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Immobilization of α-Amylase from *Bacillus circulans* GRS 313 on Coconut Fiber

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Abstract

A simple and inexpensive method for immobilizing α-amylase from *Bacillus circulans* GRS 313 on coconut fiber was developed. The immobilization conditions for highest efficiency were optimized with respect to immobilization pH of 5.5, 30°C, contact time of 4 h, and enzyme to support a ratio of 1:1 containing 0.12 mg/mL of protein. The catalytic properties of the immobilized enzyme were compared with that of the free enzyme. The activity of amylase adsorbed on coconut fiber was 38.7 U/g of fiber at its optimum pH of 5.7 and 48°C, compared with the maximum activity of 40.2 U/mL of free enzyme at the optimum pH of 4.9 and 48°C. The reutilization capacity of the immobilized enzyme was up to three cycles.

Index Entries: Coconut fiber; adsorption; response surface methodology; amylase; *Bacillus circulans* GRS 313.

Introduction

In previous works, α-amylase has been immobilized onto/into a large variety of supports such as controlled pore glass *(1,2)*, collagen *(3)*, Sephadex and Sepharose *(4)*, and polyaminostyrene *(5)*. In recent years, the enzyme has been immobilized on nitrocellulose membrane *(6)* and zirconium dynamic membrane *(7)*. However, reports on utilization of natural fiber such as coconut coir for immobilization of amylase are scarce.

Coconut coir is a product of the coconut industry that has numerous industrial applications including automobile upholstery, bedding and mattresses, drainage filters, insulation, packaging, brush and broom manufacturing, reinforcement of thermoplastics, marine cordage and fishnets, and in horticulture as a growing medium/soil substrate. Coir has a water-holding capacity and a cation-exchange capacity of 39–60 meq/100 g *(8)*.

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These properties and the fact that it is widely available make coconut fiber a potential hydrophilic support that may be used for immobilization of enzymes by physical adsorption. However, its application has been restricted to the mentioned applications and it has not been tested as an immobilization matrix for adsorption of enzyme. Thus, an attempt was made to test coconut fiber as an adsorption matrix for immobilization of an amylase from *Bacillus circulans* GRS 313.

Materials and Methods

Chemicals

Glutaraldehyde was purchased from BDH, England, and dinitrosalicylic acid (DNS) from Lanchester, England. All other chemicals used were of analytical reagent grade.

Production of Enzyme

α-Amylase was produced from *B. circulans* GRS 313 by submerged fermentation at 46°C and initial pH of 5.8 for 24 h with 0.5% (v/v) inoculum (containing 10^7 cells/mL). The medium contained 48.4 g/L of soybean meal, 15.8 g/L of yeast extract, 28.4 g/L of wheat bran, 0.01 g/L of $CaCl_2$, and 0.01% (v/v) Triton-X. The enzyme was purified by acetone fractionation, Sephadex G-100 gel filtration, and CM-Sephadex column chromatography. The culture filtrate was concentrated using cold acetone in the ratio of 1:2. The concentrate was dialyzed against 0.01 *M* acetate buffer, pH 4.0. The concentrated enzyme was loaded onto a Sephadex G-100 column (2.6×64 cm) equilibrated with the same buffer. The amylase-active fractions were pooled and concentrated by lyophilization. The lyophilized enzyme solution was loaded onto a CM-Sephadex column (2.6 × 14.5 cm) equilibrated with 0.1 *M* acetate buffer (pH 4.0). After the column was washed with the same buffer, the enzyme was eluted with 0.1 *M* phosphate buffer (pH 8.0). The pooled fractions were dialyzed against 0.05 *M* acetate buffer (pH 6.0) and used as the purified enzyme. The specific activity of the purified fraction was 197.8 U/mg containing 0.12 mg/mL of protein with an activity of 23.73 U/mL.

Preparation of Immobilized Enzyme

The coconut fibers were separated and boiled in water containing 0.01% (w/v) sodium dodecyl sulfate for 1 h. The fibers were dried completely at room temperature. Ten milliliters of enzyme solution, having a total activity of 230 U, along with 10 g of the fiber was placed in a shaking water bath at 30°C for a contact time of 2 h. After adsorption the enzyme solution was decanted and saved for subsequent assay. The unbound α-amylase was washed off with distilled water until no activity was detected in the washing.

