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Adsorption and Immobilization of Penicillin Acylase on Chitosan Beads

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ABSTRACT

Adsorption of penicillin acylase (PA) from a crude aqueous extract of *Escherichia coli* on chitosan beads and *N*-benzamide and *N*-phenylacetamide derivatives of chitosan was investigated. Penicillin acylase adsorbed specifically on the derivatized chitosan beads, with a strong interaction with the ligand groups. The interaction of the active site of PA with the affinity ligands was simulated by molecular modeling. The adsorbent was further used for immobilization of PA by using glutaraldehyde as the activating agent. The immobilized enzyme retained the same activity even after 20 weeks.

Key Words: Chitosan; Adsorbent beads; Penicillin acylase; Chitosan derivatives; Molecular modeling; Affinity; Immobilization.

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INTRODUCTION

Chitosan, a linear polymer of β-(1,4)-2-amino-2-deoxy-D-glucopyranose, is easily derived from chitin by deacetylation (Fig. 1). Chitin itself is the second most abundant natural polymer after cellulose.[1] Chitosan has many useful features, such as hydrophilicity, biocompatibility, and biodegradability that attract its use as an adsorbent.[2] It has been successfully used for the purification of wheat germ agglutinin and cellulase, mainly due to its remarkable natural affinity for these proteins.[3–5] Major investigations on chitosan and its derivatives have been in metal ion chelation, wastewater treatment, and removal of endotoxins.[6,7]

The affinity purification of proteins relies on specific molecular interactions between a desired protein and a ligand. Specific binding of an enzyme is with either substrate, product, or analogs, inhibitor or cofactor covalently linked to a solid matrix. Penicillin acylase (PA) (E.C. 3.5.1.11) from *Escherichia coli* has shown specific and strong interactions toward ligands containing a phenyl ring along with either amino or amide groups.[8] A free reactive amino group imparts chitosan a weak ion-exchange property. This amino group can be easily modified by derivatizing with other chemical agents. In the present studies, *N*-benzamide and *N*-phenylacetamide derivatives of chitosan beads were selected on the basis of their expected affinity interaction with PA.[8] The enzyme is of great pharmaceutical importance for its applications in manufacturing of 6-aminopenicillinic acid (6-APA), which is a key intermediate for manufacturing of semisynthetic penicillins. No reports are available in the literature on adsorption of PA on chitosan or its derivatives. The interaction of the derivative groups with the active site of the enzyme was investigated separately by using molecular simulation. The use of adsorbent beads for immobilization of the enzyme also was explored along with the parameters, such as cross-linking agent concentration, time, pH, and stability of the immobilized enzyme.

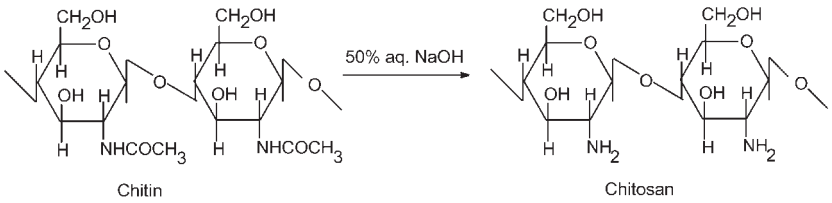

Figure 1. Chemical structures of chitin and chitosan.

MATERIALS

Yeast extract, sucrose, phenylacetic acid (PAA), nutrient agar, and bovine serum albumin (BSA) were obtained from Himedia Laboratory Ltd., Mumbai, India. 4-Dimethylaminobenzaldehyde was procured from Spectrochem Laboratories, Mumbai, India. 6-APA and penicillin-G were obtained from Hindustan Antibiotic Ltd., Pune, and Merck (I) Ltd., (Mumbai, India) respectively. All other chemicals, buffer reagents, sodium hydrogen orthophosphate, disodium hydrogen phosphate, chitin, methanol, glutaraldehyde, glacial acetic acid, copper sulfate, and sodium hydroxide were obtained from s.d. Fine Chemicals Ltd., Mumbai, India, and were of analytical grade.

EXPERIMENTAL PROCEDURES

Hydrolysis of Chitin

Chitin (10 g) was added to 50% (w/v) aqueous NaOH solution (300 cm^3) in a 500-cm^3 flask, with vigorous stirring. The reaction was allowed to proceed for 4 hr at 110°C under heterogeneous conditions. After hydrolysis, the insoluble product was separated by filtration by using a sintered glass funnel and was washed thoroughly to remove residual alkali. The product was dried at 50°C and was further characterized for the degree of deacetylation and molecular weight. The product was soluble in acetic acid, indicating the presence free amino groups. The degree of deacetylation was determined to be 68% by using infrared (IR) spectroscopy.[9] The molecular weight of chitosan was estimated to be 3.4×10^4 by using the Mark–Houwink equation from the viscosity data of chitosan solutions in 0.1 mol dm^{-3} acetic acid + 0.2 mol dm^{-3} sodium chloride solution.[10]

Preparation of Chitosan Beads

The pH-dependent solubility behavior of chitosan was used for the preparation of beads. Various parameters, which govern the physical characteristics of the beads, were optimized. The optimum concentration of chitosan in solution was 5% (w/v). Above this concentration, the viscosity of the solution was too high to pour easily, and, at lower concentrations, the beads showed poor mechanical properties. The optimum concentration of acetic acid required to completely dissolve chitosan was 3% (w/v). Chitosan was dissolved in acetic acid, and the solution was centrifuged to remove insoluble material. The supernatant was added dropwise to a NaOH–methanol solution

by using a syringe and was kept overnight in the solution. NaOH (2 mol dm^{-3}) and methanol, in 80 : 20 volume ratio, rendered almost spherical shape to the beads. The beads were washed thoroughly with distilled water and then were stored in 0.02% (w/v) aqueous sodium azide solution to prevent the growth of microorganisms.

The average diameter of the beads was approximately 1.5 mm as determined by the sieving method. The wet density of beads was 0.545 g cm^3 and water content, 88%. The beads were highly hydrophilic in nature and had to be stored in a wet state.

Synthesis of Chitosan Derivatives

N-Benzamide Derivative

The beads (5.45 g) were taken into 20 cm^3 of 0.5 mol dm^{-3} aqueous sodium hydroxide solution, and 2 cm^3 of benzoyl chloride was added dropwise, with stirring, over a period of 30 min. The mixture was further stirred for another hour. At the end of the reaction, the beads were separated by filtration and washed thoroughly with distilled water to remove excess reagent(s).

N-Phenylacetamide Derivative

A *N*-phenylacetamide derivative of chitosan was synthesized in the same way as the *N*-benzamide derivative by addition of 2 cm^3 phenylacetyl chloride (PAC) to 5.45 g of beads in the reaction mixture. PAC was synthesized separately in the laboratory by reaction of PAA with thionyl chloride.[8] The reaction mixture was refluxed for 2 hr. At the end of reaction, excess thionyl chloride was distilled off at 80°C.

