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Isolation, production, and characterization of an extracellular lipase from *Trichoderma harzianum* isolated from soil

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Abstract: This is the first report about the characterization of *Trichoderma harzianum* lipase. A novel strain of *Trichoderma harzianum* IDM14D was isolated from soil. The isolated strain was cultivated for lipase production in shake flasks at 30 °C for 7 days. For lipase production, it was determined that the best carbon source was glucose and the best nitrogen source was peptone. Maximum biomass was produced at a concentration of 1.25 g/L, in 7 days. The optimum pH and temperature for activity of the enzyme were 8.5 and 40 °C, respectively. The lipase was stable at a pH range of 8.0-10.0 and at 40 °C for 60 min. Ca^{2+} and Mn^{2+} enhanced lipase activity but it was determined that other metallic ions did not affect the enzyme activity. The K_m and V_{max} values of the crude enzyme for *p*-nitrophenyl butyrate hydrolysis were found to be 7.15 mM and 7.067 mM/min, respectively.

Key words: *Trichoderma harzianum*, extracellular lipase, enzyme characterization

Topraktan izole edilen *Trichoderma harzianum* hücre dışı lipazının izolasyonu, üretimi ve karakterizasyonu

Özet: Bu çalışma, *Trichoderma harzianum* lipazının karakterizasyonu hakkında ilk rapordur. Yeni bir suş olan *Trichoderma harzianum* IDM14D topraktan izole edildi. İzole edilen bu suş, lipaz üretimi için çalkalamalı kültürde 30 °C'de 7 gün inkübe edilmiştir. Lipaz üretimi için en iyi karbon kaynağı glukoz, en iyi azot kaynağının pepton olduğu belirlendi. Maksimum biyokütle üretimi 7 gün sonunda 1,25 g/L olarak belirlendi. Enzimin en iyi aktivite gösterdiği pH 8,5, sıcaklık 40 °C olarak belirlendi. *T. harzianum* lipazı pH 8,0-10,0 aralığında 40 °C'de 60 dakika kararlılığını koruduğu belirlendi. Ca^{2+} ve Mn^{2+} iyonlarının lipaz aktivitesini artırdığı, fakat diğer metal iyonlarının enzim aktivitesini etkilemediği gözlendi. *p*-nitrofenil butirat hidrolizi ile ölçülen ham enzimin K_m ve V_{maks} değerleri sırasıyla 7,15 mM ve 7,067 mM/dk olarak belirlendi.

Anahtar sözcükler: *Trichoderma harzianum*, hücre dışı lipaz, enzim karakterizasyonu

Introduction

Lipases (acylglycerol hydrolases, 3.1.1.3) are enzymes, the biological function of which is to catalyze the hydrolysis of triacylglycerols to fatty acids, mono-, di-, and triacylglycerols, and glycerol. Lipases show optimum enzymatic activity at the oil-water interface contrary to other esterases, which have optimum activity in homogeneous media with soluble substrates (1).

Lipases catalytic potentials are enormous. Lipases of microbial origin which are used in the food, diary, cosmetic, detergent, and tanning industries are particularly attractive. In view of their current and potential applications, lipases are considered to be a promising class of industrial enzymes (2). After proteases and amylases, lipases are considered to have the third highest sales volume, up to billions of dollars, showing their application versatility, which makes them especially attractive for industrial applications (3-5). Today, lipases are the choice of biocatalysts because they show unique chemo-, regio- and enantioselectivities, which enable the production of novel drugs, agrochemicals, and fine chemicals (6-9).

Lipases are produced by animals, plants, and microorganisms, but the majority of lipases used for biotechnological purposes have been isolated from bacteria and fungi. Filamentous fungi are preferred sources of lipases because they produce extracellular enzymes (7). The most productive species belong to the genera *Aspergillus*, *Rhizopus*, *Mucor*, *Penicillium*, and *Geotrichum* (10-12).

Potential industrial applications of lipases comprise food additives (modification of aromas), fine chemicals (esters synthesis), detergents (hydrolysis of fats), wastewater treatment (decomposition and removal of leaginous substances), leather (fat removal from animal skin), and pharmaceutical and the medical area (medicines and digestive enzymes for diagnosis) (13,14).

