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Chitosan and its amino acids condensation adducts as reactive natural polymer supports for cellulase immobilization

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ABSTRACT

Cellulase [1,4-(1,3;1,4)-β-D-glucan 4-glucanohydrolase], was immobilized directly on chitosan, chitosan–L-glutamic acid and chitosan–4-amino butyric acid by covalent attachment and cross-linking methods. The properties of the immobilized cellulase were investigated and compared with those of the free one. For the assays carried out via cross-linking method at 25 °C and pH 7, the retained activities were found to be 65.52%, 85.32% and 63.19% for chitosan, chitosan–L-glutamic acid and chitosan–4-aminobutyric acid cross-linked with 1% of glutaricdialdehyde (GDA), respectively. The immobilized cellulase had better stability and higher retained activities with respect to pH, temperature and storage stability than the free one. In the repeated use experiments, the immobilized cellulase using chitosan–GDA (1%) retained about 60% from its original activity after 6 times. In contrast, the activities of immobilized cellulase on chitosan–L-glutamic acid–GDA (1%) and chitosan–4-aminobutyric acid–GDA (1%) did not change after 10 and 7 times respectively. The retained activities after 25 times reusability were 70% and 50% from their original activity for the aforementioned carriers respectively.

1. Introduction

Enzymes are largely used as biocatalysts in chemical, pharmaceutical and food industries. Since the recovery and the reusability of the free enzyme are quite limited (Bulmus, Kesenci, & Piskin, 1998), attention has been paid to enzyme immobilization which may offer advantages over free enzymes; for example, possibility of continuous process, rapid termination of reactions, controlled product formation, ease of enzyme removal from the reaction mixture, and adaptability to various engineering designs (Cao, Langen, & Sheldon, 2003).

The basic idea behind enzyme immobilization is to entrap the protein in a semi-permeable support material, which prevents the enzyme from leaving while allowing substrates, products, and cofactors to pass through. Although the exact requirements for the immobilizing matrix are dictated by the type of enzyme and the intended application, it is clear that the material should at least be compatible with the enzymes. The process for immobilization should also be mild enough so as not to denature the enzyme during preparation (Ehab & Mansoor, 2004). When an immobilized enzyme is used in vivo, the support material should also prevent immune recognition, especially if the enzyme is of non-human origin (Liang, Li, & Yang, 2000).

Methods available for enzyme immobilization can be divided into two general classes: chemical methods, where covalent bonds are formed with the enzyme, and physical methods, where weak interactions between support and enzyme exist. The procedures involved in physical adsorption are quite simple, making it one of the most widely used methods of enzyme immobilization (Panzavolta et al., 2005). Protocols for covalent enzyme immobilization often begin with a surface modification or activation step. The surface can be bound to aldehyde groups using glutaricdialdehyde (Krajewska, Leszko, & Zaborska, 1990).

Cellulase [1,4-(1,3;1,4)-β-D-glucan 4-glucanohydrolase, EC 3.2.1.4] (Chittra, Vasanti, & Mala, 1983), can be used as the biocatalyst for cellulose hydrolysis. The cost of cellulase-based technology can be reduced by increasing the enzyme reusability and its stability. These may be accomplished by enzyme immobilization on suitable carriers (Jain & Wilkins, 1987).

As carriers for enzyme immobilization, polymeric membranes have been used extensively since they can have various functional groups and be easily modified chemically (Godjevargova, Konsulov, & Dimov, 1999). One of the most important polymeric supports for enzyme immobilization is the natural ones such as agarose and chitosan which have been reported in previous studies in case of β-amylase and α-amylase, respectively (Abd El-Ghaffar and Hashem, 2009; Abd El-Ghaffar et al., 2003). Chitosan, a cationic biopolymer consisting of (1,4)-linked 2-amino-deoxy-β-D-glucan, is a deacetylated derivative from chitin, the second most abundant polysaccharide in nature after cellulose (Amorim, Melo, Carneiro-da-Cunha, Ledingham, & Campos-Takaki, 2003). Chitosan

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is an inexpensive, inert, hydrophilic, biocompatible support, and is thus attractive for enzyme immobilization (Kumar, 2000; Yazdani-Pedram, Retuert, & Quijada, 2000). The presence of amino groups facilitates covalent binding of enzymes (Şenay and Öztop, 2003).

This study outlines the preparation of chitosan amino acids condensation products, immobilization of cellulase into chitosan and its modified compounds by covalent attachement and cross-linking methods, and investigation the operational, thermal and storage stability of the immobilized cellulase.

2. Experimental

2.1. Materials

Acid cellulase (EC 3.2.1.4 from *Aspergillus niger* with activity 108 U/g) and sodium dihydrogen phosphate anhydrous from Fluka, Chitosan flakes having 85% degree of deacetylation was purchased from Sigma, 4-aminobutric acid, glutaricdialdehyde (25 wt.% solution in water) and bovine serum albumin were purchased from Aldrich, xylene from Merk, L-glutamic acid, glucose and potassium sodium tartrate were purchased from BDH, carboxymethyl cellulose (CMC) was purchased from Mallinckrodt, 3,5-dinitrosalicylic acid (DNSA) was purchased from Panreac, Folin reagent (2N) was purchased from Fluka and sodium phosphate dibasic anhydrous from El Gomhouria Co., Egypt.

2.2. Methods and techniques

2.2.1. Methods

2.2.1.1. Modification of chitosan. Chitosan and each of the amino acids [L-glutamic acid and 4-aminobutyric acid] were mixed in stoichiometric amounts [equimolar ratios] and subjected to condensation reaction using Dean–Stark apparatus in the presence of xylene until the theoretical amount of water was separated. Chitosan amide products were separated by filtration, washing several times with methanol and hot distilled water, dried in an electric oven at 50 °C and weighed. The nitrogen content for chitosan and its amino acid adducts was determined using Keldal method.

2.2.1.2. Preparation of glutaricdialdehyde supporters. Chitosan and chitosan–amino acids condensates [amide adducts] (0.1 g) were added to GDA solution (10 ml) with different concentrations (0.1, 0.5 or 1 ml) in 100 ml phosphate buffer 7.0, kept in a shaking water bath for 4 h at 25 °C and left at that temperature overnight. The cross-linked supporters were separated and washed 3 times with (5 ml) phosphate buffer pH 7.0.

2.2.1.3. Immobilization of enzymes. Covalent attachment method

Chitosan, and chitosan–amino acids condensates [amide adducts] (0.1 g) were added to the enzyme solution [(1, 2, 3, 4 or 5 mg) in (1 ml, 0.02 M) phosphate buffer pH 7.0] and the immobilization reaction was carried out for 24 h at 25 °C in a shaking water bath. The precipitates were filtered and the unbound enzyme was removed by washing 3 times with (5 ml) phosphate buffer. The immobilized enzymes were stored at 4 °C until use.

