enzyme preparation was obtained by precipitation with ammonium sulfate from 10%-100% fractionation (Saxena *et al*., 2003). Initially, the crude protein was dialyzed by protein dialysis kit to get concentrated enzyme free from salt and metal ions. The precipitate was collected by centrifugation 8,000 rpm for 30 min at 4ºC. Sephadex G-75 was used for gel filtration. 3mL of protein precipitate suspension was carefully loaded on the top of packed column pre-equilibrated with the buffer (20 mMTris–HCl, 500 mM NaCl, pH 8.0). T. The protein-containing fractions are assayed for lipase activity. The molecular mass of the purified lipase was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli, (1970).

Characterization of purified enzyme

Effect of temperature and pH on lipase activity: The effect of reaction temperature and pH on lipase activity was measured by assaying at temperature range of5- 65°C and pH3-11.pH stability of lipase was examined by pre-incubation of lipase with0.05M sodium citrate-citric acid pH 4-6.5; 0.05M Potassium Phosphate pH 8-8.5; 0.05M Tris –HCl pH 8-8.5 and Glycine-NaOH pH 9.0-14.0 for 1 h at 30°C.The relative lipase activities were determined by using standard lipase assay as described previously.

Effect of inhibitors, metal ions and solvents on lipase activity: The effect of selected inhibitors; EDTA, PMSF and SDS, at 10mM concentration was determined by incubating purified enzyme and inhibitor for 1 h at 30ºC. The reaction mixture was also subjected to 10mM concentration of different salts (Co^{2+}, Ca^{2+}, Na^{1+}, Mn^{2+}, Cu^{2+}, Mg^{2+}, Hg^{2+}, Zn^{2+} and Cd^{2+}), separately for 1 hour at 30°C. The effect of various solvents, methanol, ethanol, propanol, butanol, hexane, benzene, chloroform, DMSO (Di-methyl sulfoxide) and acetone, on the lipase stability was also examined. The relative lipase activities were measured using standard assay conditions.

Application of lipase: 0.1 M potassium phosphate buffers (pH 7.0), 0.5 g of oil and 3 ml of crude enzyme solution was added in 100 ml erlenmeyer flask and it was incubated in orbital shaker at 30°C for 4 days at 150 rpm. The reaction was stopped by the adding 20 ml of ethyl alcohol and free fatty acid liberated was titrated against 0.1 KOH solutions. A control was carried out in the same way, however; lipase was added later the addition of ethanol. The control value was deducted from experimental, thus acid value was calculated. With the following formula hydrolysis ratio was determined (Kamini *et al*., 2000).

The hydrolysis ratio was calculated as follow:

Hydrolysis Ratio (%) = Acid value *100/ Saponification value

Statistical analysis: All results are expressed as mean of three independent biological repetitions and standard deviations was carried out using Microsoft excel software.

Results and Discussion

The industrial need for effective enzymes is relentlessly increasing; therefore, it is needed to explore for novel enzyme with desired characteristics. In the current study, extracellular lipase from *G. candidum* was purified. Lipase producing *G. candidum* strains were isolated from indigenously fermented milk product.

Purification of lipase: After 48 hours of incubation, yeast culture of UCMA 91(ATCC 204307) was separated from fermentation broth by process of centrifugation (20 min at 8,000 rpm). Then, Lipase enzyme was purified at ammonium sulphate concentration 60%, respectively. Precipitates showed 6.63 purification fold having specific activity of 5.77 U/mg for lipase. After that, lipase was further purified through jel filtration chromatography by running dialyzed enzyme. Results revealed that 62.36 fold purification was attained with specific activity of 54.26 U/mg. Detail of purification procedure of extracellular lipase is tabulated in (Table 1). A consistent increase in the purification fold and specific activity at each step was observed.