The industrial demand for highly active preparations of lipolytic enzymes continues to stimulate the search for new enzyme sources. *Trichoderma harzianum* is used as biocontrol agent. It has been reported that *Trichoderma harzianum* produces some hydrolytic enzymes such as β-glucanase, cellulase, chitinase, and amylase (15,16). The first isolation of *T. harzianum* lipase was performed in Turkey by Topal et al. (17). Although they determined the lipolytic activity of *T. harzianum*, the enzyme was not characterized. This report describes for the first time, the characterization of a novel extracellular lipase from *Trichoderma harzianum* IDM14D isolated from soil in Turkey. Therefore, the enzyme was obtained from a new and productive source.

Materials and methods

Microorganisms

The various strains of lipolytic fungi were isolated from soil. Soil samples were collected from different regions around Rize in Turkey (18). The organism used, *Trichoderma harzianum* IDM14D, was identified according to the method of Samson and Hoekstra (19) by Prof. Dr. İsmet Hasenekoğlu (Atatürk University, Erzurum, Turkey). The fungus was stored at –20 °C in a 20% glycerol solution.

Culture conditions and optimization

The organism was cultured in 100 mL of basal mineral medium ((g/L): 12 NaH_2PO_4, 2 KH_2PO_4, 0.330 $CaCl_2.2H_2O$, 0.030 $ZnSO_4.7H_2O$, 0.030 $MgSO_4.7H_2O$, 0.005 $FeSO_4.7H_2O$), in a 250 mL conical flask shaken at 150 rpm at 30 °C for 7 days. The effects of various nitrogen (peptone, yeast extract) and carbon sources (glucose, olive oil, and soybean oil) were estimated in relation to enzyme yield. The initial pH of the media was adjusted to 6.2 using 1M NaOH.

Biomass dry weight

The medium biomass was determined according to its dry weight. The growth media was filtered through pre-weighted filter paper (Whatman No.1) to extract the biomass, which was subsequently dried in an incubator at 30 °C for 24 h (20).

Crude lipase preparation

In order to remove fungus cells and spores, the culture was filtered through filter paper (Whatman No.1) and then the filtrate was centrifuged at 10,000 rpm at 4 °C for 15 min. The supernatant was collected to perform lipolytic activity assays (21). The crude extract was stored at –20 °C until used.

Determination of lipase activity

The lipolytic activity assay was performed for the enzyme that is responsible for lipid hydrolysis according to Lee et al. (22). The lipolytic activity of the enzyme was determined by measuring the increase in absorbance at 405 nm for *p*-nitrophenyl acetate (*p*-NPA), *p*-nitrophenyl butyrate (*p*-NPB), and *p*-nitrophenyl palmitate (*p*-NPP). The amount of 1 mM *p*-nitrophenolate released per minute was defined as 1 unit of lipase activity.

Protein quantification

The protein quantity of the crude enzyme extract was determined by the Lowry method using bovine serum albumin as standard (23).

Electrophoresis

A 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using extracellular crude extracts according to Musidlowska-Persson and Bornscheuer (24) on a Thermo Scientific Owl P81 Series electrophoresis unit (New York, USA). The gel, which consisted of 0.5% Triton X-100 dissolved in 0.1 M Tris-HCl buffer at pH 7.5, was incubated in a renaturation solution for 1 h. For activity staining, the gel was incubated for 30 min in a 1:1 ratio mixture of solution A (0.1 M α-naphthyl acetate suspended in acetone, which was then added to Tris buffer at pH 7.5) and solution B (0.1 M Fast Red solution dissolved in Tris buffer at pH 7.5) (25).

Effect of pH on the activity and stability of lipase

Lipolytic activity of the enzyme was determined using *p*-nitrophenyl butyrate as a substrate with ethanol in a buffer solution at a pH range of 4.0-11.0. The measurement of the lipase activity was performed at 60 °C by using 50 mM of sodium acetate (pH 4.0-5.5), potassium phosphate (pH 6.0-8.0), and NaOH-glycine buffer (pH 9.0-11.0) respectively (22). The pH stability of the enzyme extract was determined by using the same buffers with 0.5 pH value increments. The enzyme extract and the buffers were mixed in a 1:1 ratio and incubated for 24 h at 4 °C. The residual activity of the enzyme was subsequently determined with 0.3 mL of this mixture using *p*-NPB as a substrate. The residual lipase activity was measured as a percentage (%) by comparison it with the unincubated enzyme (21,23,24,26,27).