Cross-linking method

The previous cross-linked supporters (0.1 g) were added to the enzyme solution [(1, 2, 3, 4 or 5 mg) in (1 ml, 0.02 M) phosphate buffer pH 7.0] and the immobilization reaction was carried out for 4 h at 25 °C in a shaking water bath. The precipitates were filtered and the unbound enzyme was removed by washing 3 times with (5 ml) phosphate buffer. The immobilized enzymes were stored at 4 °C until use.

2.2.1.4. Determination of the amount of immobilized cellulase. The Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951) was used to determine the cellulase content in solution. After the immobilization process, the supernatant and the washing solutions were collected. The cellulase concentration was determined by comparing with the standard curve constructed using bovine serum albumin with known concentrations. The amount of immobilized cellulase was determined from the initial cellulase amount present in the cellulase coupling solution subtracting the final total cellulase amount present in the remaining coupling solution (Rosevaer, 1984). The coupling yield (%) of the cellulase was then calculated from the amount of cellulase coupled on the polymeric carriers by the initial total amount of the cellulase present in the coupling solution according to the following equation:

$$\text{Enzyme coupling yield}\,(\%) = \frac{\text{amount of cellulase coupled}}{\text{amount of cellulase introduced}} \times 100$$

2.2.1.5. Assay of cellulase activity. The activity of cellulase was measured by two methods:

1- Activities of free and immobilized cellulase were determined by incubating the enzyme for 3 min with 0.5% (CMC) in phosphate buffer (0.02 M, pH 7.0) at 25 °C using DNSA reagent as color developing agent (Miller, 1959). The reducing sugar produced was measured spectrophotometrically at 540 nm [using UV/visible recording spectrophotometer (Shimadzu UV-2401 PC double-beam)] with glucose as a standard (Somogyi, 1952) and (Nelson, 1944). One unit (*U*) of activity was defined as the amount of enzyme that produces 1 μmol of glucose equivalent per minute under above conditions. The specific activity of free and immobilized cellulase was calculated according to the following equation:

$$\text{Units/mg} = \frac{\mu\text{mol glucose released}}{3\,(\text{min}) \times \text{mg of cellulase in reaction mixture}}$$

2- Activities of free and immobilized cellulase were determined by incubating 1 ml enzyme buffer indicator solution with 10 μl of sample solution for 10 min in waterbath at 37 °C. The reducing sugar produced was measured spectrophotometrically at 500 nm with glucose kit standard. The specific activity of free and immobilized cellulase was calculated according to the following equation:

$$\text{Glucose concentration}\,(\text{mg/dl}) = \frac{\text{unknown absorbance}}{\text{standard absorbance}} \times 100$$

Conversions: 1 mg/dl glucose equals 55.5 μM, 0.001% or 10 ppm.

The retained activity yields of the immobilized cellulase on the different polymeric supports were calculated according to the following equation:

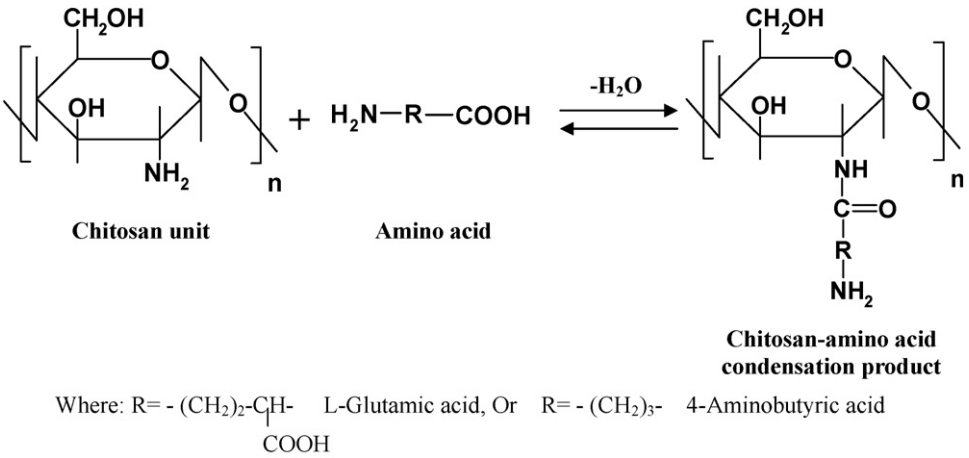
$$\text{Retained activity yield}\,(\%) = \frac{\text{specific activity of immobilized cellulase}}{\text{specific activity of free cellulase}} \times 100$$

All activity measurement experiments were carried out at least twice (generally 3 times) and the relative standard deviations were found to be less than 1%.

2.2.1.6. Parameters affecting cellulase activity. Effect of pH on activity of free and immobilized cellulase

The effect of pH on cellulase activity was investigated in the range 3–9 for both free and immobilized one.

Effect of temperature on activity of free and immobilized cellulase



Scheme 1. The possible reaction between chitosan and the amino acids.

The effect of temperature on cellulase activity was investigated in the range of 30–100 °C for both free and immobilized one.

Storage stability of free and immobilized cellulase

This experiment was carried to determine the stabilities of free and immobilized cellulase after storage in phosphate buffer (0.02 M, pH 7.0) at 4 °C for 90 days. The retained activities were then determined as described above and the activity of each preparation was expressed as a percentage of its retained activity compared to its initial activity.

Reusability of the immobilized cellulase

To evaluate the reusability of the immobilized cellulase, the matrices were washed with water and buffer after use and then suspended again in a fresh reaction mixture to measure the enzymatic activity.

