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ORIGINAL CONTRIBUTION

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Immobilization of diastase onto acid-treated bentonite clay surfaces

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Abstract The immobilization of diastase onto acid-treated bentonite surface has been investigated and the relative activity of immobilized enzyme has been examined under varying experimental conditions. The effect of various factors such as concentration of enzyme solution, pH and temperature of immobilization medium, presence of salts and organic solvents has been observed on both the extent of immobilized enzyme and its relative activity. Various thermodynamic parameters were also evaluated.

Keywords Diastase · Immobilization · Dynamics · Acid-treated · Bentonite

Introduction

There has been tremendous interest in the adsorption of enzymes from aqueous solutions due to their practical applications [1, 2, 3]. For preparing bioreactor, immobilization of enzymes by physical interaction is considered to be the easiest way. The major objectives of immobilization are the economic application of enzyme systems in various industrial and technological processes [1]. Immobilized enzymes are currently the subject of considerable research because of their advantages over soluble enzymes especially in industrial preparations. Because the recovery yield and the reusability of free enzymes as industrial catalysts are quite limited, attention has been paid to enzyme immobilization on solid supports [2, 3], which offers advantages over free enzymes in the possibility of running enzymatic reactions continuously, rapid termination of reactions, controlled product formation, ease of enzyme removal from the reaction mixture (thus saving time of a purification step for removing enzyme from the product system), adaptability to various engineering designs, prolonged activity, and obtainability of a concerted or sequential reaction of several enzymes by the use of mixed or stratified beds [4, 5, 6, 7].

The properties of immobilized enzyme preparations are governed by the properties of both the enzyme and the carrier material. The interaction between the two provides an immobilized enzyme with specific chemical, biochemical, mechanical, and kinetic properties, Table 1.

There are several reasons to use immobilized enzymes. In addition to the convenient handling of enzyme preparations, the two main targeted benefits are: (i) easy separation of enzyme from the product, and (ii) reuse of the enzyme. Easy separation of the enzyme from the product simplifies enzyme applications and permits reliable and efficient reaction technology. Enzyme reuse provides a number of cost advantages, which are often an essential prerequisite for establishing an economically viable enzyme-catalysed process.

Furthermore, the interest in the immobilized enzymes and their application to bioprocessing [8, 9], analytical systems [10], and enzyme therapy [11] has steadily grown in the past decade. Thus, many approaches to the preparation of water-soluble enzymes have been explored [12, 13, 14, 15] to study the enzyme reaction in biphasic systems similar to those existing *in vivo*. Various methods exist for immobilization of enzymes [16, 17] and these may be divided into physical methods based on molecular interactions between the enzyme and carrier, and chemical methods based on formation of covalent bonds.

Although polymeric systems have largely been employed as carriers of immobilized enzymes [18, 19, 20],

Table 1 Immobilization parameters

Parameter	Value
(i) Adsorption coefficient (K)	0.65 mg^{-1} ml
(ii) Rate constant of adsorption (k_1)	4×10^{-2} min^{-1}
(iii) Rate constant of desorption (k_2)	6.15×10^{-2} min^{-1} mg^{-1} ml
(iv) Standard Gibb's free energy change ($\Delta G°$)	1.62 KJ/mol
(v) Standard enthalpy change ($\Delta H°$)	13.38 KJ/mol
(vi) Standard entropy change ($\Delta S°$)	0.039 KJ/mol

however, a great deal of work has also been done on the enzyme-soil interaction which is also an important natural phenomenon in terrestrial and ecosystems [20]. Bacteria and fungi involved in the biodegradation of the organic matter found in these natural environments secrete extracellular enzymes which interact with the soil surfaces.

Quiquampoix et al [21] adopted enzyme activity and cation exchange as tools for the study of the conformation of proteins adsorbed on mineral surfaces, such as montmorillonite. The authors found that both electrostatic and the hydrophobic interactions played significant role in ascertaining immobilization of proteins (*A. niger* β-D-glucosidase, BSA and β-D-glucosidase) onto the mineral surfaces.

Thus, the adsorption of proteins and enzymes onto mineral surfaces is a subject of great significance and, therefore, deserves more attention. Realizing the need for a careful investigation on enzyme-mineral interaction, the proposed study involves to undertake the immobilization of diastase onto bentonite mineral surfaces.

