Appl Biochem Biotechnol (2011) 165:785–794
DOI 10.1007/s12010-011-9296-x

Isolation, Characterization, and Catalytic Properties of a Novel Lipase Which Is Activated in Ionic Liquids and Organic Solvents

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Received: 22 June 2010 / Accepted: 25 May 2011 /
Published online: 5 July 2011

Abstract A novel extracellular lipase with organic solvent tolerance was isolated from a local *Pseudomonas* species. The lipase gene was cloned and expressed in *Escherichia coli* as a heterologous host and purified by affinity chromatography. The activity of purified lipase was investigated in the presence of imidazolium-based ionic liquids (ILs) such as EMIM[Cl], BMIM[Cl], and HMIM[Cl]. It has been found that the activity of treated lipase with ILs was higher than untreated control in the hydrolysis reaction. Also, the results indicated that the enzymatic activity strongly depends on IL concentration in reaction media. The best concentration of the IL was 30%, 45%, and 50% (*v*/*v*) for HMIM[Cl], BMIM[Cl], and EMIM[Cl], respectively. Additionally, the enzyme exhibited excellent stability in the presence of 25% of *n*-hexane, toluene, acetone, and *t*-butanol. The optimum values of pH and temperature were determined 10 and 55 °C, respectively. The K_m and V_{max} values were calculated 0.4 mM and 1.92 U/ml, respectively, using *p*-nitrophenyl palmitate as substrate. With respect to the biochemical properties of the newly isolated lipase such as high-level stability and noticeable activity in the presence of organic solvents and ionic liquids, the newly isolated lipase seems to be a good candidate for environmental and industrial processes carried out in non-aqueous media.

Keywords Lipase · Biochemical characterization · Organic solvent · Ionic liquid · *Pseudomonas* sp.

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Introduction

Lipases (EC 3.1.1.3) belong to a group of enzymes which catalyze hydrolysis and synthesis of glycerol esters preferably with long-chain fatty acids at the interface generated by a hydrophobic lipid substrate in a hydrophilic aqueous medium [1]. Lipases are widely spread in nature, but their microbial counterparts have been interested in the last decades since they represent a wide range of biotechnological applications. These enzymes utilize variety of substrates and also exhibit high stability towards extremes of temperature, pH, and organic solvents. Since many of lipases are produced readily in large quantities from microorganisms, they also do not usually require cofactors nor catalyze side reactions, a strong quest exists for lipase research especially from microbial sources [2, 3]. Recently, ionic liquids (ILs) and organic solvents have received increasing attention as attractive solvents in chemical and biotechnological applications [4, 5]. Schöfer et al. showed that transesterfication occurs in different ILs using some lipases and esterases [6]. Lozano et al. showed that the conversion efficiency of lipase from *Candida antarctica* (CAL) and *Pseudomonas* sp. (PSL) was enhanced in the presence of ILs [7].

In this study, the screening and production of a novel lipase from a newly isolated *Pseudomonas* sp. are described. In next step, the lipase hydrolysis activity is studied in presence of various ILs (1-alkyl-3-methylimidazolium-based ILs) and organic solvents in order to evaluate its potential application as an industrial biocatalyst. In addition, the kinetic parameters of the lipase were determined in various temperature and pH conditions.

Materials and Methods

Chemicals

p-Nitrophenyl palmitate (*p*-NPP) as the lipase substrate and ionic liquids were purchased from Sigma (St. Louis, USA). The media components, olive oil, tributyrin, and the solvents were supplied from Merck (Darmstadt, Germany) and used without further purifications.

Isolation of Lipase Producing Microorganisms

Samples were collected from different oil-contaminated regions and inoculated in enrichment medium containing olive oil. Cells were grown at 30 °C and 150 rpm for 2–3 weeks [8]. After enrichment, nutrient agar plates containing olive oil (2.5%) and Victoria blue B (4 mg/100 ml) were used for colony selection. Growing colonies with blue color zones were isolated and further cultivated in liquid medium for lipase production [9].