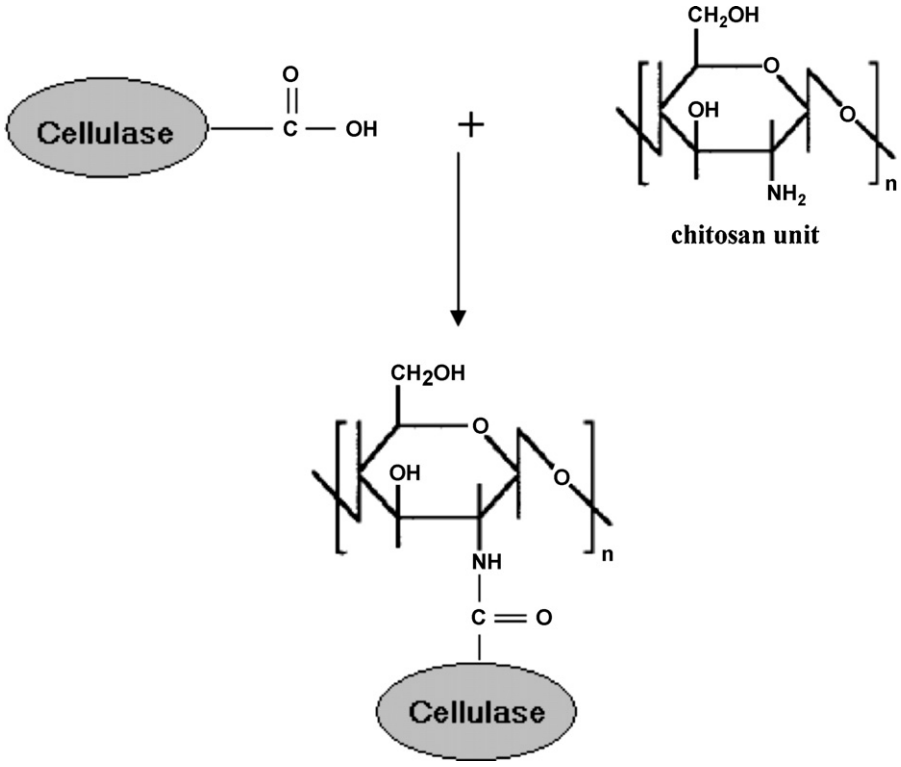
2.2.2. Techniques

FT-IR was measured using FT-IR spectrometer (Nicolet 670, range from 400 to 4000 cm^{-1}, USA), TGA was measured using TGA 7 series (Perkin Elmer, USA), the shapes of the particles were scanned using SEM (JXA-840A Electron probe microanalyzer, Jeol) and UV/Visible spectrum were measured using Shimadzu UV-2401 PC double-beam spectrometer.

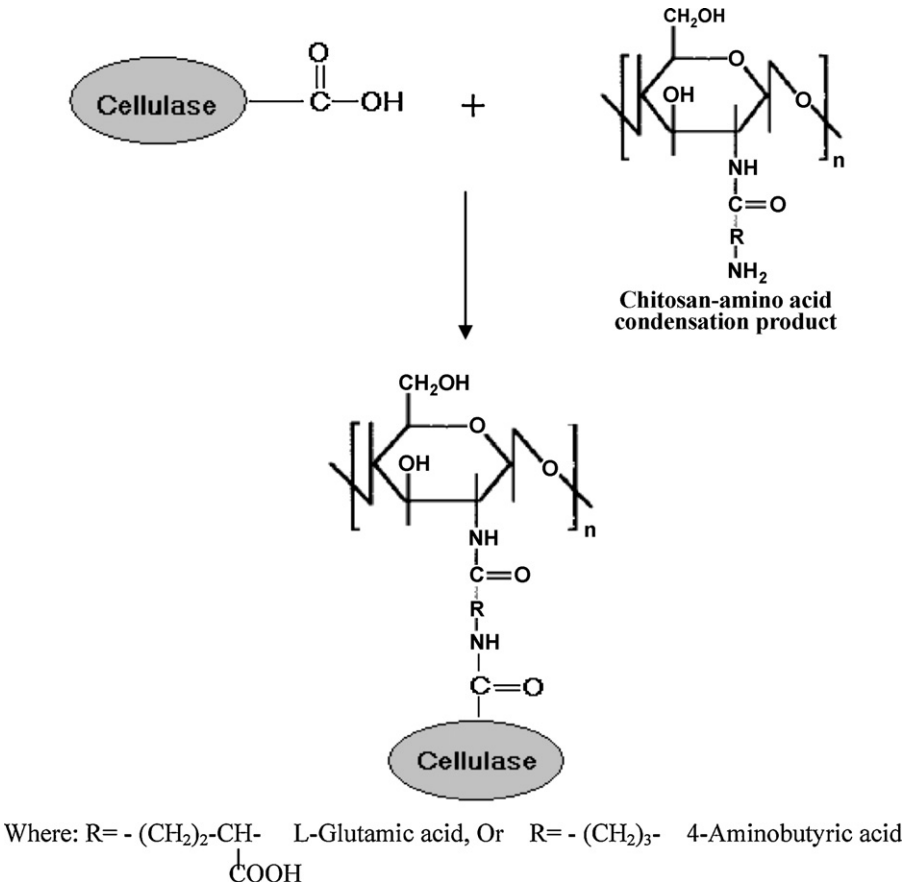
3. Results and discussion

3.1. Modification of chitosan and its amino acids condensation adducts

Stoichiometric amounts of condensation reactants of chitosan (0.025 mol calculated for one unit) with each of L-glutamic acid



Scheme 2. The possible reaction mechanism between chitosan and cellulase via covalent attachment method.



Scheme 3. The possible reaction mechanism between chitosan–amino acids adducts and cellulase via covalent attachment method.

(0.025 mol) and 4-aminobutyric acid (0.025 mol) were used. The reactions were carried out using Dean and Stark apparatus until the theoretical amounts of water were separated (≈0.5 ml). These results are similar to those obtained by (Esparza & Gomez, 2003), where they reacted chitosan with glycine, L-lysine, isoleucine and L-glutamic acid in the presence of dehydrating agent (concentrated H_2SO_4). Scheme 1 shows the proposed chemical reaction of chitosan with the previously mentioned amino acids. These results were confirmed by the data obtained from the nitrogen content which showed; 6.99, 10.06 and 13.19 for chitosan, chitosan–L-glutamic acid adducts and chitosan–4-aminobutric acid adduct respectively.

It can be seen from Scheme 1 that chitosan and chitosan–amino acids adducts have free amino groups (–NH_2) and free carboxylic groups (–COOH) which were reacted directly (both or one of them) with the carboxylic terminal residue in the enzymes. The reaction of chitosan directly with the enzymes, is similar to that was carried out by Abd El-Naby, Isamil, and Abdel-Fattah (1999) but in case of dextranase. On the other hand the literature is scanty concerning the reaction between enzymes and chitosan–amino acids condensation adducts. Schemes 2 and 3 show the possible reactions mechanism between chitosan and chitosan–amino acids adducts with cellulase.

The amino groups in chitosan and chitosan–amino acids adducts were reacted with GDA as a cross-linking agent and then cellulase was bounded to them. The reaction of chitosan and GDA is similar to those have been made by Şenay and Öztop (2000), Wu, Hung, Giridhar, and Chiou (2003), Juang and Min-Yun (2005), Gamze and Senay (2007), and the proposed reactions mechanism between (a) chitosan and (b) chitosan–amino acids with GDA and cellulase are summarized in Scheme 4.

