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Glucoamylase immobilized on montmorillonite: Synthesis, characterization and starch hydrolysis activity in a fixed bed reactor

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Abstract

Glucoamylase from *Aspergillus Niger* was immobilized on montmorillonite clay (K-10) by two procedures, adsorption and covalent binding. The immobilized enzymes were characterized using XRD, surface area measurements and ^{27}Al MAS NMR and the activity of the immobilized enzymes for starch hydrolysis was tested in a fixed bed reactor (FBR). XRD shows that enzyme intercalates into the inter-lamellar space of the clay matrix with a layer expansion up to 2.25 nm. Covalently bound glucoamylase demonstrates a sharp decrease in surface area and pore volume that suggests binding of the enzyme at the pore entrance. NMR studies reveal the involvement of octahedral and tetrahedral Al during immobilization. The performance characteristics in FBR were evaluated. Effectiveness factor (η) for FBR is greater than unity demonstrating that activity of enzyme is more than that of the free enzyme. The Michaelis constant (K_m) for covalently bound glucoamylase was lower than that for free enzyme, i.e., the affinity for substrate improves upon immobilization. This shows that diffusional effects are completely eliminated in the FBR. Both immobilized systems showed almost 100% initial activity after 96 h of continuous operation. Covalent binding demonstrated better operational stability.

Keywords: Glucoamylase; Immobilization; Montmorillonite; Adsorption; Covalent binding; Fixed bed reactor; Michaelis constant

1. Introduction

Immobilized enzymes have been used in food technology, biotechnology, medicine and also analytical chemistry. They provide various advantages over free enzymes including easy separation of the reactants and products from reaction media, easy recovery of the enzyme and repeated or continuous use [1]. Glucoamylase (α-1,4-D-glucan glucohydrolase, EC 3.2.1.3) an exo enzyme acts on the 1,4-glucosidic linkages from the non-reducing ends of amylose, amylopectin and glycogen in a consecutive manner liberating D-glucose. It also hydrolyses α-1,6- and α-1,3-glucosidic linkages but at a much slower rate compared to its action on α-1,4-linkages. Glucoamylase is an industrially important enzyme and is used for large-scale saccharification of malto-oligosaccharides into glucose and various syrups required in the food and beverages industry [2]. Studies on immobilization of glucoamylase are in rapid progress and many supports have been utilized. These include ceramic membranes [3], polymer microspheres [4,5], magnetic supports [6], etc. Inorganic supports for enzyme immobilization are of great interest because of their durability and high mechanical strength for usage in packed or fluidized bed reactors and relatively low cost. In addition, immobilization by adsorption is economically feasible and attractive.

In the present study, we have immobilized glucoamylase on montmorillonite clay via two methods, i.e.,

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adsorption and covalent binding. Montmorillonite, which is a 2:1 dioctahedral smectite, has been successfully used as a support as well as a catalyst for the past few decades [7]. The swelling ability of these naturally occurring phyllosilicate minerals provide unusual properties and appreciable surface area for adsorption of organic molecules [8]. A large number of modifications on clay that provide a variety of textural and catalytic properties are possible which include acid treatment, cation exchange, pillaring with robust metal ions, intercalation of polymeric organic moieties, etc. Depending on the type of modification, the properties of clay can be tuned in such a way as to suit specific applications. Here we have employed montmorillonite K-10, which is the acid activated form. Since clays are aluminosilicates, they possess acidic sites that are capable of interaction with amino groups of enzymes leading to ionic binding. This linkage is much stronger than mere physical binding and hence the enzyme will be retained on the support for a larger duration. The immobilized glucoamylase was characterized by XRD, surface area measurements and NMR spectroscopy and the activity for starch hydrolysis was tested in a packed bed reactor. K_m and V_{max} were calculated from the Hanes–Woolf plot. The effectiveness factor η was also determined from a study of kinetics of the reaction. Operational stability in continuous mode was tested for 100 h.

2. Experimental

2.1. Materials used

Glucoamylase from *Aspergillus Niger*, Montmorillonite K-10, 3-amino propyl triethoxy silane, glutaraldehyde and bovine serum albumin were purchased from Sigma Aldrich Chemicals Pvt. Ltd., Bangalore. All other chemicals were of high purity available commercially.

