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ORIGINAL ARTICLE

Partial purification, characterization and immobilization of a novel lipase from a native isolate of *Lactobacillus fermentum*

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ABSTRACT

Background and Objectives: Due to the widespread use of lipase enzymes in various industries, finding native lipase producing microorganisms is of great value and importance. In this study, screening of lipase-producing lactobacilli from native dairy products was performed.

Materials and Methods: Qualitative evaluation of lipolytic activity of lipase-producing lactobacilli was performed in different media containing olive oil. A clear zone observation around the colonies indicated the lipolytic activity. The strain with the highest enzymatic activity was identified. Determination of optimal pH and temperature of lipase activity was measured by spectrophotometry using *p*-nitrophenyl acetate (*ρ*-NPA) substrate. Partial purification of lipase enzyme was performed using 20-90% saturation ammonium sulfate. Eventually, lipase was immobilized by physical adsorption on chitosan beads.

Results: Among screened lipolytic bacterial strains, one sample (5c isolate) which showed the highest enzymatic activity (5329.18 U/ml) was close to *Lactobacillus fermentum.* During characterization, the enzyme showed maximum activity in Tris-HCl buffer with pH 7, while remaining active over a temperature range of 5°C to 40°C. The results of the quantitative assay demonstrated that the fraction precipitated in ammonium sulfate at 20% saturation has the highest amount of lipolytic activity, with a specific activity of 22.0425 ± 3.6 U/mg. Purification folds and yields were calculated as 8.73 and 44%, respectively. Eventually, the enzyme was immobilized by physical adsorption on chitosan beads with a yield of 56.21%.

Conclusion: The high efficiency of enzyme immobilization on chitosan beads indicates the suitability of this method for long-term storage of new lipase from native 5c isolate.

Keywords: Lipase; Dairy products; Olive oil; *Lactobacillus fermentum*; Adsoption; Chitosan

INTRODUCTION

Lipases (triacylglycerol acylhydrolases EC 3.1.1.3) are enzymes that catalyze the hydrolysis of ester bonds at a lipid-water interface into fatty acids and glycerols. Lipases are produced by many microorganisms and eukaryotes. The ability to remain active in organic solvents, high yields, ease of genetic manipulation and substrate specificity, region and enantioselectivity, make microbial lipases a better choice than other sources of enzymes (1, 2). Finding native microorganisms that produce lipase enzyme is of significant value and relevance due to the extensive usage of lipase enzymes in several sectors, particularly in the food and pharmaceutical industries (3).

Despite the importance of lactic acid bacteria in the manufacture of fermented foods, little is known about lipases produced by this group of bacteria. This is likely due to the lesser synthesis of such enzymes by these microorganisms, especially when compared to other microorganisms (4). On the other hand, lipase and esterase from lactic acid producing

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bacteria participate in the hydrolysis of milk fat, disintegration of the alcoholic bond and the sterilization process, which control the biological taste of products. Therefore, the synthesis of lipolytic enzymes by dairy bacteria, such as *Lactobacillus* sp. might be interesting to investigate (5, 6).

Enzymes are best employed in an immobilized condition for many purposes. Immobilized lipases offer greater stability, reuse, and continuous functioning, allowing for better reaction control and therefore more advantageous economic considerations (7, 8). Lipases have been immobilized on various supports either by physical adsorption, covalent binding, ionic interactions or entrapment (9). Chitosan, a chitin derivative, is the most appealing immobilization method for its numerous advantages, including a wide range of physical forms (flakes, porous beads, gel, fiber and membrane); low biodegradability; ease of handling; high affinity for proteins and, above all, non-toxicity (10, 11). In addition, earlier experiments using chitosan to immobilize lipase have had positive results.

In this study, isolated strains from native dairy products were screened for production of lipolytic enzymes. After selecting the superior isolate, the biochemical properties of the enzyme were studied. Finally, the partial purification of the enzyme as well as the possibility of enzyme immobilization on the chitosan carrier was investigated.

