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Byssus Thread: A Novel Support Material for Urease Immobilization

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Abstract Byssus threads are tough biopolymer produced by mussels (*Mytilus viridis*) to attach themselves to rocks. These were collected from mussels in their natural habitat (N) and from animals maintained in laboratory condition (L) as a novel support. Byssus thread surfaces were characterized by SEM analysis, chemically modified and used for adsorption of urease. The efficiency of the immobilization was calculated by examining the relative enzyme activity of free and the immobilized urease. The pH stabilities of immobilized urease were higher (0.5 unit) than free enzyme. Immobilized enzymes on byssus (both N and L) when stored at 6 °C retained 50% of its activity after 30 days, but they were more stable in dry condition. The optimum temperature of immobilized enzymes was found to increase (25 °C). A Michaelis–Menten constant (K_m) value for immobilized urease was also elevated (2.08 mol).

Keywords Immobilization · Urease · Byssus thread · *Mytilus* · Adsorption · SEM

Introduction

Compared to chemical catalysts, enzymes as biocatalysts exhibit a number of features that make their use advantageous. But the use of enzyme in chemical, medical, and waste-processing industries is often limited due to their high cost, relatively fragile structures, instability, and the limited possibility of economic recovery of enzyme from a reaction mixture. Immobilization of enzymes can solve some of these problems. The immobilization of enzymes on to solid support has long been studied and has been put into practice. For the expression of activity by the immobilized enzyme it is necessary to retain the structural

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integrity of the enzyme without altering the amino acid residues at the site of catalysis. If the amino acid residues at the active center or the tertiary structure change their location, the catalytic activity may adversely affect enzymatic activity and specificity [1–3]. A number of methods exist for the immobilization of enzymes such as entrapment, physical adsorption, co-polymerization, and covalent attachment. It is important to choose a proper support for a particular enzyme immobilization since its interaction with enzyme may have remarkable influence on the stability and kinetics of it [4, 5]. Physical and chemical properties of polymeric supports play very important roles in maintaining the activity of immobilized enzymes. Many support materials, however, lack biocompatible surface, leading to rapid denaturation and to a sharp decrease in the activity of surface-bound enzymes. Polymers, such as cellulose, polymeric latex particles, agarose, chitosan, etc., have been widely reported for immobilization of proteins/enzymes and cells due to their biocompatibility, biodegradability, and hydrophilicity [6–10]. Various fibrous materials, including cotton cloth, polyester fleece, polystyrene fibers, and natural silk fibroin fibers, have been explored to date [11–15]. Urease is used in various fields like in chemical and clinical analysis, environmental protection, biochemical reaction engineering, biomedical engineering, feed and beverage production, and water reclamation in spacecraft. Jack bean urease is widely used member of the urease family in biotechnology. Immobilizations of enzymes have been studied for the application in diverse analytical, medical, industrial, and biotechnological processes and have been implemented on a larger scale. Urease is among enzymes most extensively studied for immobilizations and practical applications [16, 17].

Urease from different sources (jack bean, pigeon pea, water melon, etc.) has been studied extensively for immobilization [18–21]. There is no report on immobilization of urease on byssus threads, a tough fibrous biomaterial which is biocompatible polymer. Mussel byssus is a complex structure divided in three regions: the stem, the thread, and the plaque. Stem is the structure that supports each byssal thread, whereas plaque is the adhesive pad located at the end of thread [22]. In genus *Mytilus*, byssus is product of secretions from nine distinct glands formed by fusion of retractor muscles located at the base of the foot via the stem and provides an anchor point allowing a certain degree of rotation and tension control. The thread consists of a core of collagen-like proteins stiffer at one end than at the other, as they are block copolymers with either elastin (proximal) or silk-like (distal) domains [23]. Studies performed on byssal threads from *Mytilus edulis* mussels [24, 25] found three different forms of collagen, namely byssal precollagen-P, byssal precollagen-D, and precollagen-NG.

In this work, urease is immobilized on two sets of byssus thread, a naturally obtained thread collected from bivalve *Mytilus viridis* (N) from the coast of Gujarat, India (lat 22°25′ N, long 69°04′ E) and byssus thread collected from the *Mytilus* reared in laboratory aquarium (L).

Byssus was characterized and then immobilized after chemical modification under relatively mild conditions. The effects of factors such as substrate concentration, temperature, storage, and pH were systematically studied.

