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Immobilization of carbonic anhydrase enriched microorganism on biopolymer based materials

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ABSTRACT

The whole cell of *Bacillus pumilus* was immobilized on different chitosan based materials while attempts were also made to immobilize carbonic anhydrase (CA) enzyme. The screening of materials for esterase activity resulted in the selection of biopolymer based beads as the potential material for whole cell and CA immobilization. The materials were characterized by Fourier transform infrared (FTIR) spectroscopy, X-ray diffraction (XRD), scanning electron microscopy (SEM), while physical characteristics like BET surface area, particle size etc. were also determined. Esterase activity and other parameters, like effect of swelling, were determined on immobilized cells. After cell immobilization, the esterase activities of chitosan-NH_4OH beads, multilayered beads, and sodium alginate were found to be 42, 36, and 30.5 U/ml, respectively, as compared to 27.15 U/ml for the free organism.

1. Introduction

The rising carbon dioxide (CO_2) emission leading to global climate change is one of the greatest environmental challenges that the world faces today [1]. The link between the anthropogenic CO_2 emissions and its increased atmospheric concentration resulting in global average temperature rise and consequent sea level rise is well established. The CO_2 mitigation can be achieved generally by three means: first by improving energy efficiency, second by CO_2 capture and sequestration and the third option is use of alternative clean fuels (hydrogen, biodiesel etc.). Efforts are being made at our institute (NEERI) to mimic the reaction of fixation of anthropogenic CO_2 into calcium carbonate using carbonic anhydrase (CA) as a biocatalyst. In nature, CO_2 is being sequestered by converting it into naturally occurring minerals like dolomite, calcite etc but this is happening over a geological time frame. Calcium carbonate can be precipitated from aqueous solution, given a suitable saturation of calcium and carbonate ions, and so the issue to be addressed is to produce carbonate ions rapidly from CO_2 and H_2O, a process which first requires the formation of bicarbonate ions. This problem of enhancing the rate of reaction is being facilitated using carbonic anhydrase as the enzyme and is termed as biomimetic sequestration of carbon dioxide [2,3].

Carbon dioxide is an inert and stable compound and its chemical conversion requires high pressure and temperature. However, in nature, the reduction of carbon dioxide into useful molecules is being efficiently carried out using various enzymes. Carbonic anhydrase is one of the enzymes, which plays a crucial role in various physiological processes such as respiration and photosynthesis and is responsible for rapid inter-conversion of CO_2 into carbonate in the cells. Carbonic anhydrase is a Zn containing enzyme having 3 histidine residues at the active site. The turnover number of this enzyme is very high thus making it a suitable candidate for the conversion of CO_2 into carbonate [4,5]. Carbonic anhydrase catalyzes the reversible hydration of CO_2 to form a bicarbonate anion and a proton.

$$CO_2 + H_2O \leftrightharpoons HCO_3^- + H^+$$

Extensive work is being carried out to enhance the rate of reaction using either pure carbonic anhydrase or CA enriched microorganisms.

Immobilization is a process by which enzymes/whole cells are confined to a phase distinct from the one in which the substrates and the products are present. It allows the ease of removal of the product, reuse of the enzymes, and enhanced effectiveness due to the reuse of enzyme. Various materials such as chitosan, sodium alginate have been used to immobilize enzyme and microorganisms.

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Chitosan is the product obtained from deacetylation of chitin, which is the second most abundant biopolymer after cellulose. Chitosan has been used as a raw material for biomedical applications such as surgical sutures, artificial skin and immune-suppressant [6]. One of the most important applications of chitosan is as support for immobilization of enzymes or whole cells as it is non-toxic and user-friendly, and has protein affinity [7]. Enzymes/cells that have been immobilized on functionalized and non-functionalized chitosan based matrices include pepsin on chitosan beads [8], hydrolases onto chitosan micro particles [9], amylase, glucoamylase on chitosan-clay composite beads [10], etc.

Alginate is also one of the most extensively used polymers for various biomedical applications and in pharmaceutical industries as gelling agent [11]. Alginate has the capacity to extract metal ions from dilute aqueous solutions. Reported cell immobilizations on sodium alginate beads include, immobilization of *Acidithiobacillus ferrooxidans* on PVA and sodium alginate [12], and *Saccharomyces cerevisiae* invertase on chitin-sodium alginate beads [13].

There are few reports available on the immobilization of carbonic anhydrase enzyme on different materials [14–17]. However, not much is reported on the immobilization of carbonic anhydrase enriched microorganism. The present study describes methods to develop various materials based on biopolymer materials for effective immobilization of microbial cells and carbonic anhydrase for their applications in the conversion of CO_2 to mineral carbonates. The active site of the enzyme is responsible for the acceleration of CO_2 hydration as well as for the hydrolysis of esters. Therefore, esterase activity has been used as a screening tool to determine the activity of CA by using pNPA (para nitrophenyl acetate) as a substrate which gives a yellow product (para nitrophenol) at 348 nm.

2. Materials and methods

All chemicals used in the study were of analytical grade and were purchased from Merck, India Ltd., Loba Chemicals, India, BDH, Germany and Sigma Chemicals Co. (USA).

2.1. Synthesis of materials

2.1.1. Chitosan-NaOH beads (N-1)

A chitosan solution was prepared by dissolving 3 g of chitosan flakes in 100 ml of 5% acetic acid. It was stirred for 1 h till it formed a viscous solution of chitosan. This viscous chitosan solution was added dropwise into 250 ml of 1 M NaOH solution with continuous stirring to form chitosan beads. The beads were allowed to stabilize in NaOH solution for 1 h at room temperature. The chitosan beads were filtered and rinsed with distilled water several times till the pH of the supernatant was 7.0. The beads were finally dried in an oven at 60 °C for 6 h.

