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Enhanced activity and stability of α-amylase immobilized on alumina

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Abstract

α-amylase was immobilized on alumina via adsorption. The support and the immobilized enzymes were characterized using XRD, IR spectra and N_2 adsorption studies. The efficiency of immobilized enzymes for starch hydrolysis was tested in a batch reactor. The effect of two different calcination temperatures on properties of the support as well as on immobilization was studied. From XRD, IR and N_2 adsorption studies it was confirmed that the enzyme was getting adsorbed only on the external surface of the support. pH, buffer concentration and substrate concentration had a significant influence on the activity of immobilized enzyme. K_m for immobilized α-amylase was found to be higher than the free enzyme, which may be due to interparticle diffusional mass transfer restrictions. The immobilized enzymes showed enhanced pH stability than the free enzyme.

Keywords: α-Amylase; Immobilization; Immobilized enzymes; Alumina; Adsorption; Starch hydrolysis

1. Introduction

Biotechnology is currently considered as a useful alternative to conventional process technology in industrial and analytical fields. The demand for industrial enzymes is ever increasing owing to their applications in a wide variety of processes. Enzymatic reaction can be favored over chemical catalysis under such circumstances where thermal degradation of labile compounds is minimized and use of chemicals with a potential for pollution can be avoided [1]. However, the common drawbacks associated with the enzymatic processes are the inhibition of enzyme at high concentration of substrate or product [2] and instability of enzyme under the reaction condition [3] as well as in repetitive usage [4]. Immobilized enzymes are preferred over the native ones owing to their multiple and repetitive use. In addition, the reaction product is not contaminated with the enzyme (especially useful in the food and pharmaceutical industries). Furthermore, the immobilized enzyme has a longer half-life and predictable decay rate. Amylases see a great deal of use in food and fermentation industries. The hydrolysis of starch to low-molecular weight products using α-amylase is widely applied in the food, paper, textile, distillery, and brewing industries. Hydrolysis of starch can be achieved using either acid or enzyme catalysts [5]. α-Amylase (EC.3.2.1.1;1,4-D-glucan glucanohydrolase) hydrolyses α-1,4 glucosidic bonds in amylase, amylopectin and glycogen in an endo fashion but the α-1, 6 glucosidic linkages in the branched polymers are not hydrolysed [6]. Enzymes are immobilized by physical adsorption, ionic binding, covalent binding, cross-linking and entrapment methods [7]. In the past few decades, many immobilization methods and carrier materials have been investigated [8,9]. There have been many reports about immobilization of α-amylase used for the hydrolysis of starch and production of maltose. Organic polymeric carriers are the most widely studied materials because of the presence of rich functional groups, which provide essential interactions with the enzymes. However, the organic supports suffer a number of problems such as poor stability towards microbial attacks and organic solvents and dis-

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posal issues. In contrast, inorganic materials such as silica gels, alumina, and layered double hydroxides are known to be thermally and mechanically stable, non-toxic, and highly resistant against microbial attacks and organic solvents [10]. Inorganic supports provide better stability characteristics. Porous silica is found to be good medium for immobilization [11]. Various enzymes have been immobilized on clays [12,13]. α-Amylase has been immobilized to collagen [14], Kaolin [15] and sand. Bacterial amylase has been covalently bound to silica carriers using glutaraldehyde or titanium chloride [16]. Solid acid supports can be used to immobilize enzymes since the acidic sites can act as centres of immobilization via the amino groups of enzymes. Reports on immobilization of enzymes to metal oxides are limited.

In the present study, the hydrolysis of starch to low molecular weight carbohydrates is carried out using α-amylase immobilized on alumina via adsorption. The procedures involved in adsorption are quite simple, making it one of the most widely used methods of enzyme immobilization. This property of reversibility of binding has often been used for the economic recovery of the support. The support and the immobilized enzymes were characterized using XRD, IR spectra and N_2 adsorption studies. The activity for starch hydrolysis was studied in a batch reactor. The effect of different calcination temperatures on the properties of support before and after immobilization was studied. The effect of pH on activity and stability of free and immobilized enzymes were studied. The effect of buffer concentrations on immobilization was also studied. The kinetics of the reaction was determined at various substrate concentrations and the kinetic parameters (K_m and V_{max}) were calculated from the Hanes–Woolf plot.