MATERIALS AND METHODS

Chemicals. Olive oil, chitosan and *ρ*-nitrophenyl acetate (*ρ*-NPA) were purchased from Sigma–Aldrich (St. Louis, USA). Other chemicals used in the present study including NH_4Cl (Merck), $MgSO_4.7H_2O$ (Merck), K_2HPO_4 (Merck), $CaCO_3$ (Merck), MRS agar (Merck) and MRS broth (Merck) were used without any further purification.

Isolation and identification of potential lipase-producing lactobacilli from native dairy products. In the previous study, screening of acid tolerant strains in the presence of acidic phosphate buffer (pH=2.5) led to the cultivation of 18 isolates of *Lactobacillus* spp. from different traditional dairy products (12). All the isolates were inoculated into olive oil-MRS agar, Tween 80-MRS agar and Tween 20-MRS agar. The plates were then incubated at 37°C for 24 hours. The presence of a clean zone surrounding the colonies suggested lipolytic activity (13). Colonies showing clear zones were screened out, purified on olive oil agar plates and transferred to agar slants. The levels of lipase production were determined by growing the isolates with clear zones in a liquid culture. The isolates with higher lipolytic activity were characterized and identified. Bergey's Manual was used to identify the isolates based on their morphological, cultural, and biochemical features. The implemented tests were: Gram reaction, catalase test, gas production from glucose, growth at 15 and 45°C in 1 week; acid production from carbohydrates (1% w/v) and ammonia production from arginine. Molecular identification of lipase-producing Lactobacilli was also accomplished in a prior work using 16S rDNA sequencing (14).

Enzyme production. Each isolate was cultured in MRS broth and incubated in an anaerobic jar at 37°C for 24 h. The 0.5 McFarland turbidity standard was prepared from overnight cultures and inoculated at 2% on nutrient broth medium containing 1% olive oil as a preculture medium. The preculture medium was incubated at 37°C for 24 hours on a rotary shaker (150 rpm), and was inoculated into the production medium at 2%. The composition of the production medium used in this research was: (g/L) NH_4Cl; $MgSO_4.7H_2O$ 0.25; K_2HPO_4 0.5; $CaCO_3$ 0.5; Olive Oil 20; Egg yolk 10. Submerged microbial cultures were incubated at 37°C in 500 ml Erlenmeyer flasks containing 100 ml of liquid medium on a rotary shaker (150 rpm). After 18 hours of incubation, the culture was centrifuged at 5,000 rpm for 30 min at 4°C. We gently removed the top oil layer with a syringe and used the cell free culture supernatant fluid as the sources of crude enzyme extracts (15).

Enzyme assay. Lipase activity was determined spectrophotometrically at room temperature using *ρ*-NPA as substrate. The *ρ*-NPA solution was prepared by adding the solution A (0.001 g *ρ*-NPA in 1ml isopropanal) into solution B (0.01 g gum arabic, 0.02 g Sodium deoxycholate, 50 µl Triton X-100 and 9 ml of 50 mM Tris-HCl buffer, pH 8) at volumetric ratio of 1 to 9 with stirring until all was dissolved. The reaction mixture was composed of 2.5 ml *ρ*-NPA solution, 2.5 ml solution B and 1ml of crude enzyme extract. Solution B was used as blank. Then the absorbance measured at 410 nm for the first 10 min of

reaction. Molar extinction coefficient for ρ-nitrophenol was experimentally determined as 5487.25 M^{-1} cm^{-1}. One unit of lipase activity was defined as the amount of enzyme that released 1 µmol of ρ-nitrophenol per minute (15). Specific activity was calculated as unit of lipase activity per mg of protein in certain volume.

Determination of optimal temperature and pH for lipase assay. For determination of optimal temperature, the reaction mixtures containing the crude enzyme extract in 50 mM Tris–HCl buffer (pH 8.0) were incubated at different temperatures from 5°C to 55°C for 10 min. The crude enzyme used for assay was the culture broth after separation of cells and particles. The optimum pH for the lipase assay was determined by incubating crude enzyme extract at 37.7°C in buffer solutions of pH values ranging from 5 to 11: 50 mM sodium acetate buffer (pH 5-6), 50 mM sodium phosphate buffer (pH 6-7), 50 mM Tris–HCl buffer (pH 7-9), and 50 mM Disodium hydrogen phosphate –NaOH buffer (pH 10-11) (16). Lipase activity was measured using the standard assay as described previously.

