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Immobilization of Lipases from *Candida rugosa* for efficient Hydrolytic and Esterification activities

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Immobilized lipases are widely used in the catalysis of esterification reaction in organic media. Different immobilization techniques were used to immobilize lipases produced from *Candida rugosa.* The lipases immobilized with different matrices were compared for their hydrolytic and esterification activities. Entrapment in calcium alginate was found to be the best immobilization technique for the purpose. Calcium alginate immobilized lipase possessed 928 hydrolytic units per gram and after partial drying, the *Candida rugosa* catalyzed beads formation of pentyl butylate at the rate of 51 μmole/min/g.

Keywords: Entrapment, matrix, ester, calcium alginate, hydrolytic.

Modification of biotechnological processes, using immobilized enzymes, has recently gained attention of scientist world wide. Immobilization of enzymes on a solid support can offer several advantages including repeated usage of the enzyme, ease of product separation, improvement of enzyme and operational stability[1,2], higher efficiency of catalysis[3,4], reduction in enzyme autolysis and continuous operation in packed-bed reactors[5,6,7]. Enzymes may be immobilized on solid carriers by various techniques such as carrier binding, cross linking or entrapment. A variety of carriers have been tested with different enzymes. Some of these include alginate, magnetite, fuller earth, silica gel, vermiculite and polyurethane etc[8,9]. Methods compatible with the enzyme, substrate or cofactor should be selected and most compatible methods include adsorption, covalent bonding, entrapment and encapsulation.

The use of enzymes in organic synthesis brings about significant challenges. The operational conditions in these processes are not often suitable for bio-molecules. Enzyme may be denatured due to solvent effects and mechanical shear forces. Recovery of enzymes from reaction mixture/solutions and separation of enzyme from substrate and product are in general, very difficult. The productivity of enzymatic processes is often due to substrate and product inhibition. These problems can be perfectly tackled by immobilization of enzymes. The immobilization of enzymes on solid supports is carried out in order to increase their function and stability in response to organic solvents or increased temperature. Enzymes may be stabilized by chemical and physical processes; with chemical methods, they are immobilized by strong covalent bonding but this often results in changes in the three dimensional structure of the enzyme. Alginate gels have been proved to be a very successful medium for entrapping lipases, especially when formed as beads of gel[10]. The number of methods in which alginate has been used for cell or enzyme immobilization, on laboratory or large scale, has increased dramatically in the last few years[11,12].

The purpose of the present investigation was to study the immobilization of lipase from *Candida rugosa* for higher catalytic and esterification activities using different entrapment techniques with matrices such as calcium alginate, silica gel, fuller's earth, hydroxyapatite and alumina.

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MATERIAL AND METHODS

Collection of material

Lipases from *Candida rugosa* in its pure powder form was obtained from Biotech research lab, Department of Chemistry, GC University, Lahore. The supporting materials such as calcium alginate, silica gel and fuller's earth etc were used to immobilize the enzyme. The fatty acids, butyric acid and acetic acid and fusel oil as a major source of amyl alcohol and other alcohols were also used. Olive oil, having low acidity, was used for the purpose of lipase assay and was obtained from the local market. Solvents and other reagents used were of standard laboratory grade and calibrated lab ware was used in the studies.

Immobilization of lipase

One gram sodium alginate, very light brown colored in powder form was dissolved completely in hot distilled water under continuous agitation conditions. Lipases from *Candida rugosa* (0.2 gram) was dissolved in cold distilled water to avoid denaturing of the enzyme. Mixing of lipase was carried out under agitation by magnetic stirrer as long as emulsion type homogeneous solution was formed. It took about 1 to 2 hours for the formation of desired solution. Lipase emulsion solution was dropped in to the 5% calcium chloride solution through syringe (drop wise). As the drops fell into $CaCl_2$ solution, very fine beads of drop size were formed. These beads have lipase spreaded throughout the interior and exterior surface of beads. Further experiments were carried out to determine appropriate lipase loading by using a fixed amount of support for different amounts of the enzyme. Similar method was used for immobilization, using other support materials such as silica gel, fuller earth and alumina.

Hydrolysis assay

Hydrolytic activities of free and immobilized lipases were assayed by the olive oil emulsion method according to the modification proposed by Soares[13]. One unit of enzyme activity was defined as the amount of enzyme necessary to produce 1 µmole of free fatty acids per minute under the assay conditions (37^{o}C, pH 7.0 and 150 rpm). Olive oil emulsion was prepared by taking 12 gram of ground state Gum Acacia in 100ml of distilled water. When the gum Acacia was completely dissolved in hot water, it was filtered by cotton. Then about 8 g olive oil was dissolved into 100 ml filtrate and using stirrer at about 60 rpm, emulsion was formed. Now 10 ml Gum emulsion was taken in each 100 ml flasks. 5 ml buffer solution of acetic acid sodium acetate was dissolved in each flask. Then 0.1 gram lipase in ground state was taken in 2 ml buffer solution and dissolved in one flask and let the 2nd one remained without enzyme, so that the hydrolytic activity of enzyme was measured to hydrolyze olive oil. Sample was drawn out after fixed time duration and titrated with 0.1 N alcoholic KOH solution by using Thymol Phthylien (pH 0.2-10) as indicator. It gives blue coloration in basic media and colorless in acidic media. Hydrolytic activities of free and immobilized lipases (using different immobilization supports such as silica gel, fuller's earth, calcium alginate and alumina) were studied.