2. Experimental

2.1. Materials

α-Amylase from *Bacillus subtilis* was procured from Sigma–Aldrich Chemicals Pvt. Ltd., Bangalore. Aluminium nitrate was purchased from BDH Chemicals, Mumbai and ammonium hydroxide was from Qualigens Fine Chemicals, Mumbai. Starch was obtained from SRL Chemicals, Mumbai. All other chemicals were of highest purity commercially available.

2.2. Preparation of the catalyst

Sufficient amount of ammonium hydroxide (1:1 v/v in deionized water) was slowly added to 0.1 M aluminium nitrate under vigorous stirring at a temperature of 70 °C and the pH was adjusted to 10. The precipitate was kept for stirring at this temperature for 1 h and stirring continued for 24 h at room temperature. The precipitate was then aged for 24 h and washed with distilled water until free of nitrate, filtered, dried in an air oven at 120 °C for 12 h and calcined at 500 and 700 °C, respectively, for 12 h.

2.3. Immobilization of α-amylase on oxide carriers

For adsorption, 1 g of alumina powder was mixed with equal volumes of 0.1 M phosphate buffer and α-amylase solution. It was shaken in a water bath shaker at required temperature for one and a half hour and then filtered. The filtrate was tested for enzyme protein using the spectrophotometric method of Lowry et al. [17] using Folin–Ciocaltaue's phenol reagent and measuring the absorption at 640 nm in a Shimadzu 160 A UV–Vis spectrophotometer [18].

Notation of the catalysts.

A represents alumina, AA represent α-amylase adsorbed on alumina, the numbers 1 and 2 represent calcination temperatures of 500 and 700 °C, respectively.

2.4. Characterization of immobilized enzymes

Powder XRD of the immobilized enzyme and the supports were taken on a Rigaku D max-C system with Ni filtered Cu K_α radiation (λ – 1.5406 Å) within the 2θ range 2–80° at a speed of 2° min^{-1}. A Micromeritics Gemini 2360 surface area analyser was used to measure the nitrogen adsorption isotherms of the samples at liquid nitrogen temperature. The specific surface area was determined. Prior to the measurement the samples were degassed at room temperature for 12–16 h in nitrogen flow. The IR spectrum of the samples was obtained using a Nicolet Model Magna IR 560 spectrophotometer using KBr disc method. Changes in the absorption bands were investigated in the 500–4000 cm^{-1} region. The resolution and acquisition applied were 4 cm^{-1} and 50 scans, respectively.

2.5. Free and immobilized enzyme activity for starch hydrolysis

The activities of the free and immobilized enzymes were tested in a batch reactor. Immobilized enzyme 0.1 g (1 ml enzyme solution) was mixed with buffered 5% starch solution and shaken in a water bath shaker. One milliliter of the product was mixed with 5 ml iodine solution and the absorbance was read at 610 nm. One unit of enzyme activity is defined as the amount required to hydrolyse 1 mg starch per minute under the assay conditions. All results are presented in a normalized form with the activity under optimum conditions being assigned a value of 100%. Influence of pH on activity was determined by carrying out the reaction at varying pH in the range (4–8) and keeping all other reaction conditions constant. The pH stability was determined by pre-incubating the catalyst and buffer for different time intervals between 15 min and 20 h at 30 °C followed by the reaction, as described earlier. The influence of buffer concentration was determined by carrying out the reaction in buffer of concentration range

0.1–0.01 M. The Michaelis–Menten kinetics was established from a study of effect of substrate concentration (1–5%) and the Hanes–Woolf plot was employed to calculate K_m and V_{max}.