2.2. Immobilization of glucoamylase

In case of adsorption, montmorillonite K-10 was first mixed with deionized water and vigorously stirred for 6 h. It was filtered, dried at 120 °C for 12 h and calcined at 350 °C for 12 h. This calcined clay was mixed with equal volumes of 0.1 M buffer solution and enzyme solution of required concentration and shaken for 1 h in a thermostated water bath shaker at room temperature. It was then centrifuged in a cooling centrifuge for 1 h. The centrifugate was tested for presence of protein by developing colour using Folin Phenol Ciocaltaue's reagent [9] and measuring the absorbance at 640 nm in a Shimadzu 160A UV–Vis spectrophotometer. A standard calibration curve was plotted using bovine serum albumin and the amount of un-adsorbed protein was calculated. The residue was washed several times with deionized water; each time the amount of protein in solution was measured. The immobilized enzyme was stored in 0.1 M buffer at 5 °C for further use. In order to covalently bind the enzyme, calcined montmorillonite K-10 was functionalized by mixing with a 10% (v/v) solution of 3-amino propyl triethoxy silane in acetone and vigorously stirred for 3 h at room temperature [10]. It was filtered, washed several times with acetone until the washings became colourless and later dried at 80 °C for 12 h. This silanized clay was treated with 10% aqueous solution (v/v) of glutaraldehyde and stirred vigorously for 3 h. It was filtered, washed free of excess glutaraldehyde and dried at 60 °C for 12 h. This functionalized clay was used for immobilization as per the procedure described above. The covalently bound enzyme was stored in 0.1 M buffer at 5 °C for further use.

2.3. Characterization

A *Micromeritics* model Gemini 2360 surface area analyzer was used to measure the nitrogen adsorption isotherms of the samples at liquid nitrogen temperature. The specific surface area and pore volume were determined from the BET plot ($p/p_0 = 0.05$–0.95). Prior to the measurement, the samples were degassed at room temperature for 12–16 h in nitrogen flow. Powder XRD of the immobilized enzyme systems and the support were taken on a *Rigaku* D/Max-C system with Ni filtered Cu Kα radiation ($\lambda = 1.5406$ Å) within the 2θ range 2–15° at a scanning rate of 0.5°/min at room temperature. Solid-state ^{27}Al MAS-NMR experiments were carried out over a *Bruker* DSX-300 spectrometer at a resonance frequency of 78.19 MHz for ^{27}Al. For all experiments a standard 4 mm double-bearing Bruker MAS probe was used. The sample spinning frequency was 8 kHz with a single pulse excitation corresponding to $\pi/2$ flip angle. The pulse length for the experiments was 10 μs whereas the pulse delay was 2 s. The spectra were externally referenced with respect to a dilute solution of $AlCl_3$. XWINNMR software operating in a UNIX environment on a silicon graphics computer was employed to acquire and retrieve data.

2.4. Catalytic activity measurements

A silica glass tube of 1.2 cm id and 25 cm length was used as the reactor. Provision for water circulation was also made available. The immobilized enzyme (0.5 g) was packed into a bed at the middle of the reactor, which was filled with glass beads. The substrate was fed from the top of the reactor using a *Cole Palmer 74900 series* syringe pump and the products were collected at the bottom. The reactor was operated at a space velocity of 3.26 h^{-1}. After the reaction time, an aliquot (1 mL) of the product was removed from the

reaction mixture and analyzed colorimetrically. Colour was developed using iodine solution and the absorbance read at 610 nm. The results were compared with absorbance of standard starch solution and the amount of starch converted was calculated. All the results are presented in a normalized form with the activity under optimum conditions given a value of 100. One unit of enzyme is defined as the amount of enzyme required to hydrolyze 1 mg starch per minute. The kinetic parameters (Michaelis constant K_m and maximum rate V_{max}) were calculated by measuring the rates of reaction at various substrate concentrations. The values were substituted into the Hanes–Woolf equation to obtain K_m and V_{max}. The effectiveness factor η was measured by obtaining the rates at a substrate concentration high enough to eliminate inhibition effects (5% starch solution w/v). Operational stability was tested by packing 1 g of the immobilized enzyme into the reactor and the substrate was introduced at a particular flow rate so as to get a space velocity of 2 h^{-1}. The reactor was operated continuously for 100 h. The activity is represented as percentage of initial activity retained.