Experimental

Materials

Diastase (specific activity, 1300 U/mg) was supplied in powdered form by Research Lab, Mumbai (India) and used as received. Bentonite was supplied by Loba Chemie, India and used after pretreatments as described later. Other chemicals used were of AR grade and throughout the experiments only doubly distilled water was used.

Method

Preparation of acid-treated bentonite

The bentonite powder was immersed in 0.2 N HCl for 72 h, washed several times with distilled water, then dried at room temperature for a week. The total surface area, average particle diameter, mesh size, and cation exchange capacity were found to be 400 m^2 g^{-1}, 40 μm, 200–300, and 90 meq/100 g, respectively.

Method of immobilization

Among various methods available for immobilization of enzymes onto solid carriers the adsorption is the most economic and simple process to perform and, therefore, has been widely employed for immobilization purposes. There are four procedures that have been used for the immobilization of enzymes by adsorption, namely the static process, the dynamic batch process, the reactor loading process, and the electro deposition process.

The dynamic batch process is most frequently employed for laboratory preparations of immobilized enzymes and in the present study too the method has been used for immobilizing diastase onto acid-treated bentonite surfaces. A typical immobilization procedure is as follows: 200 mg of acid-treated bentonite was suspended in 25 ml of 0.1% diastase solution at constant pH 6.9 at definite temperature. The suspension was mildly agitated for 90 min. which was found to be an adequate time for equilibrium adsorption as determined by preliminary experiments. A complete care was taken to ensure that the shaking was not so vigorous that the carrier will be abraded and disrupted. After shaking, the suspension was centrifuged at 6000 ×*g* at room temperature for 20 min. and the supernatant was analyzed for remaining diastase by a spectrophotometric method [22] as described below:

To an aliquot of enzyme solution were added 2 ml of 1% starch solution, and 1 ml each of 5% KI and 0.036% KIO_3 solution. A time period of 5 min. was allowed for iodine to react with the starch and the mixture diluted to 100 ml and read at 620 nm (Systronics, No.102, India).

The amount of diastase adsorbed was calculated with the well known mass-balance equation as given below,

$$\text{Adsorbed enzyme} = \frac{(C_o - C_e)\cdot V}{w} \tag{1}$$

where C_o and C_e are the initial and equilibrium concentrations of enzyme solution (mg per ml), V is the volume of the enzyme-acid-treated bentonite suspension, and w the weight of the adsorbent (g).

Measurements of relative activity

The catalytic activity of diastase was measured using three procedures [23]. Procedure A is a measurement of the enzyme activity without treated bentonite. Procedure B measures the enzyme activity in the presence of the treated bentonite and Procedure C measures the activity of the supernatant after centrifugation and represents the contribution of non-adsorbed enzyme to the over-all activity measured by procedure B. From these values the relative activity of the enzyme, i.e., the ratio of the activity due to the fraction immobilized and that of an equal quantity of enzyme in solution, was calculated as

$$R = \frac{(B - C)}{(A - C)} \times 100 \tag{2}$$

The substrate used was starch and the catalytic activity at 20 °C was measured according to the above procedure.

Results and discussion

Immobilization of diastase

Although operationally the adsorption technique is simple, the forces involved are the most complex. It is the surface activity of support acting in concert with a functional moiety or characteristic group on the surface of the enzyme protein that is responsible for the bonding or immobilization of the enzyme. The bonds that exist between the enzyme protein and the carrier depend on the nature of the carrier and the nature of the enzyme surface. These bonds may be ionic, hydrogen, covalent,

hydrophobic, etc. An enzyme may be immobilized by bonding to either external or internal surface of the carrier.

Before discussing the results on the immobilization of diastase, it is essential to look into the way the enzyme molecule binds to the bentonite surface. As montmorillonite forms the major constituent of the clay, we may consider its crystal structure to explain the mode of the diastase adsorption. As far as the crystal structure of montmorillonite is concerned (Fig. 1) it possesses a basal surface with a negative charge that arises from isomorphous charge substitution in the crystal lattice that is compensated by exchangeable cations from the external medium. Likewise, the edge face has a positive charge when the pH is less than 7, because Al-OH groups undergo protonation. Also, the edge face has a negative charge when the pH is >7. It is to note here that the acid treatment of bentonite has been done, the aluminols of the edge face undergo the following protonation equilibria:

$$AlOH + H^+ \rightleftharpoons AlOH_2^+$$

Thus, the protonated aluminols of the edge face offer active sites for the adsorption of diastase which are other than the negatively charged basal plane.