Partial purification of lipase enzyme. The crude enzyme extract was fractionally precipitated using 20-90% saturated ammonium sulfate and dialyzed (MWCO 12-14 kDa) with 50 mM Tris–HCl, buffer pH 8 (11). Then the resulting dialysate were checked for enzymatic activity and protein concentration.

Total protein estimation. Using the Bradford technique and bovine serum albumin as the standard, the total protein content was calculated (17).

SDS polyacrylamide gel-electrophoresis. We first prepared the Laemmli SDS polyacrylamide gel (12% separating gel, pH 8.8 and 4% stacking gel, pH 6.8) (18). The prepared proteins were mixed with 5× sample buffer. The samples were then loaded into the electrophoresis wells after being boiled for 5 min. Electrophoresis was carried out in running buffer at room temperature with 110 mV, until the tracking dye ran off. Proteins were visualized after staining by Coomassie brilliant blue R250 and silver nitrate.

Immobilization of lipase on chitosan beads. Lipase was immobilized by physical adsorption on chitosan beads using tripolyphosphate (TPP) as the gelling counter ion (7). In order to prepare chitosan dispersion, after preparing a uniform solution of 2% chitosan in 1% acetic acid, 2 ml of lipase extract was added to a 8 ml chitosan solution. Bubble-free mixture was extruded drop-wise through a syringe (22 gauge) into 20 ml TPP solution prepared in 0.05 M Tris-HCl buffer (pH 7.2) which was placed on a streerer at 50 rpm. The chitosan beads were cured in the TPP solution for 75 min and then washed four times using 3ml of 0.05 M Tris-HCl buffer (pH 7.2). The TPP solution, washing buffers and obtained beads were collected for lipase activity assessment. Instead of enzymatic extract, 2 ml of 0.05 M Tris-HCl buffer (pH 7.2) was used to form the control beads.

RESULTS

Isolation and identification of potential lipase-producing lactobacilli. In this research, putative lipase-producing lactobacilli were screened from a variety of conventional dairy products. The development of a clear zone surrounding the colony served as the foundation for screening, and five colonies were ultimately chosen from the 18 isolates. The quantity of enzyme activity in the production medium containing olive oil was tested in order to choose the optimal isolate. Finally, the 5c isolate with the greatest enzymatic activity was chosen (Fig. 1). The reference data showed that, the 5c isolate is closely related to *Lactobacillus fermentum* with the following properties: Gram (+), catalase (-), oxidase activity (+), glucose fermentation (+), arginin hydrolysis (+), CO_2 production (-), growth at 10°C (+) and growth at 45°C (-). Molecular identification using 16S rDNA sequencing revealed a 98% identity to *L. fermentum*, which has

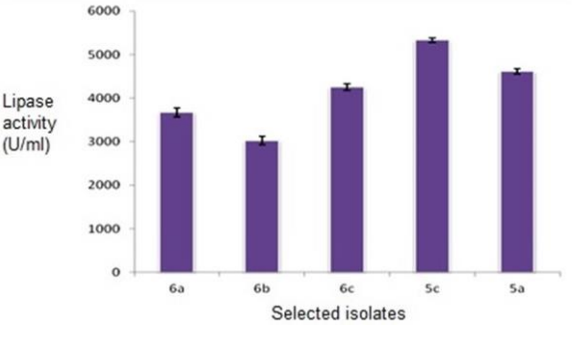

Fig. 1. lipolytic activity of selected isolates in basal production medium after 18 hours of incubation at 37°C, 150 rpm and pH = 7 (Mean ± SD, n:3)

been deposited in the Gene Bank under accession number MH333208 (5c) (14).