Table 1
Two-Factored CCD for Studying Effects of pH and Temperature on Activity of Immobilized Enzyme and Free Enzyme[a]

Run no.	X_1 (I. Enz) (temperature)	X_2 (I. Enz) (pH)	X_1 (F. Enz) (temperature)	X_2 (F. Enz) (pH)
1	55 (1)	4.5 (–1)	55 (1)	4.2 (–1)
2	55 (1)	5.5 (1)	55 (1)	4.8 (1)
3	45 (–1)	4.5 (–1)	45 (–1)	4.2 (–1)
4	45 (–1)	5.5 (1)	45 (–1)	4.8 (1)
5	50 (0)	5.0 (0)	50 (0)	4.5 (0)
6	50 (0)	5.0 (0)	50 (0)	4.5 (0)
7	50 (0)	5.7 ($\sqrt{2}$)	50 (0)	4.9 ($\sqrt{2}$)
8	50 (0)	4.3 ($-\sqrt{2}$)	50 (0)	4.0 ($\sqrt{2}$)
9	57 ($\sqrt{2}$)	5.0 (0)	57 ($\sqrt{2}$)	4.5 (0)
10	43 ($-\sqrt{2}$)	5.0 (0)	43 ($-\sqrt{2}$)	4.5 (0)
11	50 (0)	5.0 (0)	50 (0)	4.5 (0)
12	50 (0)	5.0 (0)	50 (0)	4.5 (0)

[a]I. Enz = immobilized enzyme; F. Enz = free enzyme. Numbers in parentheses are the uncoded values of the variables.

Enzyme Assay

Amylase activity was measured by the modified Bernfeld *(9)* method. The reaction mixture, containing 2.5 mL of 2% (w/v) starch solution in acetate buffer (0.1 *M*, pH 4.5) and 0.25 g of coconut fiber, was incubated at 50°C in a water bath shaker. After the enzymatic reaction had proceeded for 10 min, 0.5 mL of the digested product was assayed for amylase activity using DNS reagent. One unit was defined as the amount of amylase that produced 1 µmol of reducing sugar under assay conditions/g of fiber.

Determination of Immobilization Efficiency

Immobilization efficiency was determined from the difference in enzyme activity in the solution before and after the immobilization *(10)*:

$$\text{Immobilization yield (\%)} = (I/A - B) \times 100$$

in which A is the added enzyme (U/g of fiber), B is the unbound enzyme (U/g of fiber), and I is the immobilized enzyme (U/g of fiber).

Statistical Analyses

Response surface methodology (RSM) with two factors, pH and temperature, at five levels each was used to study the simultaneous effects of these reaction parameters on the amylase activity and to evaluate the optimum pH and temperature for the enzymatic reaction. The amylase activity of the adsorbed and free enzyme was regarded as the response value, and 12 experimental runs were performed according to a 2^2 factorial central composite experimental design *(11,12)* (Table 1). Preliminary trials enabled