3. Results and discussion

3.1. Surface area measurement

Montmorillonite functionalized with 3-APTES and glutaraldehyde shows a decrease in surface area and pore volume (Table 1). This may be because the silane and glutaraldehyde molecules are bound to the clay matrix within the inter lamellar space. Direct adsorption of glucoamylase reduces the surface area, but the drop is not so drastic and surface area decreases with increasing amounts of enzyme. It is the case with pore volume also. The covalently bound glucoamylase demonstrates a very sharp decrease in surface area from 145 to 25 $m^2 g^{-1}$ even for a low enzyme loading of 10 mg g^{-1} clay. A similar observation is seen with pore volume which reduces drastically. Covalent binding occurs via the glutaraldehyde spacer group which is present within the inter layer space. Hence linkage with enzyme leads to complete blockage of pores and so the surface area and pore volume drop sharply. Since glucoamylase is of very large size, a small enzyme concentration is enough to bring about complete pore blockage for the covalently bound systems.

Table 1
Surface area and pore volume data for immobilized glucoamylase

Catalyst	Langmuir surface area ($m^2 g^{-1}$)	BET surface area ($m^2 g^{-1}$)	Pore volume ($\times 10^{-6} m^3 g^{-1}$)
M	352	201	0.2511
SGM	266	145	0.1745
A_{10}	247	156	0.2196
A_{50}	215	132	0.2050
A_{100}	180	105	0.1919
C_{10}	53	25	0.0743
C_{50}	40	19	0.0582
C_{100}	30	15	0.0432

M, montmorillonite K-10; SGM, montmorillonite functionalized with silane and glutaraldehyde; A, adsorbed glucoamylase; C, covalently bound glucoamylase. Values in subscript depict enzyme concentrations in mg g^{-1} clay.

3.2. X-ray diffraction

The XRD patterns for immobilized glucoamylase are shown in Fig. 1. For parent montmorillonite, there is a peak at $2\theta = 8.9°$ corresponding to a d spacing of 0.998 nm. This peak represents the d_{001} plane analogous to the inter-layer spacing. After functionalization with 3-APTES and glutaraldehyde, this peak shifts partly to lower 2θ with a d spacing of 1.531 nm demonstrating an expansion of layers thereby indicating that the functionalization takes place within the clay layers. The intensity of the new peak is very high which shows that most of the clay layers are intercalated with silane and glutaraldehyde molecules. In the case of adsorbed glucoamylase, the peak of native montmorillonite is retained while a low intensity peak appears at lower 2θ region. This also suggests that intercalation occurs

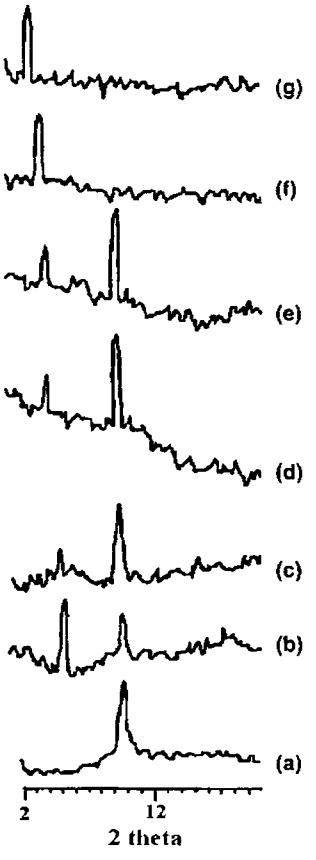

Fig. 1. XRD patterns for (a) montmorillonite (b) functionalized montmorillonite, adsorbed glucoamylase with (c) 10 (d) 50 and (e) 100 mg enzyme, covalently bound glucoamylase with (f) 10 and (g) 50 mg enzyme.

