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Full Length Research Paper

Production, purification and partial characterization of lipase from *Trichoderma Viride*

M. Akram Kashmiri, Ahmad Adnan* and Beenish Waseem Butt

Department of Chemistry, G C University, Lahore, Pakistan

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A new strain of lipolytic *Trichoderma viride* was isolated from the soil on a selective medium that contained olive oil as the only source of carbon and energy. The isolated strain was cultivated for lipase production in shake flasks at 30±1°C and the fermentation pattern was studied. The maximum extracellular lipase activity of 7.3 U/mL and the maximum intracellular activity of 320 U/g mycelium were noted after 48 h. Although maximum fungal biomass was present at 13.6 g/L at 60 h but highest specific growth rate was observed between 6 and 18 h. The extracellular lipase present in the broth was purified 134-folds with an overall yield of 46% through purification procedure of ammonium sulphate precipitation, ion exchange and gel permeation chromatography. The K_m value of the purified enzyme for triolein hydrolysis was found to be 1.229 m.mole/L.

Key words: Fermentation, specific growth rate, fungal lipases, Michaelis constant, yield.

INTRODUCTION:

Lipases are glycerol ester hydrolases (E.C. 3.1.1.3), which hydrolyze ester linkages of glycerides at water-oil interface. During hydrolysis lipases pick acyl group from glycerides forming lipase-acyl complex, which then transfers its acyl group to OH group of water. However, in non-aqueous conditions, these naturally hydrolytic enzymes can transfer acyl groups of carboxylic acids to nucleophiles other than water (Martinelle and Hult, 1995). Thus lipases can acylate alcohols, sugars, thiols and amines synthesizing a variety of stereo-specific esters, sugar esters, thioesters and amides (Singh et al., 2003; Dellamora-Oritz, 1997). To employ a lipase on synthetic job, it must be immobilized because soluble lipases lose their activity in non-aqueous reaction media (Bruno, 2004; Dosanih and Kaur, 2004). These synthetic properties allow wide spread applications in various field of biochemical and organic conversions (Poonam et al., 2005; Hsu et al., 2002). It is well known that lipases are the most widely used enzymes in organic synthesis and more than 20% biotransformations are performed with lipases (Gitlesen et al., 1997). In addition to their role in synthetic organic chemistry, these also find extensive applications in chemical, pharmaceutical, food and leather industries (Gulati et al., 2005; Gunstone, 1999). Promising fields for the application of lipases also include the biodegradation of plastics (Gombert et al., 1999) and the resolution of racemic mixtures to produce optically active compounds (Muralidhar et al., 2001). These functions of lipases owe to their broad specificity for a wide spectrum of substrates, stability in organic solvents and enantioselectivity (Snellman and Colwell, 2004; Fadnavis and Deshpande, 2002).

In view of the variety in applications, there has been a renewed interest in the development of sources of lipases. Numerous species of bacteria, yeasts and moulds produce lipases with different enzymological properties and specificities but moulds are known to be more potent lipase producer (Choo et al., 1998). These microorganisms produce lipases both by solid substrate and submerged fermentations (He et al., 2004). Because of huge variation in applications, the availability of lipases with specific characteristics is still a limiting factor. Thus to search for new lipases with different characteristics

*Corresponding authors Email:adnan_biochem@yahoo.com. Tel: 0092-42-111 000 010 ext. 266.

Abbreviations: V_{max}, Maximum velocity; K_m, Michaelis Constant; $Y_{X/S}$, Growth yield; μ, Specific growth rate; U, Lipase unit; LSD, Least significant difference; DEAE, Diethyl amino ethyl.

and improve lipase production continue to be important research topics.

Trichoderma viride, a mould of family Hypocreaceae, order Hypocreales and class Ascomycetes, is well known for the biological control and the production of cellulase and chitinase (Bailey et al., 2004; Castle et al., 1998). In this paper we reported the existence of a lipase producing strain of *T. viride.* The paper also describes the relation between lipid consumption and formation of biomass and lipase.

MATERIALS AND METHODS

Isolation of producer microorganism

Fungal species producing lipases were isolated from soil samples by using a selective medium in which olive oil was the only source of carbon and energy. The medium consisting of (g/L) ammonium sulphate, 1.0; potassium chloride, 0.7; dipotassium phosphate, 1.0; iron (II) sulphate heptahydrate, 0.05; olive oil, 100, was spread over TLC plates and was sterilized by exposure to U.V. radiations. Samples were dusted over the plates and the plates were incubated at 30±1°C for four days. The microbial colonies were developed which were picked up and purified by streaking.

