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Hydrolysis of Rice Bran Oil Using Immobilized Lipase in a Stirred Batch Reactor

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Abstract *Candida cylindracea* lipase was immobilized by adsorption on acid washed glass beads. It was observed that protein loading of the support depends on the size of the particle, with smaller particle containing higher amount of protein per unit weight. Initial reaction rate linearly varied up to enzyme concentration of 17.25 U/mL. Amount of free fatty acids produced was linearly proportional up to the enzyme loading of 1650 μg/g of bead. Achievement of chemical equilibrium took longer time in the case of less protein loading. Degree of hydrolysis was found to decrease in second and third consecutive batch operations on repeated use of immobilized lipase.

Keywords: immobilized lipase, protein loading, free fatty acids, degree of hydrolysis and chemical equilibrium

INTRODUCTION

Lipolysis by lipase is an energy saving process that can be carried out at normal temperature and pressure without denaturation of biological substances [1]. Enzyme technological approaches for the modification of fats and oils are a subject of intensive interest. The enzymatic method yields products of better odour and colour and a cheaper overall process than the conventional uncatalyzed splitting method known as colgate-emery process [2]. It had stated that enzymatic hydrolysis could provide a useful method of generating fatty acids from highly unsaturated fatty acid residues [3,4].

A large amount of literature is available on the hydrolysis of fats like olive oil, tallow and palm oil by lipase [5-11]. Use of tallow, a cheap raw material for fatty acids production is banned at several places in India. Hence, there is a need to search for cheaper raw materials for the production of fatty acids. Rice bran oil is one such raw material available in plenty in India as India is the second largest rice producing country in the world.

However, studies on rice bran oil, which contains high proportion of fatty acids are rather limited [12]. In the present work, effect of particle size and concentration of attachment solution on immobilized enzyme loadings, influence of enzyme concentration on initial reaction rate, effect of lipase loadings on production of fatty acids and repeated batch operations of immobilized lipase were studied.

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MATERIALS AND METHODS

Materials

Crude lipase enzyme preparation from *Candida cylindracea* (285 U/mg) was obtained from Sigma Chemicals Co. (St. Louis, MO, USA). This preparation was used without further purification to prepare the immobilized enzyme. Glass beads (2 mm, 1 mm spherical, acid washed, Sigma) were used as enzyme support material for immobilization. 3-Aminopropyltriethoxysilane used for generating functional groups on glass beads was obtained from Acros organics (NJ, USA). All chemicals used in this work were reagent grade and were products of Nice Chemicals (Cochin, India). Rice bran oil (saponification value=180, iodine value= 90, FFA=0.3% (w/w)) was obtained from Sri Jayasakthi Rice & Oil mills (Salem, India).

Immobilization of Lipase Enzyme

Lipase enzyme from *Candida cylindracea* was immobilized on acid washed activated glass beads (2 mm and 1 mm) based on the method developed by Wu and Weng [13]. The acid washed glass beads were treated with 3-amino propyltriethoxy silane (APTES) to produce functional amino groups on the surface. The APTES solution for the activation step was prepared as 5% (by volume) in toluene. The activation was performed with 50 mL silanizing solution/gram of support and carried out in a stirred vessel for 24 h. The solution was then decanted and the support was washed with toluene to remove APTES followed by acetone wash to remove toluene. The support was then allowed to dry in air before placing in an oven overnight at 110°C.

Every gram of the silanized support was mixed with

20 mL glutaraldehyde (10% aqueous solution) and shaken at 37°C. After 36 h, the glutaraldehyde solution was decanted and the support was washed with water to remove all excess glutaraldehyde attached on to the support. Different concentrations of lipase enzyme attachment solutions were prepared by dissolving the lipase enzyme in pH 7.2 phosphate buffer. To every gram of activated carrier, 20 mL of lipase attachment solution was added and it was refrigerated at 4°C for 2 days. The immobilized lipase enzyme was then filtered and washed twice with distilled water.