Materials and Methods

Materials

Crude urease from jack bean meal (Hi-Media Laboratories, India), urea, Nessler's reagent (Qualigen's fine chemicals, India), Folin's reagent, 1,4-butenediol diglycidyl ether (Sisco,

India), buffers, and glutaraldehyde (Acros, USA) were used. All other chemicals used were of AnalaR grade and were utilized without any further purification.

Collection and Characterization of Byssus

Byssus threads are secreted by *M. viridis* from their foot. They start producing threads immediately when transferred in water tanks and attach themselves to sand, corals, stones, shells, glass walls, or to each other. The natural byssus threads were collected from the animals in their native habitat (natural—N) and appear old, rough, and tough. Byssus threads from the animals after maintaining under laboratory condition (L) were also collected.

Byssus threads were washed several times with tap water first, followed by distilled water three times. They were then dried completely and used for SEM analysis (LEO S-440i, Cambridge, UK) before being immobilized.

Preparation of Immobilized Urease on Byssus Thread

Dry byssus threads of *M. viridis* were collected and treated with 1% sulphuric acid to remove the dirt, washed with distilled water, and dried at room temperature.

These threads (N and L) were then activated by 1,4-butenediol diglycidyl ether, and 0.6 N NaOH was added with stirring for 3 h. These were left undisturbed for a day and were washed with chilled double-distilled water to stop reaction.

For immobilization, a solution of crude urease (4 mg/ml) was added to 1% glutaraldehyde solution (10 ml) and threads with intermittent shaking for 24 h. The reaction was terminated by washing the threads with distilled water followed by phosphate buffer (pH 7.2). Immobilized byssus thus prepared was stored in phosphate buffer at 6 °C, and few were allowed to dry (at room temperature) for storage study.

Protein Content Determination and Enzyme Assay

The protein content of free and immobilized urease was determined using Folin–Ciocalteu reagent by Lowry's method [26], using BSA as standard. The amount of bound protein was estimated by subtracting the amount of protein determined in the washing after immobilization from the amount of protein used for immobilization. Free urease (dissolve in buffer) and immobilized urease fibers (15) dipped in 1 ml (0.2 M) phosphate buffer were incubated with 0.5 M urea for 30 min at 55 °C. The amount of ammonia liberated in a fixed time period was determined using Nessler's reagent. The reaction was stopped by placing the reaction mixture in ice bath, and 0.66 N H_2SO_4 was added. A calibration curve was drawn with different concentrations of ammonia, and this was used for the estimation of ammonia formed. Activity of free and immobilized urease was measured colorimetrically by using UV spectrophotometer (Elico Co., India) at 405 nm [27].

The pH Profile of Free and Immobilized Urease

The pH-dependent activity profiles of both immobilized and free urease were determined at 55 °C with 1 ml urease solution (3%) in 1 ml buffer (0.2 M, pH 7). The effect of pH on enzyme activity (soluble as well as immobilized) was investigated in pH range, 4–9. Three different buffers were used for this study: 0.1 M citrate phosphate buffer (pH 4.0–6.6), 0.2 M phosphate buffer (pH 6.6–7.6) and 0.1 M Tris–acetate buffer (pH 7.2–9.0). The

relative enzyme activity was determined at each pH by the method described for enzyme assay above.

Temperature Activity of Free and Immobilized Urease

The temperature activity of free and immobilized urease was determined by measuring the enzymatic activity (free and immobilized) by incubating solution for 30 min at temperatures ranging from 10 to 80 °C.

Reusability of Immobilized Urease

The immobilized urease was tested for its reusability by checking the activity using assay after every use, the threads were washed properly with 0.1 M Tris–acetate buffer, pH 7.2 and stored in buffer at 6 °C, until further use. Results are plotted in the form of a graph.

Enzyme Activity on Storage

For storage stability, the immobilized enzyme was kept at room temperature in phosphate buffer and in distilled water for comparative study. Few threads of each size were also kept at 6 °C for 1 month, and their enzyme activity was measured after every 5 days.