Effect of temperature on the activity and stability of lipase

A temperature gradient was employed in order to determine the lipolytic activity of the enzyme. The buffer mixture, substrate solution, and crude enzyme extract were incubated over range temperatures between 20 to 80 °C for 20 min at the observed optimum pH. The relative enzyme activity was determined spectrophotometrically at a specific temperature as rapidly as possible. The thermal stability of the enzyme was determined by incubating the crude enzyme extract at temperatures between 20 and 80 °C with 10 °C increments for 1 h. Samples were then rapidly cooled down to room temperature. Once reached, the enzyme activity assay was performed at an optimum pH value under the standard reaction conditions (28,29).

Effect of substrate concentration on lipase activity

A substrate saturation curve was plotted by the final substrate concentration in the range between 0 and 30 mM versus the activity of lipase in the presence of *p*-NPB as a substrate. The Michaelis-Menten constant (K_m) and the maximum velocity of the reaction (V_{max}) were determined from the Hanes-Wolf plot.

Effect of metal ions on lipase activity

The effect of metal ions on the activity of the enzyme was verified by the addition of 1.0 mM chloride salts Na^+ and K^+ for monovalent ions, Cu^{2+}, Ca^{2+}, Ba^{2+}, Mn^{2+}, Fe^{3+}, Cd^{2+}, and Co^{2+} for divalent ions, and Cr^{3+} for trivalent ions. Each metal ion solution was mixed with the enzyme extract at a 1:1 ratio and the mixture was then incubated at room temperature for 20 min. The relative enzyme activities were expressed in percentages by comparison of them with the standard assay mixture, with no metal ion added at optimum pH (22).

Results and discussion

Culture conditions and optimization

Different types of carbon and nitrogen sources were tested in order to determine their effects on the growth of the fungus and production of lipase. The best growth was obtained with carbon and nitrogen sources glucose and peptone, respectively (Figure 1). Maximum lipase activity was determined as 0.24 U/mL on the 7th day in a glucose plus peptone medium. Minimum lipase activity was determined as 0.15 U/mL on the 7th day in a glucose plus yeast extract medium (data not shown). Similar results were obtained by Ohnishi et al. (30). They reported that lipase activity tended to increase as the glucose concentration increased from 0% to 4%. Dalmau et al. (31) also stated that the glucose addition was a direct

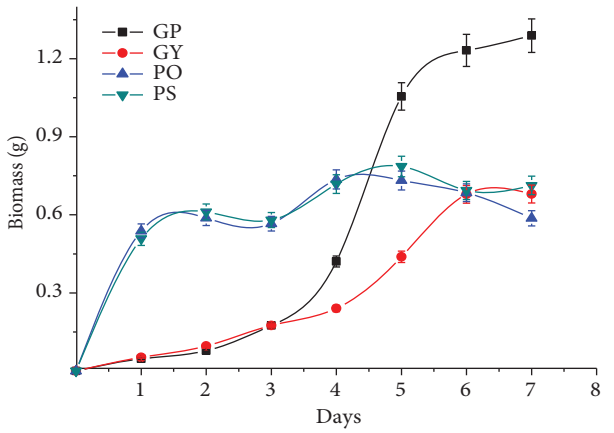

Figure 1. Determination of the effect of different carbon and nitrogen sources on the growth of the fungus and the production of *T. harzianum* IDM14D. (GP: Glucose + Peptone; GY: Glucose + yeast extract; PO: Peptone + Olive oil; PS: Peptone + Soy bean oil).

consequence of the glucose effect on lipase secretion. In another study (32), it was determined that there was no difference between glucose and olive oil for lipase production. Moreover, the lipase activities of many other fungi, such as *Aspergillus wentii* (33), *Mucor hiemalis* (34), and *Burkholderia cepacia* (35) are also stimulated by the addition of glucose to the production medium.