3.2. Characterization of chitosan and its amino acids condensation adducts

3.2.1. FT-IR studies

The FT-IR spectra of the chitosan and its amino acids condensation adducts are shown in Figs. 1–3. Fig. 1 shows characteristic peaks at 1029 cm^{-1} and 1149 cm^{-1} chracteristic for saccharide structure, and two other absorption bands at 3353 cm^{-1} and 3200 cm^{-1} characteristic for the amino group. Figs. 2 and 3 show peaks at 1576 cm^{-1} and 1591 cm^{-1} which indicate the formation of amide bond from the condensation reaction of chitosan with each of L-glutamic acid and 4-aminobutyric acid respectively. The FT-IR spectra of the products formed from condensation reaction of chitosan and its amino acid adducts with glutaricdialdehyde are shown in Figs. 4–6. It can be seen from these figures that there have been significant bands at 1626 cm^{-1}, 1633 cm^{-1} and 1630 cm^{-1},

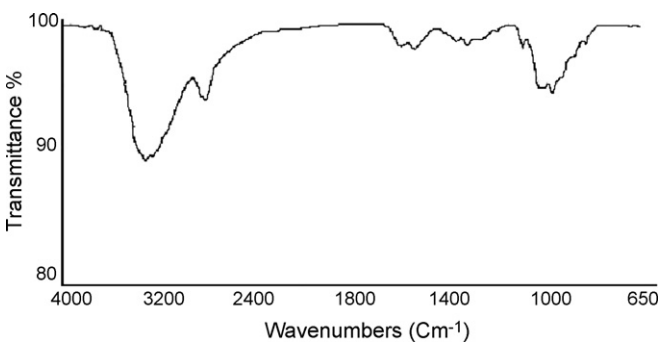
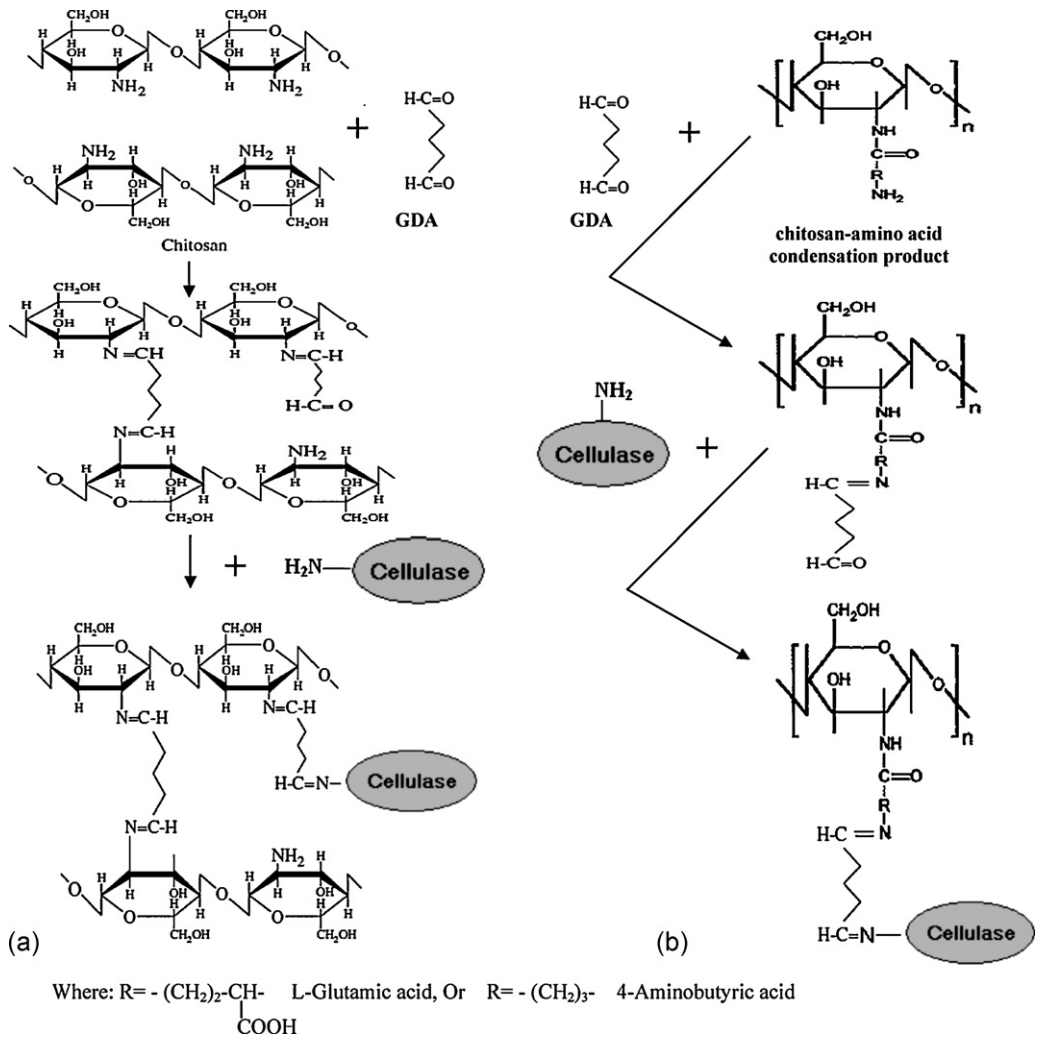


Fig. 1. FT-IR spectrum of chitosan.



Scheme 4. Possible immobilization mechanism of cellulase on (a) chitosan and (b) chitosan–amino acids adducts cross-linked with GDA.

which can be attributed to the characteristic peak of C=N for chitosan, chitosan–L-glutamic acid and chitosan–4-aminobutyric acid condensates with glutaricdialdehyde respectively.

3.2.2. Thermogravimetric analysis (TGA)

Thermogravimetric analysis (TGA) has been measured for chitosan and its amino acids condensation adducts. It is illustrated from Figs. 7 and 8 that, there are small loss in the weight of the samples (7.248% and 9.586%) from 50 °C to 233.63 °C and 200 °C for chitosan and chitosan–4-aminobutyric acid respectively, after which sharp degradation takes place till 325.44 °C

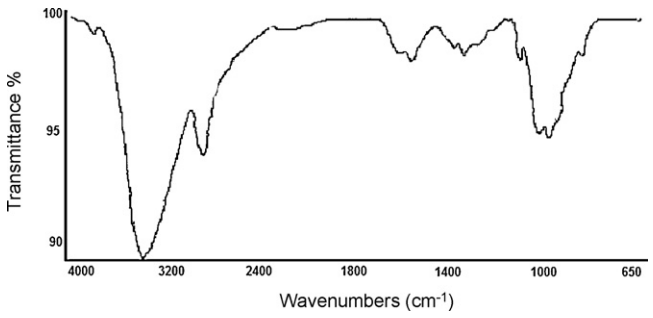


Fig. 3. FT-IR spectrum of chitosan–4-aminobutyric acid.