3. Results and discussion

3.1. Characterization studies

The XRD pattern of A-1 (Fig. 1) shows two peaks at $2\theta = 32°$ for the single phase material together with two peaks at 46° and 67° which are the characteristic peaks corresponding to the (220), (400) and (440) reflections of γ-Al_2O_3 phase [19]. In the case of A-2, the same distinct peaks as that of A-1 were present but with higher intensity and an additional peak appeared at $2\theta = 37°$ which is one of the characteristic peak of γ-alumina. The planes corresponding to the gamma phase are more prominent in A-2. After adsorption of α-amylase in the case of AA-1, the peak corresponding to 100% intensity $2\theta = 67.6°$ shifts to 66.8°. In the case of AA-2, the peak at $2\theta = 32.7°$ shifts to 31.3°. Adsorption of α-amylase leads to a decrease in the intensity of all characteristic peaks, which may be due to the strain developed as a result of interaction with enzyme. From the above results it is seen that α-amylase is adsorbed only on the external surface of the support. The spectrum appears much broader after adsorption, which indicates that the enzyme is interacting with the support. None of the peaks disappear after adsorption of α-amylase since the concentration of the enzyme taken is only 10 mg/g of the support.

The surface area of the metal oxides and that of the immobilized systems are shown in Table 1. It is seen that the surface area decreased after immobilization, which shows that the enzyme is getting adsorbed at the external surface of the support.

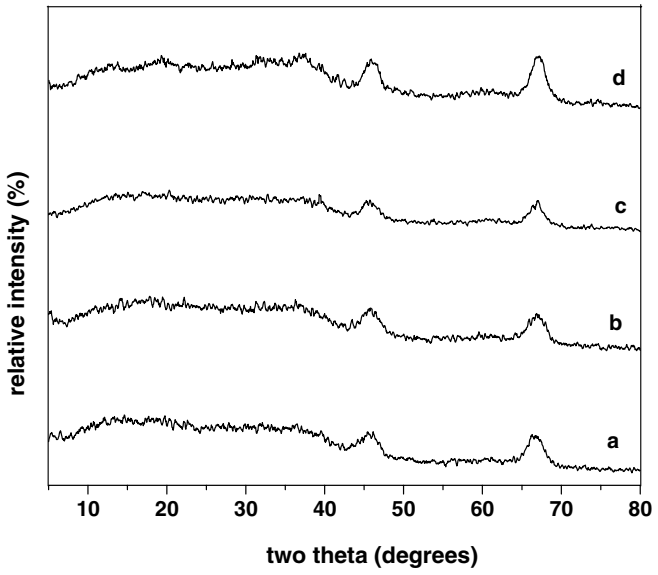

Fig. 1. XRD patterns of support and immobilized α-amylase: (a) A-1 (b) AA-1 (c) A-2 and (d) AA-2 with enzyme concentration 10 mg/g alumina.

Table 1
Surface area and pore volumes of alumina and the adsorbed systems

Catalyst	Surface area (m^2/g)	Pore volume ($\times 10^{-6}$ m^3/g)
A-1	241	0.1274
AA-1	209	0.1244
A-2	193	0.1114
AA-2	174	0.0915

The IR spectra of A-1 and A-2 (Fig. 2) show a broad band at 3440 and 1630 cm^{-1} which are assigned to the bending and stretching vibration of the OH bond in absorbed and coordinated water. The band at 1381 cm^{-1} belongs to the absorption of non-bridging OH groups [20]. There were two more additional bands at 2925 and 2846 cm^{-1} attributed to the OH stretching vibration. The bands at 579 and 612 cm^{-1} are due to the absorption of Al–O–OH [21]. A-1 and A-2 showed two broad bands at 590 and 720 cm^{-1} [22] suggesting the transformation of