The lipase activity of the *T. harzianum* IDM14D crude extract was determined spectrophotometrically using *p*-nitrophenyl acetate, *p*-nitrophenyl butyrate, and *p*-nitrophenyl palmitate. Among these substrates, the best lipase activity was observed in the presence of *p*-nitrophenyl butyrate (data not shown). Enzyme activity was confirmed in a native gel by activity staining using α-naphthyl acetate and Fast Red dye (Figure 2).

Effect of pH on the activity and stability of Lipase

The pH-relative activity (%) profile of lipase activity was determined by using *p*-NPB as a substrate and the optimum pH was found to be 8.5 (Figure 3a). Most microbial lipases have their optimum activity at a pH range of 7.0-9.0 (36). An optimum pH of 7.0 for *Mucor hiemalis* f. *hiemalis* lipase (37), pH of 8.5 for *Aspergillus carneus* (38), and pH 9.0 for *Penicillium caseicolum* lipase (38) has been reported.

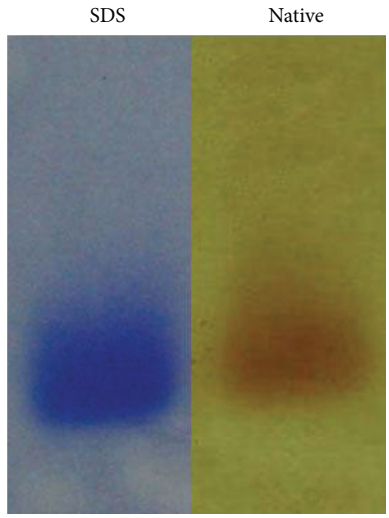

Figure 2. Detection of *T. harzianum* IDM14D lipase activity after electrophoresis in SDS and native conditions.

The residual activity of lipase from *Trichoderma harzianum* IDM14D was determined after 24 h of incubation at various pH values ranging from 4.0 to 11.0 at 4 °C (Figure 3b). The *T. harzianum* lipase was extremely stable at an optimal pH and retained at least 70% of its original activity at a pH range of 8.0-9.0, after 24 h of incubation at 4 °C. It was reported that *Aspergillus carneus* lipase retained 52.4% of its original activity at a pH of 11.0 (38). It was also reported that *Aspergillus carneus* lipase was stable at a pH range of 8.0-10.0 (38).

Effect of temperature on the activity and stability of lipase

The thermal activity profile for crude lipase from *T. harzianum* is shown in Figure 4. The optimum temperature of *T. harzianum* lipase was found to be 40 °C and it was observed that enzyme preparation had very high activity at temperatures ranging from 30 to 50 °C. Similar results were reported to be 40 °C for *Mucor hiemalis* f. *hiemalis* lipase (37), 40 °C for *Fusarium solani* lipase (40), and 45 °C for *Ophiostoma piliferum* lipase (41).

The thermostability of the enzyme was examined by measuring the residual activity after 1 h incubation at different temperatures, at a pH of 8.5 (Figure 5). The enzyme was quite stable at 20-40 °C and retained 50% of its original activity at 50 °C for 1 h. However, 1 h incubation at 60-80 °C almost completely destroyed the lipase activity from *T. harzianum*. It

