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A comparative performance evaluation of jute and eggshell matrices to immobilize pancreatic lipase

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ABSTRACT

A pancreatic lipase was immobilized on readily available and inexpensive jute and eggshell matrices. The purity of extracted enzyme was confirmed by SDS-PAGE. The maximum protein load for eggshell was 10.23 mg/g, and for jute, it was 5.7 mg/g. The free enzyme activity retention was greater than 80% for eggshell and 43% for jute. The immobilized lipase was stable over a pH range from 7 to 8 for eggshell and 7.5 to 8.5 for jute with over a temperature range from 25 to 45 °C for eggshell and 37 to 40 °C for the jute. FTIR data indicated new bonds on the jute upon immobilization. Although no new bond was observed, immobilization data on eggshell fit well with the Langmuir adsorption isotherm model. The model constants, Γ_{max} and K_l, were 13.92 mg/g and 0.382 mL/mg, respectively. Mixed adsorption with both ionic and hydrophobic interactions was observed. Lipase adsorption was reduced significantly in presence of Tween 80, whereas the effect was less in case of ionic strength, pH and temperature. For both matrices, scanning electron microscopy (SEM) was used to demonstrate the changes in surface morphology after immobilization. The performance of eggshell was better than that of jute as a matrix for immobilizing pancreatic lipase.

1. Introduction

Immobilization is the process of attaching or entrapping an enzyme to an insoluble material, the matrix, using physical or chemical interactions. The primary objective of immobilization is to reduce the process cost by enhancing enzyme stability and reusability [1]. For further cost minimization, finding a cheap and readily available matrix that can retain maximum enzyme activity is a major challenge in commercial enzyme immobilization applications. Enzymes can be immobilized by several methods, which range from carrier-bound to carrier-free techniques [2,3]. Galan et al. [4] extensively reviewed different enzyme immobilization strategies and indicated that immobilization not only allow us to reuse the enzyme but also to improve its performance by enhancing stability, activity specificity, etc. Various methods have been extensively used for enzyme immobilization, including entrapment using alginate [5], agarose, polyacrylamide, gelatin, sol–gel and k-carrageenan; adsorption using alumina, macro porous resin, silica, celite and calcium carbonate; as well as covalent binding using chitosan [6] and certain biopolymers such as jute [7]. Glutaraldehyde is used as a cross-linking agent to stabilize the enzyme–matrix interaction [8,9]. The selection of matrix and method of immobilization are critical for retaining maximum enzyme activity and improving enzyme stability. Iyer and Ananthanarayan reviewed the enhancement in enzyme stability after immobilization in aqueous and non-aqueous environments [10]. Multipoint covalent attachment of an enzyme molecule to a support matrix aids in increasing stability. However, due care must be taken to avoid distorting the enzyme structure [11]. Enzyme active site inaccessibility or conformation distortion upon immobilization causes mass transfer limitations, which results in reduced enzyme activity [12,13]. On the other hand, it is possible that activity may be lost during the reaction because the enzyme is leached from the matrix [14]. Modifying the area near enzyme active site during immobilization is reportedly beneficial for activation of certain enzymes such as lipases [11,15,16]. Changing the enzyme orientation on the support material is an additional approach employed for effective immobilization [17,18].

Lipases (glycerol ester hydrolase, E.C. 3.1.1.3) are important enzymes that break down oils and fats into fatty acids and glycerol. Lipases act at the oil–water interface and maintain good activity on hydrophobic substrates for catalysis of reactions such as hydrolysis, interesterification and transesterification [19]. Lipases have a "lid" in its structure that covers the active site of the enzyme. The enzyme contacts the oil–water interface, the lid moves away to provide access for the substrate to enter the active site [20]. The kinetic mechanism does not depend on the type of reaction. It was hypothesized that the mode of action for lipase is similar to a serine

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protease [21]. In a hydrolytic reaction, one mole of oil or fat reacts with three moles of water to produce three moles of fatty acids and one mole of glycerol. Recently, lipase demand increased due to its extensive use in a transesterification reaction for biodiesel production [22]. Lipase obtained from various sources (from microbes to the human and porcine pancreas) was immobilized primarily on hydrophobic supports using various strategies. Porcine pancreatic lipase (PPL) has been widely used for the transesterification due to its low price, easy availability and high stability. The enzyme does not require any cofactor. It was observed in most of the reports that, the substrate conversion was less than 70% because of the specificity of PPL toward 1 and 3 positions in the triglyceride. Higher conversion was also reported by some researchers and it was might be due to the migration of acyl group from 2 positions to 1 or 3 position in the monoglyceride [23]. The materials used for lipase immobilization include resins [24], alginate [25], eggshell [26], sol–gel [27,28], sepabead [29] and chitosan [30]. *Candida antarctica* lipase immobilized on an acrylic resin is currently commercially available (Novozyme 435) and widely used for biodiesel production [24].