Esterification assay

The reaction mixture consisted of non aqueous solvent n- hexane (92 ml) and fusel oil as a major source of amyl-alcohol and butyric acid with equi-molar ratio. An immobilized lipase (2 g) was added as a catalyst in the reaction mixture. The mixture was incubated at 37^{o}C for 24 h with continuous shaking at 150 rpm. The amount of product formed was estimated within certain duration of time by titration method. It was observed that the volume of titrant used against acidic solution was reduced continuously with the passage of time which indicated the product formation. Activity was expressed as µmole of amyl butyrate formed per gram of dry support. Esterification activities of free lipase were also compared with those of calcium alginate lipase and mycilial lipase[14].

RESULTS AND DISCUSSION

Hydrolytic activities of free and immobilized lipases

The lipase (from *Candida rugosa*) was partially purified, vacuum dried and stored at 4°C. It was analyzed before immobilization and was found to have about 6000 U/g. Different immobilization supports such as silica gel, fuller earth, calcium alginate and alumina were evaluated the activities of free and immobilized lipases were compared Table 1. The calcium alginate was found to absorb maximum amount of lipase as compared to other supports. After washing, it retained 928 U/g of lipase activity while silica gel, fuller's earth and alumina showed 744, 340 and 98 U/g, respectively. So the hydrolytic activities of calcium alginate, silica gel, fuller's earth and alumina immobilized lipases were about 15.4, 12.4, 5.6 and 1.6% of free lipase (6000 U/g), respectively. The results resembled quantitatively to those of Heung Chae *et al.*[15], who have reported the calcium alginate as the best support for lipase adsorption. However, these results differ from Furukawa *et al.*[16] who found the cellulose and silica gel as better adsorbent than calcium alginate.

Esterification activities of free and immobilized lipases

The data of table 2 shows the esterification activities of free and immobilized lipase from *Candida rugosa*. It was found that the free lipase had very low esterification activity and a negligible yield (2.05%) of the ester. Significance of direct application of soluble (free) lipase in ester synthesis was further reduced when the ratio of its synthetic activity to hydrolytic (28/6000 U/g) was compared to those of immobilized lipases such as 72/300 for mycelial lipases and 51/928 for calcium alginate lipase. Low synthetic activity of soluble lipase in non aqueous media is well documented, as is due to the direct exposure of the enzyme to un-natural organic solvent and reactants instead of natural aqueous environment of the living system. An effective esterification by immobilized lipase has also been reported[17]. The enzyme immobilization prevented the direct exposure and made the enzyme protein resistant toward adverse conditions of low pH, water scarcity and un-natural environment. The comparison of two immobilized lipases indicated that the calcium alginate adsorbed lipase, in spite of having higher hydrolytic activity in the aqueous system had lower esterification activity in non-aqueous systems. Mycelial lipase on the other hand had an efficient esterification activity of 72 µmoles/min/g. The yield of ester obtained in 24 hours was 24% in case of calcium alginate adsorbed lipase as compared to 48% with mycilial lipase. Lower yield with calcium alginate may be due to the enzyme inactivation during the reaction in organic solvent, in addition to lower initial rate of esterification. The mycilial lipase on the other hand, was found to have high efficiency and good operational stability during the esterification in organic medium.

Effect of temperature on lipase mediated esterification

The effect of temperature on both the synthetic activity and thermostability of lipase

Table 1. Comparison of hydrolytic activities of free and immobilized lipases

Support medium for immobilization	Hydrolytic activity (U/g)
Calcium Alginate	928±4
Silica gel	744±3
Fuller's earth	340±2
Alumina	98±1
Hydroxyapatite	278±2
Free lipase	6000±13

Table 2. Comparison of hydrolytic and esterification activity and ester yield of free and immobilized lipases

Enzyme	Hydrolytic activity (U/g)	Esterification activity (µmoles/min/g)	Yield (%)
Free	6000 ± 13	28 ± 1	2
Immobilized	928 ± 4	51 ± 2	24
Mycelial	300 ± 2	72 ± 2	48

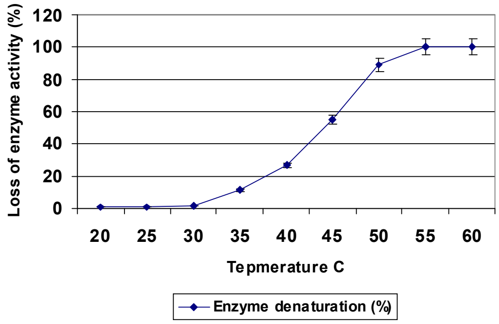

Fig 1. Thermostability of immobilized lipase produced from *Candida rugosa*

(from *Candida rugosa*) was determined. The influence of temperature on the enzyme activity was studied over a range of 20°C to 60°C. Results presented in table 3 indicate that as the incubation temperature was raised from 20°C to 60°C, the %age loss of lipase (denaturization) was increased and became 100% at 60 °C. While at temperatures 25°C , 30°C , 35°C, 40°C , 45°C, 50°C and 55°C, the losses in activity of lipase were 1.0%, 1.6%, 11.5%, 27.0%, 55.0%, 89.0%, 100% and 100% respectively.

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