2.1.2. Chitosan-KOH beads (N-2)

The method for the synthesis of the chitosan-KOH beads was the same as above (refer N-1) except for using KOH solution of concentration 1 M, instead of NaOH.

2.1.3. Chitosan-NH_4OH beads (N-3)

The method for the synthesis of the chitosan-NH_4OH beads was the same as above (refer N-1) except for using NH_3 solution of concentration 3.2 M, instead of NaOH.

2.1.4. Sodium alginate beads (N-4)

4% sodium alginate solution was prepared in distilled water with vigorous stirring for 1 h. The solution was added dropwise in 150 ml of 4% $CaCl_2$ solution with stirring and allowed to harden for 30 min. The alginate beads were rinsed with distilled water, filtered and dried an oven at 60 °C for 6 h.

2.1.5. Chitosan-sodium alginate-$CaCl_2$ beads (N-5)

A chitosan 3% solution, prepared same as above (refer N-1) and 4% $CaCl_2$ solution were mixed together with constant stirring. A sodium alginate 4% solution was prepared same as above (refer N-4) and then added dropwise to the chitosan-$CaCl_2$ solution with constant mild stirring at room temperature. It was further stabilized for 30 min. The beads obtained were filtered, washed with distilled water and dried in an oven at 60 °C for 6 h.

2.1.6. Multilayered beads (N-6)

The alginate beads were prepared, same as above (refer N-4), and then immersed in 3% chitosan solution to form a layered structure around the alginate beads and were allowed to stabilize for 30 min. The beads were filtered, rinsed with distilled water and dried in an oven at 60 °C for 6 h.

2.1.7. Chitosan-polyvinyl alcohol (PVA) beads (N-7)

First 6 g PVA was dissolved in 100 ml of distilled water at 70 °C and stirred for 5 h. A chitosan 3% solution was prepared and left for 24 h at room temperature for aging. Chitosan and PVA solutions were mixed and maintained at 70 °C for 2 h. The resulting solution was agitated for 48 h at room temperature to make a homogeneous solution. The solution was added dropwise into 500 ml of 0.5 M NaOH solution and the beads formed were filtered, washed with distilled water and dried in air.

2.1.8. Chitosan-clay beads (N-8)

Bentonite clay (2 g) was added to a 3% chitosan solution and stirred vigorously. This mixture was added dropwise to 50% (v/v) NH_3 solution and stabilized for 1 h. The ammonia solution was prepared by admixing NH_3 solution (25%) with water in 1:1 ratio thus obtaining a ammonia solution having molarity 3.2 M. The beads obtained were filtered, washed with distilled water, and then dried at 80 °C for 4 h.

2.1.9. Chitosan-meso alumina beads (N-9)

Aluminium nitrate (4.6 g) was dissolved in 20 ml distilled water and added to 3% chitosan solution with constant stirring at room temperature. The chitosan to alumina molar ratio was maintained between 1.5 and 2. The chitosan-alumina solution was added dropwise to 50% (v/v) NH_3 solution with vigorous stirring. The gel macrospheres were taken out, washed with distilled water and dried at 50 °C for 6 h.

2.1.10. Chitosan-NaOH-glutaraldehyde crosslinked beads (N-10)

Chitosan solution (3%) was added dropwise into 250 ml of 1 M NaOH solution with continuous stirring. The chitosan beads thus formed were allowed to stabilize in the NaOH solution for 1 h at room temperature and then filtered. The wet beads were left in 5% glutaraldehyde solution and left for 24 h. The beads were filtered, washed with distilled water and dried in an oven at 60 °C for 6 h.

2.1.11. Chitosan-NH_4OH-glutaraldehyde crosslinked beads (N-11)

Chitosan solution (3%) was added dropwise to 250 ml 50% (v/v) NH_3 solution with vigorous stirring. The chitosan beads thus formed were allowed to stabilize in the NH_3 solution for 1 h at room temperature and then filtered. The wet beads were left in 5% glutaraldehyde solution and left for 24 h. The beads were filtered, washed with distilled water and dried in an oven at 60 °C for 6 h.

2.2. Microorganism (Bacillus pumilus) and cultural conditions

A strain of *B. pumilus* (TS1) was obtained from the University Department of Microbiology, Delhi University, South Campus, New Delhi. The bacterial strain was cultivated in 5 l glucose-casein-peptone broth (g/l: glucose, 10.0: casein, 3.0: peptone, 5.0, pH 9.0)

in an Applikon (Netherlands) fermenter at 37 °C and 200 rpm for 24 h. The biomass thus obtained after was centrifuged and used for cell immobilization.

2.3. Immobilization of microorganism (B. pumilus) and CA on materials

To 500 μl culture and 100 ml broth, 1 g of material was added into tubes and incubated for 6 h at 37 °C. After incubation, the slurry was centrifuged and the adsorbed microorganism was collected and washed thrice with sterile distilled water. After washing, samples were suspended in phosphate buffer (0.1 M, pH 7). The sample was sonicated for 30 s and then centrifuged at 7000 rpm for 10–15 min. The supernatant was again centrifuged at 7000 rpm for 10 min. The esterase activity of carbonic anhydrase was estimated spectrophotometrically at 348 nm. Fig. 1 describes in detail the protocol for immobilization of microorganism.