Characterization of the Affinity Ligands

After completion of the reaction, the beads were separated by filtration and were washed with distilled water. The amount of the ligand attached to the support was calculated by mass balance between the ligand concentrations in the supernatant and the washing. The reaction mixture was treated with 2 mol dm^{-3} HCl and, subsequently, was extracted by using diethyl ether. Absorbance of benzoic acid and PAA was measured at 225 and 210 nm, respectively, by using a CHEMITO 2100 scanning ultraviolet-visible spectrophotometer (Chemito Technologies Ltd., Mumbai, India).

Production of PA

The bacterial strain *E. coli* (NCIM 2066), obtained from the National Chemical Laboratory, Pune, India, was grown on shake flask cultures in optimized media containing yeast extract (2% w/v), sucrose (1% w/v), and PAA (0.07% w/v) at pH 7.0 and temperature 37°C. The fermentation was carried out for 18 hr on a rotary shaker (175 rpm). After fermentation, the cells were collected by centrifugation at 10,000*g* for 10 min. The cells were washed with 50 mmol dm^{-3} phosphate buffer (pH 7.8) and then were suspended in a 25 cm^3 phosphate buffer solution at pH 7.8 and subjected to ultrasonication by using a "DAKSHIN" ultrasonic horn at a frequency of 20 kHz, with a power input of 120 W, in an ice bath for 15 min. The disrupted cells were clarified by centrifugation at 10,000*g* for 30 min. The clarified supernatant was used as the crude source of the enzyme.

Assay of PA

Free enzyme solution, 1 cm^3, or 1.09 g of beads with the immobilized enzyme was added to an equal volume of benzyl penicillin (2 mg cm^{-3}) in a 0.2 mol dm^{-3} phosphate buffer solution (pH 8.0) at 37°C. The mixture was subjected to a low degree of agitation on the rotary shaker. At the end of incubation period of 1 hr, a 0.5 cm^3 sample was transferred to a mixture of 2 cm^3 of acetic acid (20% w/v), 1 cm^3 of sodium hydroxide solution (0.05 mol dm^{-3}), and 0.5 cm^3 of 4-dimethylaminobenzaldehyde dissolved in methanol (1% w/v). The concentration of the product 6-APA was estimated at 415 nm.[11] One unit of the enzyme was defined as the activity producing 1 mmol of 6-APA per hour under the given assay condition.

Protein Estimation

The total protein content was determined by the modified Folin–Lowry method[12] or by a dye-binding assay.[13] A standard calibration curve was prepared by using BSA.

Adsorption Studies

The beads were equilibrated with a phosphate buffer solution (50 mmol dm^{-3}) before the adsorption; the pH of the solution was maintained at 8.0. The enzymatic extract/BSA solutions and bead suspension was subjected

to low oscillatory agitation. After the adsorption, the beads were washed with the same buffer solution to remove unbound proteins. The amounts of the protein and enzyme adsorbed on the beads were determined from mass balance.

Immobilization of PA on Chitosan Beads

An aqueous solution of glutaraldehyde ($20\,cm^3$) was added to $10\,cm^3$ of chitosan beads, suspended in a $50\,mmol\,dm^{-3}$ phosphate buffer solution (pH 8.0). After 2 hr of agitation (110 rpm), the beads were separated by filtration and were washed with buffer till no glutaraldehyde was detected in the washing. These beads then were added to a crude solution of PA and were stirred at 50 rpm for 6 hr. The beads were further washed with phosphate buffer (pH 8.0). Then sodium borohydride solution (10% w/v) was added dropwise, with agitation, to reduce the Schiff's base to a stable amine bond. After 1 hr, the beads were filtered off and were washed further with buffer and stored in $50\,mmol\,dm^{-3}$ phosphate buffer (pH 8.0) at 4°C.

The Michaelis–Menten constant (K_m) for the soluble and immobilized enzymes were determined in phosphate buffer solutions (pH 8.0) at various concentrations of penicillin-G ranging from 1 to $30\,mmol\,dm^{-3}$.

To determine the optimum pH of the free and immobilized enzymes between pH 5 to 10, the enzyme activity measurements were performed in $0.05\,mol\,dm^{-3}$ acetate, phosphate, and alkaline borate buffers in the pH ranges 5–6, 6–8, and 8–10, respectively. The stability as a function of pH was determined by measuring the residual enzyme activity at each pH after 1 hour storage at room temperature of 30°C. The immobilized enzyme was stored at 4°C, and its activity was checked at weekly intervals.

Molecular Modeling Studies

A molecular modeling software, Hyperchem 7.0, (Hypercube Inc., Florida, USA) was used to simulate the enzyme–ligand interaction. The structure of PA was obtained from the Protein Data Bank.[14]

RESULTS AND DISCUSSION

Adsorption of Proteins on Nonderivatized Chitosan Beads

The adsorption of BSA was studied at three different pH conditions. The adsorption studies could not be performed below pH 6.0 because

the pH-dependent solubility of chitosan led to the dissolution of beads. The results of adsorption are shown in Fig. 2. The solid lines show the values fitted in the Langmuir adsorption equation.

$$q = \frac{q_0 K \cdot C}{1 + KC} \tag{1}$$

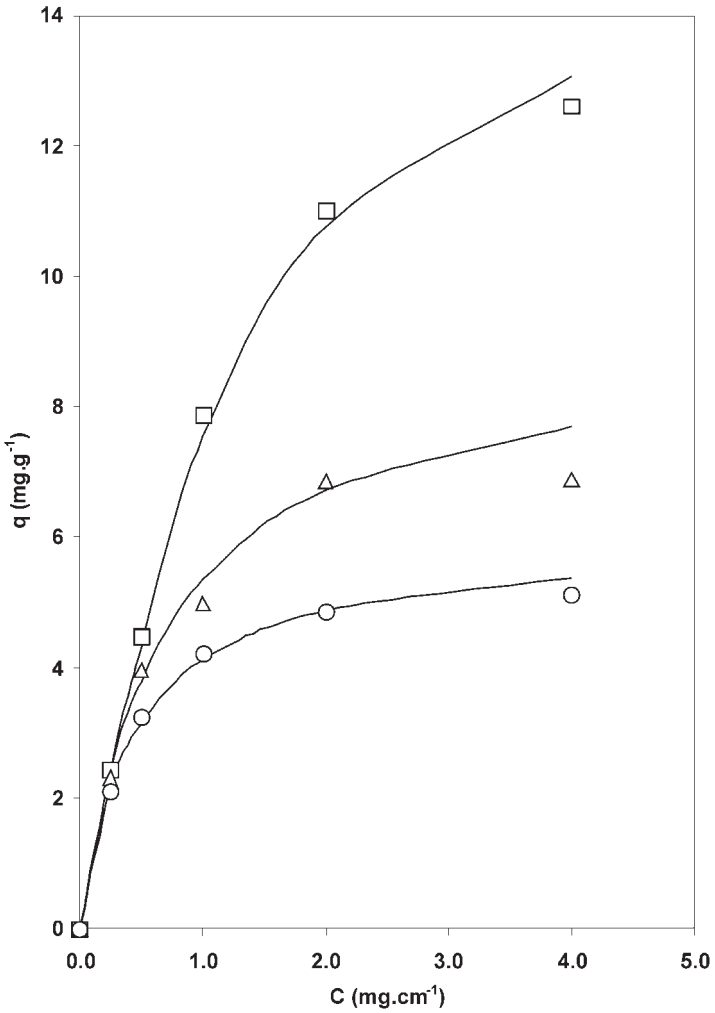

Figure 2. Adsorption isotherms of BSA on chitosan beads at different pH conditions. *Key*: □, pH 6.0; △, pH 7.0; ○, pH 8.0.