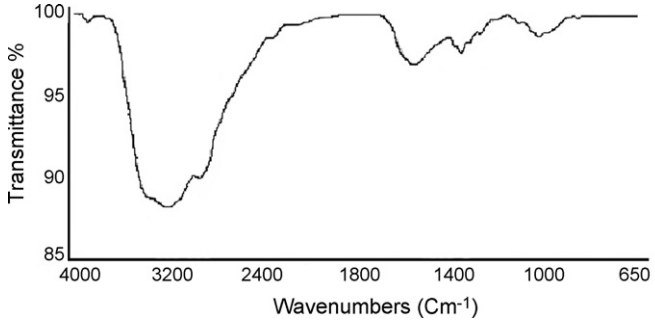


Fig. 2. FT-IR spectrum of chitosan–L-glutamic acid.

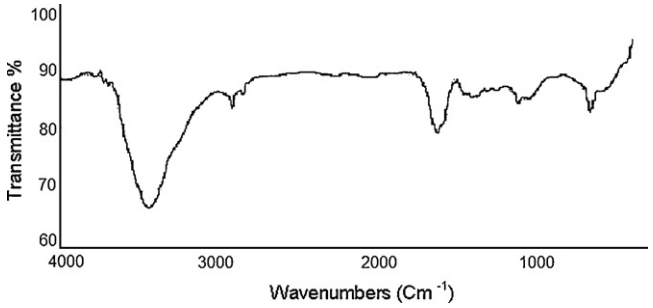


Fig. 4. FT-IR spectrum of chitosan–GDA (1%).

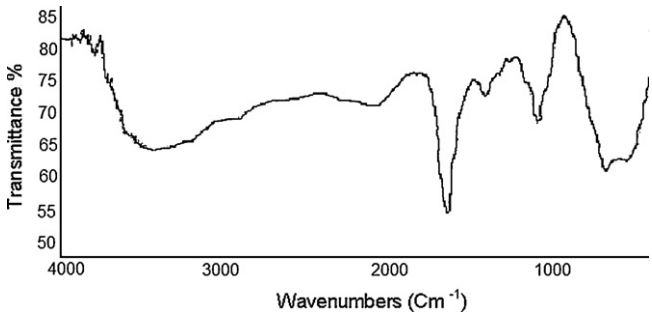


Fig. 5. FT-IR spectrum of L-glutamic acid–GDA (1%).

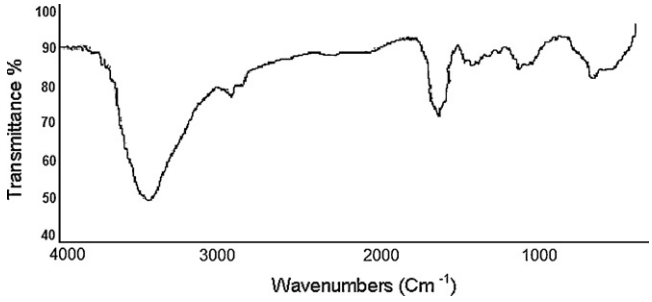


Fig. 6. FT-IR spectrum of chitosan–4-aminobutyric-GDA (1%).

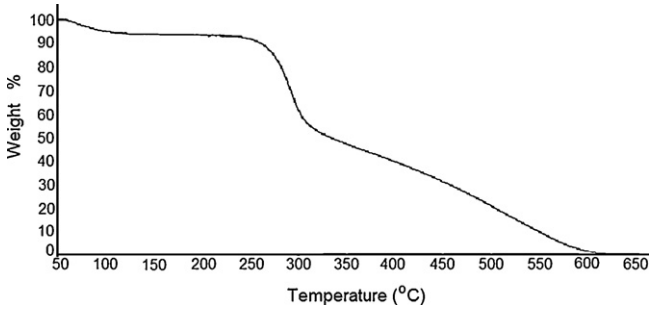


Fig. 7. TGA of chitosan.

and 333.33 °C (weight loss were 41.606% and 43.488%) for chitosan and chitosan–4-aminobutyric acid respectively. Above these temperatures gradual degradation takes place till 625.44 °C and 590.00 °C (weights loss were 51.324% and 43.774%) for chitosan and chitosan–4-aminobutyric acid respectively, after that complete decomposition occurs. In case of chitosan–L-glutamic acid, it was illustrated from Fig. 9 that there is a gradual degradation took place from 75 °C till 471.13 °C (weight loss was 59.563%), another sharp decomposition happened up to 680.6 °C (weight loss was 39.878%), followed by complete decomposition.

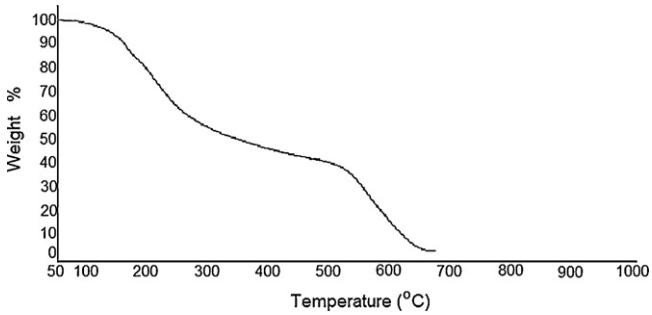


Fig. 8. TGA of chitosan–L-glutamic acid.

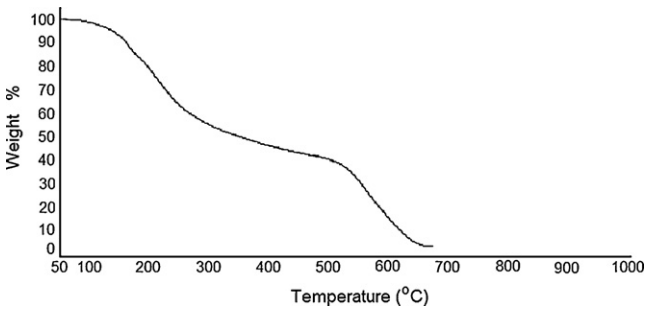


Fig. 9. TGA of chitosan–4-aminobutyric acid.

3.2.3. Scanning electron microscopy (SEM)

SEM micrographs of chitosan, chitosan–L-glutamic acid and chitosan–4-aminobutyric acid show the morphology of these polymers before and after cellulase immobilization. Photo 1a shows that the chitosan before immobilization process have flakes surface morphology, but after immobilization process took place the chitosan surface became fibrous as shown in Photo 1b. On the other hand, Photo 1c and e show that the surface morphology of chitosan–L-glutamic acid and chitosan–4-aminobutyric acid before cellulase immobilization were flaky, and after immobilization process took place, they became smooth surface as shown in Photo 1d and f. All these surface morphology observations demonstrated that the cellulase was successfully immobilized on chitosan, chitosan–L-glutamic acid and chitosan–4-aminobutyric acid.