but only in a very few of the layers. As enzyme loading increases, the intensity of the new peak also increases confirming the intercalation of enzyme into the clay layers. The d spacing increases to the 1.6–1.85 nm ranges. Covalent binding with glucoamylase also leads to enzyme intercalation. The d spacing increases in the range 2.1–2.3 nm. Complete shifting of the d_{001} peak to lower values suggests that intercalation of enzyme has taken place entirely. Enzymes are highly polymeric species of very high molecular size hence possibility of attachment within the inter lamellar space can be ruled out. But a shift in the d_{001} peak to lower values is an evidence for intercalation [11–15]. Therefore it is proposed that the whole enzyme does not get intercalated into the clay layers. It is the side chains of various amino acid groups that are responsible for intercalation. The polypeptide backbone does not enter the interlayer space but is situated at the periphery of the clay [16–18]. For glucoamylase, an enzyme loading of 10 mg was enough to bring about complete intercalation. After enzyme adsorption, the d spacing shows an increase of 0.6–0.8 nm compared to the native montmorillonite while after covalent binding also the d spacing difference with that of functionalized montmorillonite is 0.6–0.8 nm. This can be regarded as the expansion caused due to the enzyme. During adsorption and covalent binding, side chains of similar length are responsible for intercalation. The difference between the two modes of immobilization lies in the extent of intercalation alone. The enzyme is intercalated only in the initial layers of montmorillonite all through adsorption whereas intercalation occurs throughout the entire clay matrix during covalent binding.

3.3. ^{27}Al NMR studies

^{27}Al NMR shows two resonances around 0 and 70 ppm representing Al in tetrahedral and octahedral co-ordination, respectively [19–21]. Montmorillonite exhibits octahedral Al resonance at 2.8 ppm and tetrahedral resonance at 69.8 ppm (Fig. 2). A slight change in octahedral chemical shift from standard value is due to the presence of Fe^{3+}. Functionalization with silane and glutaraldehyde results in a shift of octahedral peak to −1.7 ppm while the tetrahedral peak remains unaltered indicating that only the octahedral Al atoms are involved in binding with silane and glutaraldehyde. This further infers that the binding takes place within the clay inter layer space and hence substantiates the results of XRD and surface area measurements. Enzyme adsorption leads to a shift of tetrahedral Al peak by 6–8 units keeping the octahedral peak almost constant. Thus during adsorption the enzyme interacts with the tetrahedral Al alone. When the enzyme is covalently bound to the clay matrix, the tetrahedral Al resonance is unaltered while the octahedral peaks show a shift by 3–6 ppm.

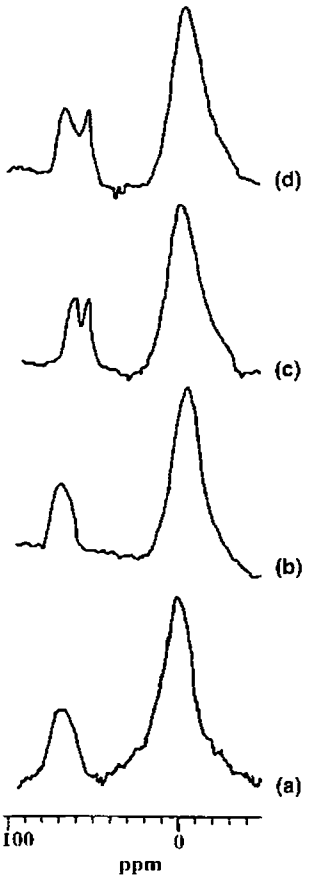

Fig. 2. ^{27}Al NMR spectra of (a) montmorillonite, (b) functionalized montmorillonite (c) adsorbed and (d) covalently bound glucoamylase.

Covalent binding takes place on the glutaraldehyde spacer and not directly on to the Al species. In spite of this, there is a sufficient shifting of octahedral peak that signifies secondary interactions between the enzyme and the octahedral Al layers. The side chains of the amino acid residues or other functional groups present in these side chains may involve in electrostatic interactions with octahedral Al species changing its chemical environment thereby causing a shift in NMR signal. The small shoulder peaks appearing may be considered to arise due to noise. An additional tetrahedral resonance is seen at 54 ppm, which is present for the adsorbed as well as the covalently bound enzyme. Glucoamylase contains the cystine residue that has the reactive –SH group in its side chain. This reactive group can interact with tetrahedral Al bringing about such a large chemical shift. The reason for a split in tetrahedral Al resonance may be that only a few of the Al are involved in interaction with cystine side chain and so they resonate at a different value than the remaining Al atoms.