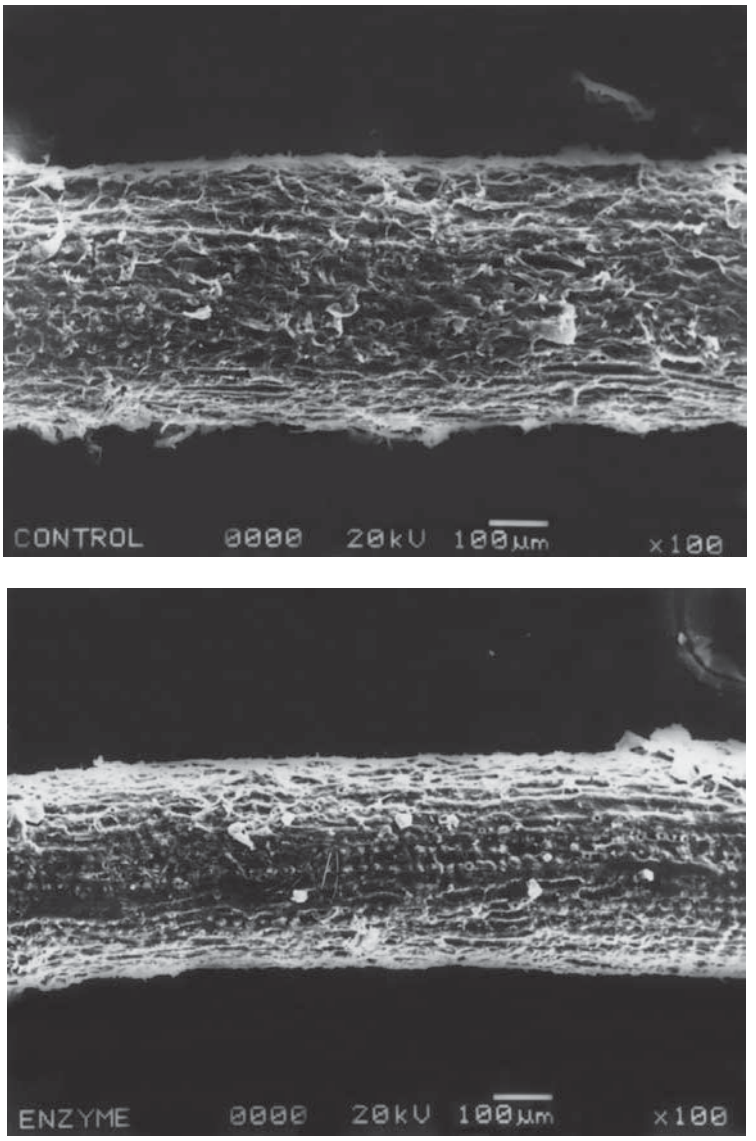

Fig. 1. **(A)** Scanning electron micrograph of coconut fiber; **(B)** scanning electron micrograph of amylase-immobilized coconut fiber.

us to fix the range of the pH from 4.3 to 5.7 and the temperature from 43 to 57°C for adsorbed enzyme. The range of variables for the free enzyme was fixed according to the preliminary trials, for a pH from 4.0 to 4.9 and an equation such that X_0 corresponded to the central value:

$$x_i = (X_i - X_0)/\Delta X_i \qquad i = 1, 2, 3, \ldots k$$

in which x_i is the dimensionless value of an independent variable, X_i is the real value of an independent variable, X_0 is the real value of an independent variable at the center point, and ΔX_i is the step change.

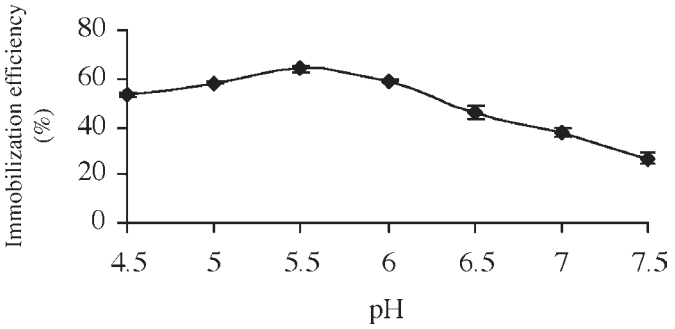

Fig. 2. Effect of immobilization pH on adsorption efficiency.

The value of the dependent response was the mean of two replications. The second-order polynomial coefficients were calculated using MATLAB software (version 4, The Math Works). The results were expressed as a contour plot. Finally, the optimum combination of pH and temperature for maximum enzyme activity was determined by a program using MATLAB.

Results

Characteristics of Coconut Fiber

The average length of the fiber was 8 cm and diameter was 1.6 μ. It had a density of 1.4 g/cm^3.

Scanning Electron Microscopy of Enzyme-Adsorbed Fiber

To observe the structure of the coconut fiber, scanning electron micrographs of the gold-coated samples were taken with a scanning electron microscope (model Can Scan, Series 2 DV). The scanning electron micrographs given in Fig. 1A,B show the surface structures of coconut fiber and enzyme-adsorbed fiber, respectively. The scanning electron micrograph of the fiber exhibited a homogeneous and highly open pore structure having a high internal surface area.

Optimization of Adsorption Conditions

Effect of pH on Adsorption Efficiency

To study the effect of pH on the adsorption efficiency, the enzyme extract was adjusted at different pHs from 4.5 to 7.5. Figure 2 shows the immobilization efficiency as a function of pH. The adsorption efficiency was found to be highest when the pH of the enzyme solution was 5.5.

Effect of Immobilization Temperature

The influence of temperature on the immobilization efficiency of amylase on coconut fiber is illustrated in Fig. 3. The highest immobilization efficiency was attained at 30°C.