where q, q_0, K, and C are the amounts adsorbed ($mg\,g^{-1}$), saturation capacity ($mg\,g^{-1}$), equilibrium coefficient ($mg^{-1}\,cm^3$), and equilibration concentration ($mg\,cm^{-3}$), respectively. The maximum adsorption of BSA was $17.19\,mg\,g^{-1}$ at pH 6 and $6.88\,mg\,g^{-1}$ at pH 8.0. In acidic conditions, the amino group of chitosan gets protonated to form—NH_3^+ as pK_b of chitosan is 7.7.[2] The isoelectric pH of BSA is ~4.8, thus, at pH > p*I*, BSA molecules are negatively charged and are adsorbed by electrostatic attractions. At pH 8, since the amino group of chitosan is in the unprotonated form, the adsorbed of BSA amount is less than that adsorbed at pH 6. The variation of adsorption capacity with pH shows that chitosan behaves as a weak anionic exchanger; BSA (58%) could be eluted by using an aqueous buffer solution of high ionic strength ($0.5\,mol\,dm^{-3}$ NaCl) in a single-stage batch mode. Thus, electrostatic interactions seem to be responsible for the adsorption of BSA on chitosan beads, which are weakened by the screening effects of ions at a higher ionic strength of solution. However, the BSA adsorption, even at pH 8, where the beads carry no or reduced charge, shows interactions other than electrostatic that also play a role in the adsorption of proteins.

Adsorption of PA was studied on underivatized chitosan beads at three different pH conditions (Table 1). PA has an isoelectric pH of 6.3.[15] The maximum amount of the enzyme was adsorbed at pH 7.0, but, above and below this pH, its adsorption decreased. Being an intracellular enzyme, PA from *E. coli* usually is contaminated with cytoplasmic proteins and nucleic acids in the crude extract obtained by breaking the cells, which might be responsible for the adsorption of enzyme with poor specificity. The protein adsorption was substantially reduced at pH 8.0 where the chitosan had no charge. The adsorption of the proteins, therefore, seems to be mainly due to electrostatic interactions. To increase the specificity of PA adsorption further, hydrophobic benzamide and phenylacetamide derivatives of chitosan beads were studied.

Table 1. Adsorption of PA on underivatized chitosan beads.

	Enzyme (total U)		Protein (mg)	
pH	Loaded	Adsorbed	Loaded	Adsorbed
6.2	17.51	5.93	17.03	13.47
7.0	17.97	9.03	17.48	14.91
7.8	18.43	5.63	15.39	6.75

Purification of PA by Using Chitosan Derivatives

Adsorption on *N*-Benzamide Derivative of Chitosan

Table 2 shows that the *N*-benzamide derivative with a ligand density of 123 $\mu g\,g^{-1}$ adsorbed almost 10.8 U of enzyme per gram of beads. The specific activity of the adsorbed enzyme was increased to 2.8, which, although not high, still suggests specific adsorption of the enzyme over other proteins when compared with the adsorption on nonderivatized chitosan beads (Table 1). The total protein adsorbed was 0.77 $mg\,g^{-1}$ of beads. This purification of PA, however, was characterized by a low capacity of the beads. Affinity chromatography supports generally present a low capacity for the protein adsorption, which should be decided by the concentration of the ligand on support and their accessibility.[16] The adsorbed PA could not be released at all by the salt solution, indicating its strong and specific adsorption. The enzyme could be partially eluted by using 0.05 mol dm^{-3} PAA, as a competitive eluent, in a 0.2 mol dm^{-3} phosphate buffer at pH 8.0. The enzyme was eluted with specific activity of 1.8, the yield was only 30% from the adsorbed amount, which is quite less, indicating a very strong interaction of the enzyme with the ligand. On increasing the ligand density (352 $\mu g\,g^{-1}$ of beads), PA was almost quantitatively bound to the *N*-benzamide derivative (Table 2). Although there was an increase in adsorption capacity with the increase in ligand density, on elution, the same results were obtained. The recovery was in the range 25%, and no elution of the enzyme with 0.5 mol dm^{-3} NaCl solution indicates a stronger interaction of the PA with the ligand. The specificity of the enzyme toward the ligand also increased the purity of the eluted enzyme. The enzyme in the adsorbed state, however, showed only 43% activity with respect to the adsorbed amount, which is probably due to the interaction of the ligand blocking the active site of the enzyme.

Table 2. Adsorption of PA on *N*-benzamide derivative of chitosan at pH 8.0.

Ligand density ($\mu g\,g^{-1}$)		Loaded	Adsorbed	Elution with 0.5 mol dm^{-3} NaCl	Elution with 0.05 mol dm^{-3} PAA
123	Enzyme (U)	25.29	10.86	0.0	3.25
	Protein (mg)	19.14	3.89	1.19	1.39
352	Enzyme (U)	21.72	23.56	0.0	5.94
	Protein (mg)	14.16	7.41	2.11	1.82

Adsorption Studies with *N*-Phenylacetamide Derivative

The *N*-phenylacetamide derivative was expected to increase the specificity of the PA adsorption, because PAA is an inducer for the synthesis of the enzyme. The adsorption results are shown in Table 3. The specific activity of the adsorbed enzyme was, indeed, increased to 5.31, indicating specific adsorption of enzyme by *N*-phenylacetamide derivative. The affinity adsorption of PA on PAC treated carrier Eupergit "C" was studied earlier by Bihari and Buccholz.[17] The elution of PA by using a method suggested by these authors, however, failed. The elution achieved with PAA solution was even lower than that from the *N*-benzamide derivative, indicating a stronger binding. The possible mechanism of a strong binding of the enzyme on these chitosan derivatives was further investigated by molecular simulation.