Effect of temperature on lipase activity. Because the *ρ*-NPA substrate is temperature sensitive, the absorption fluctuations in the absence of enzyme were investigated first at various temperatures, to allow us for deducting enzymatic activity at different temperatures. The influence of various temperatures ranging from 5°C to 55°C on the enzymatic activity of 5c isolate revealed that this enzyme has the maximum enzymatic activity between 5°C and 40°C, and that activity declines as temperature rises (Fig. 2).

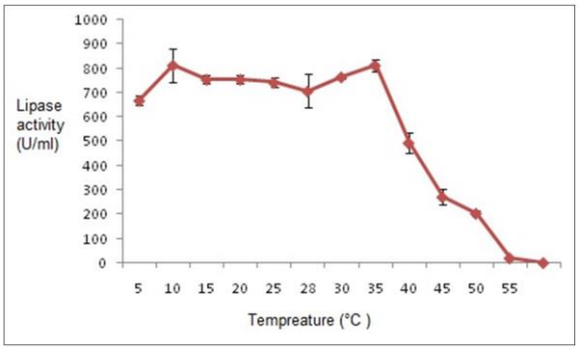

Fig. 2. The effect of different temperatures on lipase activity of 5c isolate. (Mean ± SD, n:3)

Effect of pH on lipase activity. The 5c isolate is extremely sensitive to pH level and buffers, as seen by measurements of enzyme activity at pH 5 to 11. Using a universal buffer to examine enzyme activity at various pH levels, it was found that the enzyme was only active at acidic pH values up to 5, demonstrating the relevance of buffer type for assessing enzyme activity. The enzyme is inactive in a sodium phosphate buffer. It best works in a range of pH from acidic to neutral, with the greatest activity in a Tris-HCl buffer at pH 7 (Fig. 3).

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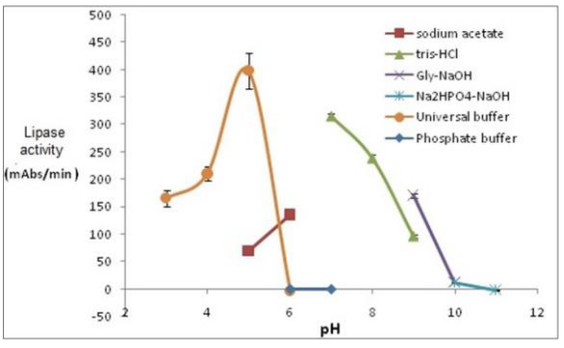

Fig. 3. The effect of buffers with different pH on the enzymatic activity of 5c isolate. (Mean ± SD, n:3)

Partial purification of lipase enzyme. The ammonium sulfate fractionation test revealed that, while there were two fractions with significant protein concentrations (Fig.4a), ammonium sulfate at 20% saturation precipitated the majority of the lipase enzyme of the 5c isolate (Fig. 4b).The maximum specific activity and hence lipase purity was obtained at 20% saturated ammonium sulfate (Fig. 4c). By further scrutinizing the results of this test we found that ammonium sulfate precipitation method can purify lipase by 8.73 times (Table 1). But the yield of purification is too low for considering this method as a successful purification step. Further analysis showed that, in comparison to crude extract, there were low molecular weight proteins as a smear in electrophoretic pattern of most fractions, including the 20% saturation fraction (Fig. 5).

Enzyme immobilization using chitosan biopolymer. Enzyme assay was performed after the immobilization of the supernatant of the production medium using chitosan beads. A second enzyme assay was performed after the prepared beads had been kept at room temperature (25°C) for 24 hours. The immobilization efficiency was calculated to be 56.21% (Table 2).The high percentage of immobilization shows that the chitosan polymer has a great capacity to capture proteins and the lipase enzyme. After 24 hours, the efficiency of stabilized lipase activity was 44.6 percent, indicating that this technique is suitable for preserving lipase enzyme for a long period.