Analytical Methods

The activity of lipase is described in terms of lipase units (U). One unit (U) of lipase is defined as the amount of enzyme required to produce one μmol of free fatty acid in one minute under assay conditions.

Free fatty acids liberated were measured by spectrophotometric method as described by Kwon and Rhee [14]. Initial rate was measured by finding the initial slope of the plot of μ mol of free fatty acids produced vs time. Ratio of this initial rate and weight of the enzyme gives the activity.

Protein measurements were performed according to a modified Lowry procedure [15,16].

Batch Hydrolysis of Rice Bran Oil by Using Immobilized Lipase

The batch stirred tank reactor was made of glass and of 120 mL capacity. The vessel had a jacket through which water at desired temperature was circulated. The contents of the reactor were stirred using a magnetic stirrer.

Rice bran oil and water (phosphate buffer) were taken in the volume ratio of 1:1 with immobilized lipase and a fine emulsion was made. Two hundred microliters of samples were collected from the reaction mixture at predetermined time intervals and the amount of free fatty acids formed was estimated. pH and temperature during hydrolysis were 7.2 and 42°C respectively. Degree of hydrolysis of the rice bran oil was calculated by the following equation:

$$\text{Degree of hydrolysis} = \frac{\mu\text{mol of fatty acids liberated}}{[\text{Saponification value}/(3 \times 56.1)][1{,}000 \times \text{g of oil}]}$$

RESULTS AND DISCUSSION

Immobilized Protein Loading on Glass Beads

Enzyme attachment solution containing 0.25–6.0 mg/mL of lipase were prepared in a 0.1 M phosphate buffer at pH 7.2. The above attachment solutions were used for immobilization on activated glass beads using the method developed by Wu and Weng [13]. The effect of varying the lipase concentration on the total protein loading for 2 mm and 1mm support particle is shown in Fig. 1. Protein loading increases rapidly as the lipase concentration is increased and leveled off at about 3.25 mg/g bead. In experiments carried out using smaller activated glass beads of 1mm in size, maximum loading of 4.25 mg/g bead was obtained with the lipase concentration of 5 mg/mL. This increase in loading is due to the increase in surface area that provides additional sites for protein attachment.

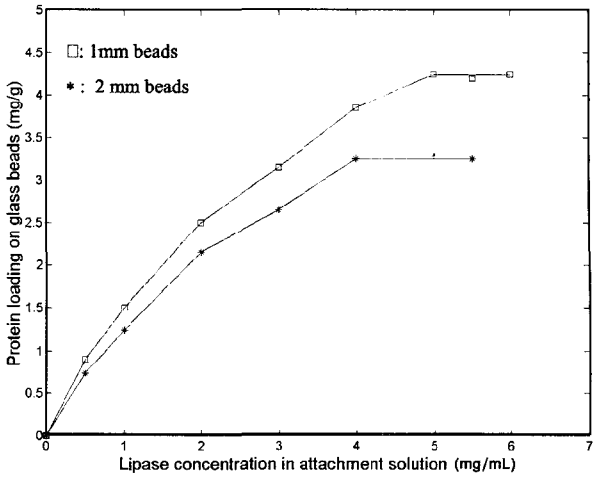


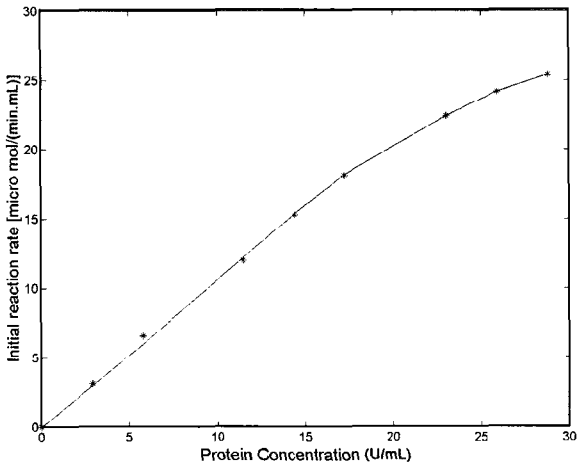
Fig. 1. Immobilized lipase loading on activated glass beads.

