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BIOCHEMICAL STUDIES ON IMMOBILIZED FUNGAL β-GLUCOSIDASE

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Abstract - β-Glucosidase from *Aspergillus niger* was immobilized on sponge by covalent binding through a spacer group (glutaraldehyde). Sponge-immobilized enzyme had the highest immobilization yield (95.67%) and retained 63.66% of the original activity exhibited by the free enzyme. The optimum pH of the immobilized enzyme remains almost the same as for the free enzyme (pH 4.0). The optimum temperature for β-glucosidase activity was increased by 10 ºC after immobilization. The activation energy (E_a) of the immobilized β-glucosidase was lower than the free enzyme (3.34 and 4.55 kcal/mol), respectively. Immobilized β-glucosidase exhibited great thermal stability and retained all the initial activity after incubation at 55 ºC for 2 h; however, the free enzyme retained 89.25% under the same condition. The calculated half-life ($t_{½}$) value of heat inactivation of immobilized enzyme at 60, 65 and 70 ºC was 213.62, 72.95 and 56.80 min, respectively, whereas at these temperatures the free enzyme was less stable (half-life of 200.0, 55.31 and 49.5 min, respectively). The deactivation rate constant at 65 ºC for the immobilized β-glucosidase is $9.5x10^{-3}$/ min, which was lower than that of the free form ($12.53x10^{-3}$/ min). The immobilization process improved the pH stability of the enzyme (immobilized and free enzyme retained 69.35 and 39.86%, respectively, of their initial activity after 45 min at pH 7.5). The effect of some chemical substances on the activity of the immobilized and free β-glucosidase has been investigated. In the presence of sodium dodecyl sulfate (SDS) and *p*-chloromercuri benzoate (*p*-CMB) the immobilized enzyme retained 36.13 and 45.34%, respectively, of the initial activity, which is higher than that of free enzyme (13.71 and 1.61%, respectively). The Michaelis constant (K_m) value of the free enzyme was 40.0 mM, while the apparent K_m value for the immobilized enzyme was 46.51 mM. The maximum reaction rate (v_{max}) of immobilized β-glucosidase was smaller than that of the free enzyme by 7.69%. Sponge-immobilized β-glucosidase was repeatedly used to hydrolyze cellobiose (5 and 8 cycles with retained activity of 67.32 and 51.04%, respectively).
Keywords: β-glucosidase; Enzyme immobilization; Stability; Reusability.

INTRODUCTION

β-Glucosidase (β-D-glucoside glucohydrolases, cellobiase E.C.3.2.1.21) is a key enzyme in the hydrolysis of cellulose to two glucoses (Karagulyan *et al*., 2008). β-Glucosidase also demonstrated activity on cello-oligomers and, although the activity varies, it generally decreases with increasing chain length. It is capable of hydrolyzing anthocyanins that are the main coloring agents found in foods of vegetable origin (Chang *et al*., 2008). β-Glucosidase can be used in the production of aromatic compounds, in the stabilization of juices and beverages, and in the improvement of the organoleptic properties of food and feed products. It is also used in biomass degradation and in the production of fuel ethanol from

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cellulosic agricultural residues (Saha, *et al*., 1994; Ortega *et al*., 1998; Karagulyan *et al*., 2008; Saritha and Lata 2012). Moreover, it can be used in the synthesis of alkyl- and aryl glycosides from natural polysaccharides or their derivatives and alcohols by reverse hydrolysis or trans-glycosylation, leading to products with applications in pharmaceutical, cosmetic, and detergent industries (Gueguen *et al*., 1999; Bhatia *et al*., 2002; Yeoman *et al*., 2010). The cellulase complex from the main fungal producers normally contain small amounts of β-glucosidase, which may limit the overall cellulose hydrolysis rate, as cellobiose inhibits activity of other enzymes of the cellulose complex (Karagulyan *et al*., 2008). β-Glucosidase is also subject to end-product inhibition, with removal of glucose significantly enhancing the enzyme activity rate (Ortega *et al*., 1998; Karagulyan *et al*., 2008). The cost of β-glucosidase-based technology can be reduced by increasing the enzyme reusability and its stability (AbdEl-Ghaffar and Hashem, 2010). Immobilization of enzymes on a carrier offers significant cost benefits for industrial processes, because it facilitates enzyme recycling, enables improvements in thermo-stability (thereby reducing enzyme inactivation), and allows for greater control of enzyme activity (El-Tanash *et al*., 2011; Alkhatib *et al*., 2012). In addition, immobilization eliminates the need to separate an enzyme from the product solution and allow these expensive compounds to be reused (Mazzuce *et al*., 2006). These advantages are in addition to those that enzymes possess over conventional catalysts such as high efficiency and regio/stereo-specificity (Lye *et al*., 1996). Various techniques have been developed for enzyme immobilization, including adsorption, covalent linking to insoluble supports, entrapment in polymeric gels, encapsulation in membranes, cross-linking with bifunctional reagents (like glutaraldhyde) and different combinations of immobilization methods are also known (Radva *et al*., 2011). If a simple and cheap immobilization technique for β-glucosidase were available, sufficient cellobiose could be converted to glucose to justify the additional cost (Ortega *et al*., 1998). Our study is the first using sponge as a suitable carrier (safe, inexpensive, readily available) for β-glucosidase immobilization that exhibits a considerable protein-binding capacity and a high activity. Protocols for covalent enzyme immobilization often begin with a surface modification or activation step. The bonds between carrier and enzyme are very strong resulting in a highly stable conjugate (AbdEl-Ghaffar and Hashem, 2010). The work reported here examined several carrier matrices for their ability to immobilize β-glucosidase by various techniques. The properties of immobilized enzyme were compared with free enzyme.