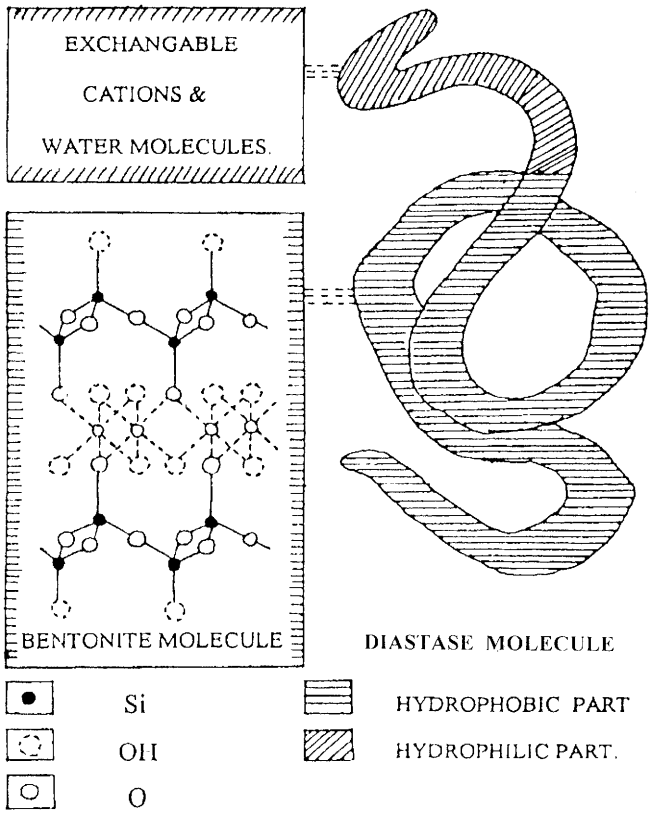

Fig. 1 A model depicting the immobilization of diastase onto acid-treated bentomite surface

Like other proteins, the hydrophilic residues in the diastase are generally found on the surface of the molecule whereas the hydrophobic residues are buried inside, protected from contact with the water. Thus, the structural features suggest for the following type of interaction: (i) Hydrophobic interaction between enzyme molecule and siloxane layer, and (ii) electrostatic interactions between negatively charged amino acid residues of the enzyme and positive charged aluminols of the edge face.

Effect of concentration of enzyme solution

When the concentrations of diastase is increased in the range 0.05 to 0.5% (w/v), it is found that immobilized diastase also increases and, after the diastase solution acquires a concentration of 0.5%, it becomes saturated. The reason for the observed behavior is that with increasing concentration of diastase solution, a large number of diastase molecules approach the interface and consequently the immobilization will increase.

For a quantitative understanding of the interaction between diastase and the treated bentonite surfaces, Langmuir isotherm can be considered. Basically, the Langmuir equation was derived for the sorption of gases on a solid surface. Nevertheless, it has been applied to include the sorption of solutes on soils. A standard mathematical representation is

$$\frac{C_e}{a} = \frac{1}{a_s K} + \frac{C_e}{a_s} \tag{3}$$

where C_e is the equilibrium concentration of the diastase solution, a is the immobilized diastase (mg g^{-1}) at any equilibrium concentration C_e, a_s is the immobilized diastase (mg g^{-1}) at saturation and $K = k_1/k_2$, k_1 and k_2 are the rate constants for adsorption and desorption respectively.

In the present study, the Langmuir isotherm is shown in Fig. 2, which clearly implies that the adsorption isotherm belongs to L2 type, i.e. the Langmuir type of isotherm, which is a widely reported isotherm in most adsorption investigations [24].

Effect of surface concentration on activity

The effect of the initial concentration of diastase on the saturated surface concentration of immobilized diastase was studied by increasing the initial concentration of diastase in the range 0.05 to 0.5% and recording the relative activity of the immobilized enzymes. The results are depicted in Fig. 3 which reveal that the amount of immobilized diastase is greatly influenced by the initial enzyme concentration. It is clearly seen that the relative