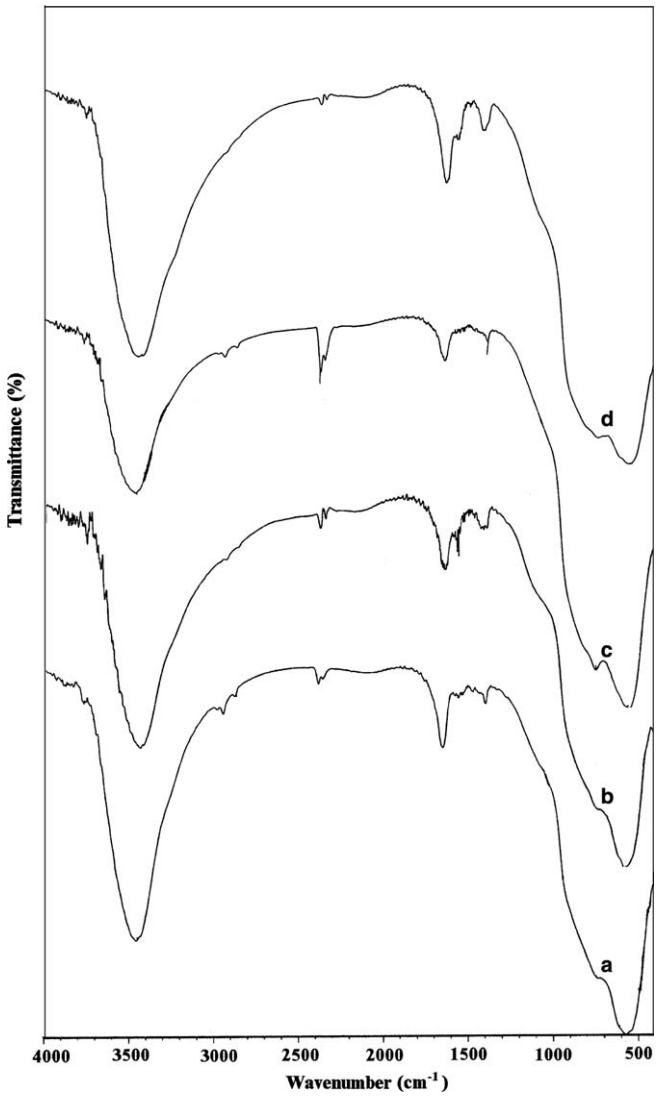

Fig. 2. IR spectrum of alumina and immobilized α-amylase: (a) A-1 (b) AA-1 (c) A-2 and (d) AA-2 with enzyme concentration 10 mg/g alumina.

AlO (OH) to gamma alumina. All the bands present in A-1 are also found in the case of A-2 except the band at 612 cm^{-1}, which disappears probably due to the decrease in the number of OH groups at higher calcination temperatures. After adsorption of α-amylase the peak position in AA-1 do not undergo any shift, but the band at 1381, 612 and in 2900 cm^{-1} disappear. The disappearance of this particular band associated with the absorption of non-bridging OH groups, AlOOH and OH stretching proves that the enzyme might be linked through the OH groups. In the case of AA-2, the band at 1374 cm^{-1} disappears. In both the cases the band at 720 cm^{-1} shifts to lower wave numbers after adsorption. The peaks in the range at 3400 cm^{-1} are broadened further confirming that the enzyme is getting adsorbed on the surface of the support via the OH groups. In addition, peaks due to the presence of characteristic groups in the enzyme structure appear in the spectrum. The peak at 1553 cm^{-1} is due to the NH bending modes and the bands at 1401 cm^{-1} is due to the CH_2 group (scissoring) attached to the amide linkage.