MATERIALS AND METHODS

Cell Growth and Enzyme Production

The fungal strain *Aspergillus niger* was obtained from the Center of Culture Collection of the National Research Center (NRC), Dokki, Cairo, Egypt, and sub-cultured every 3 weeks and stored at 4 ºC in PDA slants. To obtain the fungal enzyme, spores from *Aspergillus niger* were produced on PDA slants for 5 days at 30 ºC. One mL of spore suspension ($60x10^9$ spores) was then inoculated in 250 mL-Erlenmeyer flasks containing 50 mL production medium. The production medium was prepared from the following in 1000 mL wheat bran extract from the local market (1% w/v): NH_4Cl 3.0 g; citric acid 17.0 g; KH_2PO_4 3.0 g; $MgSO_4.7H_2O$ 0.5 g; $CaCl_2$ 0.5 g; Triton X-100 0.2 mL and milk whey 10 mL, the pH was adjusted to 7.0. The flasks were shaken at 150 rpm and 30 ºC for 7 days. The culture was filtered through cloth to remove the mycelia.

Carriers for Enzyme Immobilization

Wool, sponge, saw dust, stainless steel, Loofa and pumice were obtained from the local market. Resin (Alumina, Amberlite IR-120, Amberlite IR-400, Dowex 1x8 (20-50) mesh, DEAE-Sephadex A-50) and chitin were from Fluka Company, Switzerland. DEAE Cellulose 11 and DEAE Cellulose 53 were obtained from Whatman Biosystems Ltd. Agar, agarose and sodium alginate were obtained from BDH Chemical Ltd., Poole, England.

Assay for Cellobiase Activity

The activity of the free and immobilized β-glucosidase were determined according to the method reported by Abdel-Naby *et al*. (1999), as follows: The reaction mixture, which consists of 1 mL of 0.4% (w/v) cellobiose dissolved in 0.05 M citrate–phosphate buffer (pH 4.8) and 0.5 mL diluted enzyme solution, was incubated in a water bath at 50 °C for 30 min. The reaction was stopped by heating the reaction mixture in a boiling water bath for 10 min. The glucose released was determined by glucose oxidase–peroxidase reagent according to Huggett and Nixon (1957). One unit (U) of the enzyme activity was defined as the amount of the enzyme that releases 1 µmol glucose from cellobiose per min under the

assay conditions. At least three measurements were made for each experiment and the data given are an average of these results.

Determination of Protein

Protein content was estimated by the method of Lowry *et al*. (1951).

Immobilization Methods

Covalent Binding

One gram of each carrier was covered with 5 mL of citrate phosphate buffer (0.05 M, pH 4.0) containing 1.25% (v/v) glutaraldehyde (GA) and left for 2 h at 30 ºC. The carriers were washed with distilled water to remove excess GA and incubated with 5 mL of enzyme solution (50 U) at 4 ºC overnight as reported by Abdel-Naby *et al.* (1998).

Physical Adsorption

It was carried out according to Woodward (1985). One gram of each carrier was incubated with 50 U β-glucosidase dissolved in 5 mL of 0.05 M citrate phosphate buffer (pH 4.0) overnight at 4 ºC. The unbound enzymes were removed by washing with citrate phosphate buffer (0.05 M, pH 4.0).

Ionic Binding

One gram of each carrier was equilibrated with 0.01 M HCl and NaOH, respectively, for cation and anion exchangers, and washed with distilled water to remove the excess HCl or NaOH. Then the carriers were incubated with 5 mL of enzyme solution containing 50 U in citrate phosphate buffer (0.05 M, pH 4.0) at 4 ºC for 24 h. The unbound enzymes were removed by washing with the same buffer as reported by Ahmed *et al*. (2007).

Entrapment

In Agar and Agarose

An equal volume of enzyme solution containing 5 U and agar or agarose solution were used to obtain final concentrations of 2, 3 and 4% (w/v). The mixture was quickly cooled to 4 ºC and cut into small cubes of about 2 x 2 mm (Woodward, 1985).