The isolated cultures were screened for lipase production in 250 mL shake flasks. The medium used for screening was consisted of (g/L) olive oil, 20 (emulsified in 2% gum acacia); egg yolk, 10; ammonium chloride, 4.0; magnesium sulphate heptahydrate, 0.25; dipotassium phosphate, 0.5; calcium carbonate, 5.0. The flasks were incubated at 30±1°C for 48 h. A green colonies forming culture with a significant lipase activity was selected and identified to be *Trichoderma viride* (Bissett, 1991). The culture was maintained on potato dextrose agar slants.

Fermentation

The fermentation was carried out in shake flasks using a complex medium consisting of (g/L) olive oil, 20 (emulsified in 2% gum acacia); egg yolk, 10; ammonium chloride, 4.0; magnesium sulphate heptahydrate, 0.25; dipotassium phosphate, 0.5; calcium carbonate, 5.0. The flasks containing 40 mL fermentation medium were inoculated by 18 h old vegetative inoculum. The vegetative inoculum was developed from spore suspension prepared from 48 h old culture slants. The inoculum development and the fermentation were carried out at 30 ±1°C with orbital shaking at l00 rpm.

Lipase assay

Lipase activity in the broth or mycelia was determined titrimetrically on the basis of olive oil hydrolysis (Macedo et al., 1997). One mL sample solution or 10 mg mycelial powder was added to the assay substrate containing 10 mL of 10% homogenized Olive oil in 10% gum acacia, 2 ml of 0.6% $CaCl_2$ solution and 5 ml of 0.2 mol/L citrate buffer, pH 7.0. The enzyme substrate mixture was incubated on orbital shaker with a shaking speed of 100 rpm at 37°C for 1 h. To stop the reaction, 20 ml ethanol acetone mixture (1:1) was added to the reaction mixture. Liberated fatty acids were titrated with 0.1 mol/L NaOH. Intracellular lipase activity was expressed as units per gram mycelium and extracellular lipase activity as units per mL of the broth. One 'lipase unit' (U) was defined as the amount of the enzyme that released one µ mole fatty acid per min.

MEASUREMENT OF PROTIEN CONCENTRATION

The protein concentration was determined by the method of Bradford (1976) using bovine serum albumen (Sigma chemical Corp.) as the standard.

Measurement of lipid contents

Total lipid contents of the fermentation medium were determined by the method of Drochioiu (2005).

Dry cell mass determination

To determine the fungal biomass the mycelium was filtered through filter paper (Whatman No. 40). It was washed first with 0.I mol/L HC1 to remove $CaCO_3$, followed by washing with distilled water. The washed mycelium was dried at 105±1℃ to constant mass. It was placed in the desiccator and then the mass was determined.

Specific growth rate

To determine the specific growth rate (µ), natural log of biomass (ln X) was plotted against time (t). The slope of the line at any moment gives the specific growth rate at that moment.

Growth yield

Biomass growth yield was determined on the basis of lipid consumption. A graph was plotted between changes in mycelial dry weight and changes in the amount of the lipids consumed. The slope of the graph was $Y_{X/S}$.

Purification procedure

Step 1: Solid ammonium sulphate was added to the extract with stirring to bring the saturation to 35% and after standing it for 4 h at 4°C, the precipitates were removed by centrifugation. Lipase activity both in precipitate and supernatant was determined. Additional ammonium sulphate was added to the supernatant to bring the saturation to 60% and the mixture was left overnight. The precipitates were collected, dissolved in distilled water and the solution was dialyzed against water for 36 h using a bladder membrane.

Step2: The dialyzed solution was concentrated under vacuum and applied to a column (15 x 1.5 cm) of DEAE cellulose. The lipase was allowed to bind to the gel for 1 h. The column was eluted with linear gradient of NaCl (0.05-1.0 mole/L: 250 ml) in phosphate buffer at a flow rate of 120 mL/h. The eluent was collected in 5 ml fractions.

Step 3: The eluent was concentrated under vacuum and the concentrate was applied to a column (15 x 1.5 cm) of Sephadex G-100, which was pre-equilibrated with sodium phosphate buffer pH 7.0 and eluted with same buffer. The fractions were collected and analyzed.

Determination of V_{max} and K_m

The purified lipase was incubated with various concentrations of emulsified triolein. The final concentration ranged from 0.5 m.mol.L^{-1} to 4.0 m.mol.L^{-1}. The Michaelis constant (K_m) was calculated by

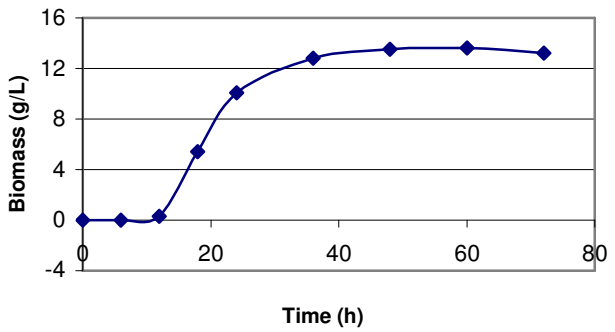

Figure 1. Shows the rate of production of fungal biomass during lipase fermentation by *Trichoderma viride* at 30±1°C.