Molecular Modeling Studies

PA from *E. coli* is a heterodimer of A and B chains of 209 and 557 amino acid residues, respectively.[18] Only a few amino acids are considered to be responsible for the active site confirmation of PA.[19] Met A142, Phe A146, Phe B57, Trp B154, and Ile B177 line the active site with Ser B67 at the closed end. The mouth of this pocket is formed by the side chains of Ser B1 and Asn B241 and by a main chain nitrogen of Gln B23 and Ala B69. A small monomer unit of glucosamine with the ligand group was considered for simulation for its interaction with the active site of enzyme. The enzyme active site and the ligand were brought together and the energy optimization of complex was performed in aqueous conditions. Periodic boundaries were applied by filling the space around the complex with discrete water molecules. Molecular mechanics or the force-field method calculates the energy as a function of the bond angle (bond stretching, angle bending, torsional energy-bond rotation) and nonbonded interactions (van der Waals and electrostatics).

Table 3. Adsorption of the PA on *N*-phenylacetamide derivative of chitosan beads.

	Loaded	Adsorbed	Elution with $0.5\ mol\ dm^{-3}$ NaCl	Elution with $0.05\ mol\ dm^{-3}$ PAA
Enzyme (U)	25.29	15.46	0.0	2.80
Protein (mg)	19.04	9.74	0.55	6.84

Note: Ligand density—$123\ \mu g\ gm^{-1}$, pH 8.0.

The energy of interaction of the enzyme with different ligands can be estimated by the difference of optimized energy of the associated complex from the total energies of the individual species.

The interaction of *N*-benzamide derivative with the enzyme active site is shown in Fig. 3. The interaction energy of the solvated complex of the enzyme was $-4467.42\,\mathrm{kJ\,mol^{-1}}$. The orientation of the phenyl group of ligand inside the pocket and its interaction with the phenyl ring of the active site of the enzyme shows that the presence of the phenyl ring imparts the affinity characteristic to the ligand. The studies showed that strong binding of the ligand to the active site of the enzyme should be responsible for its poor elution of the adsorbed condition. From the optimized structure of the complex, $\pi-\pi$ interactions also seem to be responsible for the affinity of the ligands, containing a phenyl ring, with the enzyme along with the hydrogen bonding between carboxyl O-atom of a amide group of the ligand and H of the amino acid alanine (B69).

The interaction studies with *N*-phenylacetamide derivative gave the energy minimum at $-4698.80\,\mathrm{kJ\,mol^{-1}}$ indicating a more stable interaction (Fig. 4). The binding of PAA to PA was earlier reported to induce some conformational changes in the enzyme.[20] The presence of the aromatic

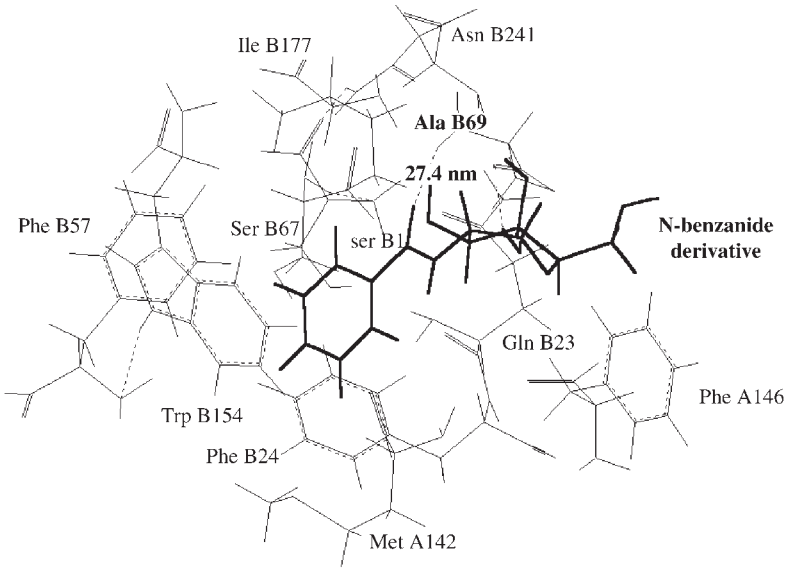

Figure 3. Optimized structure of active site of PA and *N*-benzamide derivative of chitosan by molecular modeling (Ala B69 residue of the active site shows H-bonding with carbonyl oxygen of the amide).

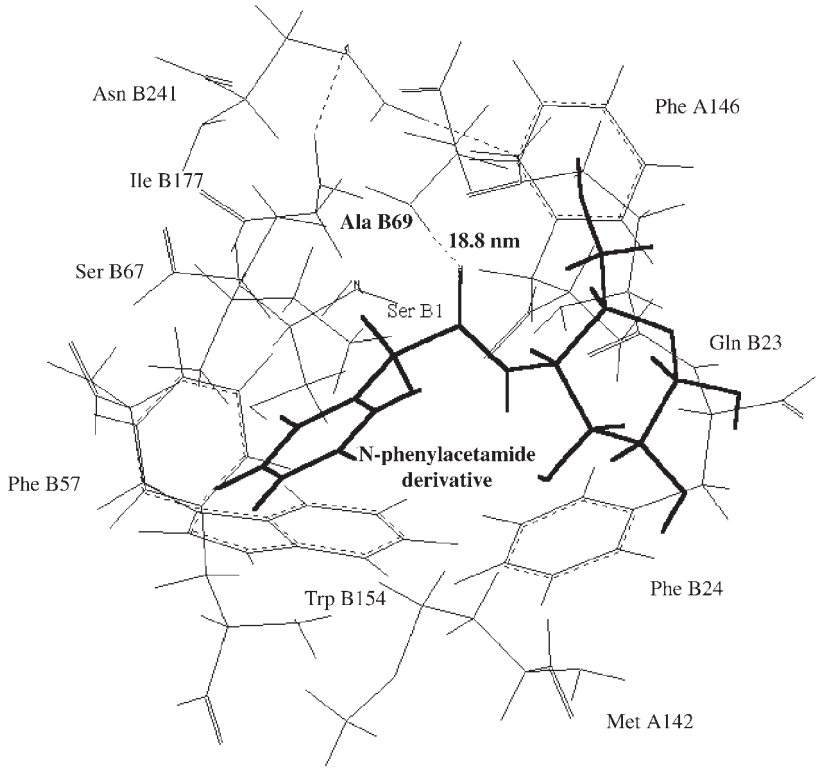

Figure 4. Optimized structure of active site of PA and *N*-phenylacetamide derivative of chitosan by molecular modeling (Ala B69 residue of the active site shows stronger H-bonding with carbonyl group of amide).

ring in the ligand is probably essential for the specific interaction. Also, experimentally, specific adsorption of the enzyme was observed with the *N*-phenylacetamide derivative of chitosan, thus, suggesting stronger interaction of the ligand with the enzyme. The optimized structure of the solvated complex also indicated strong interactions of the aromatic ring with the aromatic amino acids at the active site and H-bonding of the "O" of amide group of ligand with the alanine (B69) of enzyme. The hydrogen bond length was 18.8 nm, which is lower than that with the *N*-benzamide derivative (27.4 nm) indicates a closer interaction of the ligand with the enzyme's active site. The presence of the additional carbon chain in the derivative group gave flexibility in its interaction with the enzyme, as could be postulated from the energy minimization studies.