In Ca-Alginate Beads

Different concentrations of sodium alginate solution in 10 mL (2, 4 and 6%) were mixed with 10 mL of enzyme solution (5 U). The entrapment was carried out by dropping the alginate solution through a Pasteur pipette (1 mm diameter) into gently stirred 0.15 M $CaCl_2$ and storage at room temperature for 2 h as reported by Dey *et al.* (2003).

Temperature Profile

The effect of temperature was studied by incubating both free and immobilized β-glucosidase in 1 mL of 0.4% (w/v) cellobiose dissolved in citrate–phosphate buffer (0.05 M, pH 4.8) at different temperatures (ranging from 30 to 75 ºC) for 30 min.

pH Profile

The optimum pH for free and immobilized enzyme was determined by incubating each enzyme at different pH values using 0.05 M citrate phosphate buffer (pH 2.5-7.0) and 0.05 M phosphate buffer (pH 7.0-8.0). The reaction mixture was incubated for 30 min at 45 and 55 °C, respectively, for free and immobilized forms using 0.4% cellobiose in citrate–phosphate buffer (0.05 M, pH 4.8).

Thermal Stability

Free and immobilized enzyme was incubated at temperatures ranging from 40 to 70 ºC for times ranging from 15 to 120 min. Samples were taken at 15 min intervals, cooled, and the residual activity determined on 0.4% cellobiose in citrate–phosphate buffer (0.05 M, pH 4.0) at 45 °C and 55 °C, respectively, for free and immobilized enzyme.

pH Stability

The pH stability of free and immobilized enzyme was examined after pre-incubating enzyme samples for different times (15-60 min) at 4 ºC in the above buffer solution (0.05 M, pH ranged from 2.5 to 8.0). pH was adjusted to the value of the standard assay system (pH 4.0). The residual activity was assayed under the standard conditions for free and immobilized β-glucosidase.

Kinetic Parameters

The effect of substrate concentration on the free and immobilized β-glucosidase activity was tested at different concentrations of cellobiose ranging from 2 to 28 mM. The assays were performed under optimal pH (4.0) and temperature (45 and 55 ºC), respectively for the free and immobilized forms. The K_m

(Michaelis constant) and V_{max} (maximum reaction rate) values were determined through Lineweaver-Burk plots.

Effect of Chemical Reagents

This experiment was carried out to determine the effect of some chemical reagents (5 mM) on the activity of free and immobilized β-glucosidase. Both the free and the immobilized enzyme were incubated with the tested reagent (2 mL) at room temperature (~30 ºC) for 1 h, before mixing with cellobiose. The enzyme activity was determined at the optimum pH value and incubation temperature for each enzyme.

Repeated Batch Hydrolysis

Consecutive batch runs were performed under the optimum assay conditions using 18 mM cellobiose in 0.05 M citrate–phosphate buffer (pH 4.0) at 55 ºC for 10 min. After each cycle, the immobilized biocatalyst was separated and washed with citrate phosphate buffer (0.05 M, pH 4.0). The reaction medium was then replaced with fresh medium. The activity of freshly prepared immobilized enzyme in the first run was defined as 100%.

RESULTS AND DISCUSSION

The immobilization of enzyme onto insoluble supports has been a topic of active research in enzyme technology and is essential for their application to industrial processes. A large number of enzymes were successfully immobilized with very high activity yields on appropriate supports. These immobilized products were intended for use in the construction of artificial organs, biosensors, or bioreactors (Bayramoglu *et al*., 2003). The selection of support materials and the method of immobilization are very important for carrying out the desired enzymatic reactions. In the present study, a large variety of insoluble supports have been used as carriers for immobilized β-glucosidase via different techniques for the immobilization process. The efficiency of enzyme immobilization was evaluated by different parameters, including the retained catalytic activity and loading efficiency (immobilized activity/ gram carrier). The immobilization yield (IY) is the key parameter which is calculated as follow: IY= I/(A-B) x 100 whereas I (immobilized enzyme U/ g carrier), A (added enzyme U/ g carrier), and B (unbound enzyme U/ g carrier). The whole culture-filtrate of *Aspergillus niger* was used for β-glucosidase immobilization. The specific activity of this crude enzyme was 29.6 U/g protein.

Immobilization by Covalent Binding

Immobilization by covalent binding through a spacer group (glutaraldehyde) showed considerable bound enzyme activity (good loading efficiency) and immobilization yield (IY). This good loading efficiency for immobilization by covalent binding might be due to the formation of stable crosslinking between the carrier and the enzyme through a spacer group (GA) The bond is normally formed between functional groups present on the surface of the support and functional groups belonging to amino acid residues on the surface of the enzyme. In addition, covalent binding through a spacer group probably increased the local surface area of the carrier and, consequently, the enzyme molecule (Abdel-Naby *et al*., 1998; Chang *et al*., 2008). As shown in Figure 1, the enzyme covalently bound to sponge showed the highest IY (95.7%) and the highest retained activity (63.7%). A lower IY was obtained by Martino *et al*. (1996) while using β-glucosidase immobilized by covalent binding to cellulose PEI (18.0%). Our result is higher than that obtained by Singh *et al*. (2011) for immobilization of *Agaricus arvensis* β-glucosidase by covalent binding to silicon oxide nanoparticles with retained activity 32%.