Extracellular carbonic anhydrase from *B. pumilus* TS1 was prepared by centrifuging the culture broth at 8000 rpm for 20 min, and concentrating the CA from the supernatant by acetone (20–60% saturation) precipitation. The precipitate was lyophilized. A gram of the lyophilized powder contained 6840 units of CA. Fig. 2 illustrates the steps for immobilization of the enzyme on materials.

2.4. Esterase activity

Esterase activity was measured spectrophotometrically using p-nitrophenyl acetate as a substrate according to the method described by Armstrong et al. [18], with a slight modification. The assay system consisted of 0.2 ml enzyme (1 mg/ml) in a 1 cm spectrophotometric cell containing 1.8 ml of 100 mM phosphate buffer (pH 7) and 1 ml of 3 mM p-nitrophenyl acetate. The change

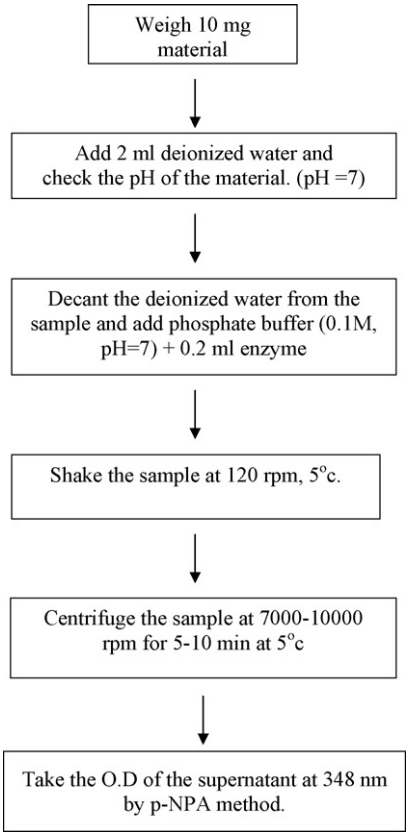

Fig. 2. Steps involved in immobilization of carbonic anhydrase on materials.

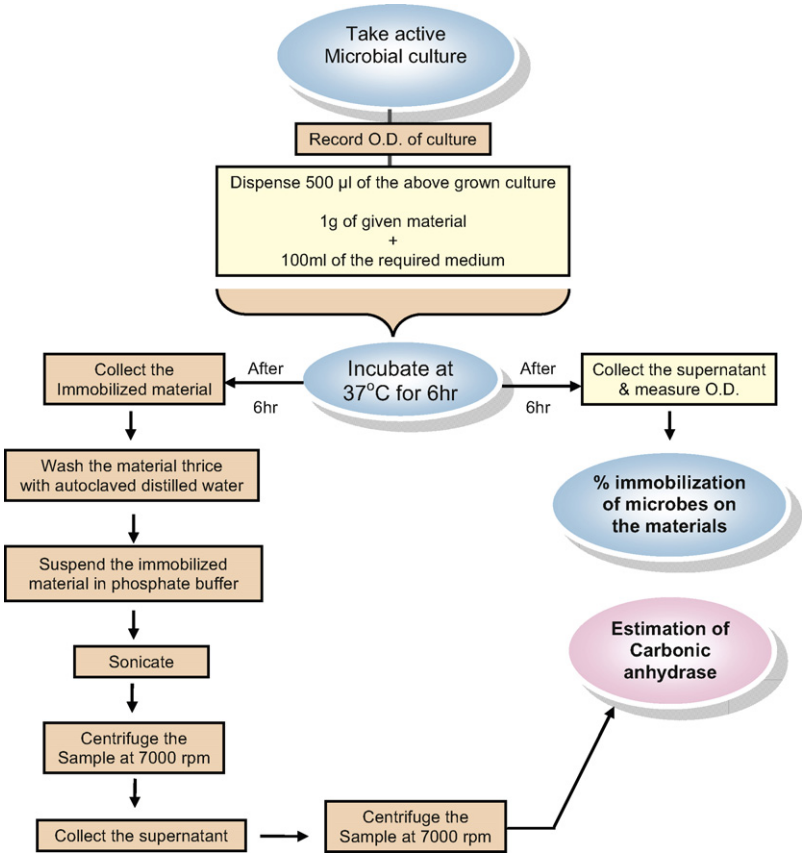

Fig. 1. Protocol for immobilization of microorganism.

in absorbance at 348 nm was measured over 5 min, before and after adding immobilized enzyme or cells. In all the immobilized enzyme preparation (N-1 to N-11) same amount has been added to the assay mixture. One unit of enzyme activity was expressed as 1 μmol p-nitrophenol released per minute at room temperature.

2.5. Determination of percent immobilization

Percent immobilization was determined from the difference in enzyme activity in the solution before and after the immobilization.

$$\text{immobilization yield}\,(\%) = \left(\frac{X}{A-B}\right) \times 100$$

where A = added enzyme, B = free enzyme, and X = immobilized enzyme.

2.6. Characterization of materials

FTIR spectra of beads (1 wt%) mixed with KBr pellets were recorded on a Bruker Vertex-70 apparatus by diffused reflectance accessory technique. Spectra of all the materials were scanned in the range 400–4000 cm^{-1}. X-ray diffraction pattern of the matrices were obtained by using a (PANalytical) X-ray diffractometer (Model no. TW 3660/50), with Cu Kα radiation (λ = 1.54060 Å) at 45 kV and 40 mA and scanned over the range of diffraction angle 2θ = 10–80°. The shape and surface characteristics of matrices were studied by scanning electron microscope (SEM). The specific surface area and pore volume of materials were measured by gas adsorption by following the BET (Brunauer, Emmett and Teller) method. A vario EL CHNS Elemental analyzer was used to determine the content of carbon, hydrogen and nitrogen in chitosan, alginate and multilayered beads. The size of chitosan-based materials was measured by the Particle Size Distribution method using CILAS 1180 Liquid instrument.