Lipase Production

The isolated bacterial colonies were precultured for 24 h at 30 °C with 150 rpm. The production medium was inoculated with 1% (*v*/*v*) of the preculture. The lipase was initially produced in a production medium pH 7.0 containing (percent *w*/*v*) peptone 0.5, yeast extract 0.3, glucose 0.1, K_2HPO_4 0.3, KH_2PO_4 0.1, $MgSO_4.7H_2O$ 0.05, $CaCl_2$ 0.01, and Tween 80 0.5 (*v*/*v*). Fifty milliliters of the production medium was taken into a 250-ml

Erlenmeyer flask, inoculated with 1% (*v*/*v*) inoculum and incubated at 30 °C for 24 h with shaking at 180 rpm. After incubation, the culture broth was centrifuged at 10,000×*g* for 20 min at 4 °C and the cell-free supernatant was used for lipase assay [10]. The optimum time needed for lipase production was measured through regular removal of culture medium with 12-h intervals followed by lipase activity assay.

Identification and Taxonomical Studies

The isolated strain was identified according to methods described in *Bergey's Manual of Determinative Bacteriology* [11]. Also, 16S rDNA sequence analysis was performed for phylogenetic recognition of the new strain.

Analytical Methods

Lipase activity was determined spectrophotometrically using *p*-NPP as the substrate. One unit of lipase activity was defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenol per milliliter per minute at 37 °C in pH 8.0 [12]. Also, lipase activity was measured according to Kwon and Rhee with olive oil emulsion as the substrate [13]. Therefore, one unit of the enzyme was defined as the amount of the enzyme which produces 1 μmol of fatty acid released per milliliter per minute at 37 °C and pH 8.0. The cell growth was determined spectrometrically at 660 nm, and the protein content was estimated using Bradford reagent [14].

Biochemical Characterization of Lipase

Recently, we have cloned and simultaneously expressed the lipase and lipase-specific foldase genes in heterologous host and then purified the recombinant protein by affinity chromatography using Ni-agarose columns. Subsequently, the purified lipase diluted in refolding buffer containing the foldase to achieve active enzyme [15]. Optimum pH of the purified lipase was determined through catalytic assay of the enzyme in a pH range from 4 to 11. The assays were performed at 37 °C using olive oil emulsion as the substrate, which is highly stable in extreme pH values.

The effect of pH on the lipase stability was investigated after preincubating the enzyme in 50 mM of different buffers (pH 6.0–11.0) for 30 min at 37 °C and then the enzyme activity was assayed using olive oil emulsion as a substrate. All buffers were prepared in 50 mM concentration with the pH of 4.0–5.0 for sodium citrate–HCl, pH 5.0–7.0 for sodium phosphate–NaOH, pH 7.0–9.0 for Tris–HCl, and pH 10.0–11.0 for glycine–NaOH. The optimum temperature of the purified enzyme was determined by standard assay method in a range of 30 to 70 °C. For thermal stability, the enzyme was pre-incubated at different temperatures (20–90 °C) for 30 min and then cooled on ice, and residual activity was determined by standard assay method .

The effect of substrate concentration (0.01–0.5 mM) on the reaction rate was also determined. The Michaelis–Menten constant (K_m) and maximum velocity of the reaction (V_{max}) were calculated by Lineweaver–Burk plot.

Various additives such as metal ions (5 mM) and chemical reagents such as SDS, sodium taurocholate, Tween 80, and Triton X-100 (1 mM each) were added to the enzyme mixture in a 1:1 ratio and incubated for 30 min at 37 °C followed by standard enzymatic assay. In all these reactions, *p*-NPP was used as the substrate and enzyme sample without any additive or treatment was taken as the control (100%).

Effect of Organic Solvents and Ionic Liquids on Enzyme Activity

Effect of various organic solvents on the lipase activity was investigated at the concentration of 25% (*v*/*v*). The enzyme used in this experiment was prepared in 20 mM phosphate buffer (pH 7) with concentration of 10 μg/ml [15]. The effect of ionic liquids was also examined on lipase activity. The enzyme (10 μg/ml in final concentration) was added into 0.05 M Tris buffer (pH 8.0) containing different concentrations (10–70% *v*/*v*) of each solvent. The remaining enzyme activity was measured after addition of *p*-NPP as the substrate. We have made a control experiment, in which the results showed that high concentrations of ILs itself hydrolyze *p*-NPP, but the rate of hydrolysis by the enzyme in presence of ILs was significantly higher, so we subtracted two rates from each other. Enzyme activity in the absence of ionic liquids was marked as the control (100%).

Statistical Analysis

All data were shown as mean±SD of independent experiments. Data were statistically compared using one-way ANOVA with Tukey post hoc, and $P<0.05$ was considered statistically significant.