Immobilization Studies

Chitosan beads can be excellent carriers for enzyme immobilization due to the free amino groups. This amino group is suitable for activation by using glutaraldehyde. Glutaraldehyde forms Schiff's base with free amino groups of chitosan and with those of the enzyme, which can be further reduced to very stable secondary amines. The unreacted aldehyde groups, if any, in the beads can be reduced by using sodium borohydride to alcohol.

Figure 5 shows that maximum activity of the immobilized enzyme was observed with 5% (w/v) glutaraldehyde. At low concentrations of

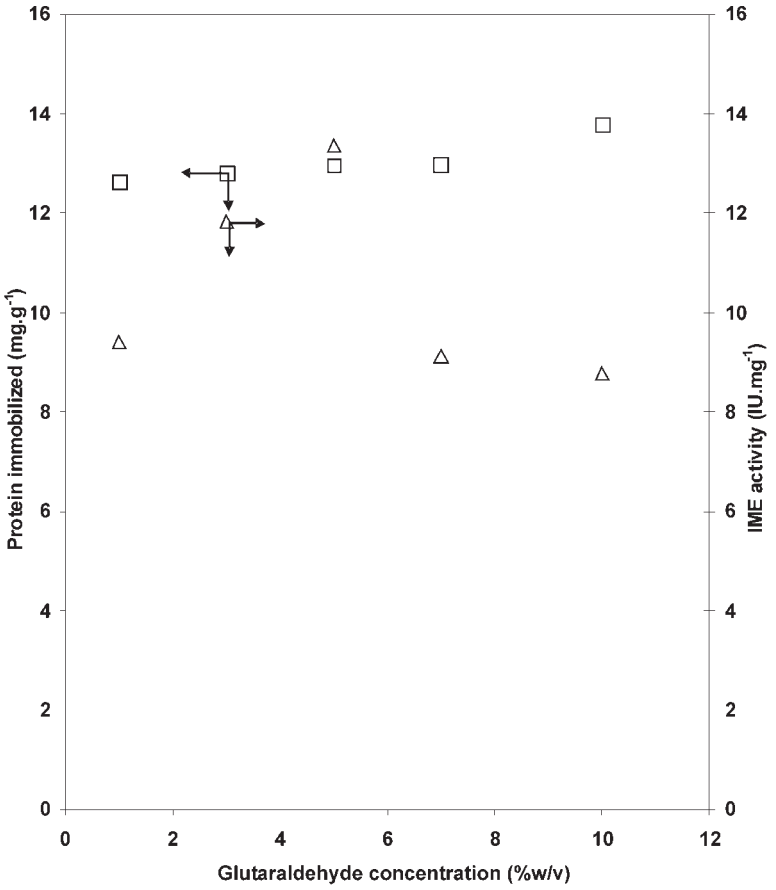

Figure 5. Effect of glutaraldehyde concentration on protein adsorption and immobilization of PA. *Key*: □, Adsorbed protein; △, immobilized PA activity.

glutaraldehyde, it is probable that both aldehyde groups involve in cross-linking with amino groups of the chitosan only, and few aldehyde groups are available for the enzyme immobilization. At higher aldehyde concentrations, more free aldehyde groups are available for the reaction. The enzyme, however, showed an ~60% loss of its activity on immobilization. Glutaraldehyde is reported to react nonspecifically with amino groups of some peptides, the sulfhydryl group of the cysteine, the α-amino group of lysine, and the phenolic and imidazole rings of the hystidine.[21] It is well documented

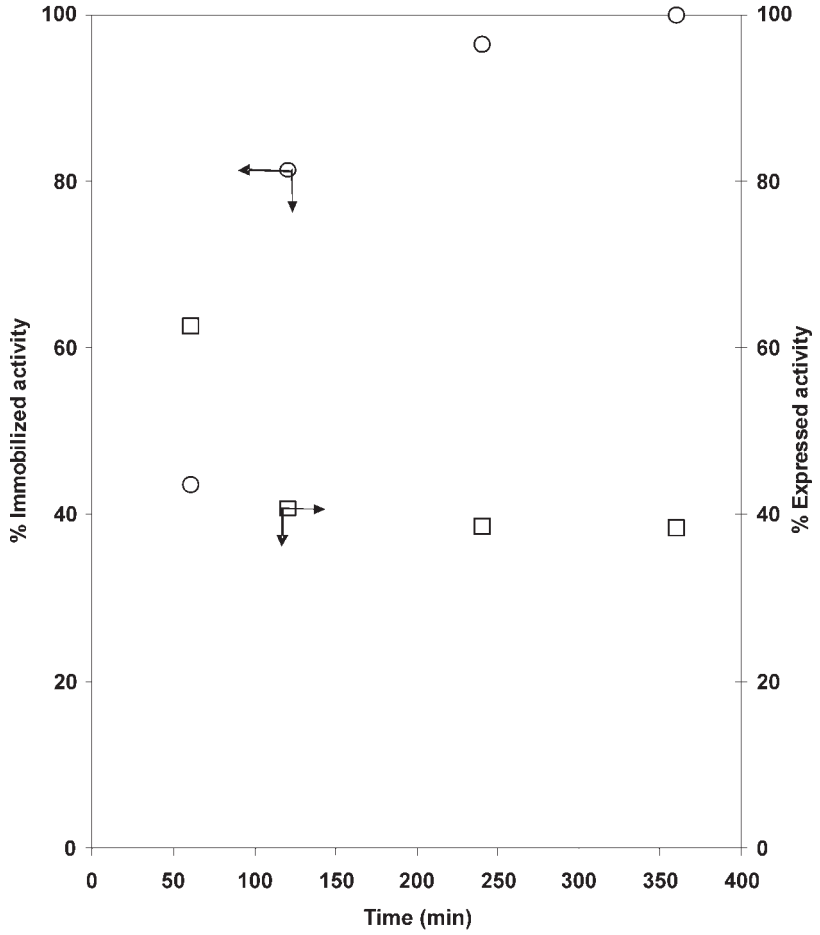

Figure 6. Effect of immobilization reaction time on PA activity. *Key*: □, Activity expressed by immobilized PA; ○, total immobilized PA.

that the active site of the PA contains amino acid residues such as lysine, tryptophan, serine, arginine, and glutamine.[19] The reaction of the glutaraldehyde with the above amino acids at the active site of PA may cause conformational changes in the enzyme itself. At higher concentrations of glutaraldehyde, the multiple point attachment increases and causes more conformational change, which might lead to more loss of the enzyme activity on immobilization.