To reduce the cost of enzymes and matrix immobilization for a high-volume, low-value product such as biodiesel and to improve the stability of enzymes, the study herein is a comparative performance evaluation of lipase immobilization on two inexpensive and readily available matrices such as jute and eggshell.

2. Materials and methods

2.1. Materials

Steapsin, a crude pancreatic lipase, was purchased from SRL (Mumbai, India). Jute and eggshell were collected from a local market and hostel canteens (IIT Kharagpur, India), respectively. The Bradford protein estimation kit and electrophoresis apparatus were purchased from Bangalore Genei Pvt. Ltd. (Bangalore, India). Tributyrin was obtained from Himedia (Mumbai, India). Sodium metaperiodate was purchased from Loba Chemie Pvt. Ltd. (Mumbai, India). An unstained protein molecular weight marker was purchased from Fermentas (Germany). All other chemicals were procured from SRL (Mumbai, India) and were analytical grade.

2.2. Methodologies

2.2.1. Preparation of the lipase solution

The crude steapsin powder contained high levels of stabilizing material. To extract the protein, the crude powder was dissolved in phosphate buffer (50 mM, pH 7) and vortexed for 15 min at room temperature. The solution was then centrifuged at 5000 rpm (approximately $3200 \times g$) for 10 min at 4 °C, and the supernatant was used as the lipase solution in further studies [31].

2.2.2. Lipase immobilization

In this study, the method described by Vemuri et al. [26] was used to immobilize the lipase on eggshell. In the first step, the eggshells were boiled in a 0.1% SDS solution for 15 min. The shells were washed thrice with distilled water to remove residual SDS and then washed with acetone three times to remove water. Acetone was subsequently removed by drying at 60 °C for 5 h. The dried eggshells were then crushed and sieved through a −16, +36 mesh to generate a uniform size. Mixtures of lipase solution containing the requisite amount of lipase and 1 g of eggshell were added to different conical flasks (50 mL) and incubated at 37 °C for specific time periods. After incubation, all of the conical flasks containing the mixture were stored at 4 °C for 16 h. The resulting enzyme-immobilized matrices were then washed with phosphate buffer (50 mM, pH 7) three times to remove the unbound enzyme.

Jute matrix-based enzyme immobilization, which is reportedly a process of chemical modification on the jute surface [7], was performed by cutting the raw jute into small pieces and boiling them in 0.05% NaOH solution for 30 min. The solution was then neutralized by a wash with water and incubated with 0.5% sodium metaperiodate ($NaIO_4$) for 3 h under dark conditions to oxidize the —OH groups into —CHO groups. The enzyme solution was added to the treated jute and incubated at 4 °C overnight. The unbound enzyme was washed with phosphate buffer (50 mM, pH 7). The immobilized enzyme was suspended and stored in the same buffer until used.

2.2.3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis

To determine the enzyme purity of the extracted lipase solution and ensure effective lipase immobilization on both matrices, an SDS-PAGE gel was generated. The immobilized preparations were boiled in 1% SDS for 15 min to elute the enzyme from the matrices. The eluted enzyme and lipase solution (described in Section 2.2.1) were analyzed using SDS-PAGE, and the relative protein positions were compared with the standard protein molecular weight marker.