Effect of Enzyme Concentration on Initial Reaction Rate

The effect of lipase concentration on reaction rate was studied in the range of 2.88-28.76 U/mL. This concentration was achieved by weighing the required amount of 2 mm size beads with 1,240 μg/g protein loading so as to get the specified enzyme activity in the reaction mixture. As shown in Fig. 2, the initial reaction rate increased linearly up to 17.25 U/mL. In general, with constant substrate concentration, as the enzyme concentration is increased, there is an increase in initial reaction rate. At higher concentration levels the rate of reaction is expected to become asymptotic in nature as the lypolysis of the rice bran oil takes place at the interfacial area of the emulsion there will be limitation on the substrate availability. Thus, for a given emulsion the maximum rate of production is limited by the concentration of enzyme that can be optimally used. Increasing the speed of agitation could minimize the mass transfer resistance. But this may lead to the deactivation of immobilized lipase [16].

Effect of Amount of Bound Lipase on the Fatty Acid Formation

Fatty acid production with lipase immobilized on activated glass beads as a function of reaction time at various bound enzyme concentration with a fixed substrate is shown in Fig. 3. The amount of fatty acids



ig. 2. Effect of Immobilized lipase concentration on initial eaction rate.

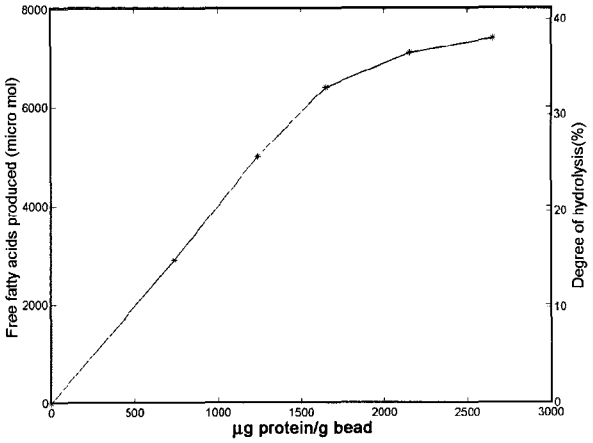
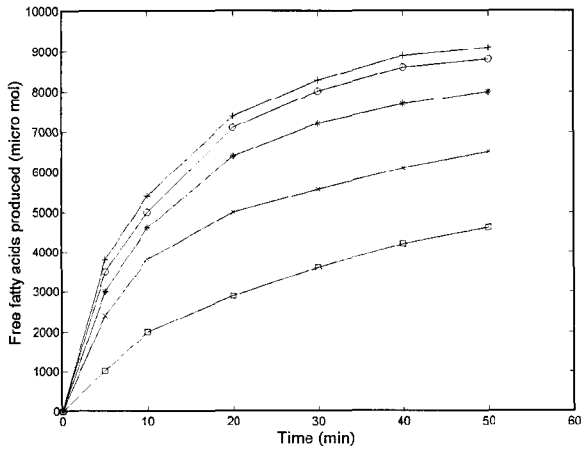


Fig. 4. Effect of amount of bound lipase on the fatty acid formation and degree of hydrolysis at fixed concentration (20 mL of 50% (v/v)) rice bran oil. Reaction time was 20 min.



ig. 3. Amount of free fatty acids produced as a function of eaction time at various bound lipase concentrations. The mount of lipase bound µg /g bead were: (+) 2,650; (o) 2,150; *) 1,650; (×) 1,240; (□) 740. 2 gms immobilized beads of each oading and 20mL rice bran oil (50% v/v) were used.