Enzyme Activity and Substrate Concentration

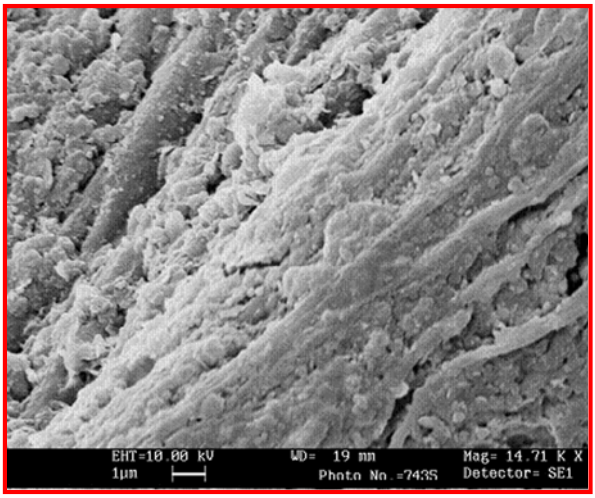
Effect of substrate concentration on free and immobilized urease activity was investigated by varying the amount of urea concentration (like 1 ml, 2 ml, and so on at 0.5 M concentration) at optimum pH (pH 7) and temperature (55 °C) for soluble and immobilized enzyme. The Michaelis–Menten constant (K_m) and V_{max} values for both free and immobilized urease were determined by Lineweaver–Burk plot.

Results and Discussion

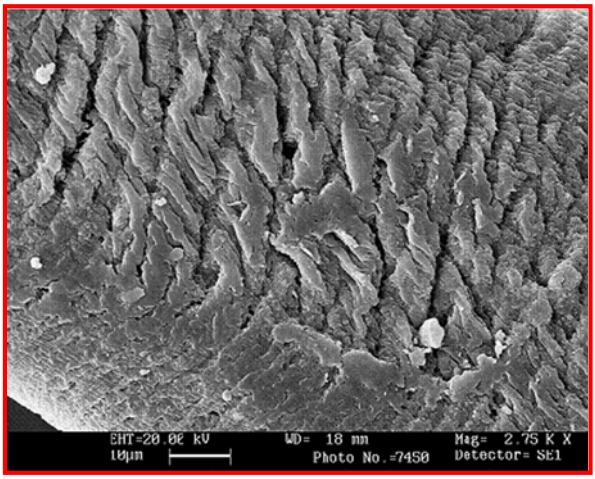
The naturally collected (N) threads had rough surfaces with many contours. Root of the naturally obtained byssus (N) threads was curved, rough in texture, and could be easily differentiated from byssus procured from laboratory (L). The SEM analysis (for natural habitat byssus N and byssus obtained from animal cultured in lab L, Fig. 1a, b) results clearly reveal this from structural texture which gives insight of molecular level. The main aim behind studying the structure of threads at SEM level was to find out whether the threads could be put to biotechnological uses; say for immobilization of enzymes. Activity obtained after immobilization in N was (78%) whereas for L byssus it was (76%).

Effect of pH on Urease Activity

The effect of pH on the activity of free and immobilized urease is shown in Fig. 2. The optimum pH of the free urease and urease immobilized on byssus (both N and L) were 7 and 7.5, respectively. However, immobilized urease maintained a higher relative activity than free urease at both lower and higher pH levels, indicating that the immobilized urease preparation is broader than that of the free enzyme, which means that the immobilization methods preserve the enzyme activity for a wider pH range [28, 29]. The pH dependence of



A SEM image of byssus obtained from laboratory aquaria (L).



B SEM image of naturally obtained byssus (N).

Fig. 1 **a** SEM image of byssus obtained from laboratory aquaria (L). **b** SEM image of naturally obtained byssus (N)

the activity of an immobilized enzyme is characteristic of the nature of enzyme and carrier, and the immobilization method. The enzymatic product (ammonium ions) is charged molecule, which may affect the pH value.

Effect of Temperature on Urease Activity

Free urease showed optimum activity at 50 °C whereas immobilized urease (both N and L) showed maximum activity at 55–70 °C (Fig. 3). At 80 °C, the free enzyme retained only 30% of activity whereas immobilized urease (both N and L) was found to be 64%. Enhanced thermal stability has been reported for urease grafted on iron oxide nanoparticle, and those immobilized on chitosan and alginate showed highest relative activity at higher

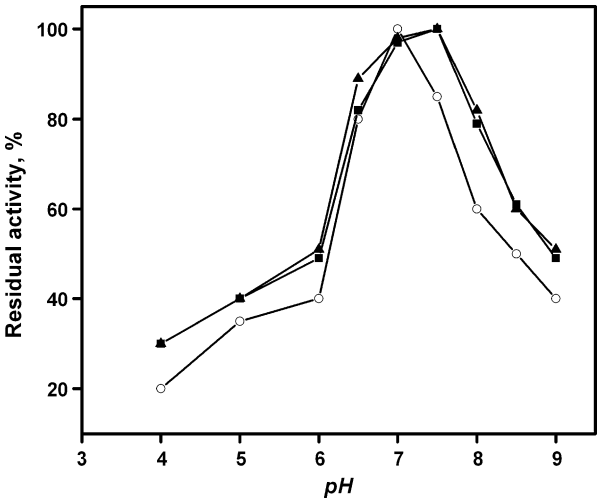


Fig. 2 Plot of percent maximum activity (percent) vs. pH profile for free (*empty circle*) and immobilized urease on byssus threads N obtained (*solid square*) and L in lab (*solid triangle*)

temperature (<55 °C) [30–32]. Improved thermal stability indicates that the immobilization of enzyme helps resist denaturation of enzymes active site.