Results

Screening of Lipase Producing Microorganisms and Identification by 16S rDNA Sequence Analysis

Among numerous isolated bacteria, ten strains which grew on olive oil agar showed better lipolytic activity. The ability of these strains for production of lipolytic enzyme was measured in liquid medium. One out of ten strains showed significant activity of 2.6 U/ml. Based on the biochemical and physiological tests performed according to *Bergey's Manual of Systematic Bacteriology*, the strain was identified as *Pseudomonas* sp. [11]. The 16S rDNA sequence was deposited in the GenBank, under accession no. EU849665. Using BLAST analysis, the nucleotide sequence was 100% identical to parts of the *Pseudomonas stutzeri* genome.

Cell growth and lipase production were studied for different incubation times at 30 °C with shaking (180 rpm) (Fig. 1). The highest lipase production was achieved after 24 h incubation, which was corresponding to the late logarithmic phase of the strain.

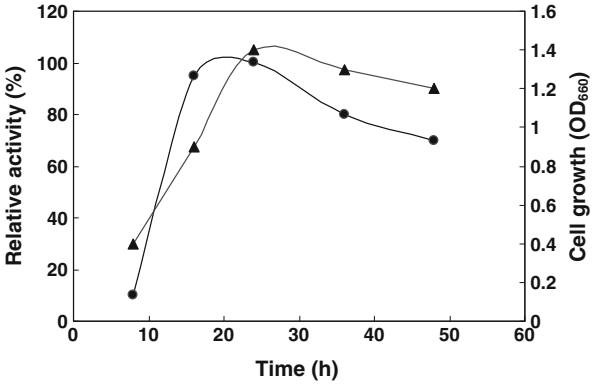

Fig. 1 Time course of lipase production. The cell growth (*circle*) and lipase activity (*triangle*) were measured at 12-h intervals during incubation. Cell growth was measured in terms of OD at 660 nm, and lipase activity was determined using *p*-NPP as the substrate

Cloning, Purification, and Biochemical Characterization of Lipase

Recombinant lipase was expressed in *Escherichia coli* BL21 (DE3) under the control of the strong T7 promoter. Then, it was purified by affinity chromatography and refolded by dilution method [15]. The enzyme thermostability was determined at various temperatures