Table 1. Purification of lipase from *G. candidum* UCMA 91(ATCC 204307) **(sample mean deviation ≤ 5%).**

Purification step	Volume (ml)	Total Protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)
Crude enzyme solution	450	743	651	0.87	1.0
Ammonium sulfate precipitation	10	66	381	5.77	6.63
Sephadex G-75	5	6.1	331	54.26	62.36

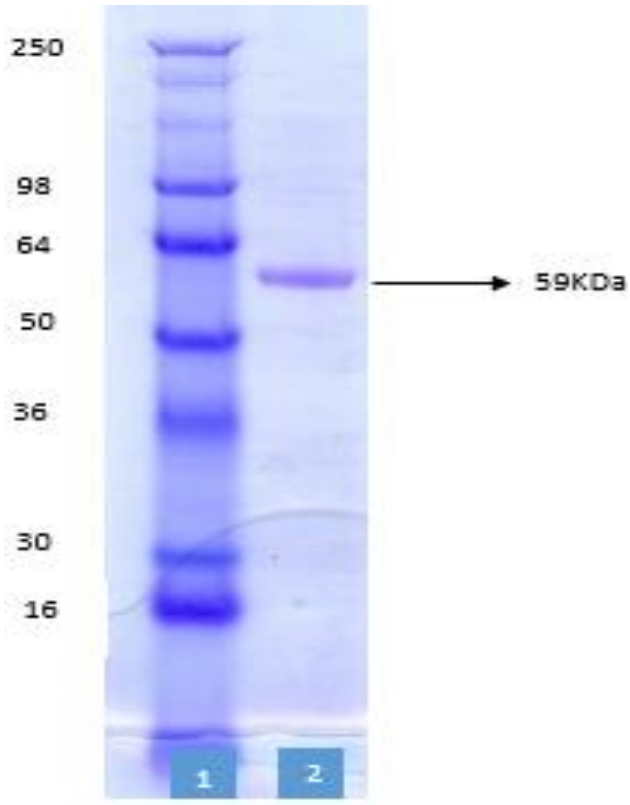

Fig. 1. SDS-PAGE analysis of *Geotrichum candidum* UCMA 91(ATCC 204307)
Lane 1, standard protein marker, Lane 2, purified Lipase

Molecular weight determination: SDS-PAGE was run and molecular weight of protein was calculated 59 KDa (Fig. 1). Our results were coherent with Shu *et al.* (2006) and Gordillo *et al.* (1995).

Lipase enzymatic properties

The influence of temperature and pH on lipase stability: The effect of temperature on the enzyme activity is a very important parameter when it comes to processes in which enzymes are used as biocatalyst, and often cannot be applied due to thermal inactivation of the enzymes (Terrasan *et al*., 2010). The thermo stability of lipase was tested by imposing temperature regimes (5-65ºC) on the enzyme. Purified lipase enzyme showed maximum relative activity of 136.37 at 55 ºC. The lipase retained most of its thermo stability from 15-65ºC. The results showed that lipase from *G. candidum* has good thermo stability and exhibit full activity even after 1 hr. incubation. Our results are coherent with lipase reported by Nawani *et al*. (1998) and Kulkarni, (2000). This thermo stability can accelerate the enzyme use in many biotechnological processes to avoid the contamination by common particularly mesophilic and thermophilic microorganisms (Fig. 2).The lipase showed maximum stability at pH range of 4-7, with maximum relative activity of 134.85% at pH 7 (Fig. 3). Any slight change in pH lessens the enzyme activity as it causes ionization of amino acids in protein molecule (Williams, and Jones, 1976). The results were in agreement with the earlier studies on lipases from *Yarrowia lipolytica* 681 (Corzo & Revah, 1999).