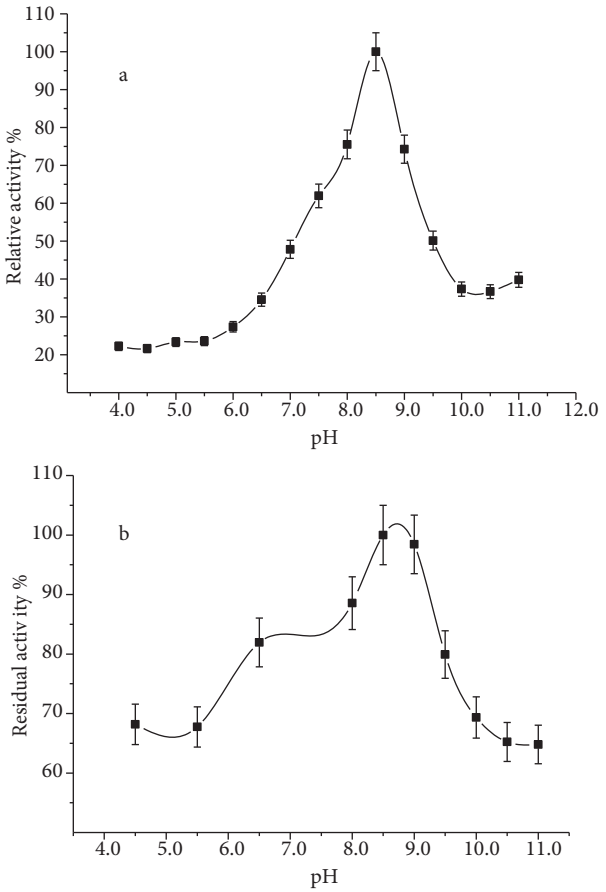

Figure 3. Effect of pH (a) on the activity of *T. harzianum* IDM14D lipase and (b) its stability.

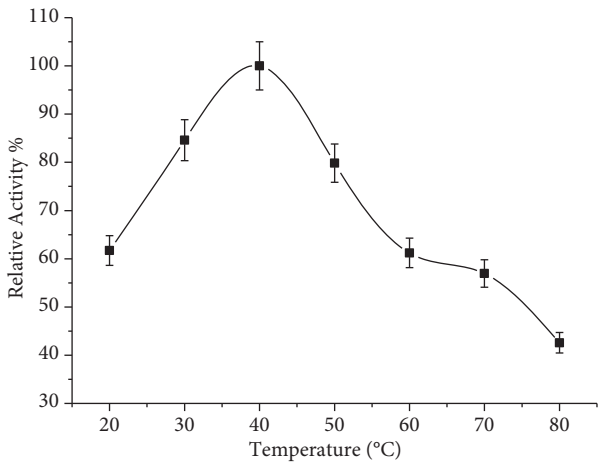

Figure 4. Optimum temperature of *T. harzianum* IDM14D lipase.

has been reported that the drop in the percentage of residual activity at high temperatures results first in some conformational changes in the tertiary structure, and then almost complete inactivation of

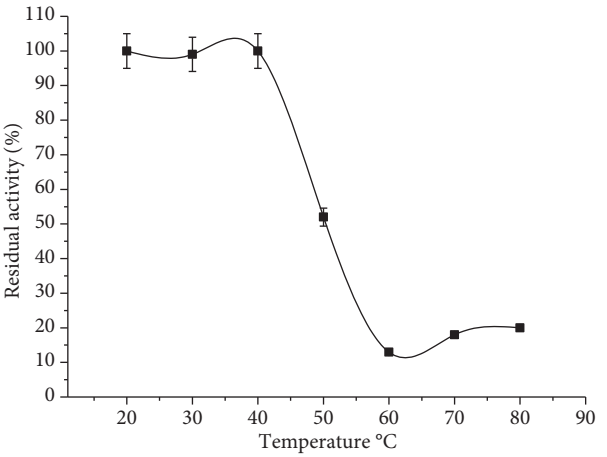

Figure 5. Thermal stability of *T. harzianum* IDM14D lipase.

the enzyme (29). Similar thermal stabilities have also been reported for other lipases (37,42,43).

Effect of substrate concentration on the lipase

Substrate saturation curves for *p*-NPB indicated that the *T. harzianum* lipase followed simple Michaelis-Menten kinetics. The substrate saturation curve was obtained by interpolating the substrate concentrations against activity values. The reaction velocity increased up to approximately 5.796 µM *p*-NPB and then reached a constant value. Michaelis-Menten constants (K_m) and maximum reaction velocities (V_{max}) were determined as 7.15 mM and 7.067 mM/min, respectively, from a Hanes-Wolf plot (Figure 6).

