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Lipolytic activity from *Rhizopus arrhizus* using solid state fermentation in biphasic system

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ABSTRACT

The hydrolytic activity of extracellular lipases (Triacylglycerol hydrolase EC 3.1.1.3) from *Rhizopus arrhizus*, in a water-organic solvent biphasic system was investigated. The purpose of the work was to examine the conditions for best lipolysis reactions by microbial lipase in microaqueous biphasic system with special emphasis on the involvement of surfactants, metal ions and a chelating agent in the system for biocatalysis and enzyme stability. The lipases were produced from a *Rhizopus arrhizus* strain, using rice bran as solid substrate, by solid state fermentation. The activity of lipases was found to be optimum at 30 °C and pH 6.5. The effect of different solvents on hydrolytic activity was carried out and isooctane was selected as the solvent of choice. The hydrolytic power exhibited by lipases in a biphasic system was compared with that displayed in aqueous system (phosphate buffer pH 6.5). The effects of various metal ions and a chelating agent on hydrolytic activity in biphasic system were also studied. Among the metal ions tested, Ca^{2+} had an activating effect, Zn^{2+}, Cu^{2+}, Co^{2+} and the chelating agent (EDTA) had little inhibitory effect, and Fe^{3+} showed the highest inhibitory effect. The activating effect of Ca^{2+} on hydrolytic activity was highest at pH 6.5. Mg^{2+}, Na^{+} and K^{+} had no significant effect on lipolysis. The K_m value for the enzyme in the solvent isooctane (K_m = 91.6 mg/ml) was less as compared to the K_m value in the buffer (K_m =110 mg/ml). Among the surfactants tested, non-ionic surfactants had the highest effect with Triton X-100.

Keywords: Solid state fermentation, rice bran, biphasic, metal ions, chelating agent, surfactants

INTRODUCTION

Lipases are defined as triacylglycerol hydrolases (EC 3.1.1.3) that catalyze the hydrolysis of fats and oils to glycerol and fatty acids (Gopinath et al., 2002; 2003). Lipases also display catalytic activity towards esterification and interesterification reactions such as ester exchange, acidolysis and alcoholysis of triglycerides. Lipases, particularly those produced by microorganisms, have recently become the focus because of their high potential for many industrial applications (Newmark, 1988; Harwood, 1989). The reasons for the enormous biotechnological potential of microbial lipases include the fact that they are stable in organic solvents, do not require co-factors, possess broad substrate specificity and exhibit high enantioselectivity (Sharma et al., 2002).

Large-scale industrial use of lipases occurs in food industry for the production of emulsifying agents and flavoring agents (McNeil et al., 1991; Debnath et al., 2005; 2006). They have also been widely used in pharmaceutical (Newmark, 1988) and laundry detergent (Tatara et al., 1985) industries. The ability of lipases to catalyze trans- and inter-esterification reactions have been used for the production of a glucoside ester for use as a biosurfactant (Ducret et al., 1995) and the production of certain anti-inflammatory and

analgesic drugs namely naproxen and ibuprofen (Bhandarkar and Neau, 2000; Sharma et al., 2002).

Chen (1989) has shown that *Candida rugosa* lipase hydrolyzed oils and fats almost completely (99 per cent) and the activity could be increased by the addition of dimethyl β-cyclodextrin. Hydrolysis of palm kernel olein by *Rhizopus arrhizus* lipase in reversed micelle, in isooctane was reported by Kim and Chung (1989).

MATERIALS AND METHODS

Microorganism, medium and inoculum

Rhizopus arrhizus species was obtained from Institute of Microbial Technology, Chandigarh, India. The culture was maintained on potato dextrose medium. The inoculum was prepared by suspending loop full of spores in 10 ml sterile distilled water.

Enzyme production by solid state fermentation

Rice bran used for the fermentation was obtained from a local rice mill and sieved. Dry rice bran powder (20 g) was mixed with distilled water (1:1, Rice bran: Distilled water) in a Rhu bottle. The additional carbon (2 % (w/w) maltose) and nitrogen (2 % (w/w) yeast extract) were added to the flask and then sterilized for 20 min at 120 °C. After cooling the sterile bran, the innoculum was added and thoroughly mixed aseptically and incubated under specified conditions. After incubation for four days, the culture was ground in mortar with acid-washed sand using minimum amount of phosphate buffer (pH. 6.5) (1:5, bran: buffer ratio). It was then filtered through muslein cloth and then centrifuged at 4 °C at 10,000 x g for 15 min. The pellet obtained was discarded and the clear supernatant used as the crude enzyme extract. All the operations were done below 5 °C. To the supernatant (culture), ammonium sulphate was added with constant stirring to achieve 50-70% saturation. The mixture was left at 4 °C overnight; the resultant insoluble precipitate was collected by centrifugation at 10,000 x g for 15 minutes and the precipitate was dissolved in phosphate buffer (pH 6.5). This fraction was used to conduct the hydrolytic studies (Sasi and Debnath, 2006).