3. Results and discussion

3.1. Immobilization studies

3.1.1. Screening of materials for immobilization of cells and partially purified enzyme

Table 1 shows the screening results of the materials with respect to cells and partially purified enzyme. In this study, the cells and the enzyme were immobilized on different materials. The table shows the maximum activity for cell immobilization in N-3 (chitosan-NH_4OH beads) followed by N-4 (sodium alginate beads) and N-6 (multilayered beads). These materials have been selected for further studies.

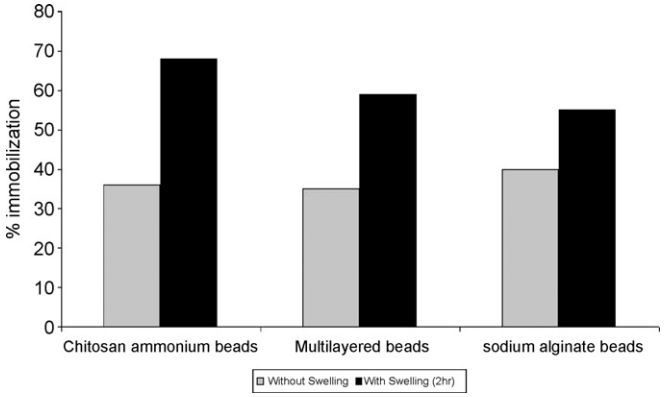

Fig. 3. Percent immobilization of immobilized cells as affected by swelling.

However, almost all materials showed reasonably good activity for enzyme immobilization with the highest activity observed for N-8 (chitosan-clay beads) followed by N-1 (chitosan-NaOH beads) and N-3 (chitosan-NH_4OH beads). N-3, N-4, and N-6 seem to be good materials for immobilization of cell as well as enzyme. N-1 and N-8 showed preference for enzyme immobilization as compared to whole cell. This may be attributed to the presence of increased level of aluminol (AlOH), silianol (SiOH) and hydroxyl groups for enzyme immobilization. However, the presence of clay in N-8 (chitosan-clay beads) material seems to suppress the activity of the material for whole cells to adhere. Clay as an admixture does not adsorb whole cell whereas it appears to adsorb the enzyme. A similar trend was observed for N-1 (chitosan-NaOH beads) and N-2 (chitosan-KOH beads) beads.

In the category of crosslinked chitosan beads, N-10 (chitosan-NaOH-glutaraldehyde crosslinked beads) and N-11 (chitosan-NH_4OH-glutaraldehyde crosslinked beads) show low enzyme immobilization. The substantially low adsorption of whole cell and enzyme on N-10 warrants further investigation. N-11 shows reasonably good adsorption for whole cell.

3.1.2. Effect of swelling on immobilization

The percent activity of immobilized cells as affected by swelling of the beads is shown in Fig. 3. The untreated and unexposed chitosan-NH_4OH beads show low percent of immobilization as compared to beads exposed to water. This may be attributed to non exposure of the group such as free amino and hydroxyl groups on the surface of the chitosan. On exposure, the beads swell and the amino and hydroxyl groups are fully exposed. This leads to more

Table 1
Comparison between CA activity of *B. pumilus* and partially purified CA after immobilization on materials.

Serial no.	Materials	Esterase activity in U/ml	
		Cell immobilization	Enzyme (partially purified CA) immobilization
0	Free culture/enzyme	27.2	59.4
Biopolymer based materials			
1	N-1	3.0	51.8
2	N-2	–	39.4
3	N-3	42.0	47.2
4	N-4	30.5	38.2
5	N-5	24.0	36.6
6	N-6	36.0	35.1
7	N-7	16.5	–
8	N-8	–	54.9
9	N-9	9.0	3.2
10	N-10	13.5	12.3
11	N-11	21.0	10.8

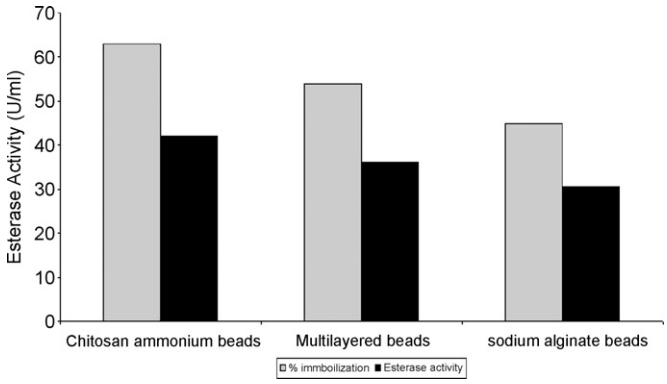

Fig. 4. Percent immobilization and esterase activity of *B. pumilus* on matrices.

adsorption of microbial cells on the surface. In other two materials, namely, alginate and multilayered beads, these groups are either absent or masked leading to less adsorption of microbial cells on the surface and hence a lower percent of immobilization.