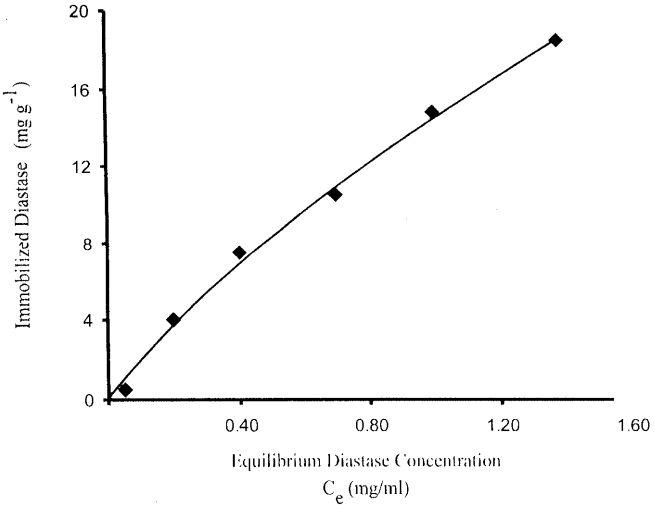

Fig. 2 A plot showing the variation of the adsorbed amount of enzyme (mg g^{-1}) with the concentration of enzyme with 0.2 g acid-treated bentonite at pH 6.9 and 25 ± 0.2 °C

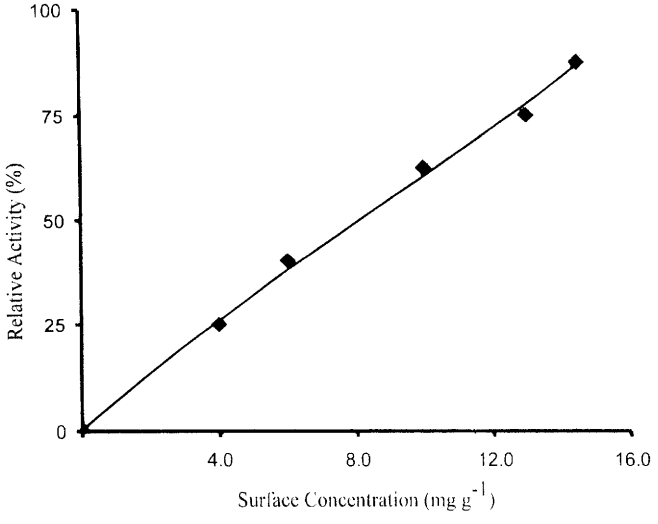

Fig. 3 Effect of surface concentration of diastase on its relative activtiy when immobilized onto acid-treated bentonite (0.2 g) at pH 6.9, and 25 ± 0.2 °C

activity (RA) of immobilized diastase increases with increasing surface concentration of diastase. The observed higher activity at higher surface concentration of diastase can be explained in terms of structural deformation of the immobilized diastase as shown in Fig. 4. It is clear from the figure that at lower surface concentration, the enzymes may not undergo strong deformation in structural conformation as the enzyme molecules will be strongly bound to the bentonite surface. Thus, the diastase will show a lesser relative activity. On the other hand, at higher surface concentration of enzyme the molecules may not be tightly bound to the mineral

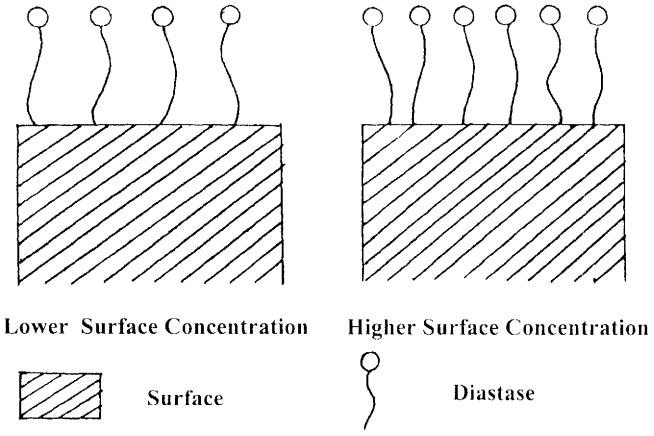

Fig. 4 A model depicting smaller activity at lower surface concentration, and greater activity at higher surface concentration

surface and, therefore, will not undergo greater structural deformations exhibiting enhanced relative activity. Similar type of results have been reported by other workers also [25].

Kinetics of immobilization

The kinetic course of the immobilization process was followed by monitoring the progress of the process at different time intervals. For this purpose several identical sets were run simultaneously and the amounts of immobilized diastase were estimated at different time intervals. The overall progress of the immobilization process is shown in Fig. 5a which clearly implies that the immobilization rate is almost constant up to 20 minutes and then gradually increases and attains saturation at 90 minutes.