3.2. Influence of pH on enzyme activity

Free enzyme exhibits maximum activity in the pH range 5–7. AA-1 and AA-2 exhibit high activity in the pH range 6–8. Fig. 3a shows a dependence of enzyme activity on pH. Maximum activity is shown at pH 6 for free and immobilized systems. Hamzehi and Pflug [23] showed that sorption of polysaccharides (i.e., cellulases, α-amylases) was highest at their isoelectric point when the enzymes had no net charge. They suggested that sorption takes place through Lifschitz van der Waal's type forces. Although individual van der Waal's forces are regarded as weak, retention may be cumulative if the enzyme is in close contact with the adsorbing surface [24]. After immobilization the optimal pH for starch hydrolysis did not change. pH value at 8 inhibits enzyme activity for two reasons: a lower loading and a possible change of the enzyme conformation due to an unfavorable charge distribution of the amino acid residues that produce a further activity decrease. Therefore, pH 6 was chosen for further studies. A change in pH will affect the intramolecular hydrogen bonding thus leading to a distorted conformation that will reduce the activity of the enzyme. The adsorbed systems show good activity in the higher pH range. The pH profile of the immobilized enzyme was broader than that of the free enzyme, which means that this method preserves the enzyme activity in a wider pH range. Similar observations, for immobilization of α-amylase and other enzymes have been reported by other researchers [25,26]. This may also be due to diffusional limitations or secondary interactions between the enzyme and the support [27]. This indicates that sensitivity to pH is reduced as a result of immobilization [28,29]. The variation of activity with pH, within a range of 2–3 units each side of the pI (isoelectric point), is normally a reversible process. The conformation of the enzyme will be more favorable in the higher pH range so that good activity is

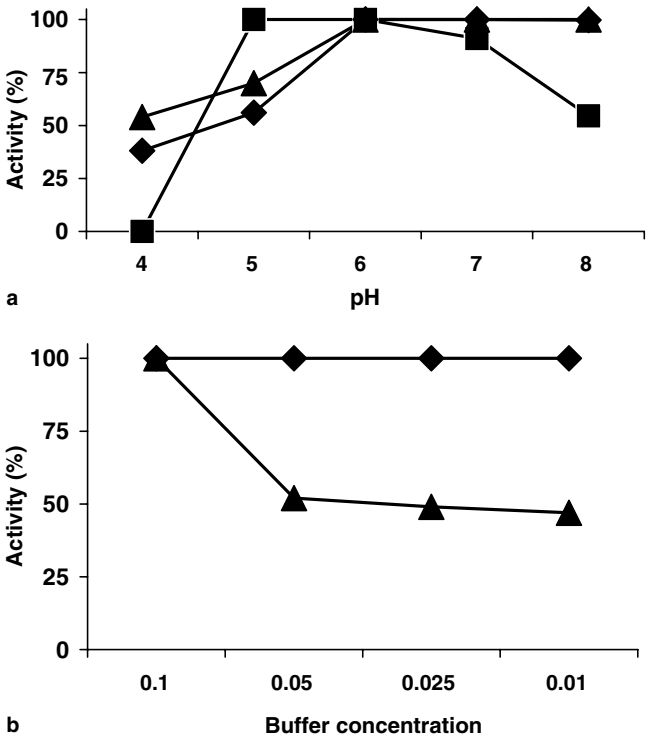

Fig. 3. (a) Influence of pH on activity of (■) free α-amylase (▲) AA-1 (◆) AA-2. Reaction conditions: starch concentration 5%, α-amylase concentration 10 mg/g alumina, immobilized enzyme 0.1 g, buffer concentration 0.1 M, reactant volume 20 ml, temperature 30 °C, time 30 min. (b) Effect of buffer concentration on activity of immobilized α-amylase (▲) AA-1 (◆) AA-2. Reaction conditions: starch concentration 5%, α-amylase concentration 10 mg/g alumina, immobilized enzyme 0.1 g, pH 6, reactant volume 20 ml, temperature 30 °C, time 30 min.

obtained. The enzyme is inactivated at lower pH values. Extremes of pH will, however, cause a time and temperature-dependant, essentially irreversible denaturation. In alkaline solution (pH > 8), there may be partial destruction of amino acid residues due to base catalysed β elimination reactions whereas in acid solutions (pH < 4), hydrolysis of the labile peptide bonds, (sometimes found next to aspartic residues) may occur. As the enzymes showed high initial activity at more than one pH values, stability measurements had to be performed in order to ascertain the optimum value.