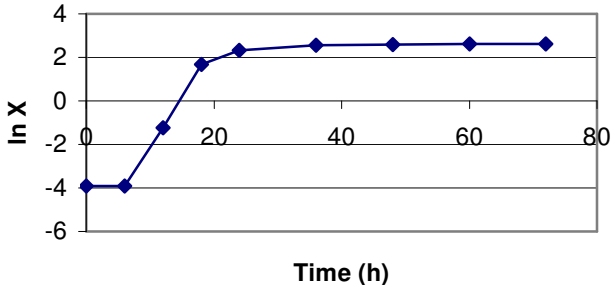

Figure 2. Specific growth rate of the fungal biomas, (μ) of 0.48 h^{-1} between 6-18 h.

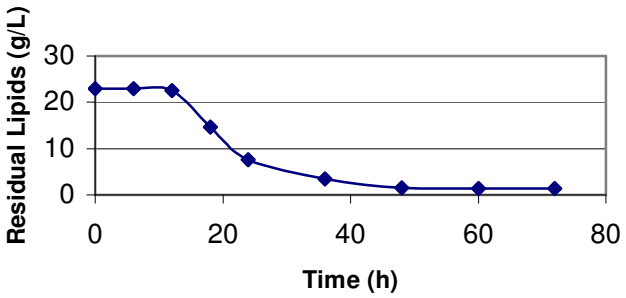

Figure 3. Consumption of lipid source with time in h.

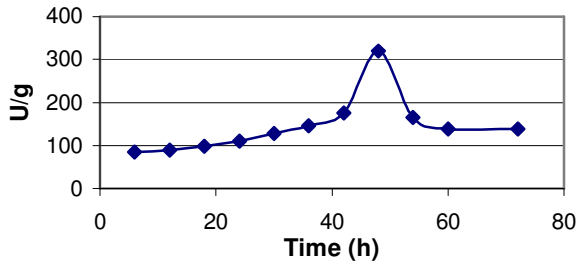

Figure 4. Intracellular lipase activity, showing maximum of 320 u/g at 48 h.

plotting 1/V against 1/[S] in Lineweaver and Burk plot.

Statistical analysis

Experiments were performed in triplicate and the results were statistically analyzed using computer software Costat cs6204W.exe. The treatment effects were compared after Snedecor and Cochran (1980) and the significant difference among replicates has been presented as Duncan's multiple range tests in the form of probability values.

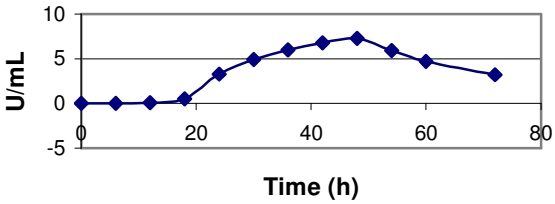

Figure 5. Extracellular lipase activity, showing maximum of 7.3 u/ml at 48 h.

RESULTS AND DISCUSSION

The rate of growth and enzyme production by microorganisms is quite important in understanding their fermentation pattern. Figure 1 shows the rate of production of fungal biomass during lipase fermentation by *T. viride* at 30±1°C for 72 h of incubation. Maximum growth rate was observed between 12 and 48 h of fermentation. At 60 h 13.6 g/L fungal dry mass was present. After 60 h, there was a slight decrease in the mycelial dry mass. Figure 2 shows that 6 h lag phase was followed by a true exponential growth between 6 and 18 h, during which a constant specific growth rate (μ) of 0.48 h^{-1} was observed. After that, although the growth rate went on increasing but the specific growth rate (slope of the curve in Figure 2) decreased. After 18 h the growth showed divergence from the exponential because in place of homogeneous growth, mycelial pellets began to form in which nutrients and oxygen supply became the growth limiting.

Total lipid content was measured throughout the experiments (Figure 3). It was observed that the fungus started using lipid source after 12 hours of inoculation but the utilization rate was low in the beginning. The extraordinary low utilization rate of lipids during early hours of fermentation and apparently very high $Y_{X/S}$ (0.8) may be due to the utilization of nutrients other than lipids, like small amount of sugars and proteins present in the egg yolk. The most rapid utilization of the lipids was observed after 18 h. After 18 h, $Y_{X/S}$ was almost constant (0.63 - 0.66 g/g). Statistical analysis showed that no significant lipid consumption was observed after 48 h and 5.7% of the total lipids were still unused at 72 h.

