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## Production of Lipase by Hyper-lipolytic *Rhizopus oryzae* KG-10 on Low-value Oil Emulsions

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**Abstract:** The aim of present study to reveal some rare facts about the indigenous lipolytic mold *Rhizopus oryzae* KG-10. Indigenous hyper-lipolytic mold *Rhizopus oryzae* KG-10 was isolated from decaying bread and vegetable samples in Jharkhand, India for the first time and was tested for its ability to produce lipase. The fungus when grown on a modified lipase assay media containing peptone and tween 20 at 37°C for 7 days produced 42 IU mL<sup>-1</sup> of Lipase (EC 3.1.1.3). The specific activity of *Rhizopus oryzae* KG-10 lipase was found to be 300 IU mg<sup>-1</sup> of total protein. The enzyme was found to be active over a broad range of temperature and pH with 37°C as optimum temperature for enzyme activity. The fungus was also screened for its ability to produce lipase on three different lipolytic substrates. It produced 37.33 IU mL<sup>-1</sup> of lipase on Tween 20 followed by 33.33 IU mL<sup>-1</sup> on low value olive oil emulsion and 18.67 IU mL<sup>-1</sup> on locally made coconut oil.

**Key words:** Lipase, *Rhizopus oryzae*, low value substrates, oil emulsions, lipolytic

### INTRODUCTION

Lipases (triacylglycerol acylhydrolases EC 3.1.1.3) catalyse the hydrolysis and synthesis of esters formed from glycerol and long chain fatty acids (Kohno *et al.*, 1994). These enzymes occur extensively in nature in animals, plants and microorganisms. Fungal lipases are commercially important and find use in diverse range of industries like detergents, pharmaceuticals, beverages, dairy etc. (Hiol *et al.*, 2000; Jaeger and Reetz, 1998; Vulfson, 1994; Wooley and Peterson, 1994). Lipids constitute an enormous part of biomass and lipolytic enzymes play an important role in the turnover of these water insoluble compounds. Lipolytic enzymes are involved in the breakdown and mobilization of lipids within the cells of an individual as well as transfer of lipids from one organism to another, which makes them unique for variety of biotransformations. Moreover lipases work at lipid water interfaces involving interfacial adsorption useful application in catalysis industry (Martinelle *et al.*, 1995; Mohanty *et al.*, 2001; Balcao *et al.*, 1996; Bornscheuer, 2000). Apart from their prevalent use in detergents, dairy foods, bakery and beverages, lipases also find use in health foods, chemicals and pharmaceuticals for transesterification and enantioselectivity (Kazlauskas and Bornscheuer, 1998; Liese *et al.*, 2000; Chatterjee *et al.*, 2001; Ducret *et al.*, 1998; Zhang *et al.*, 2001; Pabai *et al.*, 1995a). Because of their industrial significance lipases have been widely studied with main focus on enzyme action, kinetics, sequencing and cloning of lipase genes and structural characterization (Alberghina *et al.*, 1991; Arroyo and Sinisterra, 1995; Beer *et al.*, 1998; Espisan *et al.*, 1990; Nakashima *et al.*, 1988; Rapp, 1995) but the studies on using low value substrates have been left

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relatively unexplored. The present study reveals some rare facts about the indigenous lipolytic mold *Rhizopus oryzae* KG-10 isolated from decaying bread and vegetable samples in Jharkhand, India for the first time and was tested for its ability to produce lipase on two locally available lipolytic substrates along with Tween 20 as standard substrate. These substrates included local made Coconut Oil (CO) and low value Olive Oil (OL) along with Tween 20 (TT) which is known to support lipase production (Hiol *et al.*, 2000; Sharma *et al.*, 2001).