Immobilization by Physical Adsorption

The results in Figure 2 indicated that the lowest IY (54.2%) and bound enzyme (9.6 U/g carrier) were detected with Pumice. On the other hand, the highest retained activity (60.3%) and bound enzyme (30.1 U/g carrier) were found with sponge. Karagulyan *et al.* (2008) reported that physical adsorption of fungal β-glucosidase on kaolin involved weak and non-stable bonds between enzyme and carrier.

Immobilization by Ionic Binding

A series of ion exchangers was used for the immobilization of β-glucosidase by ionic binding (Figure 3). As shown by the results, immobilization by ionic binding gave a low binding of the enzyme and low IY. Dowex 1x8 was the most suitable ion exchanger for enzyme immobilization. This immobilized enzyme had the highest IY (72.2%) and highest retained activity (31.71%). The lowest IY was obtained for β-glucosidase bound to DEAE-cellulose 53 (25.3%) with the lowest bound enzyme (6.7 U/g carrier).

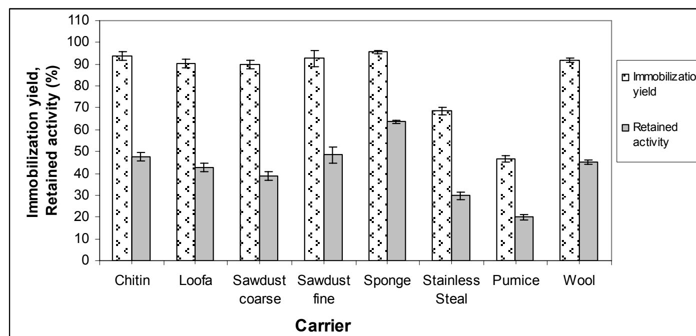


Figure 1: Immobilization of *Aspergillus niger* β-glucosidase by covalent binding.

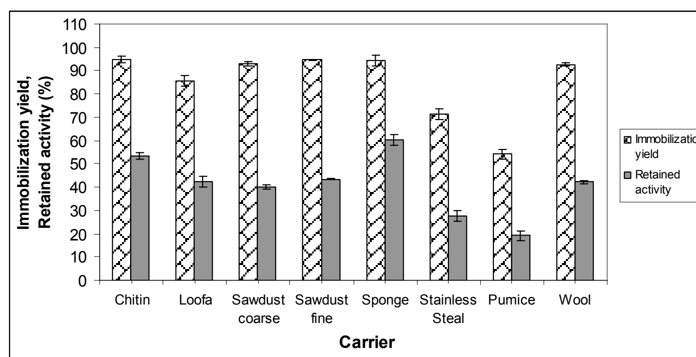


Figure 2: Immobilization of *Aspergillus niger* β-glucosidase by physical binding.

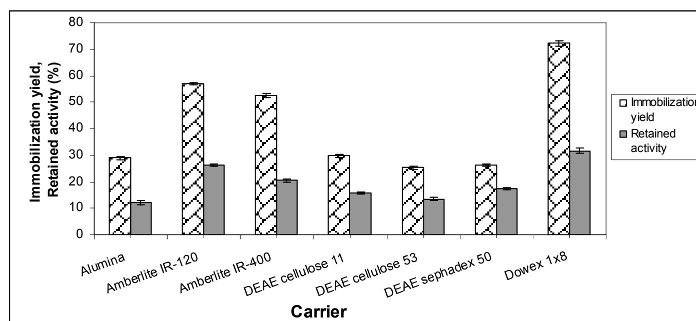


Figure 3: Immobilization of *Aspergillus niger* β-glucosidase by ionic binding.

Immobilization by Entrapment

The results obtained from the enzyme immobilization by entrapment are shown in Figure 4. The highest IY (64.4%) was observed with agar (3%), and the lowest IY (20.4%) with agarose (2%). Ortega *et al.* (1998) entrapped β-glucosidase in alginate and polyacrylamide with retained activity of 66.0%. From the results, the IY and retained activity were affected by gel type and concentration. The pore size of the gel, reflected in the viscosity of the carrier due to the size of the molecule and/or its concentration, can affect the diffusion of substrates or products and limit the reaction rates of the entrapped enzyme (Ortega *et al*., 1998). Within all supports screened, the highest result regarding IY (95.7%) was obtained by covalent binding of β-glucosidase with sponge. Beside, covalent binding is a simple immobilization technique and sponge is a cheap, available, safe and suitable carrier for industrial application. Therefore sponge covalent β-glucosidase was taken as the example of immobilized enzyme to compare its properties with free enzyme.