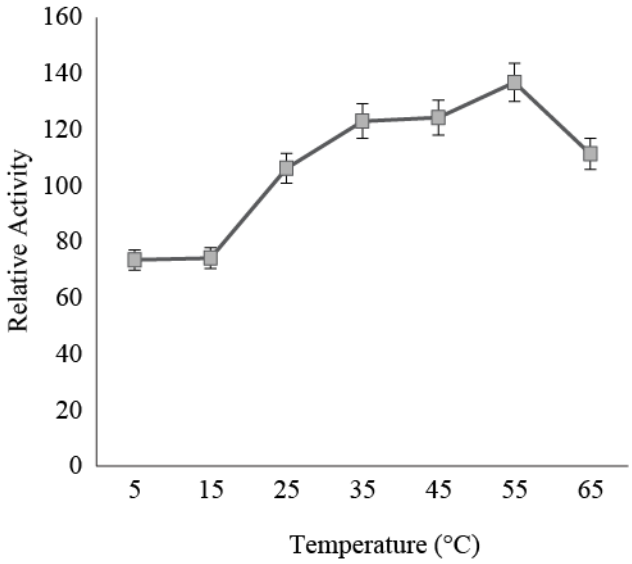

Fig. 2. Stability of lipase at various temperatures (5-65ºC).

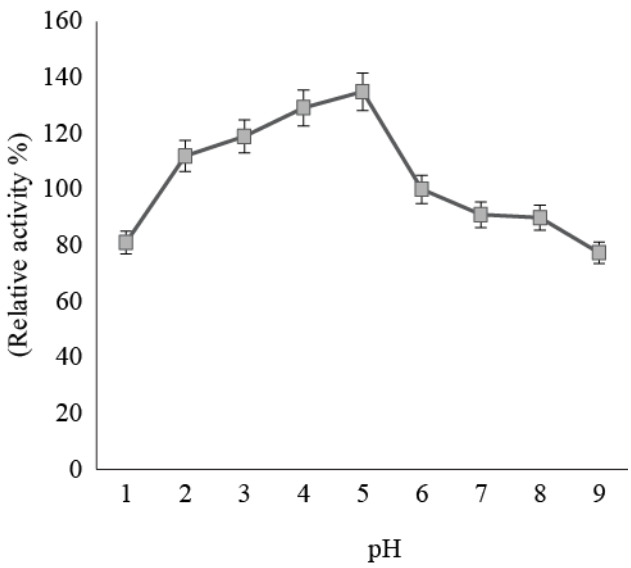

Fig. 3. Stability of lipase on various pH (3.0-11.0).

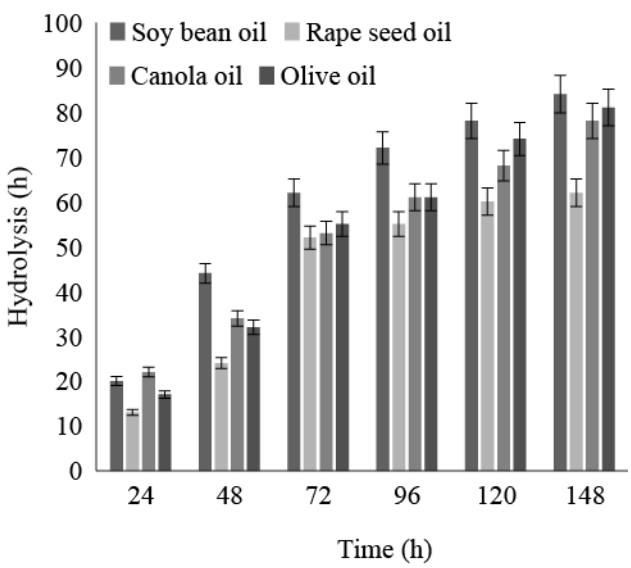

Fig. 4. Hydrolysis of vegetable oils.

Table 2. Effect of various inhibitors (10mM), Solvents (10mM) and metal ions (10mM) on the activity of lipase from *G. candidum* UCMA 91(ATCC 204307).