## MATERIALS AND METHODS

### Microorganism

Hyper-lipolytic mold *Rhizopus oryzae* KG-10 was isolated from decaying bread samples collected from local shops across the town during the period August 2006 to December 2006. After ascertaining the purity of culture the fungus was identified on the basis of cultural and morphological features with the help of suitable literature (Hiol *et al.*, 2000; Sztajer *et al.*, 1988; Kohno *et al.*, 1994). The culture was grown on Potato dextrose agar media supplemented with streptomycin and tetracycline ( $20 \mu\text{g mL}^{-1}$ ) at  $28^\circ\text{C}$  for 3-5 days and maintained at  $4^\circ\text{C}$ .

### Enzyme Production in Liquid Cultures

Erlenmeyer flasks (250 mL) containing 1 mL of each of three different lipolytic substrates along with 50 mL mineral salt solution containing (Peptone: 15 g; NaCl: 5 g,  $\text{CaCl}_2$ : 1 g, Agar: 1 g) were autoclaved at 15 psi for 20 min. These flasks were inoculated with 1 mL of spore suspension (containing  $10^4$  spores  $\text{mL}^{-1}$ ) prepared by harvesting spores from 7 days old slant in sterile water blanks and inoculated at  $37^\circ\text{C}$  for 7 days in stationary submerged fermentation. The contents were filtered through Whatman filter paper (No. 1). The culture filtrate thus obtained as centrifuged at 8000xg for 20 min and the clear supernatant was used as enzyme source.

### Enzyme Assay on Solid Media

The lipase triacylglycerol acylhydrolases, EC 3.1.1.3) assay was carried out by using modified lipase assay media (Peptone: 15 g; NaCl: 5 g,  $\text{CaCl}_2$ : 1 g, Tween 20: 10 mL, Agar: 15 g Water: 1000 mL; pH 7.0). The lipase activity was detected due to occurrence of a zone of clearance around the colony and subsequent formation of white precipitate of calcium monolaurate around the colony (Sierra, 1957; Cardenas *et al.*, 2001).

### Enzyme Activity

Lipases require oil-water interface for their action. The enzyme activity was measured by universal titrimetric method (Musantha, 1992; Cardenas *et al.*, 2001; Godfrey and West, 1996). The oil-water emulsion and enzyme extract (0.1: 9.9: 1 mL) was titrated at constant temperature against 0.1 N NaOH using phenolphthalein as indicator. A blank (9.9 mL water; 0.1 mL Tween 20 and 1 mL sterilized broth media) was previously run to find the standard deduction in titer value. Due to this phenomenon lipase releases free fatty acids from the tween 20 and causes change in pH to acidic, which was further neutralized by titrating with NaOH through constantly stirring the vessel. Each reading was taken in triplicate and the activity was measured as amount of enzyme required liberating one micromole equivalent fatty acid per  $\text{mL min}^{-1}$ .

### Effect of pH and Temperature on *Rhizopus oryzae* KG-10 Lipase

The effect of pH on lipase activity was determined by incubating 0.1 mL of protein precipitate in 0.4 mL of appropriate buffers (0.1 M citrate-phosphate buffer: pH 4 and 5; 0.1 M phosphate buffer: pH 6,7 and 8; 0.1 M Tris-HCl buffer: pH 6,7 and 8; 0.1 M Tris-HCl buffer: pH 9; 0.1 M glycine-NaOH buffer: pH 10, 0.1 M HCl-NaOH buffer: pH 11 and 12). To this, 0.5 mL of tween 20

(1% w/v) was dissolved and the reaction mixture was incubated at 50°C for 5 min. The effect of temperature was determined by incubating 0.5 mL of proper diluted enzyme and 0.5 mL of tween 20 (1% w/v in 200 mM sodium acetate buffer pH 5) for 5 min at different temperatures.