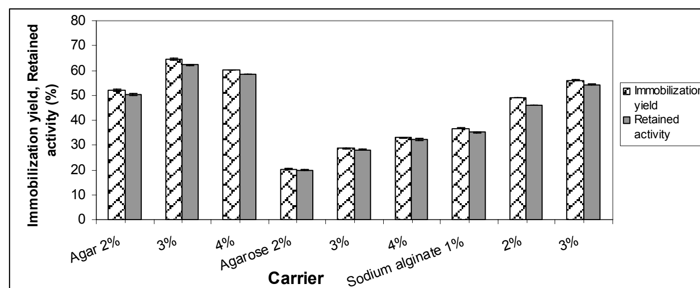


Figure 4: Immobilization of *Aspergillus niger* β-glucosidase by entrapment.

Enzyme Properties

Temperature Profile

The temperature dependence of the enzyme activity was studied in the temperature range of 30-75 ºC using 0.4% cellobiose in 0.05M citrate phosphate buffer (pH 4.8). As shown in Figure 5, the free and immobilized enzyme exhibited a difference of 10 ºC in their optimal temperature. The free enzyme lost 64% from its activity at 70 ºC, while at the same temperature, the immobilized enzyme retained activity of 53%. This result demonstrates the effectiveness of the carriers in protecting the enzyme activity under higher temperature conditions. AbdEl-Ghaffar and Hashem (2010) reported that free cellulose exhibited an optimum temperature of 50 ºC that shifted to 60 ºC for immobilized cellulose on chitosan-L-glutamic acid-GDA (1%). In general, the effects of changes in temperature on the rate of enzyme-catalyzed reactions do not provide much information on the mechanism of catalysis. However, these effects can be important in indicating structural changes in enzymes (Busto *et al*., 1997). The increase in optimum temperature was caused by changing the physical and chemical properties of the enzyme (Peng *et al*., 2005; AbdEl-Ghaffar and Hashem, 2010). The activation energy (E_a) was determined from the slope of linear plots of log enzyme activity vs 1/temperature (in Kelvin), according to the Arrhenius law: Slope = E_a / 2.303R where E_a is activation energy and R is the gas constant (1.976 cal/mol). A change in slope was noted for both free and immobilized enzyme at the same temperature, where a transition probably caused structural changes in the enzyme (Busto *et al*., 1997). From the slope of the straight lines produced in the Arrhenius plots, activation energies of 4.55 and 3.34 kcal/mol were deduced for free and immobilized β-glucosidase, respectively. A lower activation energy for the immobilized enzyme has been reported to be an indication of diffusional limitations (Allenza, 1986; EL-Tanash *et al*., 2011). The activation energy of an enzyme reaction may or may not change as a consequence of the immobilization process. For example, the E_a of immobilized enzyme was almost the same as that of the corresponding native enzyme in the case of lactase immobilized by alkylation with chloro-s-triazinyl cellulose (Sharp *et al*., 1969). Calsavara *et al*. (2001) reported that E_a of cellobiase immobilized by covalent binding on pore silica particles (11 kcal/mol) increased in comparison to that of the native enzyme (5 kcal/mol). On the other hand, a decrease in the E_a from 23.6 to 18.9 kcal/mol was observed for alkaline protease immobilized by physical adsorption on loofa (Ahmed *et al*., 2007).

pH Profile

The effect of immobilization on the initial reaction rate of cellobiose hydrolysis was evaluated within a given range (2.5-8.0). The optimal pH for both immobilized and free forms of the enzyme was 4.0 (data not shown). Roughly similar patterns, where optimum pH is not changed with immobilization, were reported previously (D'Souza and Kubal, 2002; Tu *et al*., 2006; Figueira *et al*., 2011). AbdEl-Ghaffar and Hashem, (2010) found that the maximum activity was at pH 7.0 for free cellulase and cellulase immobilized on chitosan-L-glutamic acid-GDA (1%), pH 8.0 for cellulase immobilized on chitosan-4-aminobutyric acid-GDA (1%). However, compared to the free enzyme, the immobilized enzyme showed changes in pH profiles. The immobilized enzyme is less sensitive to modification of the reaction conditions. Such effects are usually observed when the enzyme is covalently bound to a support or entrapped in a porous matrix. In general, difference in pH profiles of the immobilized enzymes depends on the applied immobilization methods and on the carriers used for the enzyme immobilization (Abdel-Naby, 1999).