3.1.3. Percent immobilization and esterase activity of B. pumilus on matrices

The esterase activity and percent immobilization on chitosan-NH_4OH, alginate and multilayered beads are shown in Fig. 4. Percent immobilization is the highest for chitosan-NH_4OH beads followed by alginate and multilayered beads. This may be due to relatively high number of free amino and hydroxyl groups in chitosan-NH_4OH beads as compared to alginate and multilayered beads, where these groups are either absent or involved in crosslinking. The presence of amino and hydroxyl groups facilitates the adsorption of enzyme and microorganisms.

3.2. Characterization of materials

3.2.1. Shape and size

The beads (N-1 to N-11) formed are spherical in shape with approximate particle diameter of 1.1–1.7 mm.

3.2.2. XRD analysis

From the X-ray diffraction results of chitosan flakes and chitosan beads as shown in Fig. 5, it is observed that there are two major peaks at $2\theta = 10^\circ$, 20° in chitosan flakes and beads. The degree of crystallinity decreases in chitosan beads as compared to chitosan

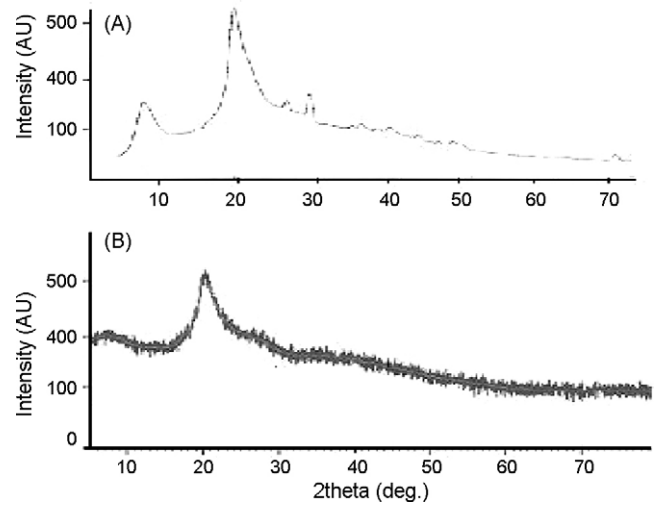

Fig. 5. XRD spectra of (A) chitosan flakes and (B) chitosan-NH_4OH beads.

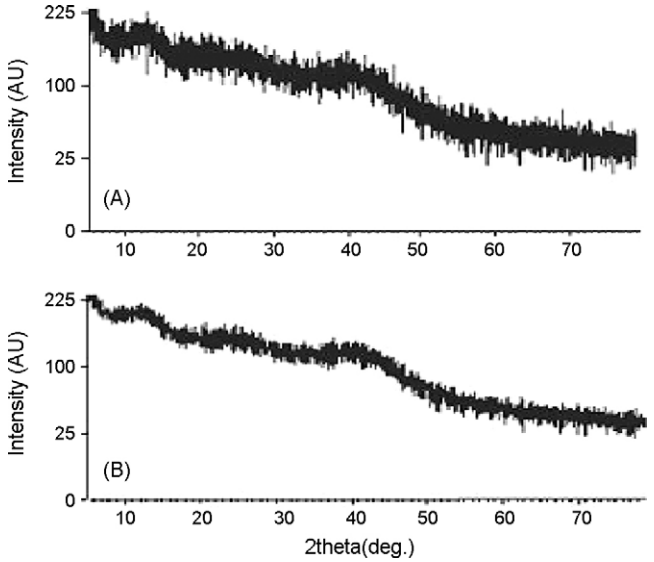

Fig. 6. XRD spectra of (A) alginate beads and (B) multilayered beads.

flakes. The XRD pattern, shown in Fig. 6, suggests that alginate and multilayered beads are largely amorphous in nature. Fig. 7 shows the low angle XRD pattern of chitosan-clay beads. The XRD pattern of chitosan-clay beads has two reflection peaks at about 6.84° and 12.18°, which may be due to the replacement of Na^+ in clay by H^+ ions in chitosan matrix. When clay is placed in weak and diluted acids, an exchange reaction of interlayer sodium ions in clay by protons in chitosan takes place. The characteristic peak of chitosan was observed at 19.87°. The Al and Si atoms in clay show characteristic peaks at $2\theta = 25.00^\circ$ and 29.36°.

3.2.3. FTIR analysis

Chitosan is a heteropolymer made up of glucosamine and acetyl glucosamine units. The functional groups of chitosan are amino and hydroxyl groups which are very important for immobilization of cells. The FTIR spectra of chitosan flakes and beads are shown in Fig. 8. The band at 3694 cm^{-1} in chitosan flakes is attributed to stretching vibration of N–H group, which shifted 3653 cm^{-1} in chitosan-NH_4OH beads. The hydroxyl group in chitosan flakes detected at 3298 cm^{-1} is shifted to 3308 cm^{-1} in beads. This shift of band may be due to the formation of weak intermolecular hydrogen bonding between amino and hydroxyl groups of chitosan. As reported by Paulino et al. [19], in chitosan-NH_4OH beads and flakes, the band at 1575 cm^{-1} has a larger intensity than that at 1676 cm^{-1}, which suggests effective deacetylation. The peaks at 2918 and 1321 cm^{-1} in chitosan flakes and at 2888 and 1407 cm^{-1} in chitosan beads are attributed to C–H stretching vibration in polymeric backbone and C–H bending respectively.