2.2.4. Enzyme loading and activity assay

The enzyme concentration was quantified using the Bradford protein estimation method with bovine serum albumin (BSA) as the standard [32]. Enzyme activity was determined using the tributyrin method [33]. First, a 2% polyvinyl alcohol (PVA) solution was prepared in distilled water. Next, a PVA solution and tributyrin were mixed at a 9:1 ratio to form an emulsion. A mixture comprising 1 mL emulsion, 0.8 mL phosphate buffer (50 mM, pH 7) and 0.2 mL free enzyme solution (containing 10 mg enzyme) was then prepared and incubated at 37 °C for 30 min. For the immobilized enzyme, instead of free lipase, 2 g of lipase-immobilized jute or 1 g of lipase-immobilized eggshell was used. The quantity of immobilized jute and eggshell was determined such that the amount of enzyme was approximately equal in both immobilized systems (using the data for the enzyme loading capacity of jute and eggshell). The reaction was terminated after 30 min by adding an acetone:ethanol (1:1) mixture and then titrated with 0.1 N sodium hydroxide solution using phenolphthalein as indicator. The enzyme activity was expressed as butyric acid produced per unit time per mg lipase. The maximum activity was considered 100% in calculating the percentage of relative activity for the enzyme.

2.2.5. Variation of pH and temperature

To determine whether immobilization changed the optimum pH or temperature for maximum enzyme activity, a series of experiments with varying conditions was performed using both matrices and the free enzyme. In the first set of experiments, the pH value of the reaction mixture was varied from 6 to 8.5, but the temperature was maintained at 37 °C. Acetate, phosphate and tris–HCl buffer was used to maintain the desired pH. To determine the effect of temperature, the reactions were performed at the temperature range that varied from 25 to 50 °C and pH 7. All the experiments were performed in triplicate, and the data are shown with the standard deviation.

2.2.6. Immobilization time

The incubation period is an important parameter for effective immobilization. For cellulosic fibers, overnight incubation was reported [34]. Thus, for jute, the samples were incubated overnight for efficient immobilization. For the eggshell, lipase dissolved in phosphate buffer (50 mM, pH 7) was mixed with 1 g of eggshell matrix and incubated for different time periods ranging from 30 min to 10 h at 37 °C. The mixtures were incubated at 4 °C for 16 h and then washed with same buffer thrice to remove unbound enzyme. The enzyme immobilized for different time periods was tested for enzyme activity using the tributyrin method, which was expressed as a percentage of the relative activity.

2.2.7. Fourier transforms infrared spectroscopy

The Fourier transform infrared (FTIR) spectroscopic study was performed in a Nexus-870 spectrometer (Thermo Nicolet Corporation, Wisconsin, USA). For the jute matrix, three samples were analyzed, including the untreated jute, chemically treated jute and enzyme immobilized jute. Similarly, for the eggshell, the matrix samples were analyzed before and after immobilization. To generate a pellet, 2 mg of each sample was mixed with 100 mg of FTIR grade potassium bromide (KBr). The spectrometer was used in the transmission mode over a wave number range from 4000 to 400/cm. The potassium bromide pellet, which does not absorb in the wave number range from 4000 to 400/cm, was used as blank for the study.

2.2.8. Experimental design for adsorption studies

To study the adsorption of pancreatic lipase on eggshell, 1 g of eggshell was mixed with different concentrations of the enzyme in 50 mL conical flasks, and the mixture was incubated at 37 °C for 2 h followed by 4 °C for 16 h. The mixtures were then filtered through Whatman No. 1 filter paper, and the matrix was washed with phosphate buffer (50 mM, pH 7) to remove unbound enzyme. The total protein content in the filtrate was measured using the Bradford method [32]. The difference between the initial protein concentration and the concentration after filtration indicated the amount of enzyme immobilized onto the matrix. The experimental data on the initial enzyme concentration and the mass of the enzyme immobilized on eggshell were fit into the Langmuir adsorption isotherm model using MicroCal Origins 8.0 software (MicroCal Software Inc. USA). The linear Langmuir model from Arami et al. [35] is described in Eq. (1).

$$\frac{1}{\Gamma} = \frac{1}{\Gamma_{max}} + \frac{1}{\Gamma_{max} \times K_l} \times \frac{1}{c} \quad (1)$$

Here, Γ is the mass of the adsorbed enzyme, Γ_{max} is the maximum enzyme uptake, c is the initial enzyme concentration and K_l is the Langmuir constant.

To determine the manner of adsorption, eggshell-immobilized lipase was mixed with a salt solution (NaCl) comprising 0.5 and 1 N and a non-ionic detergent (Tween 80) between 1 and 5%. The mixture was then incubated at 37 °C for 1 h and washed with phosphate buffer (50 mM, pH 7) to remove the unbound enzyme from the matrix surface. The activity of the immobilized enzyme was measured and compared with that of the untreated immobilized preparation.