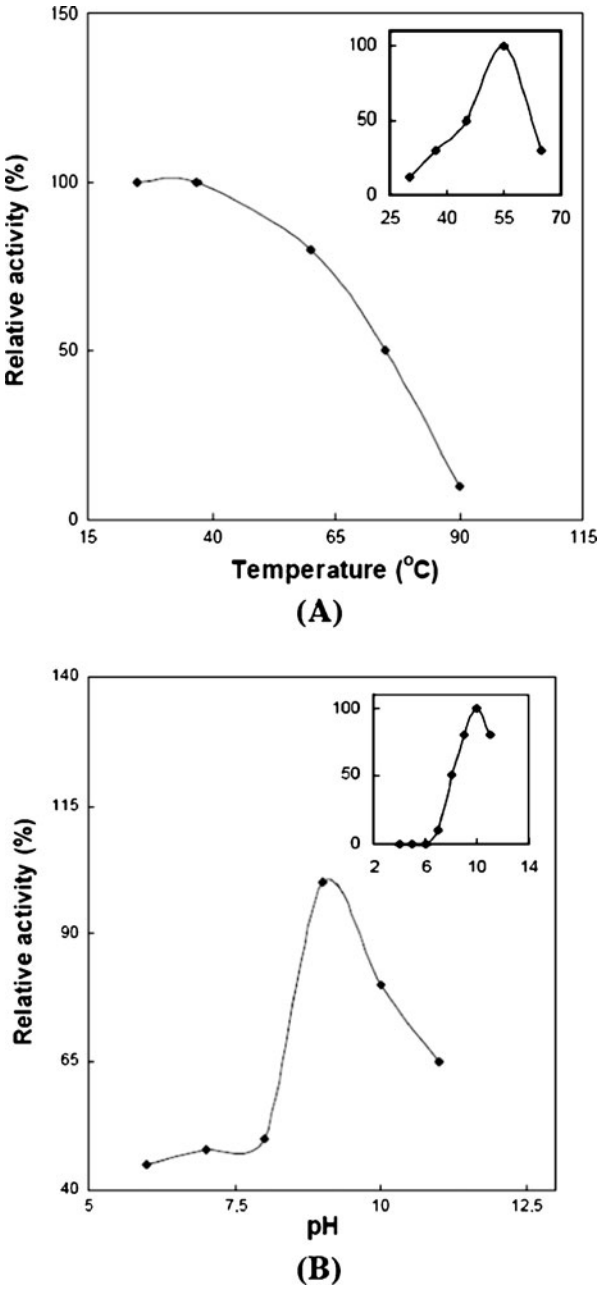

Fig. 2 Effects of temperature and pH on enzyme activity and stability. Optimum temperature for enzyme activity (Fig. 1a, *inset*) and stability of the enzyme (Fig. 1a) was monitored at different temperatures (from 30 to 90 °C) under the standard conditions using *p*-NPP as substrate. Optimum pH for lipase activity (Fig. 1b, *inset*) and pH stability of the enzyme (Fig. 1b) was measured at 37 °C in 50 mM buffer with variable pH using substrate solution of olive oil (for more details, please see "Materials and Methods")

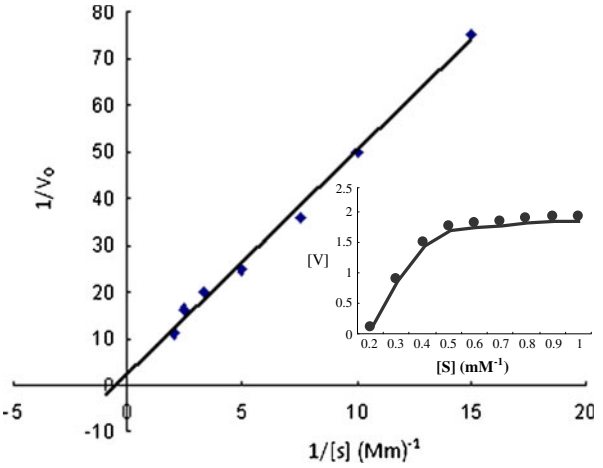

Fig. 3 The Michaelis–Menten constant (K_m) and maximum velocity of the reaction (V_{max}) were calculated by Lineweaver–Burk plot using *p*-NPP as substrate (for more details, please see "Materials and Methods")

ranging from 20 to 90 °C and the pH stability evaluated within a pH range of 5 to 11. The results indicated that lipase retained 100%, 96%, 78%, and 1% of its basal activity after being incubated at 37, 45, 60, and 90 °C for 30 min, respectively. The enzyme was stable over a range of alkaline pH (8–11) after being incubated at 37 °C for 30 min (Fig. 2). The optimum pH and temperature for the lipase activity was measured 10 and 55 °C, respectively (Fig. 2, inset). Kinetic parameters of the purified enzyme were obtained at room temperature using different concentrations of *p*-NPP. The purified lipase had a V_{max} and K_m of 1.9 U/ml and 0.4 mM, respectively, for hydrolysis of this substrate (Fig. 3).

The lipase activity was also investigated under standard conditions in the presence of different metal ions (5 mM) and compared to the control with no additives. None of the metal ions could stimulate lipase activity. Divalent ions such as Fe^{2+}, Ba^{2+}, Mn^{2+}, and Hg^{2+} inhibited the enzyme activity. The lipase activity was slightly affected in presence of monovalent metal ions, Na^{+} and K^{+}, but Ca^{2+}, Cu^{2+}, and Mg^{2+} had no effect on the enzyme activity (Table 1).

Table 1 Effect of various factors on lipase activity

Metal ions	Relative activity (%)	Chemical reagents	Relative activity (%)
Control	100±0.0	Control	100±0.01
Fe^{2+}	72±0.03**	Tween 80	100±0.01
Mg^{2+}	98±0.01*	Triton X-100	80±0.02**
Ba^{2+}	70±0.01**	SDS	100±0.05
Hg^{2+}	67±0.02**	Sodium	100±0.01
Ca^{2+}	100±0.01	Taurocholat	
Cu^{2+}	96±0.05**		
K^{+}	95±0.02**		
Na^{+}	88±0.01**		

Purified enzyme supplemented with the metal ions and chemical reagents at final concentrations of 2.5 and 0.5 mM, respectively; the enzyme was incubated at 37 °C for 30 min on a rotary shaker (150 rpm). Then, remaining activity was determined by a standard method with *p*-NPP . Final enzyme activity was calculated relative to the control activity with no additives. Data are expressed as mean±SD of three independent experiments