Reusability

The most important practical utility of immobilized enzymes is their reusability. The reusability of the immobilized enzyme preparations was ascertained by quantifying their activity in nine consecutive cycles, each of 30 min duration. The study revealed that the immobilized byssus threads (both N and L) retained almost 50% of its original activity after nine uses (Fig. 4). Stability of byssus threads after repeated use was found to be quite stable with respect to their texture, shape, etc. The observed decrease in activity is likely a result of desorption of the enzyme. Also, some loss of activity of the immobilized preparation in repeated use is a common phenomenon [33].

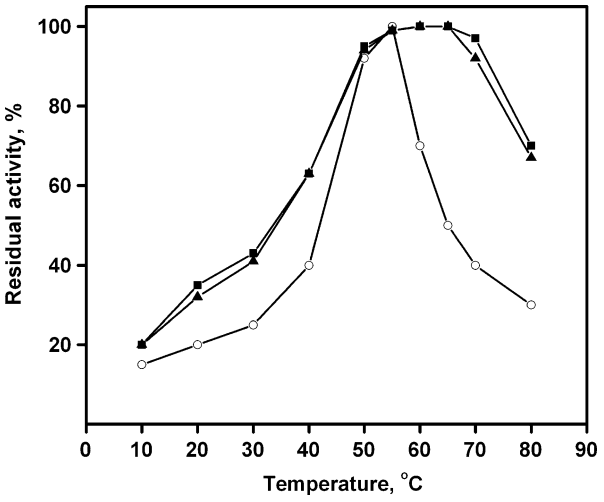


Fig. 3 Plot of percent maximum activity vs. temperature stability for free (*empty circle*) and immobilized urease on byssus thread N obtained (*solid square*) and L in lab (*solid triangle*)

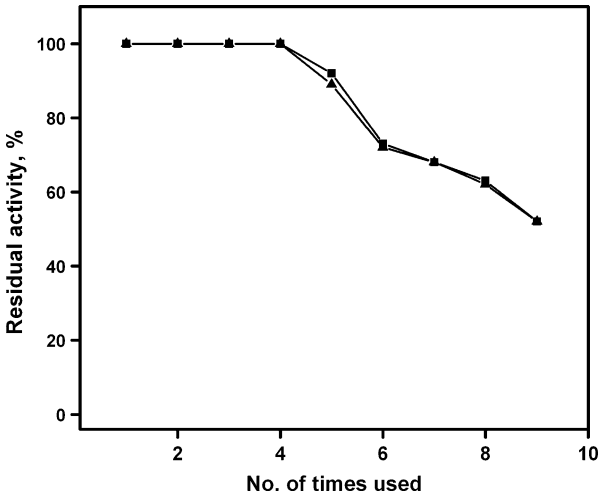


Fig. 4 Plot of residual activity (percent) vs. number of times used for reusability studies of free and immobilized urease on byssus threads N obtained (*solid square*) and L in lab (*solid triangle*)

Effect of Storage Stability on Urease Activity

The enzyme immobilized on byssus threads preserved in buffer retained the 95% activity for 10 days and about 40% of activity after a month. When the threads were preserved in distilled water, only 30% of its original activity was retained after a month. When preserved under dry condition, the enzyme retained 50% of its activity. Free enzyme retained its original activity for about 5 days, after which it slowly decreased and was lost completely after a month. The improved storage stability of the immobilized urease could be attributed to the improved resistance to the conformational change as a result of covalent attachment of urease on the byssus threads. It is concluded that dry immobilized threads show better result (Fig. 5).

