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# EFFECT OF PH AND TEMPERATURE ON THE ACTIVITY AND STABILITY OF LIPASE *ISOLATED FROM TRICHODERMA HARZIANUM*

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## *ABSTRACT:*

The main aim of this study is to investigate the effect of pH and temperature on the activity and stability of lipase *isolated from Trichoderma harzianum.* 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. 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. . 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.

***KEYWORDS:*** *Lipase, Trichoderma harzianum, Optimal pH, Optimum temperature.*

## *INTRODUCTION:*

The use of enzyme-mediated processes can be traced to ancient civilizations. Today, nearly 4000 enzymes are known, and of these, about 200 are in commercial use. The majority of the industrial enzymes are of microbial origin. Until the 1960s, the total sales of enzymes were only a few million dollars annually, but the market has since grown spectacularly (Wilke, 1999). Because of improved understanding of production biochemistry, the fermentation processes, and recovery methods, an increasing number of enzymes can be produced affordably. Also, advances in methods of using enzymes have greatly expanded demand. Furthermore, because of the many different transformations that enzymes can catalyze, the number of enzymes used in commerce continues to multiply.

The world enzyme demand is satisfied by 12 major producers and 400 minor suppliers. Around 60% of the total world supply of industrial enzymes is produced in Europe. At least 75% of all industrial enzymes (including lipases) is hydrolytic in action. Proteases dominate the market, accounting for approximately 40% of all enzyme sales. Major fields of applications of enzymes are summarized in Table 1. Lipases are represented in most of these fields of applications.

Lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3) are ubiquitous enzymes of considerable physiological significance and industrial potential. Lipases catalyze the hydrolysis of triacylglycerols to glycerol and free fatty acids. In contrast to esterases, lipases are activated only when adsorbed to an oil–water interface (Martinelle et al., 1995) and do not hydrolyze dissolved substrates in the bulk fluid. A true lipase will split emulsified esters of glycerine and long-chain fatty acids such as triolein and tripalmitin. Lipases are serine hydrolases. Lipases display little activity in aqueous solutions containing soluble substrates. In contrast, esterases show normal Michaelis–Menten kinetics in solution. In eukaryotes, lipases are involved in various stages of lipid metabolism including fat digestion, absorption, reconstitution, and lipoprotein metabolism. In plants, lipases are found in energy reserve tissues.

Lipase-producing microorganisms have been found in diverse habitats such as industrial wastes, vegetable oil processing factories, dairies, soil contaminated with oil, oilseeds, and decaying food (Sztajer et al., 1988), compost heaps, coal tips, and hot springs (Wang et al., 1995). Lipase-producing microorganisms include bacteria, fungi, yeasts, and actinomyces. A simple and reliable method for detecting lipase activity in microorganisms has been described by Sierra (1957). This method uses the surfactant Tween 80 in a solid medium to

identify a lipolytic activity. The formation of opaque zones around the colonies is an indication of lipase production by the organisms. Modifications of this assay use various Tween surfactants in combination with Nile blue or neet's foot oil and $Cu^{2+}$ salts. Also, screening of lipase producers on agar plates is frequently done by using tributyrin as a substrate (Cardenas et al., 2001) and clear zones around the colonies indicate production of lipase. Screening systems making use of chromogenic substrates have also been described (Yeoh et al., 1986). Wang et al. (1995) used plates of a modified Rhodamine B agar to screen lipase activity in a large number of microorganisms. Other versions of this method have been reported (Kouker and Jaeger, 1987; Hou, 1994). Lipidic carbon sources seem to be generally essential for obtaining a high lipase yield; however, a few authors have produced good yields in the absence of fats and oils.