The FTIR spectra of alginate and multilayered beads are shown in Fig. 9. The bands at 3775 and 3707 cm^{-1} in both the spectra are attributed to O–H groups. The peak at 2889 cm^{-1} in alginate is attributed to stretching vibration of C–H group is slight shift to 2894 cm^{-1} in multilayered beads may be due to interaction between alginate and chitosan. The bands at 1723 cm^{-1}, and 1674 cm^{-1} for alginate beads and multilayered beads are attributed to COO^- stretching vibration. The band at 1591 cm^{-1} for alginate beads has shifted to 1601 cm^{-1} in multilayered beads; which may be due to chitosan interaction at the surface of alginate beads [10].

The FTIR spectra of chitosan beads and chitosan-clay beads are shown in Fig. 10. Both the spectra show the strong absorption peak at $\lambda = 3691$ cm^{-1} which corresponds to the vibration of protonated amine group (δNH_3). This correlation strongly suggests that the $-NH_3$ group in the chitosan intercalated with the negatively

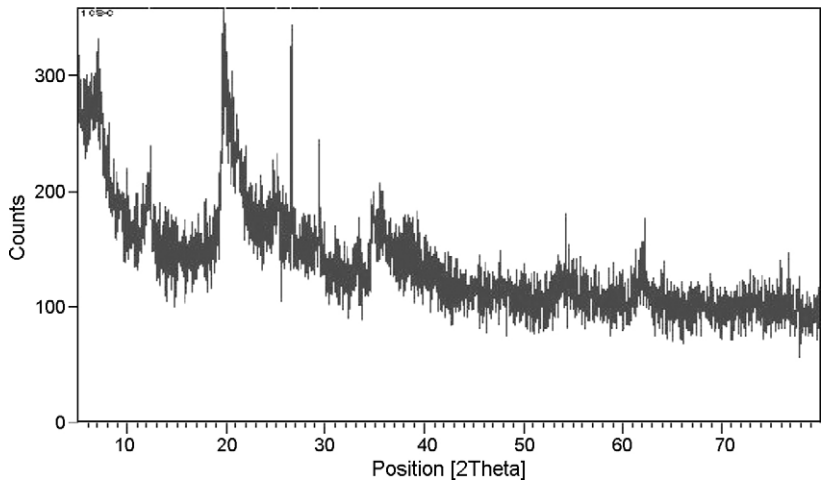

Fig. 7. XRD spectra of chitosan-clay beads.

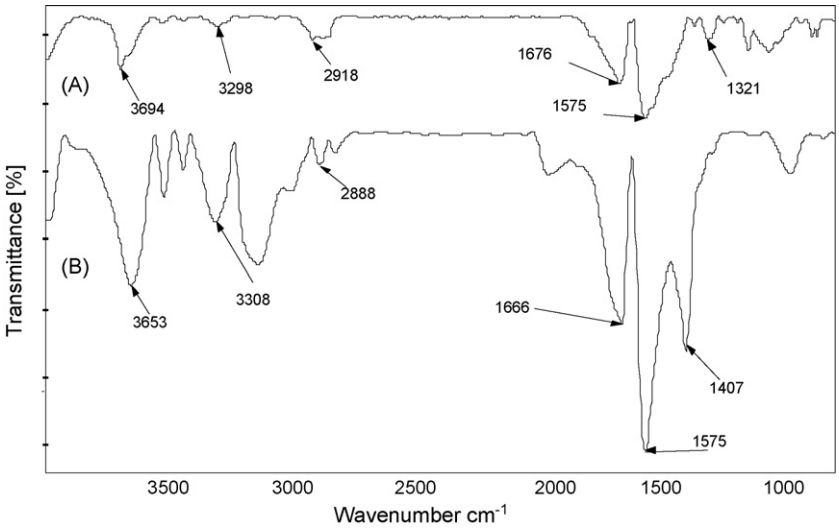

Fig. 8. FTIR spectra of (A) chitosan flakes and (B) chitosan-NH_4OH beads.

charged site of clay surface. The broad band at 3528–3295 cm^{-1} may be attributed to O–H stretching vibration, with increased hydrogen bonding between hydroxyl and organic groups in chitosan and clay matrix. The FTIR spectra of chitosan-clay beads shows strong band at 1629 cm^{-1} due to H–O–H bending, 896 cm^{-1} due to Al–OH and 530 cm^{-1} due to Si–O bending vibration.

Chitosan has one amino group and two hydroxyl groups in the repeating unit. This is substantiated by the above observed IR peaks

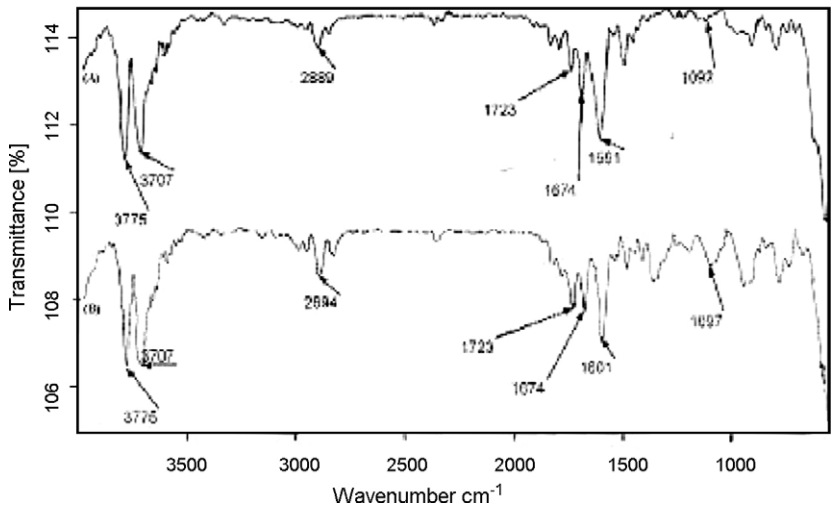

Fig. 9. FTIR spectra of (A) alginate beads and (B) multilayered beads.