3.3. Effect of buffer concentration on the activity of immobilized α-amylase

To determine the buffer concentration upon activity, the adsorbed systems were studied at optimum pH and concentration range 0.01–0.1 M using 5% starch solution. The effect of buffer concentration upon activity of the immobilized systems is presented in Fig. 3b. The ionic strength of the solution is an important parameter affecting enzyme activity. Thus both the binding of charged substrates to enzymes and the movement of charged groups within the catalytic 'active' site will be influenced by the ionic composition of the medium. In the case of AA-1, the conversion

decreases initially and then remains constant. In the case of AA-2, there is 100% activity in all the concentration range studied. At higher buffer concentration, the ionic strength of the solution is high and, therefore, it shields the charge effects on the support, thereby diminishing pH differences between the carrier and bulk [30]. As the buffer concentration is lowered, the ionic strength decreases reducing the shielding effect and hence the difference in pH between the carrier and the bulk increases lowering the activity. In the case of oxides calcined at 500 °C ,there is greater number of OH groups and the surface charge developed influence the pH difference between the microenvironment and bulk and hence decrease in activity is observed. At higher calcination temperature the number of OH groups decreases and hence the charge effects on the support may be reduced so that the increase or decrease in ionic strength has no influence on the pH in the bulk and the microenvironment thereby increasing activity.

3.4. pH stability

Free enzyme shows maximum stability at pH 6 (Fig. 4a). Hence, the optimum pH of free α-amylase was taken as 6. AA-1 and AA-2 also exhibited increased stability at pH 6 even though at pH 7–8 it exhibited 100% initial activity. In the active centre protonation is a reversible process, while changes in the charge of structure supporting residues may cause irreparable damage to the native conformation. As time passes by higher pH causes changes in the protonation at the active centre of the enzyme, which is an irreversible process and hence may cause irreversible damage of the native structure. Reversible process may not show any changes in the activity, the pH stability curve exhibits a plateau in this region, dropping at both ends as a result of irreversible inactivation, making the curve wider than the pH optimum curve [31]. At pH 6 (Fig. 4a) and 7 (Fig. 4b) there is reversible denaturation of the enzyme while at pH 8 (Fig. 4c) there is irreversible denaturation of the enzyme. Hence, the optimum pH for free and adsorbed α-amylase on alumina was taken as 6. Though the pH optimum remains the same for AA-1 and AA-2, the maximum stability is shown in the case of AA-2. This may be due to the increase in crystallite size and more distinct phase separation at higher calcination temperature, which is confirmed from the XRD studies.

3.5. Kinetic parameters

The K_m value for free enzyme was 2.51×10^{-4} mol/ml whereas the V_{max} value was 1.02×10^{-4} mol/ml/min (Table 2). The kinetic parameters of the adsorbed α-amylase were determined in a batch reactor. The K_m values for AA-1 and AA-2 were approximately 1.8 and 2.8-fold higher than that of the free enzyme. In general, K_m of an immobilized enzyme is different from that of the free enzyme due to diffusional limitations, steric effects and ionic strength [32].