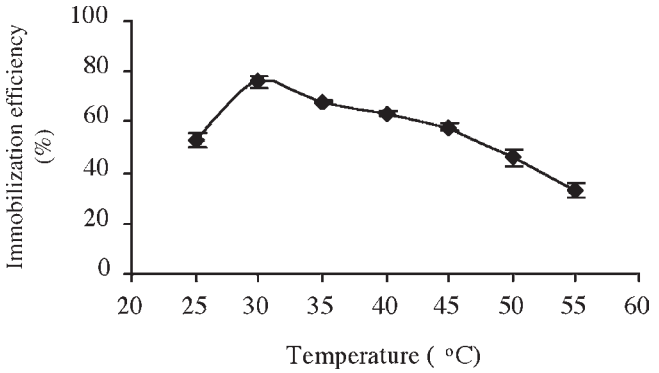

Fig. 3. Effect of immobilization temperature on adsorption efficiency. Immobilization pH = 5.5.

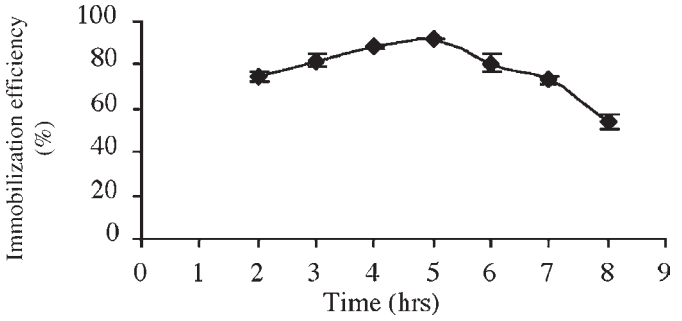

Fig. 4. Effect of contact time on adsorption efficiency.

Effect of Contact Time on Adsorption Efficiency

The time required for adsorption of amylase on coconut fiber was optimized by keeping the coconut fiber in contact with the enzyme solution at pH 5.5 and 30°C for a period of 2–8 h. According to the results, a time period of 4 h was necessary for maximum immobilization efficiency (Fig. 4). Beyond 4 h there was no improvement in immobilization efficiency. A fall in enzyme activity was observed after 5 h.

Enzyme-to-Support Ratio

Maximization of the amount of enzyme to be immobilized onto the fiber was attempted by varying the ratio of the enzyme amount to the weight of fiber. As shown in Fig. 5, a volume of 10 mL of enzyme solution, containing 1.2 mg of protein, adsorbed to 10 g of coconut fiber was best suited for maximum product formation.

Glutaraldehyde Treatment

Glutaraldehyde was used as a crosslinking agent and its effect on the activity of the immobilized enzyme was studied. The activity of the immobilized enzyme declined in the presence of glutaraldehyde even at a low

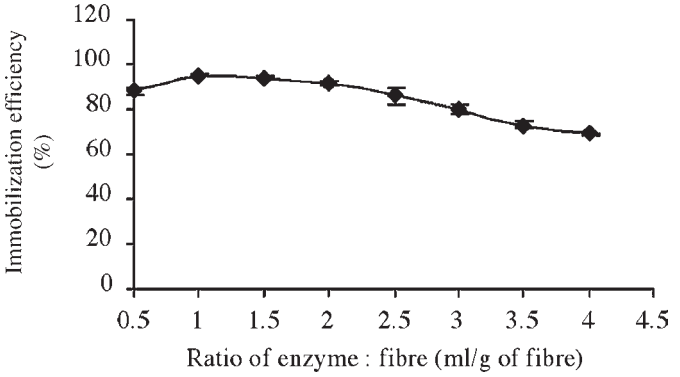

Fig. 5. Effect of enzyme-to-support ratio on adsorption efficiency.