Figure 4 shows the intracellular lipase activity during lipase fermentation. The intracellular lipase activity after 6

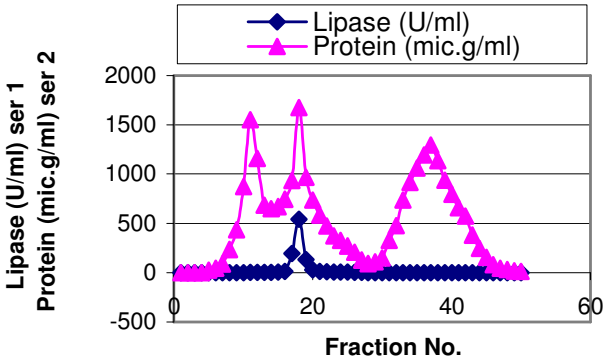

Figure 6. Lipase purification by DEAE Cellulose Column Chromatography.

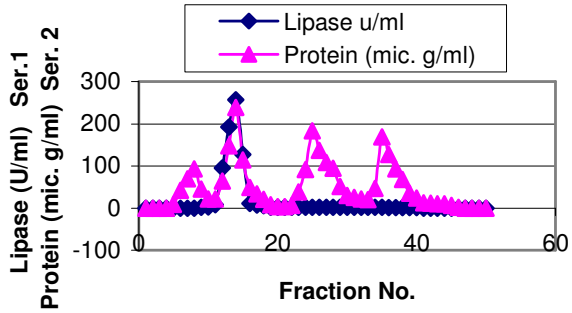

Figure 7. Lipase purification by Gel permeation Chromatography.

h of incubation was 85 U/g. Then it gradually increased and reached to 320 U/g after 48 h. The rate of lipase accumulation inside the mycelia was significantly higher between 42 and 48 h. During this period the rate of lipase formation exceeded the rate of its secretion into the broth. After 48 h the secretion dominated over its formation and the cell-bound lipase activity fell down. Figure 5 shows the extracellular lipase activity during the fermentation. The rate of lipase secretion into fermentation broth was the highest between 18 to 24 h and then the rate gradually decreased. The level of extracellular lipase was the maximum till 48 h i.e. 7.3 U/mL. Then its level slightly decreased due to denaturation of the formed enzyme.

The results are similar to that of Kamimura et al. (1999) and Burkert et al. (2004) who worked on *Geotrichum* sp. and reported that tin shake flasks maximum lipase activity was achieved in 48 h. However, Kamimura et al. (1999) also reported that only 10 h were needed to get maximum lipase activity in stirred fermenter using a complex medium containing corn steep liquor, olive oil and ammonium nitrate.

Once the maximum extracellular lipase activity had been reached, the mycelium was filtered and the filtrate was found to have 7.3 lipase U/mL. The specific activity of the filtrate was noted to be 11.0 units/mg protein. The enzyme was salted out using ammonium sulphate at 35-60% saturation. Precipitates were found to have 329 U/ml. These precipitates were dissolved in and dialyzed against distilled water and loaded on the DEAE cellulose column (Figure 6). Fraction numbers 17, 18 and 19 were lipase active. These fractions were pooled and found to

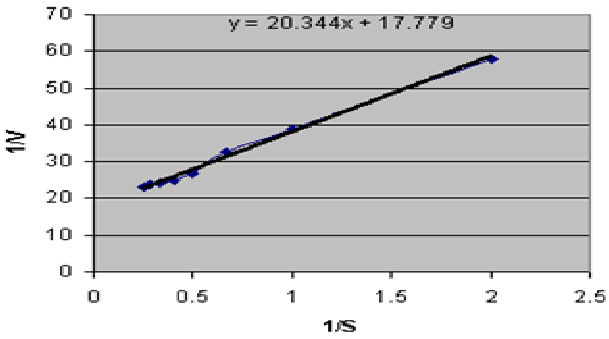

Figure 8. Determination of Km of Lipase activity.

have the lipase activity 298 lipase U/ml. The specific lipase activity in these fractions was 328 U/mg proteins.

The lipase active fractions were concentrated again under vacuum and were further purified by gel permeation. Fraction numbers 12, 13, 14 and 15 was lipase active as shown in the Figure 7. The purified enzyme had lipase activity 168 U/mL and a specific lipase activity in these fractions was 1479 U/mg protein. A representative profile is summarized in the Table 1.

The purified lipase was incubated with various concentrations of triolein in the emulsion and the final concentrations of the triolein in the reaction mixture ranged from 0.5 to 4.0 mM. The Michaelis Constant (K_m) was determined from the Lineweaver and Burk plot (Figure 8), by dividing the slope of the line with the intercept. The V_{max} was determined as the reciprocal of the intercept. The enzyme was found to have K_m of 1.14 m.mol. L^{-1} and V_{max} of 0.056 m.mol.$L^{-1}min^{-1}$.

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