Determination of Lipolytic activity in aqueous phase

Lipase activity was assayed by Cupric-acetate method of Lowry and Tinsley (1976), as modified by Kwon and Rhee (1986).

Samples containing 2.0-5.0 moles of free fatty acid (oleic acid) were prepared by dissolving in isooctane. Then, 1.0 ml of cupric-acetate-pyridine reagent was added and mixed vigorously for 90 s using a vortex mixer. The mixture was allowed to stand for about 10-20 min until the aqueous phase was separated clearly from the organic phase (isooctane and fatty acid). The standard plot of concentration of free fatty acid versus the optical density was determined by measuring the absorbance of isooctane layer at 715 nm against the control, which contains no free fatty acids.

One milliliter of olive oil and 1 ml of buffer (pH 6.5) were mixed vigorously for 90 s, until the water-oil solution become emulsified and 1 ml of enzyme was subsequently added to the mixture and incubated at 30 °C for 1 hr at 100 rpm. After the reaction was stopped by adding 1 ml of 6N HCl, 5 ml of isooctane was added, mixed and then the mixture was centrifuged. The isooctane layer containing fatty acids was drawn off and then mixed with 1 ml of cupric acetate-pyridine reagent. The amount of free fatty acid that dissolved in isooctane layer was determined spectrophotometrically by measuring the absorbance at 715 nm and interpolating from the standard calibration curve according to the method described above. One Unit of enzyme activity is defined as one μmol of fatty acid produced per minute at 30 °C and at pH of 6.5.

The substrate (olive oil) was dissolved in organic solvent and the enzyme was in the aqueous phase (phosphate buffer, pH 6.5). For the determination of stability, lipase enzyme was incubated separately with organic solvents (chloroform, hexane, cyclohexane, n-heptane, octane and isooctane) for up to 8 hr at 30 °C at 100 rpm and assayed. Stability of enzyme with respect to temperature was carried out by incubating the enzyme over temperatures ranging from 30 to 70 °C and for different time interval ranging from 30-120 min. After incubation, enzyme activity was assayed. Lypolysis was also carried out with varying substrate (olive oil) concentrations (10%, 20%,

30%, 40% and 50% v/v) in order to study the kinetic parameters: V_{max} (maximum velocity constant) and K_m (Michaelis-Menten constant) values of the enzyme.

Biocatalytic transformation of olive oil was carried out by replacing buffer (1.0 ml) with different solvents and studying the effect of different organic solvents like chloroform, hexane, cyclohexane, n-heptane, octane and isooctane. After finishing the enzymatic reaction, each solvent was evaporated and the lipolytic activity was determined.

The effects of various metal ions (Ca^{2+}, Mg^{2+}, Fe^{3+}, Zn^{2+}, Cu^{2+}, Co^{2+}, Na^{+} and K^{+}) and chelating agent (EDTA) on lipolytic activity were examined by assaying the residual activity after incubating the enzyme with 1 mM salts of metal ions and EDTA separately for 1 hr at 35 °C in the presence of isooctane.

The effects of various surfactants on lipolytic activity in the presence of organic solvent were tested at the concentrations of 0.1% (w/v). The surfactants used were cationic (cetrimide), anionic (Sodium dodecane sulfonate) and non-ionic surfactants (Triton X -100, Tween 80, Span 60 and Span 80). The relative activity of the enzyme was compared with that in the absence of surfactants.

RESULTS AND DISCUSSION

Lipase assay conditions were optimized. The optimum pH, temperature and time of incubation were found to be 6.5, 30 °C and 60 minutes, respectively (Tables 1, 2, 3). Stability of lipase in different organic solvents showed that the residual activity of the enzyme remained at about 95-98% when the enzyme was incubated for 60 minutes regardless of the solvent used (Figure 1). The lipase was relatively unstable in chloroform, isooctane, moderately stable in n-heptane and very stable in hexane, cyclohexane and n-octane. Therefore, the lipase activity must be measured within time range in which the activities of lipase do not appreciably change with the solvent. The result of the thermal stability experiment is as shown in Figure 2. Enzyme activity is lost rapidly at temperatures between 50-70 °C.