The Fig. 5b also reveals that the activity of immobilized enzyme also increases with time as the increased amount of immobilized diastase will result in a greater relative activity of the enzyme.

Electrolyte effect

In all the immobilization systems where a macro ion contacts an electrically charged surface, the immobilization is greatly affected by the presence of external electrolytes. In aqueous solution, charged sorbent surfaces and enzyme macro ions are surrounded by counter ions and in such systems immobilization of enzyme molecules to the charged solid will involve a redistribution of charge in the interfacial region.

In the present study, the electrolyte effect has been observed by adding uni, bi, and trivalent anions to the external solution in the concentration range 0.02 M to

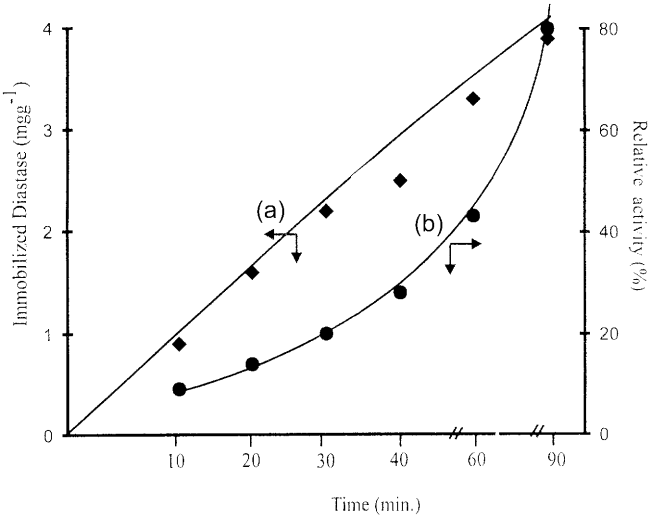

Fig. 5 Variation in the amount of immobilized diastase with time at [Diastase] = 0.5% w/v, acid-treated bentonite = 0.2 g, pH = 6.9, temp. = 25 ± 0.2 °C

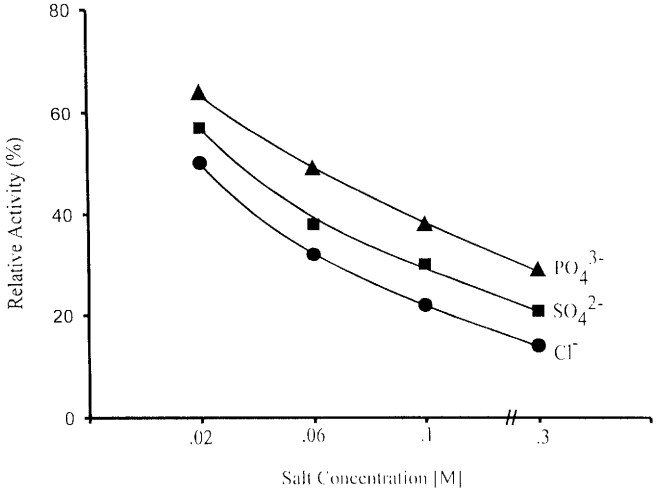

Fig. 6 Effect of addition of anions of varying valencies on the relative activity of immobilized diastase at [Diastase] = 0.5% w/v, acid-treated bentonite = 0.2 g, pH = 6.9, temp. = 25 ± 0.2 °C

0.3 M. The results (not shown) reveal that in the concentration range 0.02 M to 0.3 M of the added salts, the immobilization decreases and the order of effectiveness of the added anions follow the sequence,

$$Cl^- > SO_4^{2-} > PO_4^{3-}$$

The observed decrease in amounts of immobilized enzyme may be explained by the fact that added anions diffuse into the pores of the bentonite and cause repulsive forces to operate between the bentonite surface and approaching enzyme molecules, which, in turn, suppress the adsorbed enzymes. Since the chloride ions are the smallest among the three, they diffuse rapidly into the adsorbent surface and produce greater repulsion forces and consequently result in the least immobilization.

It is also revealed by Fig. 6 that the relative activity of immobilized activity is suppressed by the addition of electrolyte and the activity decreases with increasing concentration of added salts. Moreover, the order of suppression of activity is as follows:

$$PO_4^{3-} < SO_4^{2-} < Cl^-$$

The results can be explained on the basis of the changes in conformation of the diastase molecule upon addition of electrolytes. As Cl^- ions are the smallest ions, they diffuse into the pores of the bentonite and cause change in conformation of the immobilized enzyme molecules which as a consequence, result in a suppressed activity. It is also obvious that greater the concentration of added electrolyte, larger would be the conformational charges and, therefore, greater would be the fall in the relative activity.