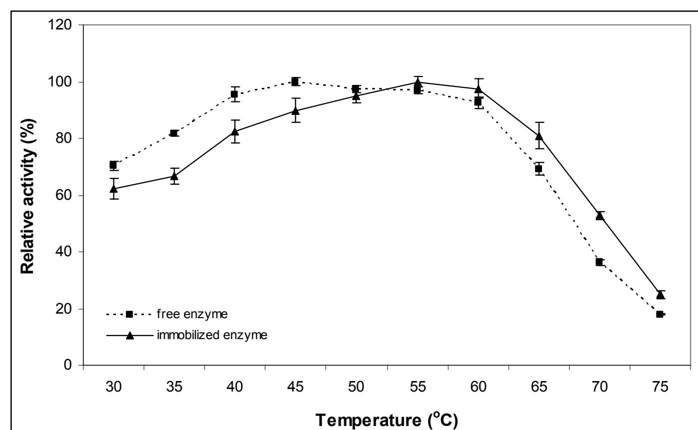


Figure 5: Effect of temperature on the activity of free and immobilized β-glucosidase by covalent binding to sponge.

Thermal Stability

The kinetic stability of the free and immobilized enzyme was compared. The free enzyme lost its activity completely after incubation at 70 ºC for 1 h. The immobilized enzyme was highly stable in comparison to the free form (Table 1). A significant improvement in the stability of β-glucosidase was noted upon immobilization (Tu *et al*., 2006; Itoh *et al*., 2010; Singh *et al*., 2011). At 65 ºC, free enzyme retained 20% of its initial activity after 1h, whereas the immobilized enzyme conserved 42% of its activity in the same conditions. The immobilization support can have a protecting effect from heat when enzyme inactivation occurs (Chang *et al*., 2008; Figueira *et al*., 2011). Figueira *et al*. (2011) reported that immobilization causes an increase in enzyme rigidity, which is commonly reflected by an increase in stability towards denaturation upon raising the temperature (Table 2). The time required for the enzyme to come to 50% of its initial activity is indicated by $t_{½}$, which can be calculated from the slope (K) of a semi-log plot as $t_{½} = 0.693/K$. The half-life ($t_{½}$) for the free enzyme was 55.31 and 49.50 min at 65 and 70 ºC, respectively, whereas the immobilized enzyme at the same temperatures had the $t_{½}$ of 72.95 and 56.80 min. Calsavara *et al*. (2001) reported that $t_{½}$ of free and immobilized cellobiase at 65 ºC were 2.1 and 21.3 h, respectively. This is probably due to the formation of multiple covalent bonds between β-glucosidase and the support that reduce conformational flexibility and thermal vibration, thus preventing the immobilized protein from unfolding and denaturing (Mateo *et al*., 2000; Wang *et al*., 2009; Figueira *et al*., 2011). The deactivation rate constant at 65 ºC for the immobilized β-glucosidase is $9.5x10^{-3}$/ min, which is lower than that of the free form ($12.53x10^{-3}$/ min). A similar result was previously reported for *A. niger* cellobiase with a decrease in deactivation rate constant after immobilization from $36.0x10^{-2}$/ h to $7.9x10^{-2}$/ h (Abdel-Naby, 1999).

Table 1: Thermal stability of free and immobilized β-glucosidase by covalent binding to sponge.

Time (min) / Temperature (ºC)	Relative activity (%)															
	Free enzyme								Immobilized enzyme							
	15	30	45	60	75	90	105	120	15	30	45	60	75	90	105	120
40, 45,50	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
55	100	100	100	100	100	100	98.1	89.3	100	100	100	100	100	100	100	100
60	100	94.8	87.0	77.7	73.0	61.1	50.9	43.3	100	100	95.1	83.3	79.1	63.9	55.2	48.1
65	60.4	53.1	40.7	20.2	11.1	0.0	0.0	0.0	76.3	68.4	54.1	42.0	34.9	19.2	9.6	0.0
70	21.6	15.3	8.1	0.0	0.0	0.0	00	0.0	33.3	26.4	18.5	9.1	0.0	0.0	0.0	0.0

Table 2: Comparison between the thermal properties of free and immobilized β- glucosidase.

Enzyme kinetics	Free enzyme	Immobilized enzyme
Optimum temperature (ºC)	45	55
Activation energy (Ea, kcal/mol)	4.55	3.34
Half-life ($t_{½}$, min)		
60 ºC	200.0	213.6
65 ºC	55.3	72.9
70 ºC	49.5	56.8
Deactivation rate constant (min^{-1})		
60 ºC	1.55×10^{-3}	1.45×10^{-3}
65 ºC	5.60×10^{-3}	4.25×10^{-3}
70 ºC	6.26×10^{-3}	5.46×10^{-3}
K_m (mg)	40.0	46.5
V_{max} (U /mg protein)	416.7	384.6

pH Stability

The pH stability was improved following immobilization (Table 3). As shown from the results, the pH of greatest activity (pH = 4) is in the range of stability for both forms of the enzyme. Immobilized enzyme was stable in pH ranging from 3.5 to 6.5 with no change in activity, while its stability decreases for more alkaline or acidic conditions (retaining 39% and 72% of the initial activity at pH 8.0 and 3.0 after 1h). In contrast, the free enzyme was less stable regarding pH (retained 38% of the initial activity at pH 7.5 after 1 h). Chellapandian (1998) reported the same observation for immobilized alkaline protease on vermiculite, which exhibited a higher stability under acidic and alkaline conditions, compared to the free enzyme. In our study, the activity of immobilized enzyme was unaffected by pH 5.0 after 1 h. However, at the same pH β-glucosidase entrapped in lentikats retained 65% of its activity (Figueira *et al*., 2011).