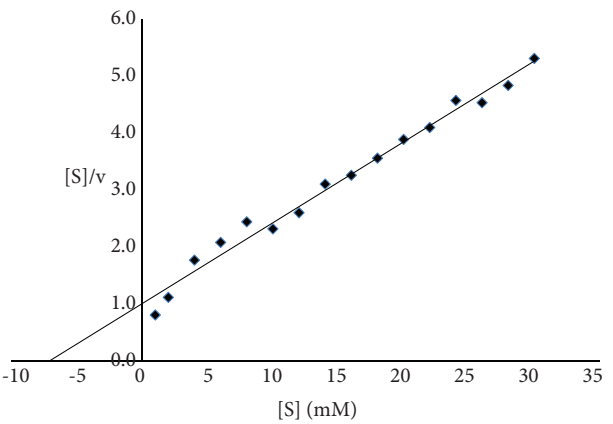

Figure 6. Hanes-Woolf plot for *T. harzianum* IDM14D lipase as substrate *p*-NPB.

Effect of the various metallic ions on the lipase activity

It is known that metal ions have a significant function to continue the enzyme in active and stable structure by binding to amino acid residues with negative charge in specific sites (28). The behavior of the *T. harzianum* IDM14D lipase in the presence of some metal ions was investigated by using chloride salts of each metal at a concentration of 1 mM (Table). These results showed that Ca^{2+} and Mn^{2+} ions increase lipase activity up to 25% and 15%, respectively. *T. harzianum* IDM14D lipase activity is partially stimulated by these metal ions.

Table. Effects of the various metallic ions on lipase activity.

Metallic ion (1 mM)	Relative activity %
Control	100 ± 1
Na^{+}	93 ± 2
K^{+}	78 ± 2
Ca^{2+}	125 ± 3
Ba^{2+}	93 ± 1
Cu^{2+}	90 ± 1
Cd^{2+}	98 ± 2
Co^{2+}	95 ± 2
Mn^{2+}	115 ± 4
Cr^{3+}	79 ± 2
Fe^{3+}	93 ± 1

The lipase activity was slightly inhibited by K^{+} and Cr^{3+} but was not affected by other metallic ions. Similar results were obtained from different *Mucor* species (37,44). Hasan et al. (46) had reported that metal ions generally would form complexes with ionized fatty acids, changing their solubility and behavior at interfaces. The release of fatty acids to the medium is rate determining and could be affected by metal ions. However, the effects of metal ions depend on the particular lipase. The activity of lipase from *Rhizopus japonicus* NR400 was not affected by the addition of 1 mM metal ions (47). The activity of *Rhizopus chinensis* lipase was enhanced 24% by 1 mM Ca^{2+} (48). On the other hand, the lipase of *Aspergillus oryzae* was inhibited by 77% by 5 mM Ca^{2+} (30).

In this study, we identified a new strain of *Trichoderma harzianum* IDM14D, which excretes an extracellular lipase. This is the first report describing the production of lipase by this strain of *T. harzianum* IDM14D isolated from soil in Turkey. Although lipases from different species of fungi have already been characterized, there is only one report about *Trichoderma viride* (45).

Lipase isolated from *T. harzianum* was characterized and determined from some biochemical properties. The pH curve for the *T. harzianum* lipase was similar to other different microbial lipase studied. Lipases are usually stable at a neutral pH, or near the neutral pH range of 6.0 and 7.5, and have considerable stability at an acidic pH down to 4.0 and to an alkaline pH of up to 8.0 (44,45). The optimum pH value was determined as 8.5. *T. harzianum* lipase showed high stability at 40 °C. Although the majority of lipases are from plants and animals, they completely lose activity at temperatures above 40 °C and some microbial lipases are known to be resistant to heat inactivation. The enzymes produced by *M. hiemalis* (44) and *Mucor* sp. (37) are stable at 45 °C and thermotolerant *Bjerkandera adusta* excreted a lipase that is stable at 60 °C (49).

This extracellular crude preparation was investigated in terms of substrate specificity, pH, temperature optima, stability, and kinetic parameters. The biochemical properties of lipase from *T. harzianum* on account of stability of the enzyme at 40 °C showed a wide range of pH and high thermostability of the enzyme, which suggested its application in detergents and other products that require a high stability at room temperature. Purification of the *T. harzianum* IDM14D lipase is in progress in our laboratory.

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