Solvent effect

The effect of addition of water-miscible alcohols on the immobilization of diastase was investigated by adding various alcohols (10% v/v) to the immobilization system. The results are shown in Fig. 7 which clearly reveals that the immobilized diastase constantly decreases with an increasing number of carbon atoms in the aliphatic chain of the alcohols. Thus, the order of increasing depression in the amount of immobilized diastase was:

$$MeOH < EtOH < iso - PrOH < n - BuOH$$

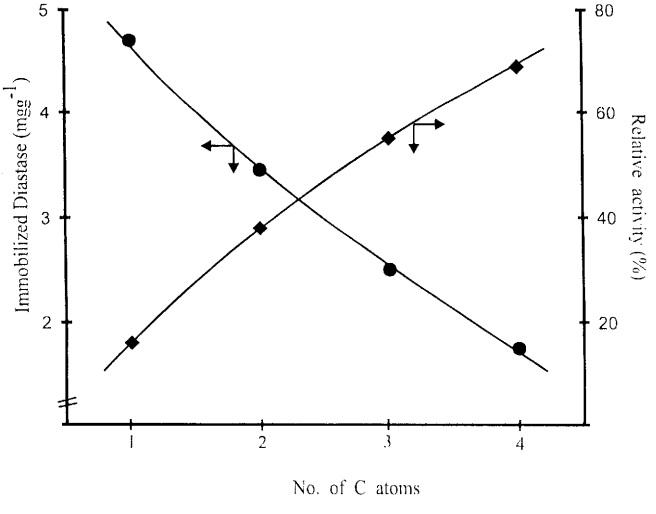

Fig. 7 Effect of addition of aliphatic alcohols on the amounts of immobilized diastase and their relative activity at [Diastase] = 0.5% w/v, acid-treated bentonite = 0.2 g, pH = 6.9, temp. = 25 ± 0.2 °C

It is evident from the previous discussions that hydrophobic interactions are mainly responsible for the immobilization of diastase molecules. Upon addition of aliphatic alcohols to the suspensions, the hydrophobic portion of aliphatic chains interacts with the hydrophobic siloxane layer of the clay surface and may be immobilized. Thus, due to a decrease in number of active sites on the hydrophobic region of the treated bentonite clay surface, the immobilization of diastase molecules will decrease. Moreover, since the hydrophobic character of added alcohols increases with increasing number of carbon atoms, this accounts for the order of effectiveness of added alcohol.

Temperature effect

For investigating the effect of temperature on immobilization, the immobilization experiments were performed in the range 5–45 °C. The results indicate that the extent of immobilization increases with increasing the temperature of the medium.

This can be explained by the following facts:

(a) Since the immobilization is normally a diffusion controlled process, it may be postulated that with increasing temperature, the mobility of the diastase molecules increases and, thus results in greater immobilization.

(b) It is worth mentioning here that at higher temperature, the electrostatic attractions are normally weakened and, therefore, a lower immobilization should be expected. However, a higher immobilization definitely points out that besides electrostatic forces some other forces must be contributing towards immobilization. These forces may be the hydrophobic forces as suggested elsewhere [26].

(c) It is also likely that with increasing temperature the number of active sites also increase on the bentonite surface and, therefore, the amount of immobilized diastase increase. Similar type of results were also reported by other workers [27].

Thermodynamic parameters

We have also calculated following thermodynamic parameters for immobilization process.

(i) Standard free energy change (ΔG°)

The standard free energy change (kJ/mol) was calculated by using the following equation:

$$\Delta G^\circ = -RT \ln K$$

where K is the equilibrium constant of the immobilization process. The value of ΔG°, as calculated above, has been found to be 1.62 KJ/mol.

(ii) Standard enthalpy change (ΔH°) for immobilization

The apparent heat of reaction – enthalpy ΔH° (KJ/mol) was estimated using the following equation:

$$\ln \frac{k_2}{k_1} = \frac{\Delta H^\circ}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right)$$

The value of ΔH° has been calculated to be 13.38 KJ/mol.

(iii) Standard entropy change (ΔS°)

It was calculated by following equation:

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$$

and the value of ΔS° was found to be 0.039 KJ/kmol.