Effect of Substrate (Cellobiose) Concentration

The effect of cellobiose concentration on the activity of free and immobilized enzyme is highlighted in Figure 6. Lineweaver-Burk plots of data were obtained in experiments where the rates of the reactions catalyzed by native and immobilized β-glucosidase were monitored at varying cellobiose concentrations (figure not shown). The Michaelis constant (K_m) value of the free enzyme was 40.0 mM, while the apparent K_m value for the immobilized enzyme was 46.51 mM. However, the K_m value for the immobilized enzyme was higher than that of the free enzyme by a factor of 1.16. This is in agreement with results reported by other authors, who stated that the apparent K_m was increased when the enzyme was immobilized (Kamboj *et al*., 1996; Ortega *et al*., 1998; Tu *et al*., 2006; Singh *et al*., 2011). In all, the increase in the K_m values of immobilized enzymes may be due to reduced accessibility of the substrate to the active site by enzyme fixation to the support. Comparison of the K_m value for free and immobilized enzymes provides information about interaction between an enzyme and its support. An increase in K_m once an enzyme has immobilized indicates that immobilized enzyme has an apparently lower affinity for its substrate than a free enzyme does, which may be caused by steric hindrance of the active site by the support, the loss of enzyme flexibility necessary for substrate binding or diffusion resistance to solute transport near the beads of the support (Chang *et al*., 2008). On the other hand, the maximum velocity (v_{max}) of immobilized β-glucosidase (384.62 U/mg protein) was 7.69% smaller than that of the free enzyme (416.67 U/mg protein). These results agree with the amount of active enzyme that was immobilized. Chang *et al*. (2008) reported that the v_{max} of immobilized β-glucosidase is smaller than that of free enzyme. This is because immobilization on a carrier treated with glutaraldehyde reduces enzyme activity and may prohibit substrate diffusion to the enzyme. The change in the affinity of enzyme for its substrate is probably caused by structural changes in the enzyme due to the immobilization procedure or by the lower accessibility of substrate to the active site of immobilized enzymes. In contrast of our result, Tu *et al*. (2006) found that v_{max} of immobilized β-glucosidase was increased from 296 to 2430 μmol/mg/min.

Table 3: pH stability of free and immobilized β-glucosidase by covalent binding to sponge.

pH \ Time (min)	Relative activity (%)							
	Free enzyme				Immobilized enzyme			
	15	30	45	60	15	30	45	60
2.5	48.7	48.1	47.8	46.8	65.9	63.0	62.4	59.0
3.0	70.8	70.1	69.9	69.7	77.3	76.5	74.1	72.1
3.5, 4.0, 4.5, 5.0	100	100	100	100	100	100	100	100
5.5	100	100	98.0	96.0	100	100	100	100
6.0	100	100	97.0	93.8	100	100	100	100
6.5	85.9	85.3	80.6	74.0	100	100	100	100
7.0	75.6	60.4	56.7	55.9	100	98.6	91.5	87.7
7.5	45.3	41.3	39.9	37.7	84.8	72.6	69.4	36.7
8.0	36.4	36.0	34.4	33.6	52.0	45.4	40.8	38.7

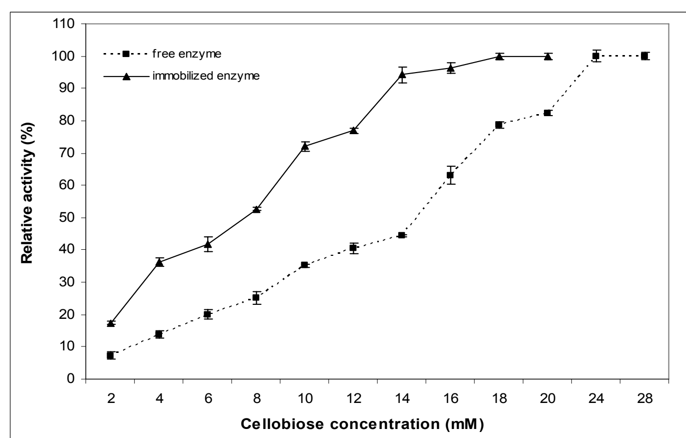


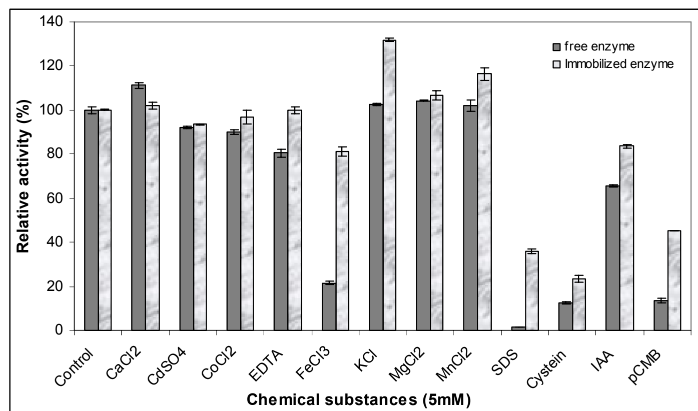
Figure 6: Effect of substrate concentration on the activity of free and immobilized β-glucosidase by covalent binding to sponge.