3.3. Cellulase immobilization

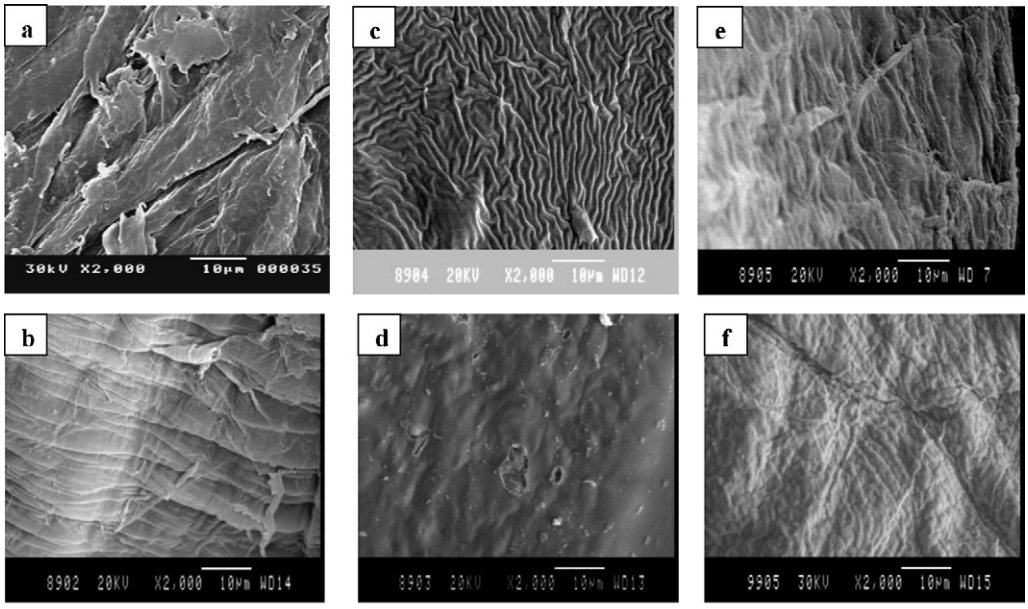
The immobilization of enzymes onto insoluble polymeric supports has been a topic of active research in enzyme technology and is essential for their application to industrial processes. A large number of enzymes were successfully immobilized with very high activity yields on appropriate supports. These immobilized products were intended for use in the construction of artificial organs, biosensors, or bioreactors (Bayramoglu, Akgol, Bulut, Denizli, & Arica, 2003). The selection of support materials and the method of immobilization are very important for carrying out the desired enzymatic reaction. In the present study, a large variety of natural supports have been used as a carries for cellulase immobilization via covalent attachment and cross-linking methods as a two different techniques for the immobilization process.

3.3.1. Covalent attachment method

[Chitosan, chitosan–L-glutamic acid, and chitosan–4-aminobutyric acid] 0.1 g were used for immobilization of cellulase [from 1 mg to 5 mg (108–540 U)] in phosphate buffer pH 7 at 25 °C for an incubation time 24 h. The maximum amount of cellulase activity was reached at concentration of 3 mg (324 U). The effect of cellulase concentration on the immobilization using the previous mentioned supports is shown in Table 1. The retained activities were: 58.09%, 76.17% and 55.40% for chitosan, chitosan–L-glutamic acid and chitosan–4-aminobutyric acid, respectively.

3.3.2. Cross-linking method

0.1 g of chitosan–GDA (0.1%, 0.5% and 1%), chitosan–L-glutamic acid–GDA (0.1%, 0.5% and 1%) and chitosan–4-aminobutyric acid–GDA (0.1%, 0.5% and 1%)] were used for immobilization of cellulase [from 1 mg to 5 mg (108–540 U) in phosphate buffer pH 7 at room temperature for an incubation time 4 h. The maximum amount of cellulase activity was reached at concentration of 3 mg (324 U). The effect of cellulase concentration on enzyme immobilized on the previous mentioned supports is shown in Tables 2–4. The retained activities were: 65.52%, 85.32% and 63.19%



(a) chitosan, (b) chitosan-GDA (1%)-cellulase, (c) chitosan-L-glutamic acid, (d) chitosan-L -glutamic acid- GDA (1%)-cellulase, (e) chitosan-4-aminobutyric acid, (f) chitosan-4-aminobutyric acid- GDA (1%)-cellulase.

Photo 1. SEM of chitosan and its condensation adducts before and after cellulase immobilizations via cross-linking method. (a) Chitosan, (b) chitosan–GDA (1%)–cellulase, (c) chitosan–L-glutamic acid, (d) chitosan–L-glutamic acid–GDA (1%)–cellulase, (e) chitosan–4-aminobutyric acid, (f) chitosan–4-aminobutyric acid–GDA (1%)–cellulase.

Table 1
Effect of cellulase concentration on immobilization using chitosan and its amino condensation adducts via covalent attachment method.

Added enzyme (mg)	Added enzyme (units)	Immobilized enzyme (mg/0.1 g support)			Activity of immobilized enzyme (U/0.1 g support)			Retained activity (%)		
		A	B	C	A	B	C	A	B	C
1	108	0.46	0.65	0.46	50.01	70.54	49.77	46.31	65.32	46.08
2	216	1.06	1.46	1.02	114.87	157.36	110.01	53.18	72.85	50.93
3	324	1.74	2.28	1.66	188.21	246.79	179.49	58.09	76.17	55.40
4	432	2.08	2.84	2.06	224.68	306.81	222.35	52.01	71.02	51.47
5	540	2.35	3.32	2.26	253.80	358.56	243.65	47.00	66.40	45.12

A = chitosan, B = chitosan–L-glutamic acid and C = chitosan–4-aminobutyric acid.

Table 2
Effect of cellulase concentrateon on immobilization using chitosan–GDA via cross-linking method.

Added enzyme (mg)	Added enzyme (units)	Immobilized enzyme (mg/0.1 g support)			Activity of immobilized enzyme (U/0.1 g support)			Retained activity (%)		
		A	B	C	A	B	C	A	B	C
1	108	0.50	0.50	0.54	54.49	54.41	58.69	50.46	50.38	54.34
2	216	1.11	1.15	1.22	120.51	124.09	132.02	55.79	57.45	61.12
3	324	1.81	1.83	1.96	195.18	198.22	212.28	60.24	61.18	65.52
4	432	2.24	2.21	2.44	241.96	239.28	263.43	56.01	55.39	60.98
5	540	2.45	2.51	2.82	264.82	271.62	304.72	49.04	50.30	56.43

A = chitosan–GDA (0.1%), B = chitosan–GDA (0.5%) and C = chitosan–GDA (1%).

Table 3
Effect of cellulase concentration on immobilization using chitosan–L-glutamic acid–GDA via cross-linking method.