The optimum time for the immobilization was determined by incubating activated chitosan beads with the enzyme solutions. Figure 6 shows that

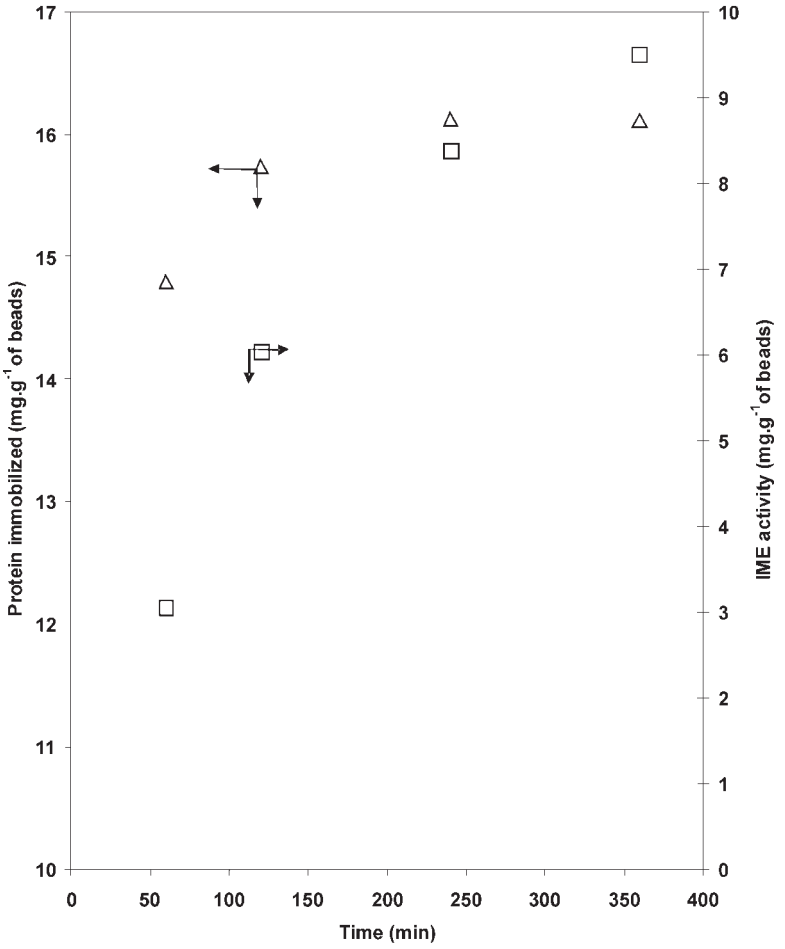

Figure 7. Effect of time on protein adsorption and immobilization on PA activity. *Key*: □, Adsorbed protein on chitosan beads; △, immobilized enzyme activity.

activity expressed by the immobilized enzyme decreases initially with the incubation time up to 2 hr and remains almost constant thereafter. With the increased incubation time, the immobilization of the proteins also increases, which might lead to steric hindrance and loss of specificity. The immobilization of proteins is shown in Fig. 7.

The experiments conducted at different pH conditions revealed that the immobilization yield also increased at higher pH. The immobilized enzyme activity was the highest at pH 8.0 (Fig. 8). A greater immobilization was

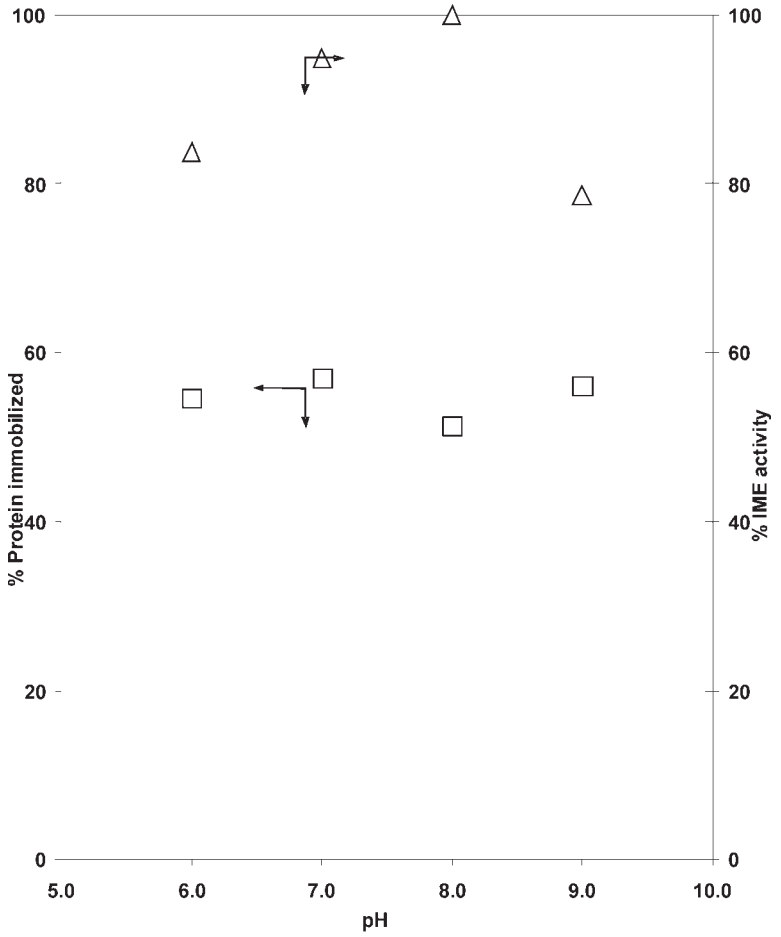

Figure 8. Effect of pH on protein adsorption and immobilization of PA. *Key*: □, Adsorbed protein; △, immobilized PA.

observed when the chitosan beads were incubated at pH 8.0, where the degree of ionization of the amino group was low and, thus, more reactive. No significant effect on the total protein immobilization was observed by change in pH, which could be related to the nonspecific binding of the proteins to the glutaraldehyde.