Effect of Various Chemicals

The effects of chemical reagents on the activity were tested and presented in Figure 7. Of the chemicals investigated, $CaCl_2$, KCl, $MgCl_2$ and $MnCl_2$ stimulated the activity of both free and immobilized enzyme. On the other hand, $CdSO_4$, $CoCl_2$, $FeCl_3$, sodium dodecylsulfate (SDS), cystein, iodoacetamide (IAA) and *p*-chloromercuribenzoate (*p*-CMB) inhibited the activity. Similar observations were obtained by Ohmiya *et al*. (1985), who reported that β-glucosidase from *Ruminococcus albus* showed similar properties against sulfhydryl-reacting reagents (*p*-CMB and IAA). In general, the inhibitory effect for the free enzyme was higher than for the immobilized enzyme. For example, in the presence of *p*-CMB the free enzyme lost 86.29% of its initial activity, which is higher than that lost by immobilized enzyme (54.66%). Moreover, the activity retained in the presence of SDS and EDTA of free enzyme was 1.61 and 80.53%, which is 22.4 and 1.24 times lower than that retained by immobilized enzyme (36.13 and 100%, respectively). The results suggested that the immobilization protected the enzyme against the inhibitory effects of some chemicals. These results are in agreement with those reported by Abdel-Naby (1999), who found that immobilization of enzyme formed stable covalent bonds that led to resistance against chemicals.

Repeated Hydrolysis of Cellobiose by Immobilized Enzyme

Immobilized β-glucosidase was used repeatedly to hydrolyze cellobiose, and reusability examined because of its importance for repeated applications in a batch or a continuous operation. Figure 8 illustrates the effect of repeated use on the activity of β-glucosidase. It can be observed that the immobilized enzyme retained 59.49 and 51.04% from its original activity after 6 and 8 reuses (every run 10 min). This result agreed with that obtained by AbdEl-Ghaffar and Hashem (2010) for cellulase immobilized on chitosan-GDA (1%), which retained 60% of the initial activity. Dinçer and Telefoncu (2007) reported that immobilized cellulase retained 52% of its initial activity after 8 cycles of reuse.



Sodium dodecylsulfate (SDS), Iodoacetamide (IAA), *p*-Chloromercuribenzoate (*p*-CMB) and Ethylenediamine tetraacetic acid (EDTA)

Figure 7: Effect of chemical substances on the activity of free and immobilized β-glucosidase by covalent binding to sponge.

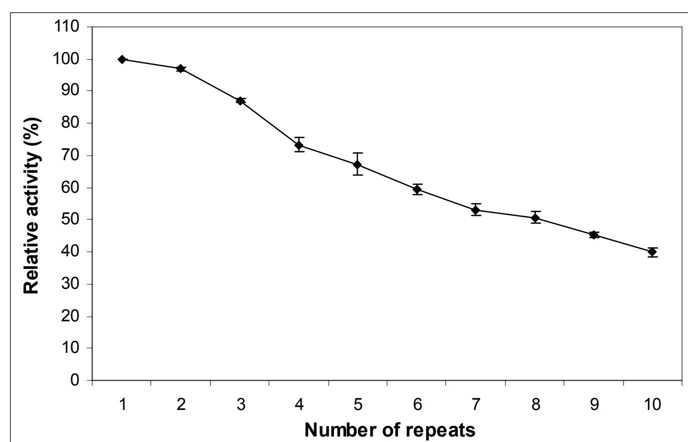


Figure 8: Repeated hydrolysis of cellobiose by sponge immobilized β-glucosidase.

CONCLUSIONS

Of the supports examined, sponge gave the best results. This support is safe, inexpensive, readily available on the market and suitable for industrial application. It is the first report of using sponge for β-glucosidase immobilization. Moreover, it exhibits a considerable protein-binding capacity and a high activity. The immobilized enzyme exhibited 95.67% immobilization yield, with a higher optimum temperature and improved thermal stability and pH stability as compared to the free enzyme. The half-life at 65 ºC showed a 131.89-fold improvement relative to the free enzyme. This study indicates that the immobilization process enhanced the thermal properties of the enzyme, besides the possibility of reusing sponge-immobilized β-glucosidase in industrial applications for 8 cycles with 51.04% retained activity.

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