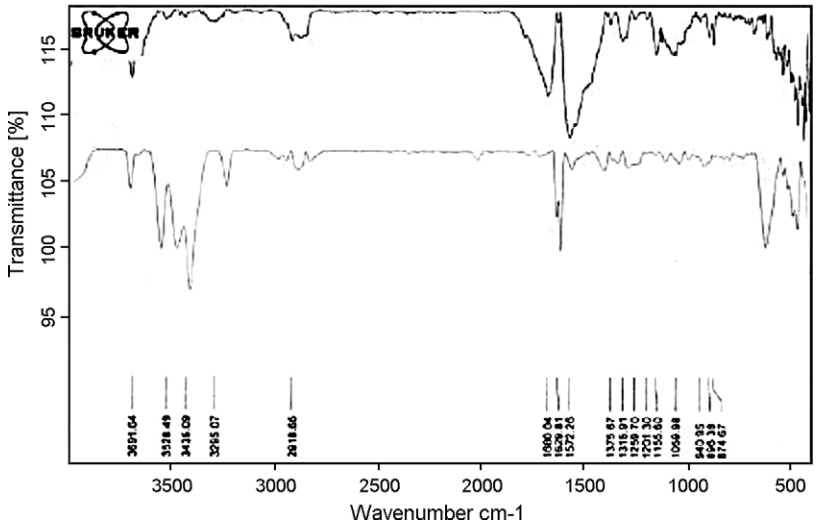

Fig. 10. FTIR spectra of chitosan-clay beads.

of 3694, 3298 cm^{-1}. It has strong intra and intermolecular hydrogen bonding between the polymer chains which decreases their reactivity towards enzymes and microorganisms. An advantage of chitosan is the high hydrophilic character of the polymer due to the large number of hydroxyl groups present on its backbone which is also substantiated by the presence of the 3298 cm^{-1} IR peaks in the above spectra. However, the low porosity of chitosan flakes is an important limiting parameter for the adsorption of enzyme and microorganisms.

This problem of low porosity has been overcome by gel bead conditioning, which decreases the polymer crystallinity, allows the expansion of porous structure network, which in turn is expected to enhance the adsorption of enzyme and microorganisms. This has been observed in the case of N-1, N-3.

Chitosan is an easily soluble polymer in dilute aqueous solutions of organic and mineral acids. Crystallinity controls polymer hydration, which in turn determines the accessibility to internal sites. The dissolving of the polymer breaks the hydrogen bonds between polymer chains and increases the number of reactive amino and hydroxyl groups for adsorption of enzyme and microorganisms. The protonation of amino groups causes the polymer to dissolve in acidic solutions. The polyelectrolyte solutions formed have limited application because bioactive agents may be affected by acid. Hence an alkaline solution treatment process is being given to remove the excess acid. Chitosan beads were, therefore, prepared in NaOH (N-1) and NH_4OH (N-3).

In chitosan beads a key feature is its positive charges on the amino groups at C-2. Chitosan binds to the bacterial outer membrane because of the membrane negatively charged carboxyl and phosphate groups. The outer membrane is thereby disrupted, leading to cellular and enzyme damage or even death of cell [20,21], which has been observed for N-1, wherein no activity was observed. A reverse trend was observed in the case of N-3, which maybe due to lesser damage to cell/enzyme. This damage to the cell could be suppressed by decreasing the amino groups by immobilizing chitosan onto clay and alginate beads. This may slightly decrease the adsorption of cell but the activity would be retained. Similar findings have been observed in the cases of N-3, N-4, and N-6. The high adsorbing ability of chitosan makes it useful for bacterial concentration while retaining viability, which is observed for N-4 and N-6. It has been reported that almost all microorganisms express surface-exposed carbohydrates [22]. The carbohydrates may be covalently bound to peptidoglycan, or non-covalently bound, as in capsular polysaccharides. Every surface-exposed carbohydrate is a potential lectin-reactive site. The ability of lectins to complex with microbial glycoconjucates has made it possible to employ the proteins and related materials like chitosan as sorbents for whole cells, which substantiates the research findings of this work.

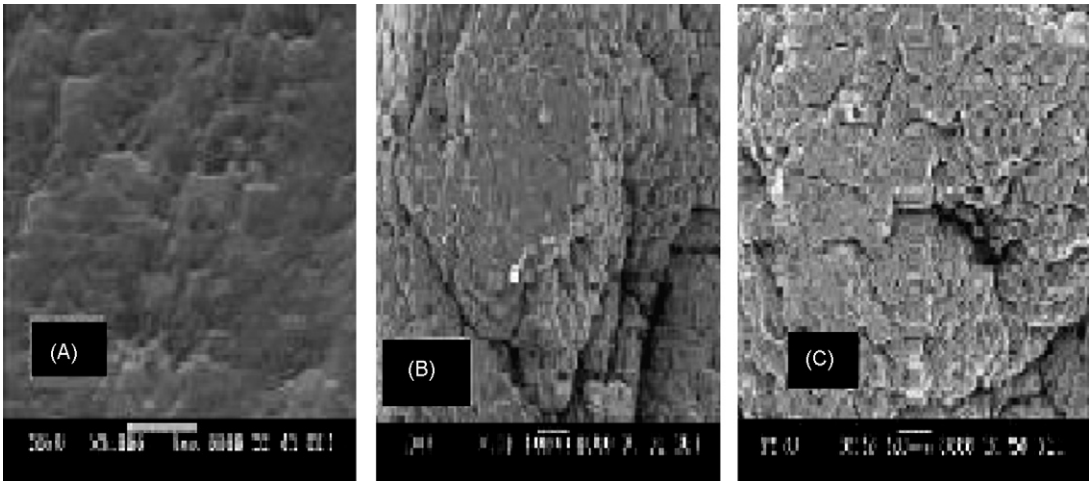

Fig. 11. SEM images of (A) chitosan-NH_4OH beads, (B) alginate beads and (C) multilayered beads.