Relative activity (%)	
Chemicals	
PMSF[a]	65.37 ± 4
EDTA[a]	3.98 ± 10
SDS[a]	94.41 ± 9
Solvents	
Methanol[b]	102.6 ± 12
Ethanol[b]	104.54 ± 7.4
Propanol[b]	106.12 ± 3
Butanol[b]	117.6 ± 9
Hexane[b]	100 ± 6
Benzene[b]	87.18 ± 11
Chloroform[b]	80.81 ± 10
DMSO[b]	102.12 ± 5
Acetone[b]	89.63 ± 3
Xylene[b]	60.90 ± 6.4
Metal ions	
$CaCl_2$[c]	41.02 ± 8.4
$MnCl_2$[c]	46.13 ± 4.3
$CuCl_2$[c]	47.44 ± 3.6
$MgCl_2$[c]	16.1 ± 5.3
$HgCl_2$[c]	34.30 ± 3.0
$CdCl_2$[c]	33.86 ± 7.5
$ZnCl_2$[c]	20.43 ± 8

Relative activity represents average of mean ± SD of triplicates
Inhibitors[a], Solvents[b], Metal Ions[c]

The influence of inhibitors, metal ions and solvents on lipase stability: Inhibition studies suggests first understanding regarding the enzyme nature, its co-factor requirements, and the active site nature. The following inhibitors were used: PMSF, SDS and EDTA. It was observed that PMSF and SDS have no inhibitory effect on the lipolytic activity reducing the relative activity by 65.37% and 94.41% respectively (Table 2). However, the enzyme activity was drastically inhibited in the presence of EDTA 3.98%. This suggests that lipase possesses three amino acid in its catalytic site. EDTA sensitive enzymes are metallolipase in nature. Our results were in line with Chakraborty & Raj (2008). Different researchers have reported that presence of metal ion plays vital role to enhance lipase activity (Adinarayana *et al*., 2004). The presence of appropriate metal ion shows a significant part in order to maintain the enzyme conformation against temperature denaturation and they play important role in biological functions (Fisher *et al*., 2005; Donaghy & Mckay, 1995). Relative activity of purified lipase was determined in various metal ions. Maximum relative activity was observed in the presence of cupric chloride, ferric chloride and manganese chloride 47.44%, 43.94 and 46.13%, respectively (Table 2). The stability and enzyme activity in organic solvents have received great consideration in current decades, as lipases are used in a varied biotechnological fields, like as catalyst in organic synthesis, biotransformations and the optical resolution of chiral compounds. However, minimum relative activity was observed in the presence of magnesium chloride. Numerous reports presented that synthesis of peptide is increased by the organic solvent addition (Gupta, 1992). Stability of purified lipase was evaluated in the presence of different solvents. However, lipase relative activity showed stability against almost all organic solvents, butanol (117.6±9%), proponal (106.12±3%) and ethanol (104.54±7.4%) (Table 2).

Hydrolysis of vegetable oils: The hydrolysis ratio of four plant oils soybean, olive, canola and rapeseed oil was determined by using lipase as biocatalyst. The decreasing order of plant oil hydrolysis ratio is Soybean >Olive>Canola>Rap seed oil (Fig. 4). The hydrolysis ratio of soybean oil reaches to 84% in the presence of lipase enzyme that justifies the lipolytic potential of *G. candidum* and its commercial significance. The hydrolysis of fatty acid and oils is important in oleo-chemical industry and it is energy saving process otherwise conventional high pressure and temperature (Yamamoto & Fujiwara, 1995; Mesbah & Wiegel, 2014). This flexibility makes lipases the potential candidate in food, textile and paper and leather industries.

Conclusions

In present study, a lipase from *G. candidum* was first purified and partially characterized. The characteristics features of the strains *G. candidum* UCMA 91(ATCC 204307) shows that produced lipase is of metallolipase. *G. candidum* lipase has several promising and favorable characteristics for industrial biotechnological applications including wide temperature and pH tolerance range, the stability of lipase in solvent. This observation indicates that strain *G. candidum* and its enzymes might be useful for the dairy industry, pharmaceuticals and biofuel industry. In future, structural study of respective enzyme will be carried out.

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Conflict of interest

The authors declare that they have no conflict of interest.

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