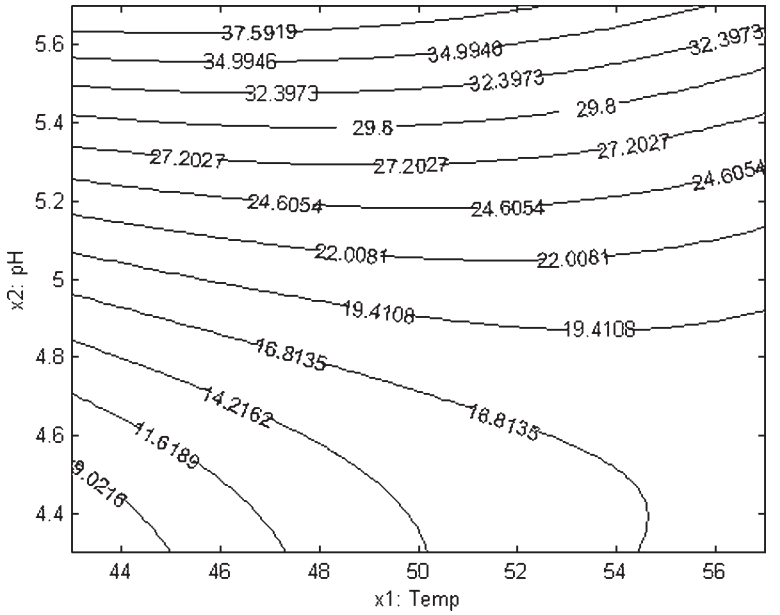

Fig. 6. Contour plot showing effect of pH and temperature on activity of adsorbed amylase.

concentration of 0.025%. Therefore, glutaraldehyde could not be used as a crosslinking agent for the present immobilized-enzyme system.

Properties of Immobilized and Free Enzyme

Optimization of Temperature and pH Using RSM

The effects of temperature and pH on the activity of the immobilized enzyme are shown in Fig. 6. The maximum activity of amylase adsorbed to coconut fiber was attained at 48°C and pH 5.7. On optimization the α-amylase activity was enhanced by 1.9-fold from 20.2 to 38.7 U/g of fiber.

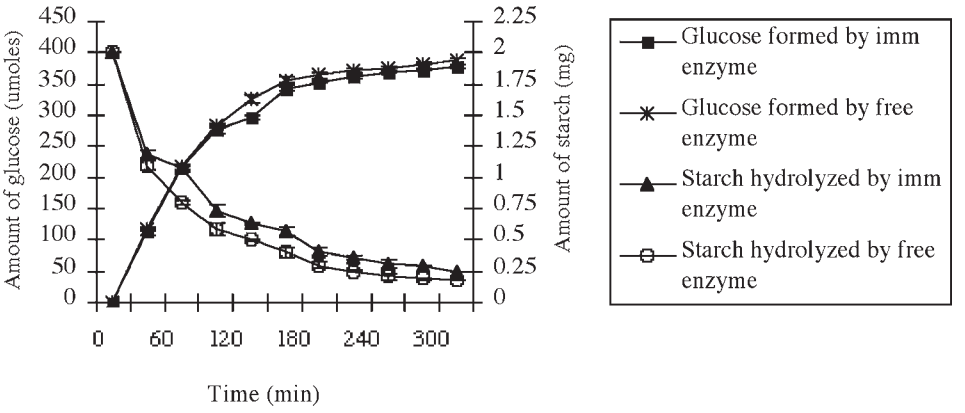

Fig. 7. Course of starch hydrolysis with immobilized and free amylase.

The optimum activity of the free enzyme was similarly optimized using RSM, and the activity was found to be maximum at 48°C and pH 4.9.

Kinetics

The activity of the free and fiber-bound amylase preparation with identical enzyme concentration was determined at a substrate concentration ranging from 1 to 4 g/100 mL and incubation at 48 and 57°C. The K_m and V_{max} were calculated for both the free and immobilized amylase from the Lineweaver-Burk plot. The K_m of immobilized enzyme was 13.07 and 14.11 mg/mL at 48 and 57°C, respectively. The values for free enzyme were 11.66 and 19.11 mg/mL at 48 and 57°C, respectively. Similarly, the V_{max} values for immobilized enzyme were 65.36 and 55.55 U/g of fiber and for free enzyme were 68.97 and 59.521 U/mL at 48 and 57°C.

The activation energy, E_a, of the enzyme was calculated from Arrhenius plot by plotting the log of the reaction rate against $1/T$. The E_a of immobilized and free enzyme was 6.5 and 7.5 kcal/mol, respectively.

Efficiency of Enzyme

A comparative study on the efficiency of the immobilized and free enzyme was performed at their respective optimum reaction conditions. As Fig. 7 shows, the percentage of starch hydrolyzed by adsorbed enzyme at the end of 5 h was 88%, and for the free enzyme was 91%.

Operational Stability

To evaluate the operational stability of the enzyme-immobilized fiber, the activity assay was repeated successively every day for the same coconut fiber up to cycle 7. The percentage activity retained for successive runs was quantified and found to be 100, 110, 90, 75, 62, 46, and 24% in cycles 1, 2, 3, 4, 5, 6, and 7, respectively. There was an increase in the activity in cycle 2. Almost 90% activity was retained up to cycle 3, but with subsequent runs there was a decline in the activity of the enzyme-immobilized fiber.