## RESULTS AND DISCUSSION

The hyper lipolytic fungus *Rhizopus oryzae* KG-10 was able to grow on all lipolytic substrates tested at 37°C (Fig. 1). Earlier reports indicate isolation of lipase producing molds in diverse habitats such as industrial wastes, vegetable oil processing factories, dairies, soil contaminated with oil, oilseeds and decaying foods (Sztajer *et al.*, 1988; Elibol and Ozer, 2001; Haas *et al.*, 1992) but *Rhizopus oryzae* KG-10 was isolated for the first time from this geographical part of eastern India was unique due to its unique lipase production profile utilizing low value substrates. The fungus when grown on mineral salt solution containing Coconut Oil (CO), Olive Oil (OL) and Tween 20 (TT) for seven days produced 18.67, 33.33 and 47.33 IU mL<sup>-1</sup>, respectively (Fig. 1).

The enzyme was active over a broad range of pH (5.0-11.0) and temperature (20 - 50°C) and the optimum pH and temperature were 7.0 and 37°C, respectively (Fig. 2 and 3). The results of

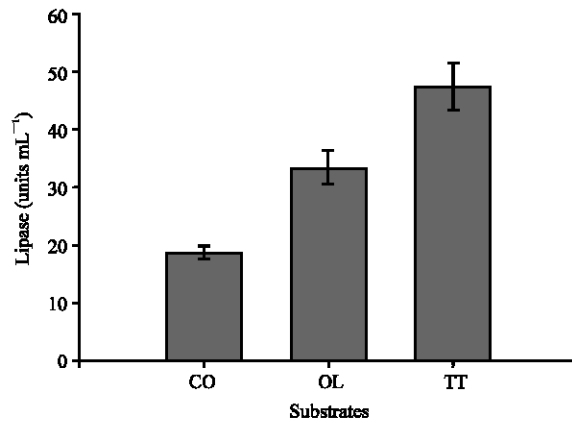


Fig. 1: Lipase production by *Rhizopus oryzae* on three low value oily substrates. (OL: Olive Oil, CO: Locally made Coconut oil, TT: Tween 20)

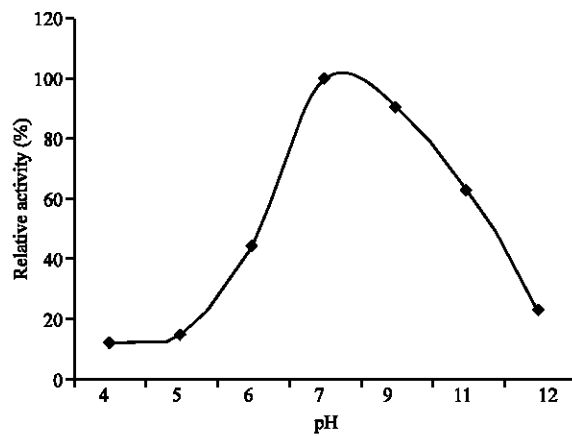


Fig. 2: Time course of lipase production by *R. oryzae* KG-10 on low value substrate