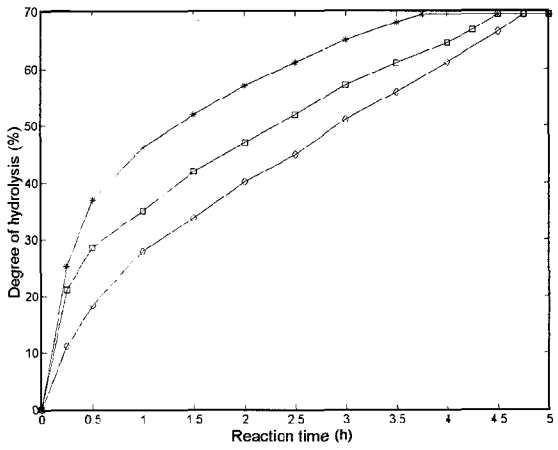


Fig. 5. Effect of the amount of bound lipase on batch hydrolysis of 20 mL of 50% (v/v) rice bran oil. The amount of lipase used per gm of glass bead were: (*) 1,650 µg; (□) 1,240 µg; (◊) 740 µg. 2 g immobilized beads of each loading were used.

roduced was linearly proportional up to the enzyme oncentration of 1,650 µg/g glass bead as shown in Fig. . At higher enzyme concentration the production rate eviates from the straight line probably because of limiation of substrate to the interface. In emulsion system he value of kinetic constants are highly dependent on ssay conditions such as interfacial area of substrate reated in emulsion.

ffect of the Amount of Bound Enzyme on hemical Equilibrium in a Batch Hydrolysis

Fig. 5 shows the time courses of the hydrolysis of 20 nL 50% (v/v) Rice bran oil using immobilized lipase of hree differently bound enzyme loadings. The maximum hydrolysis obtained in all three cases are same and found to be 69.5%. The time required for achieving this maximum hydrolysis decreased with an increase in enzyme loading during long batch operations. Fig. 5 also shows that the progress of reaction was rapid for first 1 h, and then slowed down thereafter. This can be attributed to the denaturation of lipase or chemical equilibrium between substrate and product. In our earlier paper [17], negligible amount of denaturation for this particular immobilized enzyme at the working temperature and pH was reported. It may be presumed that the slowing down in hydrolysis must be due to chemical equilibrium between substrate and product.

Repeated Use of Immobilized Lipase

Experiments were performed repeatedly using the

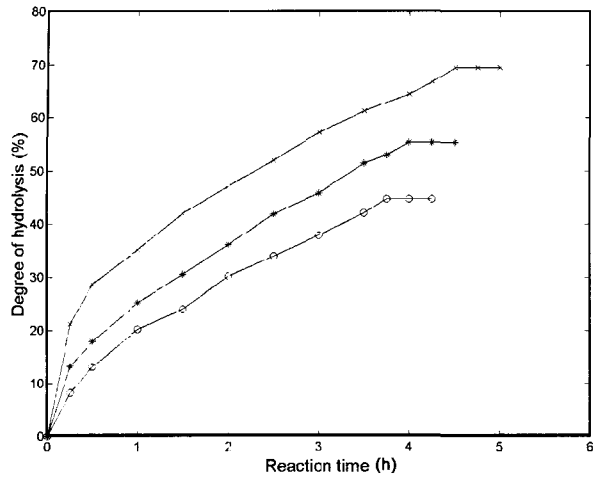


Fig. 6. Repeated use of immobilized lipase for the hydrolysis of Rice bran oil: (×) first batch; (*) second batch; (o) Third batch. 2 g of 1,240 μg/g immobilized beads and 20 mL of rice bran oil (50% (v/v)) were used.

same sample of enzyme in consecutive batches. Once reaction was completed, the enzyme was separated from product and substrate, fresh substrate added and the reaction was started again. The results from batch experiments in which the same enzyme was used in successive runs are shown in Fig. 6. They indicate that effectiveness of the catalyst falls with repeated batches during these experiments. This effect could be due to the accumulation of reaction products on the support.

CONCLUSION

Lipase enzyme was effectively immobilized on activated glass beads retaining 65% of the original activity. This immobilized preparation was found to be quite suitable for the hydrolysis of rice bran oil. Enhanced protein loadings were obtained with decrease in particle size. Equilibrium conversion levels of substrate were found to be unaffected by the amount of bound lipase. A drop of 20.4% and 35.7% in equilibrium hydrolysis were observed in the consecutive second and third batch runs respectively.

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