DISCUSSION

In most studies, solid media containing tributyrin

have been used to screen out lipase-producing strains. The use of this substrate alone cannot guarantee that genuine lipase-producing bacteria will be selected (19). Tributyrin composed of short chain fatty acid, namely butyric acid. Tributyrin can be easily broken down by esterases acting on short lipid chains (20). To avoid false positive results, ideal substrates are the long-chain triacylglycerols triolein and olive oil. Triolein is a highly specific lipase substrate; nevertheless, the high content of triolein in olive oil and its lower cost makes it most suitable in lipase activity assays (3, 21). These results are consistent with several studies on microbial lipases showing a high production of lipase in the presence of olive oil among several oils tested (22, 23).

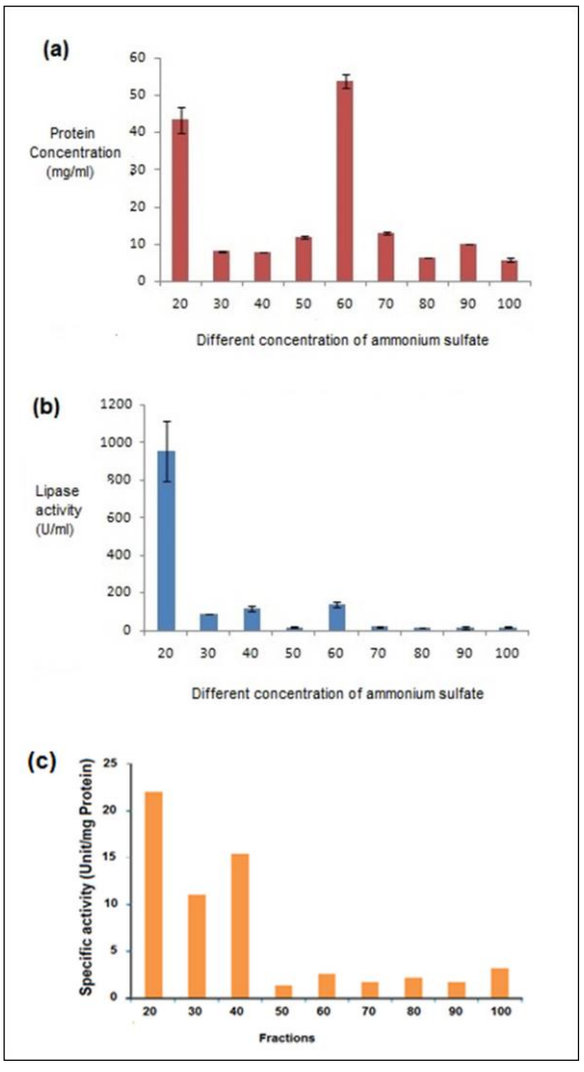

Fig. 4. Ammonium sulfate fractionation of lipase with concentrations of 20-90% saturation ammonium sulfate. (a) The average of total protein concentration, (b) The enzyme activity and (c) The specific activity of each fraction.

The bacteria studied in this work have an optimal temperature ranging from 5 to 40°C. This has been observed in lipases of *L. plantarum* with an optimal temperature of 35°C (24) and in *Lactobacillus* sp. isolated from meat where maximum activity occurs at 40°C (5).

Bacterial lipases are generally neutral or slightly alkaline (25). In this study, the enzyme showed maximum activity in Tris-HCl buffer with pH 7, which is consistent with lipolytic activity of *L. plantarum* at pH 7 (6). While, some articles have reported alkaline pHs of 8-9 as the optimal pH for *Bacillus* and *Lactobacillus lipase* activity (3, 5, 16).

Precipitation is usually used during the early stages of a purification procedure, and is followed by chromatographic separation. The most popular method of protein precipitation is precipitation by ammonium sulfate (26). Ammonium sulfate is a low-cost salt with a high water-solubility and a chaotropic agent that increases entropy in water. It enhances hydrophobic interactions and decreases protein flexibility as a result. Therefore, the third structure of the protein becomes more stable and prevents domains separation (27). However, our findings indicate that ammonium sulfate is less favorable for purifying the 5c isolate produced lipase, and that alternative techniques, such as column chromatography, should be used followed instead. The low recovery of lipase activity may reflect the fact that the addition of ammonium sulphate at high concentration can result in a drop in pH and consequently a loss in enzyme activity (28).