*$p<0.01$;**$p<0.001$, in comparison to control

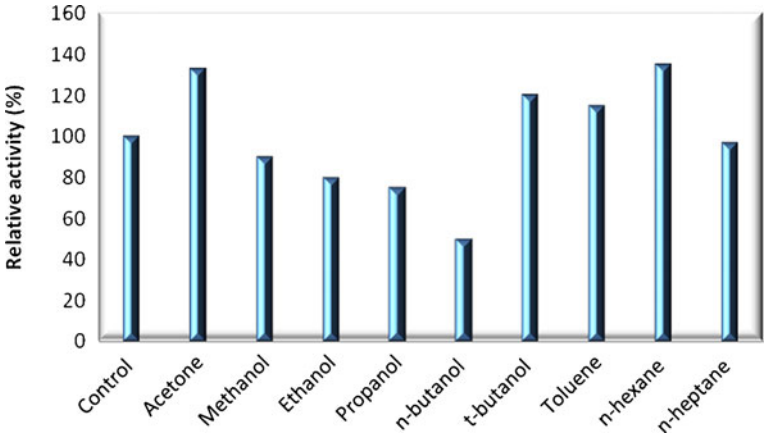

Fig. 4 Activity of purified lipase was determined in various organic solvents with different log *P* at 25% (*v*/*v*) concentration. The enzyme activity was calculated relative to control activity (for more details, please see "Materials and Methods")

Triton X-100 inhibited the activity by 20% in comparison to control, while SDS, sodium taurocholate, and Tween 80 did not show any effect on lipase activity (Table 1).

Effect of Organic Solvents and Ionic Liquids on Enzyme Activity

The effects of various solvents on purified lipase activity are shown in Fig. 4. Among organic solvents, the highest stability was achieved in acetone, *n*-hexane, *t*-butanol, and toluene. In fact, lipase was not only stable but also activated in the presence of these solvents. However, the lipase retained almost its activity in methanol and *n*-heptane but lost its activity in the presence of ethanol, propanol, and *n*-butanol by 20%, 25%, and 50%, respectively (Fig. 4).

Figure 5 shows the enzyme activity in buffers containing 10–70% (*v*/*v*) of EMIM[Cl], BMIM[Cl], and HMIM[Cl] as reaction medium at room temperature. The enzyme activity was increased drastically in the presence of ionic liquids. The enzyme activity was also highly dependent on IL concentration, and the best concentrations were determined 30%, 45%, and 50% (*v*/*v*) for HMIM[Cl], BMIM[Cl], and EMIM[Cl], respectively, while further increase in IL content reduced enzymatic hydrolysis reaction.

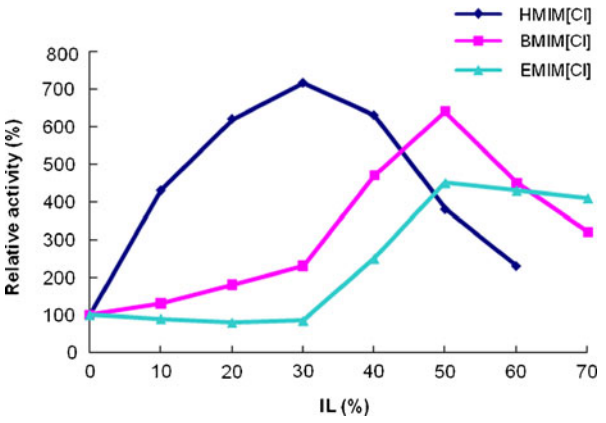

Fig. 5 Activity of purified lipase was determined in the presence of various imidazolium-based ILs with 10–70% (*v*/*v*) concentration. The enzyme activity was calculated relative to control activity (for more details, please see "Materials and Methods")

Discussion

The use of bacterial lipases for a variety of biotechnological applications is rapidly and steadily increasing which has led to the isolation and characterization of a large number of these enzymes during the last few years. In the present work, a local strain which was identified as *Pseudomonas* sp. was isolated from soil after olive oil enrichment in medium. *Pseudomonas* lipases are highly interesting biocatalysts, and their catalytic and biochemical properties make them suitable for potential applications [16, 17]. Biochemical characterization of the new enzyme showed that its optimum temperature is similar to the reported amount for lipase produced by *Pseudomonas fluorescens* HU380 [18]. Generally, thermal stability is a desirable characteristic of enzymes since the productivity of the reaction could be greatly enhanced at relatively high temperatures. It was found that optimum pH for lipase activity falls on the alkaline range which is usually observed for lipases from *Pseudomonas* genus [19]; relative thermal stability and optimum alkaline pH expect the enzyme suitable for detergent industry. As described for most of extracellular lipases, the enzyme was secreted in the culture when the bacterial cells reached the late logarithmic phase after 24 h of incubation [2]. It is claimed that accumulation of products formed in the culture media can inhibit the activity or production of the enzyme during the long stationary phase [20].