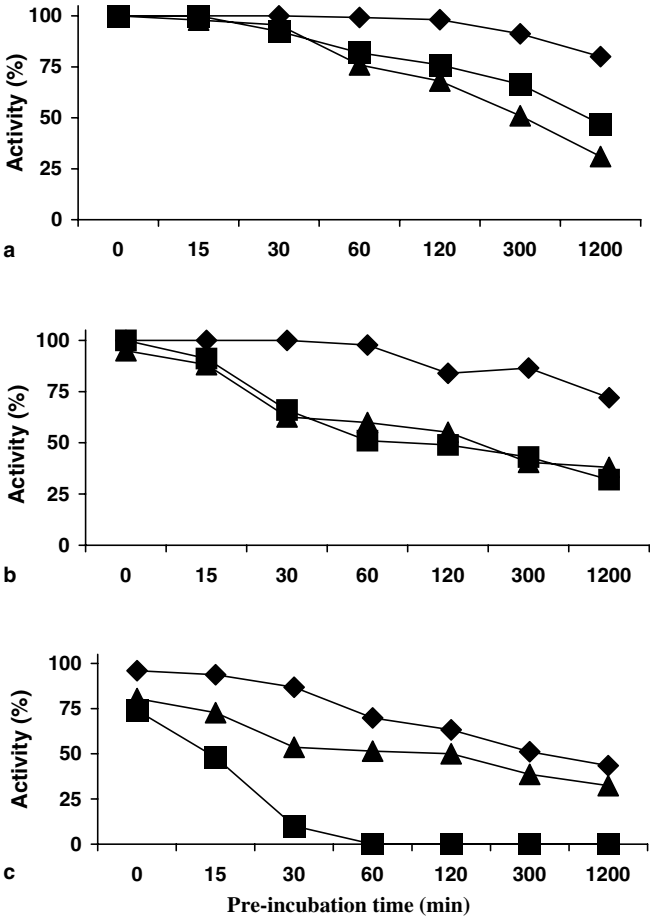

Fig. 4. Change in activity with respect to pre-incubation time for (■) free α-amylase (▲) AA-1 (◆) AA-2 at pH (a) 6 (b) 7 (c) 8. Reaction conditions: starch concentration 5%, α-amylase concentration 10 mg/g alumina, immobilized enzyme 0.1 g, buffer concentration 0.1 M, reactant volume 20 ml, temperature 30 °C, reaction time 30 min.

Table 2
Kinetic parameters for free and immobilized α-amylase

Catalyst	Michaels constant K_m ($\times 10^{-4}$ mol/ml)	V_{max} ($\times 10^{-4}$ mol/ml/min)
Free enzyme	2.51	1.02
AA-1	4.67	0.99
AA-2	7.09	0.83

The change in the affinity for its substrate is also caused by structural changes in the enzyme introduced by the immobilization procedure and lower accessibility of the substrates to the active site of the immobilized enzyme [33]. Similar results were obtained for kinetic parameters in the case of free, P(HEMA) and P(St-HEMA) bound enzymes, respectively [18]. Bayramoglu et al. [34] have reported significantly larger values of K_m (2.3 times that of free enzyme) for α-amylase immobilized on poly (HEMA-GMA-1-3) membranes. V_{max} values for the immobilized systems were lower than that of the free enzyme indicating a lowering of activity of enzyme on account of immobilization. The difference in the K_m values in our case

was not as apparent as that in other reports. This small increase in K_m may be the result of conformational changes and/or diffusional limitations to mass transfer.

4. Conclusions

α-Amylase was immobilized on alumina via adsorption. From XRD and N_2 adsorption studies it was confirmed that the enzyme is getting adsorbed only on the external surface of the support. In XRD, there was a decrease in the intensities of the peaks and a slight broadening after adsorption of α-amylase on AA-1 and AA-2. The surface area also showed a decrease after adsorption. IR studies reveal that the enzyme is adsorbed via the OH groups, as there is disappearance of the bands in the OH region. Additional bands due to the characteristic groups in the enzyme appear in the spectrum. Free enzyme and immobilized systems showed maximum activity at pH 6. The oxides calcined at higher temperatures showed 100% activity in the buffer concentration studied while in the case of AA-1 the conversion decreases initially and then remains constant. Compared to free enzyme the immobilized enzymes exhibited much more stability in the higher pH range. AA-2 exhibited higher stability than AA-1, which may be due the increase in crystallite size and the separation of phases being more distinct at higher calcination temperatures. K_m values calculated was higher than the free enzyme, which may be due to the diffusional resistances or conformational changes in the enzyme resulting from immobilization. V_{max} values for the immobilized systems were lower than that of the free enzyme indicating a lowering of activity of enzyme on account of immobilization.