The Lineweaver–Burk plots for reaction rates at various concentrations of penicillin-G, ranging from 1 to 30 mmol dm^{-3}, measured for the free and immobilized enzymes, are shown in Fig. 9. The Michaelis–Menten constant of the free and immobilized PA was 5.56 and 27.77 $mmol^{-1}$ dm^3, respectively. The K_m value of the immobilized PA was almost fivefold higher than that of

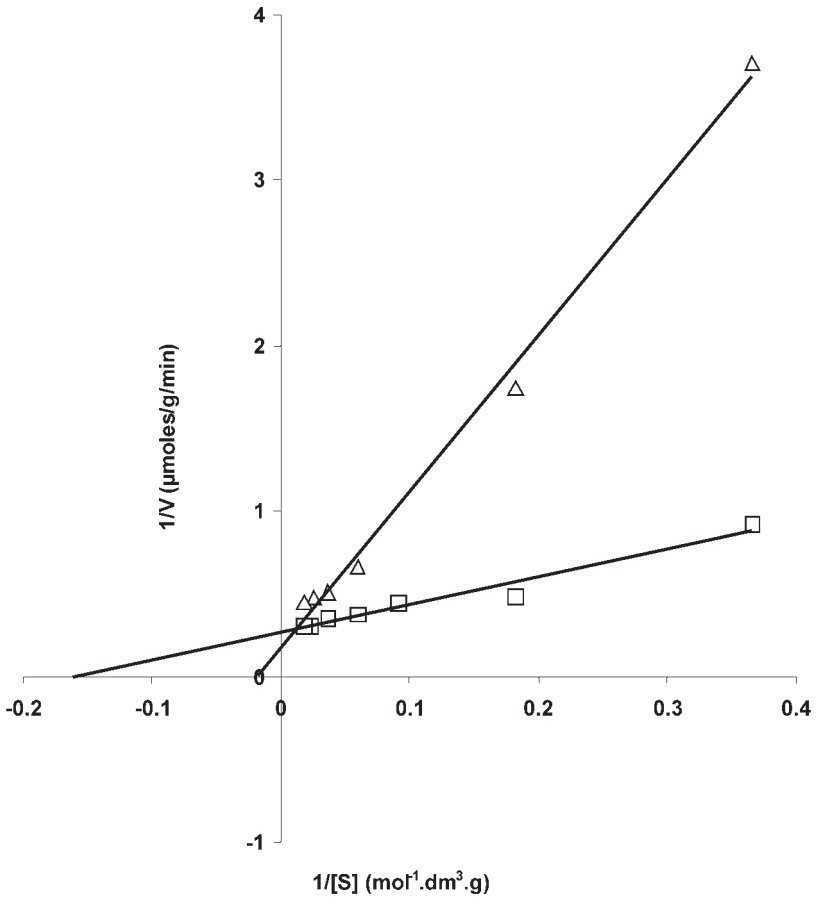

Figure 9. Lineweaver–Burk plots for free and immobilized enzyme. *Key*: □, Free enzyme; △, immobilized PA.

the soluble PA. A 10-fold increase in the Michaelis–Menton constant was reported for immobilized PA by using the same cross-linking agent of glutaraldehyde by Bihari and Buchholz.[22]

The activity profiles of the native and immobilized PAs show the maximum enzyme activity around pH 8.5 and 8.0 for the immobilized and native enzymes, respectively (Fig. 10). A change in pH profile of immobilized enzyme usually is observed when the enzyme is immobilized on a charged support or a reaction product is ionic in nature. Since the hydrolysis of

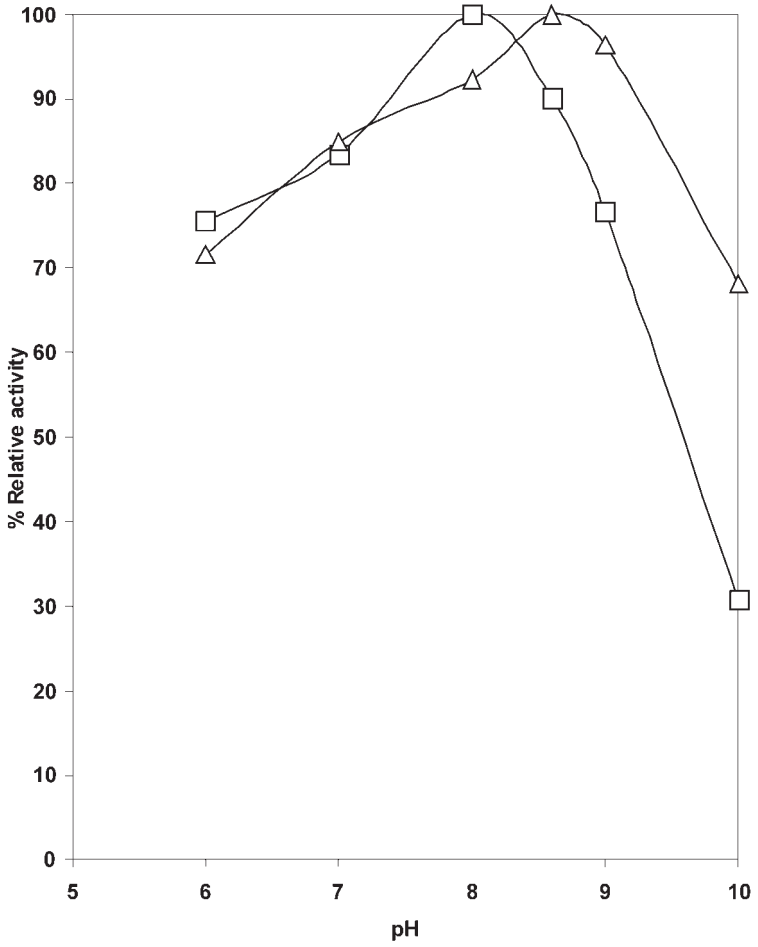

Figure 10. Effect of pH on activity of free and immobilized forms of PA. *Key*: □, Free enzyme; △, immobilized enzyme.

penicillin-G yields 6-APA and PAA, the product acid might cause local pH to fall so that a higher pH of the bulk phase is needed to maintain the same reaction rate. Another possibility is that when the matrix is ionizable, the environmental pH within the beads will be dictated by charged groups closer to the enzyme, rather than the bulk fluid. The effect of pH on PA stability expressed as the residual activity after 1 hr of storage at a given pH is shown in Fig. 11. The native and immobilized enzymes showed the complete retention of the activity in pH range 5–8, but, above pH 8, the enzyme lost substantial activity. It is worth noting the stability of the immobilized