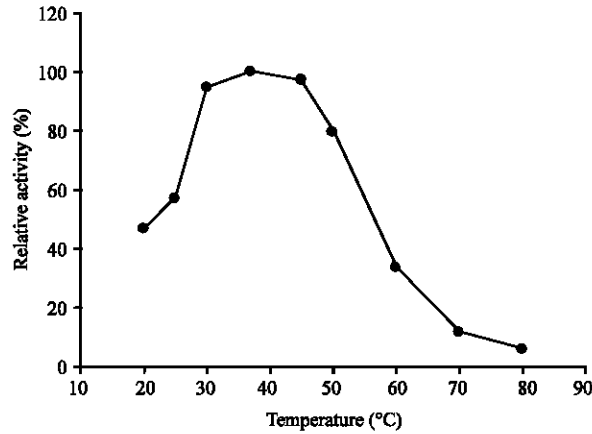


Fig. 3: Effect of pH on *R. oryzae* KG-10 lipase activity

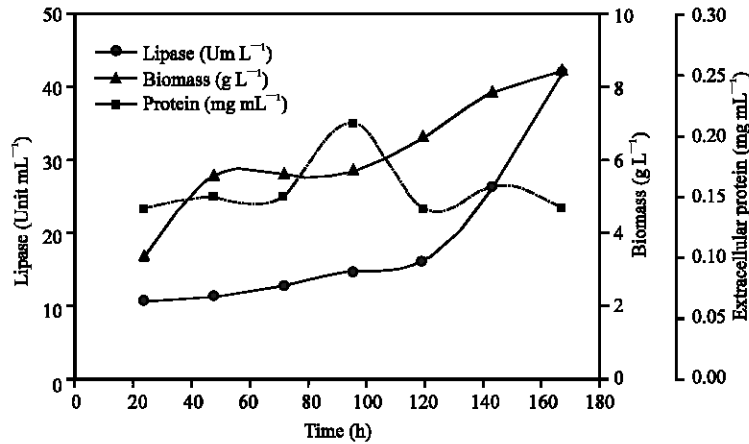


Fig. 4: Effect of temperature on *R. oryzae* KG-10 lipase activity

lipase production by *R. oryzae* KG-10 on three lipolytic substrates are summarized in represented in Fig. 1. The fungus produced 47.33 IU mL<sup>-1</sup> of lipase in a medium containing TT. It was further noticed that the fungus could also utilize two other lipolytic substrates viz., CO and OL. It produced 33.33 IU mL<sup>-1</sup> of lipase on OL and CO, respectively. It was also reported during the present study that the fungus *R. oryzae* KG-10 shows a balanced profile of enzyme production and subsequent protein released for optimal utilization of low value substrates along with the prolonged stability of the enzyme. These results showing time course of lipase production by *R. oryzae* KG-10 on low value substrate have been summarized in Fig. 4.

Lipase productions by variety of microorganisms have been reported by many workers (Sztajer *et al.*, 1988, 1989, 1993; Ghosh *et al.*, 1996). In one study 50 strains of molds were explored for their ability to produce lipase and the strain was identified as *P. wortmanii* which was determined to be the best lipase producer (Costa and Peralta, 1999) and it was reported to produce 12.5 IU mL<sup>-1</sup> in a 7 day culture using olive oil (5% w/v) as substrate. Adding to this the optimal pH and temperature for the crude lipase activity were 7.0 and 45°C in the same study. Along with this other workers have also reported the lipase production using various media components and substrates (Salleh *et al.*, 1993; Coenen *et al.*, 1997; Beer *et al.*, 1998; Essamri *et al.*, 1998; Takahashi *et al.*, 1998; Hiol *et al.*, 2000

Mayordoma *et al.*, 2000) but the utilization of low value substrates was not reported by these workers. Earlier studies on lipase production by *P. wortmanii* the lipase activity was considered maximum by altering the media components and a perusal of these results showed that the present hyper lipolytic fungus *Rhizopus oryzae* KG-10 produced a much higher level of lipase on medium containing substrates CO, OL and TT as compared to previous reports on *P. wortmanii*.

A perusal of literature indicated that *Rhizopus oryzae* KG-10 produces much higher levels of lipase and can be used as a potential strain for lipase production. Although the work for better lipase producing microbial strains was carried out by many other workers using various natural selection strategies by UV and NTG treatment (Bapiraju *et al.*, 2004) and immobilizing the enzyme (Xin *et al.*, 2001) but reports on utilizing low value lipolytic substrates seems scanty and not much explored till date. The results thus obtained indicated the ability of indigenous isolate *Rhizopus oryzae* KG-10 to grow and utilize three lipolytic substrates to produce lipase in an excellent way. Lipases from *Rhizopus oryzae* are one of the most versatile enzymes that are used in vast spectrum of industries and assume great value in high end product industries like oleo chemical industries due to their enantioselective and regioselective nature that makes them highly suited for biopharmaceutical industries. So, further studies on purification and optimization of process parameters for lipase production by *Rhizopus oryzae* KG-10 are in progress for effective utilization of this enzyme.

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