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References

[1] R.A. Sheldon, Chirotechnology: Industrial Synthesis of Optically Active Compounds, Marcel Dekker, New York, 1993.
[2] M. Markweghanke, S. Lang, F. Wagner, Enzyme Microb. Technol. 17 (1995) 512.
[3] V.V. Mozhaev, Y.L. Khmelnitsky, M.V. Sergeeva, A.B. Belova, N.L. Klyachko, A.V. Levashov, K. Martinek, Eur. J. Biochem. 184 (1989) 597.
[4] L. Giorno, E. Molinari, E. Drioli, B.P. Cesti, J. Chem. Technol. Biotechnol. 64 (1995) 345.
[5] F.W. Schenck, R.E. Hebeda, Starch Hydrolysis Products: Worldwide Technology, Production and Applications, VCH, New York, 1992.
[6] A.H. Rose, Microbial Enzymes and Bioconversions, Academic Press, New York, 1980.
[7] I. Chibata, Immobilized Enzymes, Halsted Press, New York, 1978.
[8] L. Cong, R. Kaul, U. Dissing, B. Mattiasson, J. Biotechnol. 42 (1995) 75.
[9] W. Tischer, V. Kashe, Biotechnology 17 (1999) 326.
[10] W. Tischer, F. Wedekind, Top. Curr. Chem. 200 (1999) 95.
[11] L. Cao, U.T. Bornscheuer, R.D. Schmid, J. Mol. Catal. B: Enzyme 6 (1999) 279.
[12] G. Sanjay, S. Sugunan, Catal. Commun. 6 (2005) 1.
[13] G. Sanjay, S. Sugunan, Catal. Commun. 6 (2005) 525.
[14] C.A. Groom, J.M. Meising, B.N. White, Appl. Microbiol. Biotech. 28 (1988) 8.
[15] I. Karabe, S. Mitsuda, S. Suzuki, Environ. J. Microbiol. Biotechnol. 7 (1979) 343.
[16] G.I. Kvesitadze, M.S.H. Dvali, Biotechnol. Bioeng. 14 (1982) 1765.
[17] O.H. Lowry, N.J. Rosebrough, A.L. Faar, R.J.J. Randall, J. Biol. Chem. 193 (1951) 265.
[18] H. Tumturk, S. Aksoy, N. Hasirci, Food Chem. 68 (2000) 259.
[19] J.M. Dominguez, J.I. Hernandez, G. Sandoval, Appl. Catal. A: Gen. 197 (2000) 119–130.
[20] J.S. Church, N.W. Kant, D. Trimm, Appl. Catal. 101 (1993) 105.
[21] Q. Wei, D. Whang, S. Zhang, J Chin. Ceram. Soc. 29 (2001) 392.
[22] R. Viswanath, S. Ramasamy, J. Mater. Sci. 34 (1999) 2879.
[23] E. Hamzehi, W. Pflug, Z. Pflanzenernaehr. Bodenkd. 144 (1981) 505.
[24] R.G. Burns, in: P.M. Huang, M. Schnitzer (Eds.), Interaction of Soil Minerals with Natural Organics and Microbes, SSSA, Madison, WI, 1986, SSSA Special Publication Number 17.
[25] D. Tanyolac, B.I. Yuruksoy, A.R. Ozdural, Biochem. Eng. J. 2 (1998) 179.
[26] D. He, Y. Cai, L. Nie, S. Yao, Biochem. Eng. J. 6 (2000) 7.
[27] M.Y. Arica, V. Hasirrci, J. Chem. Technol. Biotechnol. 58 (1993) 287.
[28] S.P. O'neill, P. Dunnill, M.D. Lilly, Biotechnol. Bioeng. 13 (1971) 337.
[29] I.J. Ida, T. Matsuyama, H. Yamamoto, Biochem. Eng. J. 5 (2000) 179.
[30] Z. Hui, K. Wei, C. Xiao, L. Wei, S. Jiacong, J. Chem. Technol. Biotechnol. 54 (1992) 509.
[31] K.F. Tipton, H.B. Dixon, Method. Enzymol. 63 (1979) 183.
[32] G.P. Lopez, B.D. Ratner, R.J. Rapoca, T.A. Horbett, J. Macromol. Sci. 26 (1993) 3247.
[33] M.Y. Arica, S. Senel, N.G. Alaeddinoglu, S. Patir, A. Denizli, J. Appl. Polym. Sci. 75 (2000) 1685.
[34] G. Bayramoglu, M. Yilmaz, M.Y. Arica, Food Chem. 84 (2004) 591.