Discussion

In the present study, in which there was no covalent bonding of the enzyme, the process of physical adsorption was influenced by the pH of the medium. The decline in immobilization efficiency beyond pH 5.5 may be owing to desorption of the enzyme from the carrier. Desorption of enzyme as a result of pH and ionic strength has previously been reported *(13)*. Temperature and time also affected the immobilization efficiency of α-amylase from *B. circulans* GRS 313. The immobilization efficiency was highest at 30°C, beyond which there was a decline in activity of the enzyme, which may be owing to denaturation of the enzyme at higher temperatures. Similarly, the enzyme concentration exposed to the unit weight of the carrier during the immobilization process also influenced the immobilization efficiency. The immobilized amylase reached a limiting value with 1.0 and 1.5 mL of enzyme solution adsorbed to 1 g of carrier. Higher loading of carrier with enzyme resulted in less enzyme activity. This could be owing to the fact that the amount of enzyme available for reaction was restricted since the predominant reaction occurs in the first few layers that are available to the substrate. The decline in amylase activity in the presence of glutaraldehyde may be the result of steric hindrance caused by the presence of the crosslinking agent, which may have affected the accessibility of the substrate to amylase *(14)*. Under optimum conditions for adsorption, the immobilization efficiency for the present amylase was 95%, which is higher than the reported value (20.9%), for immobilized amylase onto nitrocellulose membrane *(6)*.

A comparative study of the properties of adsorbed and free enzyme was conducted in order to evaluate the influence of physicochemical properties of the carrier on the enzymatic activity. The amylase-immobilized coconut fiber could be operated at higher temperature (48°C) compared with that reported for amylase-immobilized zirconium dynamic membrane (40°C) *(7)*. After optimization, the adsorbed amylase from *B. circulans* GRS 313 had an optimum temperature the same as that of free enzyme (48°C). On the other hand, the optimum pH for immobilized enzyme increased by 0.8 units compared with that for the free amylase. Usually, carriers with negative surface charges exhibit an apparent pH optimum higher than that observed with the free enzyme *(15)*, suggesting that the coconut fiber may have negative surface charges.

The kinetic constant, K_m, of the immobilized amylase increased with an increase in temperature and there was a concomitant decrease in the V_{max} of the enzymatic reaction. This indicates that at higher temperature the conformation of the immobilized enzyme was affected, which resulted in an alteration of the enzyme-substrate affinity. The decline in reaction rate could also be the result of desorption of enzyme molecules from the fiber at higher temperature. On comparison of the kinetic constants of immobilized and free amylase, it was observed that K_m was altered in the case of immobilized enzyme. Whenever an enzyme is bound to a solid matrix,

the kinetics of the reaction changes considerably, and these changes are attributable to an alteration in enzyme conformation, microenvironmental effects of the carrier, and bulk and diffusional effects. In the present case of adsorption of amylase on coconut fiber, the change in K_m suggests the presence of charge-charge interactions during the immobilization of the enzyme. Consequently, V_{max} of the immobilized enzyme declined compared with that of the free enzyme. Interestingly, the change in K_m of the enzyme with increasing temperature was more drastic in the case of free enzyme than in immobilized enzyme. This suggests that the enzyme in its bound state is less sensitive to conformational changes owing to rise in temperature and therefore is more stable.

E_a of the immobilized enzyme exhibited a decrease over that of the free amylase, possibly owing to the introduction of strain in the enzyme and unfavorable change of polarity in the vicinity of the active site *(16)*.

The high performance of this carrier may be the result of the presence of an effective surface area for adsorption, which was evident from the scanning electron micrographs (Fig. 1A,B).

Evaluation of the operational stability of the immobilized enzyme showed that further studies are required to improve the reutilization capacity of the enzyme.

Conclusion

An easily available and inexpensive matrix, coconut fiber, was used for adsorption of *B. circulans* GRS 313 amylase. The immobilized α-amylase displayed a slightly lower substrate affinity than free enzyme. Nevertheless, the immobilized catalyst exhibited an appreciable catalytic capability (38.7 U/g of fiber). The immobilized enzyme maintained 90% of its initial activity up to three cycles, beyond which there was a fall in enzyme activity. We conclude that coconut fiber may be used as a potential immobilization support of the enzyme provided that the rate of desorption can be limited.

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