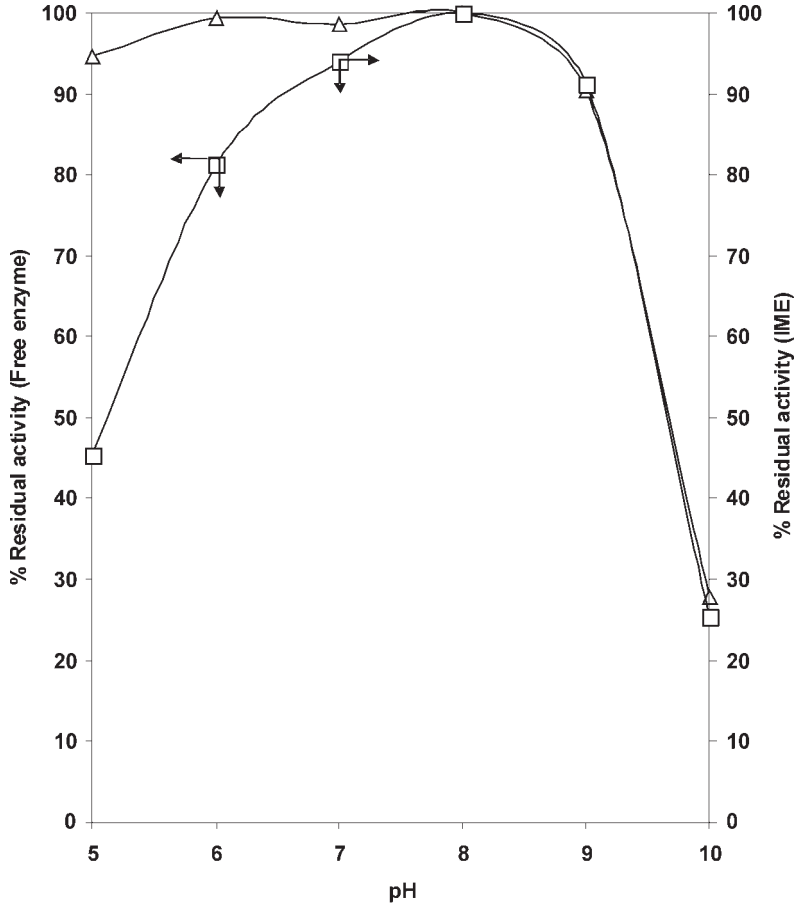

Figure 11. Effect of pH on stability of free and immobilized forms of PA. *Key*: □, Free enzyme; △, immobilized enzyme.

enzyme in the acidic range, which is important for the reverse reaction, i.e., acylation of 6-APA for the synthesis of semisynthetic penicillin.

The immobilized enzyme was stored in freezer at 4°C, and the enzyme activity was measured at weekly intervals. The immobilized enzyme displayed the same activity even after 20 weeks, indicating no leakage of enzyme and very stable immobilization.

CONCLUSION

Spherical beads of chitosan showed moderate adsorption capacity for proteins. The *N*-benzamide and *N*-phenylacetamide chitosan derivatives showed selective adsorption of PA; but, poor elution of the enzyme indicated strong and specific interaction of the ligands with the active site of the enzyme. Molecular simulation successfully predicted the affinity of the ligands toward the enzyme. A priori identification of the interactive forces could, thus, be useful for the design of more efficient ligand(s) for the enzyme separation. Immobilized PA was stable for 20 weeks, indicating its very stable immobilization.

REFERENCES

1. Singla, A.K.; Chawla, M. Chitosan: some pharmaceuticals and biological aspects—an updates. J. Pharm. Pharmacol. **2001**, *53*, 1047–1067.
2. Muzzarelli, R.A.A. *Chitin*, 1st Ed.; Pergamon: Tarrytown, New York, 1977; 45–85.
3. Senstad, C.; Mattiasson, B. Purification of wheat germ agglutinin using affinity flocculation with chitosan and a subsequent centrifugation or flotation step. Biotechnol. Bioeng. **1989**, *34*, 387–393.
4. Roy, I.; Sardar, M.; Gupta, M.N. Exploiting unusual affinity of usual polysaccharides for separation of enzymes in fluidized beds. Enzyme Microb. Technol. **2000**, *27*, 53–65.
5. Homma, T.; Fujii, M.; Mori, J.; Kawakami, T.; Kuroda, K.; Taniguchi, M. Production of cellobiose by enzymatic hydrolysis: removal of β-glucosidase from cellulase by affinity precipitation using chitosan. Biotechnol. Bioeng. **1993**, *41*, 405–410.
6. Kawamura, Y.; Mitsuhashi, M.; Tanibe, H.; Yoshida, H. Adsorption of metal ions on polyaminated highly porous chitosan chelating resin. Ind. Eng. Chem. Res. **1993**, *32*, 386–391.
7. Hirano, S. Chitin biotechnology applications. In *Biotechnology Annual Review*; EL-Gewely, M.R., Ed.; Elsevier Science B.V.: Netherlands, 1996; Vol. 2, 237–358.

8. Gavasane, M.R.; Gaikar, V.G. Aqueous two phase affinity partitioning of penicillin acylase from *E. coli* in presence of PEG-derivatives. Enzyme Microb. Technol. **2003**, *32*, 665–675.
9. Domszy, J.G.; Roberts, G.A.F. Evaluation of infrared spectroscopic techniques for analyzing chitosan. Makromol. Chem. **1985**, *186*, 1671–1677.
10. Muzzarelli, R.A.A. Chitin. In *The Polysaccharides*; Aspinall, G.O., Ed.; Academic Press Inc.: Florida, 1985; Vol. 3, 417–450.
11. Balasingham, K.; Warburton, D.; Dunhill, P.; Lilly, M. The isolation and kinetics of penicillin amidase from *E. coli*. Biochim. Biophys. Acta **1972**, *276*, 250–256.
12. Lowry, O.H.; Rosenbrough, N.J.; Farr, A.L.; Randall, R.J. Protein measurement with the folin phenol reagent. J. Biol. Chem. **1951**, *193*, 265–275.
13. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **1976**, *72*, 248–254.
14. http://www.rscb.org/pdb/result Browser cgi 13th January, 2003, 10.00 hrs).
15. Mahajan, P.B.; Borkar, P.S. Novel approach to the purification of penicillin acylase. Appl. Biochem. Biotechnol. **1984**, *9*, 421–437.
16. Klein, E. *Affinity Membranes, Their Chemistry and Performance in Adsorptive Separation Processes*; John Wiley and Sons: USA, 1991.
17. Bihari, V.; Buccholz, K. A support-spacer-ligand system for specific affinity adsorption of penicillin acylase. Biotech. Lett. **1984**, *6*, 571–576.
18. Brüns, W.; Hoppe, J.; Tsai, H.; Bruning, H.J.; Maywald, F.; Collins, J.; Mayer, H. Structure of the penicillin acylase gene from *Escherichia coli*: a periplasmic enzyme that undergoes multiple proteolytic processing. J. Mol. Appl. Genet. **1985**, *3*, 36–44.
19. Duggleby, H.J.; Tolly, S.P.; Hill, C.P.; Dodson, E.J.; Dodson, G.; Moody, P.C.E. Penicillin acylase has a single-amino-acid catalytic centre. Nature **1995**, *373*, 264–268.
20. Done, S.H.; Brannigan, J.A.; Moody, P.C.E.; Hubbard, R.E. Ligand-induced conformational change in penicillin acylase. J. Mol. Biol. **1998**, *284*, 463–475.
21. Dubey, A.K.; Bisaria, V.S.; Mukhopadhyay, S.N.; Ghosh, T.K. Stabilization of restriction endonuclease *Bam Hi* by crosslinking reagents. Biotechnol. Bioeng. **1989**, *33*, 1311–1316.
22. Bihari, V.; Buchholz, K. Kinetics and effectiveness of soluble and immobilized penicillin acylase (*E. coli*) in different forms. Ind. J. Exp. Biol. **1983**, *21*, 27–30.