Table 2
Surface area and pore volume of immobilization matrices.

Sample	BET surface area ($m^2\ g^{-1}$)	Avg. pore diameter (Å)	Pore volume ($\times 10^{-6}\ m^3\ g^{-1}$)	% Immobilization
Chitosan-NH_4OH beads	2.29	111	0.0062	63.20
Alginate beads	2.24	34	0.0027	44.19
Multilayered beads	2.34	109	0.0064	54.00

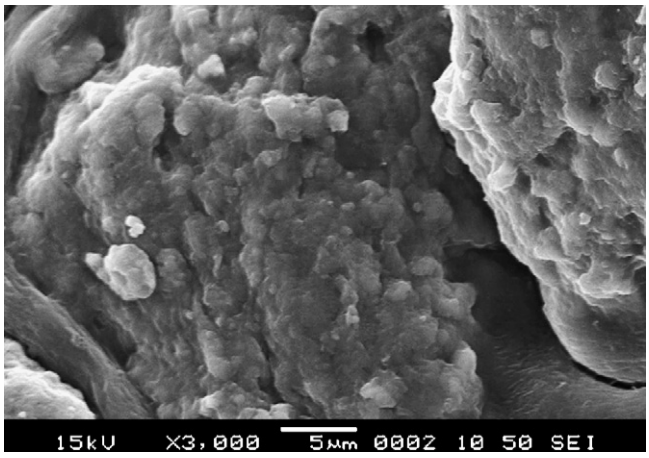

Fig. 12. SEM images of chitosan-clay beads.

3.2.4. SEM analysis

The surface morphologies of chitosan-NH_4OH, alginate, multilayered and chitosan-clay beads were studied using SEM and the images are shown in Figs. 11(A–C) and 12. It is observed that chitosan-NH_4OH beads have a spongy surface. Comparison of the SEM images of alginate beads and multilayered beads shows that the surface texture of multilayered beads changes due to the incorporation of chitosan. For the alginate beads, the surface is found to be rough as compared to the multilayered beads.

SEM of N-3 (A) shows a smooth surface with porous network formation, which substantiated the adsorption of enzyme and microorganisms. SEM of N-4 (B) shows a rougher surface as compared to N-3. SEM of N-6 (C) shows a much higher rough surface as compared to N-3 and N-4. There does not appear to be any correlation between the adsorption of enzyme or microorganism and the degree of roughness observed in the sample. However, there is a slight decline with decreasing free amino groups due to the incorporation of clay, alginate, etc.

The surface morphology of chitosan-clay beads was studied using SEM and is shown in Fig. 12. Clay forms intercalated and flocculated structure in between the chitosan matrix. The SEM image of chitosan-clay shows the flocculated structure due to hydroxylated edge–edge interaction of the silicate layer with chitosan. Chitosan unit possesses one amino and two hydroxyl groups; these functional groups can form hydrogen bonds with the silicate hydroxylated edge groups, which lead to a strong interaction between matrix and silicate layer. This strong interaction between chitosan and clay matrix results in the flocculated structure.

3.2.5. Measurement of specific surface area by using BET method

The specific surface area and pore diameter of matrices have been measured by using the BET method and the observed values are given in Table 2. The presence of functional groups like amino etc facilitates the adsorption of microorganism on chitosan-NH_4OH beads and multilayered beads.

Table 3
Elemental analysis.

Sample	Carbon (%)	Hydrogen (%)	Nitrogen (%)
Chitosan-NH_4OH beads	36.25	5.328	5.720
Alginate beads	23.73	3.139	0.000
Multilayered beads	26.86	4.199	0.594

3.2.6. Elemental analysis

The elemental analysis of carbon, hydrogen and nitrogen contents for chitosan-NH_4OH and other modified polymer beads are summarized in Table 3. The carbon and nitrogen contents in chitosan-NH_4OH beads are significantly high due to the presence of amino groups, which seem to facilitate the immobilization. The multilayered beads have lower percent immobilization due to crosslinking of chitosan with alginate [23].

4. Conclusions

CA enriched microbial cell and partially purified CA have been immobilized on different biopolymer based materials. Out of these materials, chitosan-NH_4OH beads, multilayered beads and alginate beads are showing reasonably good affinity for enzyme as well as for whole cell. The highest immobilization activity of whole cell on chitosan-NH_4OH beads (42 U/ml) is due to the presence of hydroxyl group on the surface, which facilitates the adsorption of microbial cell more as compared to other groups. These groups are either absent or crosslinked in other materials. Functionalized materials do not show significant improvement in percent immobilization of cells as compared to non-functionalized material. Further investigation to use increased loading of functional molecule and newer functional molecule is in progress. The short listed materials with better affinity for whole cell and enzyme are being studied for deducing Michaelis constant, half-life period, etc.

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