## *MATERIALS AND METHODS:*

**Soil Samples collection:**

Soil samples were collected from the agriculture fields of Seetha Ramapuram for isolation of *T. harzianum* The soil samples were collected in sterile polythene bags, taken into laboratory and stored at $4^0$C

**Isolation of *T. harzianum***

For isolation of *T. harzianum* , a serial dilution technique was followed and a $10^{-3}$, $10^{-4}$ and $10^{-5}$ dilutions of each samples were prepared. 1ml of each dilution was pipetted onto a Rose Bengal Agar (RBA) plate and incubated at 28 °C for 5days week. The culture plates were examined daily and each colony that appeared was considered to be one colony forming unit (cfu). After enumeration of cfu, individual colonies were sub-cultured onto a freshly prepared Potato Dextrose Agar (PDA) plates.

**Morphological Identification of *Trichoderma* spp:**

For visual observation, the isolates were grown on PDA agar for 3-5 days. The mode of mycelia growth, colour, odour and changes of medium colour for each isolate were examined every day. For micromorphological studies, a slide culture technique was used. Examination of the shape, size, arrangement and development of conidiophores or phialides provided a tentative identification of *T. harzianum* . Samples were compared to a taxonomic key for the genus Trichoderma.

**Maintenance of cultures**

A loopful of inoculum from sub cultured plates of *T. harzianum* transferred to Potato Dextrose Agar (PDA) slants and maintained as pure culture. For laboratory studies, the fungus was cultured on PDA medium. The medium was sterilized at 15 psi for 30 min in autoclave, poured to sterilized plates, cooled and inoculated with pure culture of the fungus under aseptic conditions. The plates were then incubated at room temperature (26±2°C) for ten days. After complete sporulation, conidia from the medium were harvested by washing them thoroughly with sterilized water containing Tween-20 (0.2%) for immediate use. Otherwise, spores were harvested with the help of a small sterile metal spatula. Harvested conidia were air dried under laminar air flow and stored in a small air tight screw cap vials (10 cm with 2.5 cm diameter) in refrigerator at 4°C before using for further studies. Colony forming units (cfu) were estimated by plating technique. Suspension of spores was made using distilled water with Tween-20 (0.2%) and filtered through a double layered muslin cloth. Spore count was made using a double rolled Neubauer's haemocytometer after necessary serial dilutions under phase contrast microscope. From the stock solution, further dilutions were made to obtain the required concentrations for further studies.

**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. 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.

**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 (Costa t al. 1999; Dharmsthiti et al. 1997; Dong et al. 1999; Ducret et al. 1998).

**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 (Elibol et al. 2001; El-Shafei et al. 1997).

## *RESULTS AND DISCUSSION:*

**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. 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.

**Table 1: Effect of pH on reactive activity**

| S.No | pH | Reactive activity |
|---|---|---|
| **1** | 4 | 20 |
| **2** | 5 | 28 |
| **3** | 6 | 35 |
| **4** | 7 | 54 |
| **5** | 8 | 88 |
| **6** | 9 | 79 |
| **7** | 10 | 40 |
| **8** | 11 | 30 |
| **9** | 12 | 32 |

**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. 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 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 the enzyme (29). Similar thermal stabilities have also been reported for other lipases (Guit et al. 1991; Ishihara ety al. 1975; Jaeger et al. 1998).

**Table 2: Effect of pH on reactive activity**

| S.No | Temperature | Reactive activity |
|---|---|---|
| **1** | 20 | 60 |
| **2** | 30 | 85 |
| **3** | 40 | 99 |
| **4** | 50 | 80 |
| **5** | 60 | 58 |
| **6** | 70 | 55 |
| **7** | 80 | 45 |

## CONCLUSION:

In this study, we identified a new strain of *Trichoderma harzianum*, which excretes an extracellular lipase. This is the first report describing the production of lipase by this strain of *T. harzianum* isolated from soil in Turkey. Although lipases from different species of fungi have already been characterized, there is only one report about *Trichoderma viride* (Janssen et al. 1999). 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. 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* and *Mucor* sp. (Guit et al. 1991) are stable at 45 °C and thermotolerant *Bjerkandera adusta* excreted a lipase that is stable at 60 °C (Kim et al. 1998).

This extracellular crude preparation was investigated in terms of substrate specificity, pH, temperature optima and stability. 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.

***Conflict of Interest:*** None declared.

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