3.4. Starch hydrolysis activity in a packed bed reactor

The adsorbed and covalently bound glucoamylase retained 95% and 100% activity of the free enzyme when

operated in a packed bed reactor. The kinetic parameters calculated using Hanes–Woolf plot (Fig. 3) are represented in Table 2. Hanes–Woolf equation is a modified form of the Michaelis–Menten equation according to which,

$$\frac{S}{V} = \frac{S}{V_{max}} + \frac{K_m}{V_{max}},$$

where S is the substrate concentration, V is the rate of the reaction, V_{max} is the maximum rate and K_m is the Michaelis constant. This plot was used because the distribution of errors is uniform in this case [22] when compared to the Lineweaver–Burk or Eadie–Hoftsee plots. The K_m and V_{max} values are very close to that of free enzyme. Therefore the immobilized glucoamylase does not show any loss in affinity for substrate making it clear that the native conformation of the enzyme is retained almost completely. Covalently bound glucoamylase exhibits a lower K_m and a higher V_{max} than the free enzyme indicating an improved activity upon immobilization. This is a very rare observation with inorganic supports. Only very few researchers have obtained a lower K_m after immobilization [23,24] but they have used polymeric supports. The effectiveness factor η is the ratio of rates of the free and immobilized enzymes given as,

$$\eta = \frac{\text{Rate (immobilized enzyme)}}{\text{Rate (free enzyme)}}.$$

Effectiveness factor provides information on the role of diffusion in the reaction. A value of $\eta = 1$ is obtained under conditions of complete diffusion, i.e., in case of homogeneous reaction with the free enzyme. Immobilization results in the use of enzymes in heterogeneous mode and therefore diffusional effects operate whose extent is given by measuring η. The kinetics of enzyme bound on a porous particle can be affected by external or internal diffusional resistances, which respectively correspond to the transport of substrate and products from the bulk solution to the outer surface of the enzyme particle, and the internal transport of these species inside the porous system [25]. In our case, internal diffusional resistances can be avoided since the enzyme is too large to enter the pores of montmorillonite. Consequently the only prevailing effect is external diffusional resistance. The effectiveness factor values are very close

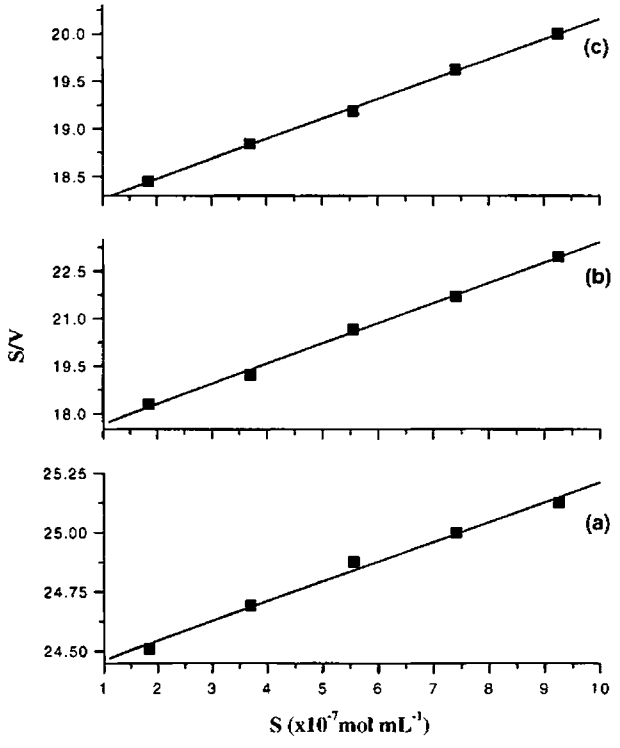

Fig. 3. Hanes–Woolf plot for (a) free (b) adsorbed and (c) covalently bound glucoamylase.