Enzyme activity increased with increase in substrate concentration as shown in Figure 3. There was no significant change in activity in substrate concentrations ranging 20-35% v/v (183.2 g/200ml), and in biphasic system 1.2-2.0-fold increase in enzyme activity was observed. Enhanced activity in solvent system may be due to dissolution of water insoluble substrate. V_{max} is almost twofold (V_{max} = 4 U/ml) and K_m value is less (K_m = 91.6 mg/ml) in solvent (isooctane) compared to buffer activity (V_{max} = 2.6 U/ml and K_m = 110 mg/ml). Less K_m value indicates the higher affinity of substrate with enzyme in organic solvent due to the dissolution of water insoluble substrate. Maximum enzyme activity was observed in organic solvents. Branched (isooctane) and cyclic (cyclohexane) hydrocarbon yielded much higher lipase activity than the group of straight chain aliphatic hydrocarbons and halogenated hydrocarbons. Isooctane was found to be the solvent of choice for lipase activity determination (Figure 4).

The results of the effects of various metal ions and EDTA on lipolytic activity showed that among the metal ions tested, only Fe^{3+} showed inhibitory effect and Ca^{2+}, showed activating effect, while other metal ions Co^{2+}, Zn^{2+} and Cu^{2+} showed little inhibitory effect. Mg^{2+}, Na^{2+}, K^{+} and EDTA did not significantly affect enzyme activity (Table 4). Calcium ion can serve as stabilizing bridge analogous to a disulphide bond. Study of metal ions effect is justified by the need to control the water activity of the enzyme reaction in presence of metal salts.

The cationic and anionic surfactants tested did not show any significant effect on enzyme activity. The non ionic surfactants on the other hand showed an activating effect. When the level of enzyme activity in the absence of detergent was taken on 100%, the activities in the presence of 0.1% polyoxyethylene octylphenyl ether (Triton X - 100), polyoxyethylene sorbitan monoleate (Tween 80), sorbitan monostearate (span 60) and sorbitan monooleate (span 80) were found to be 141.20, 108.80, 122.00 and 130.28%, respectively (Figure 5).

In the light of the foregoing, and taking into account its maximum activity in organic solvent (isooctane), resistance to metal ions and EDTA inhibition, and its enhanced activity with non-ionic surfactants, this enzyme is a good candidate for potential biocatalysis. The ultimate objective is to

enhance the performance of the enzyme activity in biphasic systems, and expand their utility in many other processes of practical interest.

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Table 1: Effect of pH on enzyme activity.

pH	Activity (U/ml)
5.5	1.6
6.0	1.8
6.5	2.3
7.0	1.9
7.5	1.0
8.0	0.7

Table 2: Effect of temperature on enzyme activity

Temperature (°C)	Activity (U/ml)
20	0.6
25	1.0
30	1.4
35	2.3
40	1.5
45	1.0
50	0.5

Table 3: Effect of incubation time on enzyme activity.

Incubation time (min)	Fatty acid (µmol/min)
15	30.08
30	57.2
45	77.8
60	125.11
80	127.5
100	126.30

Table 4: Effect of various metal ions and chelating agent on lipolytic activity in the presence of isooctane.

Metals Ions	Relative Enzyme Activity (%)
Control	100.00
Fe^{3+}	28.35
Ca^{2+}	118.58
Co^{2+}	61.00
Mg^{2+}	99.10
Na^{+}	98.77
K^{+}	96.91
Zn^{+}	63.00
Cu^{2+}	52.00
Chelating agent (EDTA)	77.20

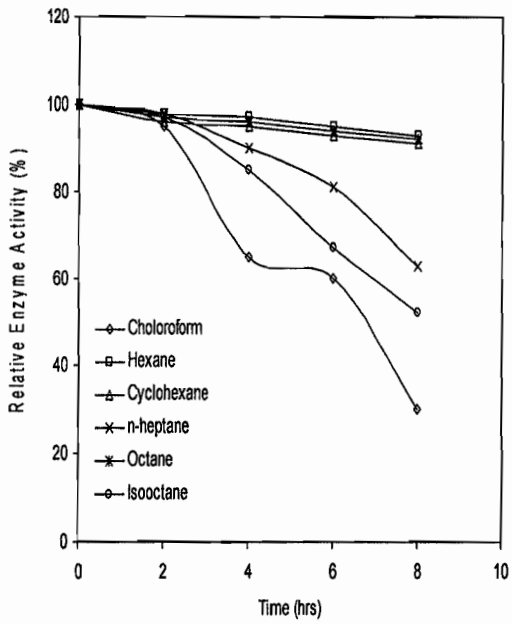

Figure 1: Stability of lipase in organic solvents. Lipase enzyme was incubated with the organic solvents for 8 hr at 30 °C, at 100 rpm. Residual activity of enzyme was determined with respect to control (without incubation and organic solvent). At regular interval of time, aliquots of the enzyme were withdrawn and assayed.