Added enzyme (mg)	Added enzyme (units)	Immobilized enzyme (mg/0.1 g support)			Activity of Immobilized enzyme (U/0.1 g support)			Retained activity (%)		
		A	B	C	A	B	C	A	B	C
1	108	0.69	0.66	0.75	74.84	71.41	80.83	69.30	66.12	74.84
2	216	1.52	1.44	1.60	163.79	155.41	173.10	75.83	71.95	80.14
3	324	2.43	2.35	2.56	262.57	253.98	276.44	81.04	78.39	85.32
4	432	2.98	2.81	3.18	321.49	303.91	343.48	74.42	70.35	79.51
5	540	3.48	3.29	3.64	375.41	354.89	393.28	69.52	65.72	72.83

A = chitosan–L-glutamic acid–GDA (0.1%), B = chitosan–L-glutamic acid–GDA (0.5%) and C = chitosan–L-glutamic acid–GDA (1%).

Table 4
Effect of cellulase concentration on immobilization using chitosan–4-aminobutyric acid–GDA via cross-linking method.

Added enzyme (mg)	Added enzyme (units)	Immobilized enzyme (mg/0.1 g support)			Activity of Immobilized enzyme (U/0.1 g support)			Retained activity (%)		
		A	B	C	A	B	C	A	B	C
1	108	0.48	0.46	0.50	51.99	50.17	54.16	48.14	46.45	50.15
2	216	1.08	1.05	1.14	116.64	113.08	123.75	54.00	52.35	57.29
3	324	1.83	1.75	1.89	199.76	188.76	204.73	61.09	58.26	63.19
4	432	2.24	2.07	2.25	241.83	224.03	243.52	55.98	51.86	56.37
5	540	2.51	2.55	2.55	270.65	246.40	275.40	50.12	45.63	51.00

A = chitosan–4-aminobutyric acid–GDA (0.1%), B = chitosan–4-aminobutyric acid–GDA (0.5%) and C = chitosan–4-aminobutyric acid–GDA (1%).

for chitosan–GDA (1%), chitosan–L-glutamic acid–GDA (1%) and chitosan–4-aminobutyric acid–GDA (1%), respectively. It can be observed from the previous results that the higher values of the retained activities of chitosan and its amino acids condensation adducts were at GDA concentration (1%).

3.3.3. Enzyme loading

From the previous results, it was found that the amounts of bonded cellulase in case of covalent attachment method were 17.40 mg, 22.80 mg and 16.60 mg per gram of chitosan, chitosan–L-glutamic acid and chitosan–4-aminobutyric acid, respectively. The retained activities were found as 58.09%, 76.17% and 55.40% for the previous mentioned supports, respectively. On the other hand, the amounts of bonded cellulase in case of cross-linking method were 19.60 mg, 25.60 mg, 18.90 mg, 27.60 mg and 22.40 mg per gram of chitosan, chitosan–L-glutamic acid and chitosan–4-aminobutyric acid, respectively. The retained activities were found to be 65.52%, 85.32% and 63.19% for the previous mentioned supports, respectively.

It was observed from the previous results that the activities of the immobilized cellulase on chitosan and its L-glutamic acid and 4-aminobutyric acid adducts cross-linked with GDA (1%), are higher than those via covalent attachment method. This result may be due to that the cross-linker (GDA) acts as spacer arms between the enzyme and the polymers which decreases the steric hindrance. Accordingly the following investigations concerned with immobilized cellulase via cross-linking method.

3.3.4. Parameters affecting cellulase activity

The activities of free and immobilized cellulase were calculated by measuring the librated glucose at 540 nm, taking into account that the retained activities of the bounded cellulase on the different polymeric materials after immobilization process would be the initial activities (relative activity 100%) of them in the following parameters studied. Effect of pH, temperature, storage stability as well as reusability on the free and immobilized cellulase activities was examined.

3.3.4.1. Effect of pH. The pH dependence of the immobilized cellulase activity was compared with that of the free one in the pH range of 3–10 at 25 °C. Fig. 10 shows the effect of pH on relative activity of cellulase in free and immobilized forms. It was found that the maximum activity was at pH 7 for free cellulase and cellulase immobilized on chitosan–GDA (1%), pH 8 for immobilized cellulase and on chitosan–4-aminobutyric acid–GDA (1%) and have almost no activity variation (relative activity ~97%) under the pH range studied in case of immobilized cellulase on chitosan–L-glutamic acid–GDA (1%). It can be seen from Fig. 10 that, at pH 10, the retention activities of the free and immobilized cellulase on chitosan–GDA (1%), chitosan–L-glutamic acid–GDA (1%), chitosan–4-aminobutyric acid–GDA (1%) were: 15.70%, 61.43%, 97.54% and 85.54%, respectively. While at pH 3, the retained activities of the free and immobilized cellulase on the previous mentioned polymers were: 30%, 68.05%, 97.15% and 74.32%, respectively.

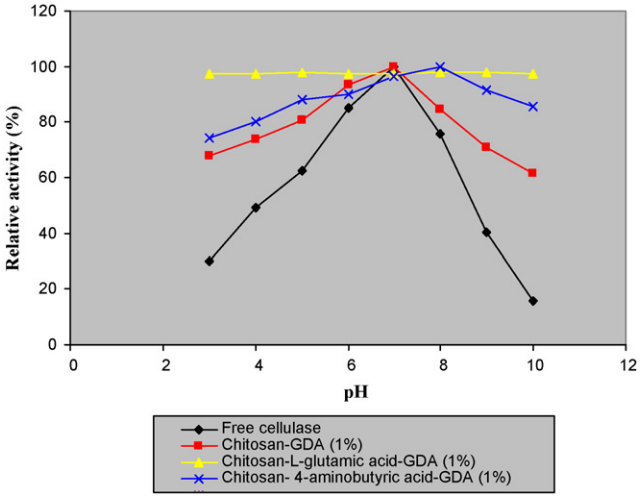


Fig. 10. Effect of pH on the relative activities of free and immobilized cellulase on chitosan–GDA (1%), chitosan–L-glutamic acid–GDA (1%) and chitosan–4-aminobutyric acid–GDA (1%) via cross-linking method.