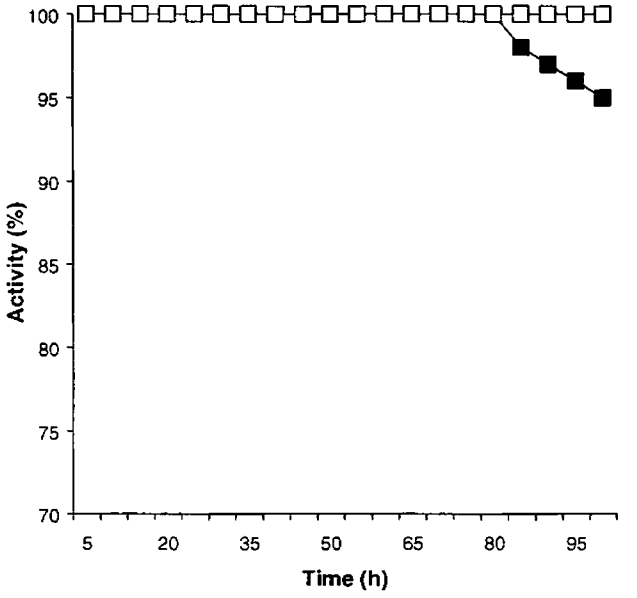

Fig. 4. Variation of activity with time during the continuous operation of (■) adsorbed and (□) covalently bound glucoamylase. {Reaction conditions: Starch concentration 5% (w/v), temperature 30 °C, space velocity 2 h^{-1}, flow rate 5 mL h^{-1}.}

Table 2
Kinetic constants for free, adsorbed and covalently bound glucoamylase

Mode of reaction	Catalyst	Michaelis constant K_m (g L^{-1})	Maximum rate V_{max} ($\times 10^{-5}$ mol mL $^{-1}$ min^{-1})	Effectiveness factor η
Batch	Free	1.58	1.20	-
Packed bed	Adsorbed	1.70	1.12	0.95
	Covalently bound	1.48	1.24	1.03

to unity suggesting that diffusional effects (external mass transfer restrictions) do not operate in this case, which is also a remarkable observation. In the continuous operation, the covalently bound glucoamylase could be used without any loss in activity for 100 h while the adsorbed form lost 5% activity after 84 h (Fig. 4). The immobilized glucoamylase demonstrated greater stability for a continuous run. Thus immobilization improves stability of the enzyme and makes it more suitable for long-term operation. Arica et al. [4] employed glucoamylase covalently bound to pHEMA/EGDMA microspheres in a packed bed reactor for 120 h and demonstrated that the immobilized enzyme lost only 9% activity at the end of the run. Glucoamylase immobilized on ceramic membranes [3] and PEI-coated cotton [26] also showed appreciable operational stability up to 120 h. Our immobilized preparations, especially the covalently bound one, also exhibit immense potential for the continuous production of glucose. The loss in activity may be due to the natural inactivation of enzyme on account of time dependent denaturation of the enzyme protein [27–29].

4. Conclusions

Glucoamylase was successfully immobilized on montmorillonite K-10 via two independent procedures namely adsorption and covalent binding. Surface area and pore volume decreased upon immobilization indicating pore blockage. This was more pronounced for covalently bound enzyme. XRD showed intercalation of the enzyme into the clay layers. The entire enzyme is not intercalated; the side chains of amino acid residues alone are responsible for intercalation. ^{27}Al NMR highlights that adsorption involved the tetrahedral Al alone while covalent binding was concerned with octahedral Al only. An additional tetrahedral resonance observed was entirely due to interaction with cystine residue that contains the reactive –SH group. NMR studies also throw light on the fact that during covalent binding the enzyme gets intercalated in the clay layers while during adsorption the extent of intercalation is very less. The packed bed reactor was best suited for application of immobilized glucoamylase in starch hydrolysis. K_m and V_{max} values suggest that substrate affinity and activity are improved on account of immobilization, which is a rare observation with inorganic supports and there is no involvement of diffusional resistances to mass transfer. The immobilized glucoamylase demonstrated high operational stability especially the covalently bound form which could be used continuously for about 100 h.

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