Effect of pH

The influence of pH on both the amounts of adsorbed diastase and their relative activity has been investigated by performing experiments in the pH range 1.4–12.8. The results are depicted in Fig. 8 which indicate that the optimum immobilization and relative activity are recorded at pH 8.6 and 10.0, respectively while on the both sides of the optimum pH, the amounts of immobilized diastase and relative activity decrease. The results can be explained as below:

Immobilized diastase

When pH of the diastase-bentonite suspension is varied in the acidic range, i.e. beyond pH 1.4, the positive charge on the enzyme molecules go on decreasing while the negative charge on basal surfaces remains unchanged as the later is pH independent. However, because of the positive charge of edge face the adsorption goes on increasing as shown in Fig. 8 also. At pH around the optimum activity pH of the enzyme a maxima in the amount of immobilized enzyme is noted which could be due to reason that at this pH the enzyme molecule becomes electrically neutral and, therefore, offers greatest attachment to the active sites of the bentonite surface. This clearly explains the optimum immobilization of diastase on the acid-treated bentonite surfaces. However, beyond this optimum pH a decrease in adsorption of diastase is observed which may be due to the fact that

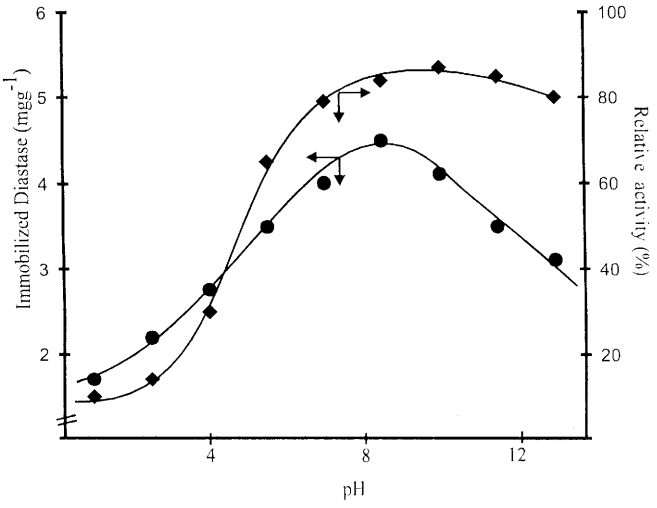

Fig. 8 Variation in the amounts and relative acitivity of immobilized (●) and free (■) enzymes with pH of the medium at [Diastase] = 0.5% w/v, temp. = 25 ± 0.2 °C

beyond the optimum pH the enzyme molecule goes on acquiring negative charge and, therefore, experience increasing force of repulsion due to negatively charged basal surface of the bentonite.

Relative activity

The adsorption of a protein on a negatively charged surface often shows a maximum near the isoelectric point (i.e.p.) of the macromolecule [28]. Several explanations of this phenomenon have been proposed, including (i) competition between the protein and protons in the solution for the negative sites in the surface below the i.e.p., and a coulombic repulsion of the protein above the i.e.p.; (ii) an increase in the lateral coulombic repulsions between adsorbed proteins above and below the i.e.p., decreasing the surface coverage [29]; and (iii) unfolding of the adsorbed protein above and below the i.e.p. as a result of internal electrostatic repulsions [26].

In the present study too the observed results support the above mentioned well reported findings. It can be clearly seen that an optimum activity is noticed at pH 8.6 which is greater than the optimum pH 6.9 of the same enzyme in the solution.

The decrease in the relative activity of the enzyme with decreasing pH indicates a progressive change in the conformation of the adsorbed enzyme. When the pH falls below the i.e.p., the positive charge on the enzyme increases and the attractive coulombic interaction with the electronegative surface (basal plan) causes an unfolding of the protein.

Conclusions

Diastase molecules are immobilized onto acid-treated bentonite surfaces via both electrostatic and hydrophobic forces and obey Langmuir isotherm equation when adsorbed onto the surfaces. The relative activity increases with increasing surface concentration of enzyme. Both the amount and relative activity of immobilized enzyme increase progressively with time. Whereas the relative activity of immobilized diastase is found to decrease with increasing concentration of added anions, it increases on addition of aliphatic alcohols to the diastase-bentonite suspension. The temperature also enhances both the amount and relative activity of immobilized diastase. The pH at optimum activity also shifts towards alkaline range when the enzyme is immobilized onto the bentonite surfaces.

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