Many studies have used adsorption process for

Table 1. Purification of enzyme lipase by ammonium sulfate at a concentration of 20%.

Purification step	Volume (ml)	Protein concentration (mg/ml)	Enzyme activity (U/ml)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude extract	230	2120.5	5353.43	2.525	1	100
20% saturation ammonium sulfate	5.73	43.292	954.26	22.042	8.73	0.44

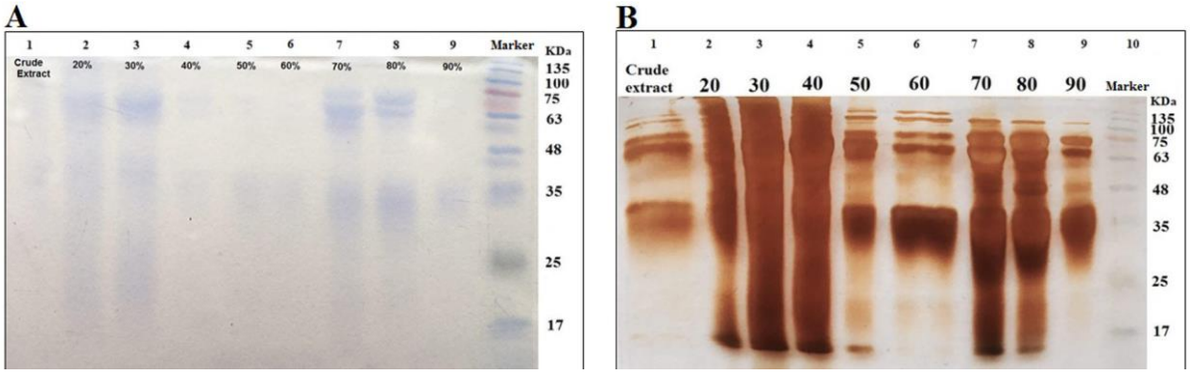

Fig. 5. SDS-PAGE analysis stained by (A) Coomassie Blue and (B) silver nitrate. Lane 1: crude enzyme extract, Lane 2 to 9: the proteins in different fractionsresulted different saturation percentages of ammonium sulfate (20 to 90%), Lane 10: protein marker

Table 2. Immobilization efficiency and activity efficiency related to immobilization of lipase enzyme of 5c isolate on chitosan carrier

Total enzyme activity (U/g carrier)		Immobilized enzyme activity (C)	Immobilization efficiency (%)	Efficiency of activity (%)
Primary (A)	Not absorbed (B)	(U/g carrier)	[(A-B)/A* 100]	(C/A) *100
3616.46	1583.58	1613.09	56.21	44.604

lipase immobilization on chitosan (29). The high stabilization effectiveness in this study might also be attributed to the enzyme's positioning in the active conformation following adsorption on chitosan. Through the creation of hydrogen bonds and hydrophobic interactions, ionic interactions have a minor effect on enzyme immobilization and absorption on chitosan. Because the pH of the immobilization buffer (pH 8) is greater than the pKa of chitosan (6.4), the free amino groups are more deprotonic and positively charged at this pH (11). Most lipases have a hydrophobic lid structure at their active site, positioned in their active space structure by immobilization on hydrophobic carriers, which is comparable to activation at the lipid-water interface (8). In some cases, like lipase B from *Candida antarctica* the lid is very small and does not cover the whole active site but in some other cases like *Bacillus thermocanetulatus* lipase, there is a double lid which simultaneously moves during interfacial activation and complicate the immobilization procedure (30).

CONCLUSION

The goal of this work was to partially purify and characterize *Lactobacillus* sp. lipase (isolated from local dairy products). The great effectiveness of enzyme immobilization on chitosan beads suggests that this technique might be useful for long-term storage of the 5c isolate lipase. Lipase is a widely used enzyme in the food and pharmaceutical industries. Chitosan is also a safe chemical that may be used for the oral administration of proteins and medicines. Therefore, this technique holds promising potentials for coating this enzyme for oral intake.

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