3.3.4.2. Effect of temperature. Fig. 11 shows the effect of temperature on the activity of free and immobilized cellulase in the range of 30–100 °C at pH 7. The free cellulase exhibited an optimum temperature of 50 °C and this is the same value for immobilized cellulase on chitosan–GDA (1%) and chitosan–4-aminobutyric acid–GDA (1%), but it shifted to 60 °C for immobilized cellulase on chitosan–L-

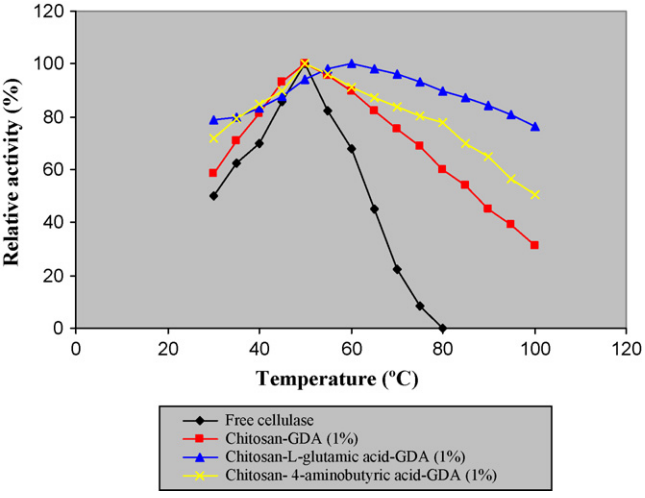


Fig. 11. Effect of temperature on the relative activities of free and immobilized cellulase on chitosan–GDA (1%), chitosan–L-glutamic acid–GDA (1%) and chitosan–4-aminobutyric acid–GDA (1%) via cross-linking method.

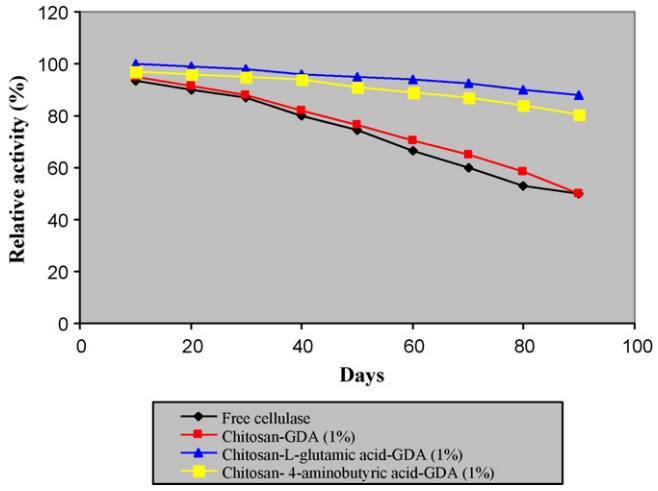


Fig. 12. Storage stability of the free and immobilized cellulase on chitosan–GDA (1%), chitosan–L-glutamic acid–GDA (1%) and chitosan–4-aminobutyric acid–GDA (1%) via cross-linking method.

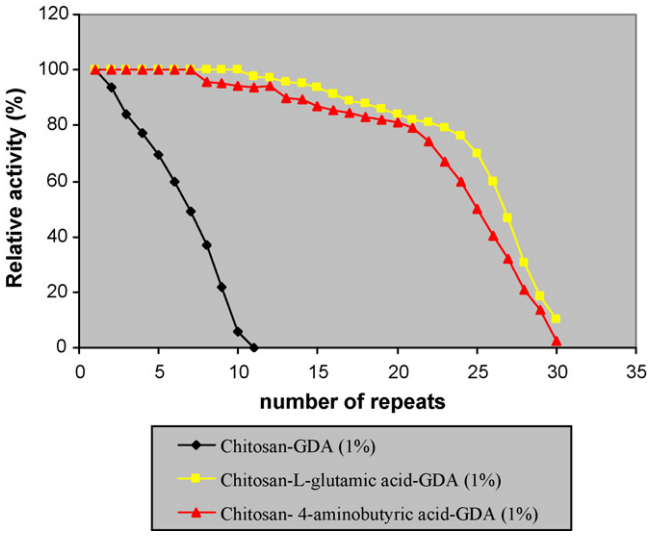


Fig. 13. Influence of repeats on the activities of the immobilized cellulase on chitosan–GDA (1%), chitosan–L-glutamic acid–GDA (1%) and chitosan–4-aminobutyric acid–GDA (1%), via cross-linking method.

glutamic acid–GDA (1%). Also it was observed that the temperature profile of the immobilized enzyme was slightly broader than that of the free one. The increase in optimum temperature was caused by changing the physical and chemical properties of the enzyme. The covalent bond formation, via amino groups of the immobilized cellulase, might also reduce the conformational flexibility and may result in higher activation energy for the molecule to reorganize the proper conformation for binding to the substrate (Arıca, Sinan, Yasemin, & Adil, 2001; Peng, Zhi-Kang, Ai-Fu, Jian, & Patrick, 2005). These results demonstrate the effectiveness of the carriers in protecting the enzyme activity under high temperature conditions. It can be seen from Fig. 11 that, the free cellulase lost its all activity at 80 °C, while at 100 °C, the retained activities of the immobilized cellulase on chitosan–GDA (1%), chitosan–L-glutamic acid–GDA (1%) and chitosan–4-aminobutyric acid–GDA (1%) were: 0%, 31.47%, 76.49% and 50.64% from their original activity respectively.

3.3.4.3. Storage stability. The storage stabilities at 4 °C in the dry state were investigated by measuring the enzyme activities at certain time intervals (0–100 days) and the results are given in Fig. 12. The enzyme activity was determined at 25 °C in phosphate buffer pH 7. It was found that the free cellulase maintained 50% from its original activity and the immobilized cellulase on chitosan–GDA (1%) maintained 50.16% from its initial activity after 90 days, while the immobilized cellulase on chitosan–L-glutamic acid–GDA (1%) and chitosan–4-aminobutyric acid–GDA (1%) maintained 87.81% and 80.44% from their initial activities after the same period, respectively.

3.3.4.4. Efficiency of reuse. Immobilized cellulase was used repeatedly to hydrolyze (CMC), and the reusability examined because of its importance for repeated applications in a batch or a continuous operations. In this study the activity of the immobilized cellulase was detected initially, and then it was washed thoroughly with phosphate buffer solution then used for the next activity measurement. Fig. 13 illustrates the effect of repeated use of the activity of cellulase. It can be observed that the immobilized cellulase using chitosan–GDA (1%) retained about 60% from its original activity after 6 reuses. In contrast, the activities of immobilized cellulase on chitosan–L-glutamic acid–GDA (1%) and chitosan–4-aminobutyric acid–GDA (1%) did not change after 10 and 7 times and retained about 70% and 50% from their original activity after 25 cycles, respectively. In comparison, the activity retained by the immobilized cellulase using the previous mentioned polymers, in the present study, is higher than the previous studies (Jain & Wilkins, 1987; Yoshimoto, Li, Fakunaga, & Nakao, 2002). These results indicate that the immobilized cellulase is relatively stable without sever lose of its activity after several repeated uses.

4. Conclusions

The present study demonstrates that chitosan and its amino acids condensation adducts are promising carriers for the cellulase enzyme. The immobilization of cellulase on chitosan and chitosan–amino acids adducts as a new matrix for cellulase immobilization, using covalent and cross-linking methods, promoted cellulase stability to pH, temperature, storage and reuse compared with the free one.

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