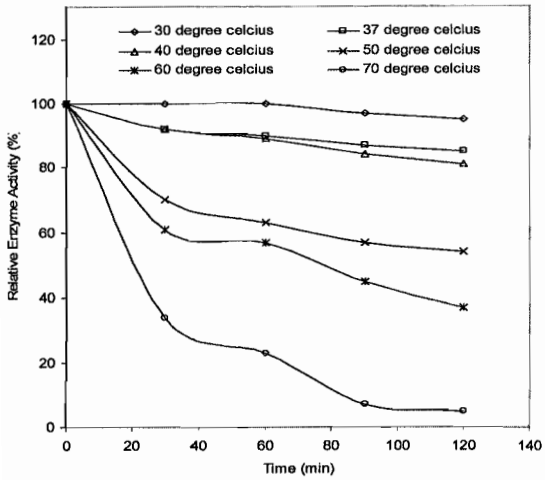

Figure 2: Effect of temperature on stability of lipase enzyme. Enzyme aliquots were incubated at various temperatures (30-70 °C) and assayed for lipase activity with respect to 0 time activity (100%).

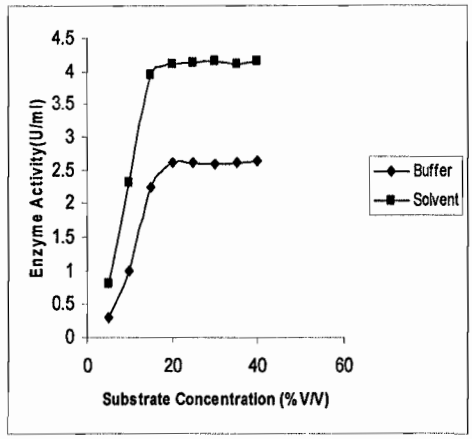

Figure 3: Effect of substrate concentrations (% v/v). This was performed in both buffer and isooctane solvent at 30 °C for 1 hr and at 100 rpm.

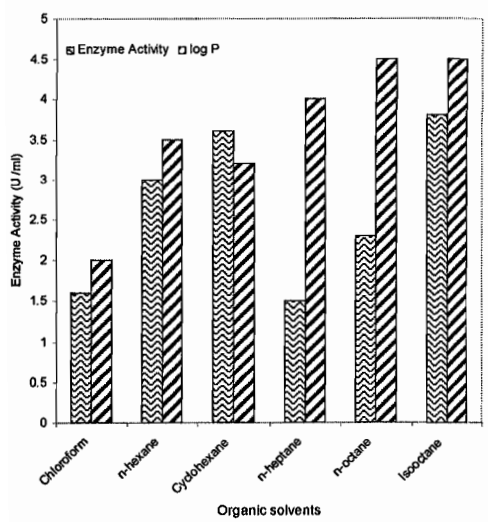

Figure 4. Effect of different solvents on lipolytic activity. Lipolysis was carried out by incubating olive oil (1 ml) in 1 ml organic solvent and 1 ml enzyme, at 30 °C for 1 hr at 100 rpm. Lipase activity was measured by cupric acetate-pyridine reagent.

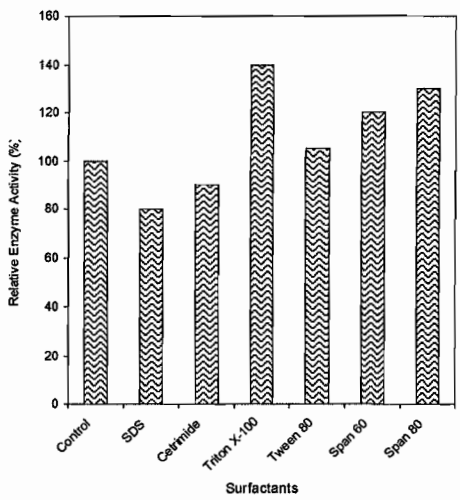

Figure 5: Effect of surfactants on lipolytic activity. Lipolytic activity was tested with different surfactants at 0.1% (w/v) concentration in the presence of organic solvent. The enzyme activity in the absence of detergent was taken as 100%.

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