The effects of various environmental conditions and chemical agents on the activity and stability of lipase were investigated in order to evaluate its potential industrial application. Experiments on the effects of different metal ions on lipase activity revealed that Fe^{2+}, Ba^{2+}, Mn^{2+}, and Hg^{2+} can inhibit the enzyme. Probably, these ions impose some conformational changes towards a less stable structure [21]. The lipase activity was slightly affected by the presence of monovalent metal ions such as Na^{+} and K^{+} as similarly reported for lipase from *Pseudomonas aeruginosa* KKA-5 [21]. Divalent ions such as Ca^{2+}, Cu^{2+}, and Mg^{2+} showed no effect on lipase activity [22, 23]. In contrast to our results, some reports have proved strong activation of lipase in the presence of Ca^{2+} since the enzyme required calcium as a cofactor for catalytic activity [24]. Contradictory to neutral effect of Cu^{2+} on the present lipase, this ion has inactivated lipase from *P. fluorescens* HU380 [18].

Activity of enzymes in organic solvents depends on the properties and concentration of the solvents and the nature of the enzymes [25]. The highest activity was achieved against acetone, *n*-hexane, *t*-butanol, and toluene. In fact, this lipase was not only stable but also activated in the presence of organic solvents. The ability of solvents to increase the solubility of substrates, thus facilitating the reaction or maintaining the active structural conformation of the enzyme, might be the cause of higher lipase activity in exposure to organic solvents [16]. Also, the results show that use of ethanol, propanol, and *n*-butanol in reaction system leads to inactivation of the lipase. Protein molecules in aqueous solution are surrounded by a hydration layer composed of water molecules associated with the protein surface. It has been suggested that organic solvents tend to displace the water molecules both in the hydration layer and in the interior of the protein, thereby distorting the interactions responsible for maintaining the native conformation of the enzymes [26]. It has been emphasized that particularly, the increase in polarity of water-miscible solvents (acetone > methanol > ethanol > propanol > butanol) leads to increase the enzyme inactivation because of essential water stripping by polar organic solvents. But in fact, solvent polarity is one of the factors determining the activity and stability of biocatalysis [16, 27].

Previous studies have indicated that enzymes activity and stability could be better preserved in the ionic media [28, 29]. For instance, lipase from *Candida rugosa* was not active in pure hydrophilic, but active in some hydrophobic ionic liquids [30, 31]. Most of

the water-miscible ILs acted as enzyme deactivating agents by stripping essential water molecules from the enzyme microenvironment in reaction media, but there are a few exceptions [30–34]. Lou et al. showed that the lipase hydrolysis activity was increased three times in the co-solvent systems with 20% (*v*/*v*) BMIM[BF4] content [35]. Dang et al. showed that the activity and stability of lipase pretreated with ILs (BMIM[BF4] and EMIM [BF4]) were higher than those of untreated lipase for the hydrolysis reaction in an aqueous medium [36]. In this study, the results indicated that the lipase hydrolytic activity was enhanced significantly in aqueous solutions of imidazolium-based ionic liquids as compared to the control system containing no IL. The ILs could interact with charged groups of the enzyme, inducing conformational changes in the enzyme's structure and making the enzyme more functional for hydrolysis activity. Also, in accordance to the elongation of the alkyl chain attached to the cation in three ILs which are used here, the hydrolytic activity significantly increased [35, 37]. But high concentrations of ILs decreased the enzymatic activity. In higher concentrations of ILs, elevated ionic strength of the reaction medium can inactivate the enzyme. Also, high viscosity of the reaction mixture might limit the diffusion of substrates and products to and from the active site of the enzyme [35]. However, more factors and further experiments toward its structure and stability in the organic solvents and ionic liquids are required to analyze the exact reason of activity reduction in the case of newly isolated lipase precisely.

In summary, we have isolated a new lipase gene from local microflora that is related to lipases from *Pseudomonas* sp. The enzyme was expressed and biochemically characterized in the presence of imidazolium-based ionic liquids in reaction media. It has been found that the activity of pretreated lipase with ILs was remarkable higher than untreated lipase in the hydrolysis reaction. Moreover, the results clearly indicate that improvement of enzyme activity in organic solvents and alkaline solution, suggesting the enzyme applicability in bioremediation processes, detergent industry, and biotransformation in non-aqueous media.

Acknowledgments We would like to gratefully thank Mehrnoosh Fathi-Roudsari for manuscript editing.

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