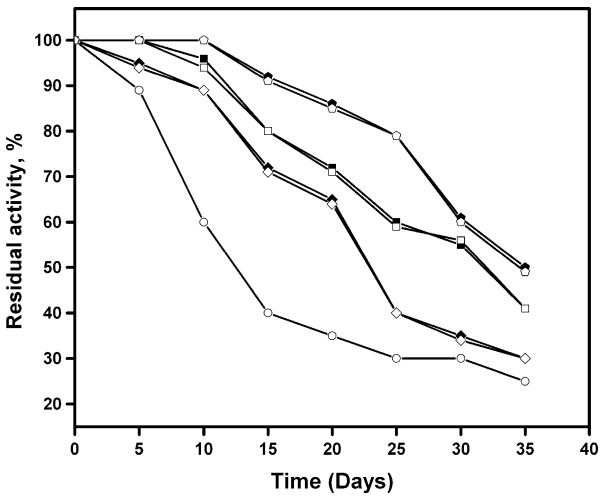


Fig. 5 Plot of percent maximum activity (percent) vs. time (days) for storage stability of free (*empty circle*) and immobilized urease on byssus threads N obtained (*empty square*) and L in lab (*solid square*), kept as dry films N (*empty pentagon*) and L (*solid pentagon*), preserve in buffer N (*solid diamond*) and L (*empty diamond*)

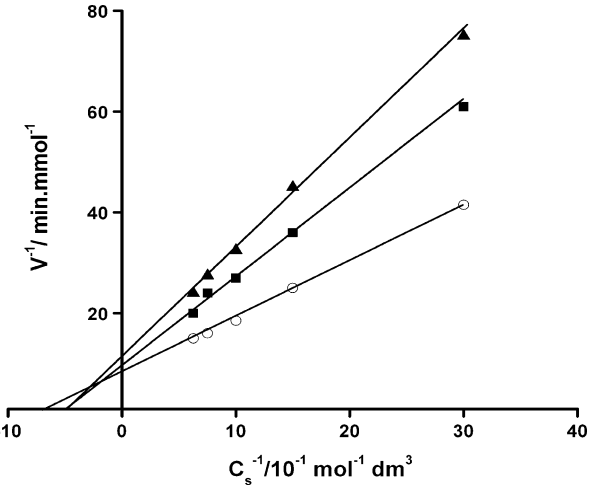


Fig. 6 Double reciprocal plot of velocity V (millimoles of NH_3 produced per minute per milligram of urease) vs. concentration of urea for free (*empty circle*) and immobilized urease on byssus thread (N) obtained (*solid square*) and (L) in lab (*solid triangle*)

Effect on Kinetic Parameter upon Immobilization

The kinetic parameters of the immobilized urease were determined from the Lineweaver–Burk graph obtained by plotting inverse values of substrate concentration against inverse values of initial reaction rates (Fig. 6). It was observed from the graph that the values of kinetic parameters of the immobilized enzyme changed from those of the free enzyme. The K_m and V_{max} value of N was 2.08 mol ($C_s^{-1}/10^{-1}$ mol^{-1} dm^3) and 0.87×10^{-1} mmol NH_3 min^{-1} mg^{-1}protein (V^{-1}/min $mmol^{-1}$) whereas for L byssus K_m was same as N obtained byssus, but there was increase in V_{max} value 1.02×10^{-1} mmol NH_3 min^{-1} mg^{-1}protein (V^{-1}/min $mmol^{-1}$). The K_m and V_{max} values of free urease were found to be 1.48 mol ($C_s^{-1}/10^{-1}$ mol^{-1} dm^3) and 1.17×10^{-1} mmol NH_3 min^{-1} mg^{-1}protein (V^{-1}/min $mmol^{-1}$). An increase in the K_m value is not an indication of reduced enzyme activity as a result of immobilization, or its affinity for the substrate is affected. Immobilization restricts the kinetic movement of enzyme in the reaction medium, while the free enzyme is not governed by such limitations. N has lower V_{max} with respect to free and L (Table 1). It may be due to the fact that enzyme was covalently attached to thread using glutaraldehyde, and during this, some of the enzyme molecules get attached via groups of active site leading to their inactivation, thus a decrease in V_{max} was observed [34, 35]. This is another result supporting the formation of conformational changes in the enzyme molecule by the immobilization. In the entire studies, the concentration of urea is very less (0.5 M); however, this concentration is sufficient to soften the threads making them supple and this induces conformational changes in the surface topography. This may also be responsible for changes in the kinetic behavior.

Table 1 Kinetic parameter for free and immobilized urease

Enzyme studies	C_s^{-1}, mol$\times10^{-1}$	V^{-1}/min $mmol^{-1}$
Native enzyme	1.48	1.17
Modified byssus threads	2.08	1.02
Naturally obtained byssus threads	2.08	0.87

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