Graphical abstract

Reinforced chitosan beads by chitin nanofibers for the immobilization of β-glucosidase

The chitin nanofibers prepared from partially deacetylated α-chitin were used as the fillers to form DEChN/CS(chitosan) composite beads towards to the application of immobilization supporters. The chitin nanofibers provided a fibrous network in the chitosan matrix that enhanced both the mechanical and porous property of DEChN/CS composite beads, which was normally considered as the contradictory material characteristics. All of these enhanced features improved the efficiency of enzyme immobilization from 40% in the chitosan (CS) beads to 67% in the DEChN/CS composite ones.

Fig. Schematic representation of the CS and DEChN/CS composite beads formation, enzyme diffusing and crosslinking towards to the further enzyme immobilization
Reinforced chitosan beads by chitin nanofibers for the immobilization of β-glucosidase

Liang Liu, Hechan Lv, Jie Jiang, Ke Zheng, Wenbo Ye, Zhiguo Wang††, Yimin Fan†

The chitosan (CS) solution were filled with the partially deacetylated α-chitin nanofibers (DEChN) and the composite (DEChN/CS) beads prepared thereof were used for the immobilization of β-glucosidase. The filling of chitin nanofibers resulted in the increase of the shearing modulus (G', G''), that indicated an improvement of the mechanical property of the DEChN/CS composite beads. Meanwhile, the specific surface area was improved from 46.47 m²/g to 229.71 m²/g when the chitosan beads were filled with the chitin nanofibers, and the prepared DEChN/CS composite beads had a broad pore size ranging from 20 nm to 60 nm. Moreover, a more porous and fibrous structure in the SEM image of DEChN/CS beads was observed than that of the sole CS ones. The chitin nanofibers provided a fibrous network in the chitosan matrix that enhanced both the mechanical and porous property of DEChN/CS composite beads, which was normally considered as the contradictory material natures. All of these enhanced features improved the efficiency of enzyme immobilization from 40% in the chitosan (CS) beads to 67% in the DEChN/CS composite ones.

Introduction

β-Glucosidase (β-D-Glucoside glucohydrolases, celllobiase E.C.3.2.1.21) is a key enzyme in the hydrolysis of cellubiose to glucoses, which were always involved in the biomass degradation and the fuel ethanol production from cellulosic agricultural residues[1]. It can also be used in the synthesis of alkyl and aryl glycosides from natural polysaccharides or their derivatives, the production of aromatic compounds, leading to products with applications in pharmaceutical, cosmetic, detergent and food industries, e.g. in the stabilization of juice and beverages, in the improvement of the organoleptic properties of food and feed products. However, the poor operational stability and difficult reusability of free β-glucosidase, as well as high production cost, have limited its large-scale industrial application[2]. Therefore, how to improve the enzymatic hydrolysis efficiency has become a focus of current research[3]. The enzyme immobilization was considered to be one of the ways to overcome the above shortcomings when the free enzyme was applied. Various techniques and carriers have been developed for enzyme immobilization, including adsorption, covalent linking to insoluble supports, entrapment in polymeric gels, encapsulation in membranes, crosslinking with bifunctional reagents (like glutaraldehyde), and different combinations of immobilization methods are also known[1]. Chitin and chitosan are natural polyaminosaccharide. Chitin is the second most abundant renewable natural structural polysaccharides after cellulose. Chemically, chitin is composed of β (1→4) linked 2-acetamido-2-deoxy-β-D-glucose units (or N-acetyl-D-glucosamine), forming a long chain linear polymer. It is insoluble in most solvents. Chitosan, the derivative of chitin, is obtained by N-deacetylation to a varying extent that is characterized by the degree of deacetylation, and is consequently a copolymer of N-acetyl-glucosamine and D-glucosamine. Chitosan is insoluble in water, but the presence of amino groups renders it soluble in acidic solutions below pH about 6.5[4]. Chitosan has great many of significant biologic and chemical properties[5]. They were always used as enzyme immobilization supports in the form of powders, flakes and gels of different geometrical configurations. But, regardless of such interesting features, usage of chitosan is usually limited due to their insufficient mechanical properties. Hence, their mechanical functionality is generally improved by blending with other polymers, such as collagen, silk, starch, and gelatin[6]. Over the past several decades, the nanofibers from chitin with appealing physical and biological features have attracted intense attentions due to their excellent properties in relation to biodegradability, biocompatibility, antibacterial activity, low immunogenicity and wound healing capacity. And in the last decades, many attempts have been paid to chitin/chitosan blended nanofibers regenerated from the...
dissolved chitin and chitosan, which may enhance both physical and biological functionality as it can take advantage of favorable properties (strength/durability, enhancement of cell attachment) of both components. However, the processes have some drawbacks such as the solvents used are toxic, the condition used are harsh which may do harm to the enzyme immobilization.[7]

In this study, the aqueous chitin nanofiber dispersions were blended with the chitosan solutions, thereby, the DEChN/CS composite beads were prepared in which the chitin nanofibers (DEChN) acted as the fillers, and were applied to the immobilization of the β-glucosidase. The filling of chitin nanofibers had the effect on the properties of not only the DEChN/CS blendings but also the composite beads, e.g. viscoelasticities, thermal curves, morphologies. As a result, the enhanced DEChN/CS composite beads improved the enzyme immobilization efficiency.

Experimental

Materials and methods

Materials. The α-chitin used for immobilization was purified from crab shell collected from Nantong, a seaside city in Jiangsu Province, China, as the following steps. Crab shell wastes were soaked in 1M HCl for 12 h followed by treating with 1M NaOH for 12 h, and these two steps were repeated for three times for full reaction. Then the obtained residual solid was decolorated by immersing in 0.5% (w/w) NaClO2 for three times for full reaction. Then the obtained residual solid was washed with deionized water at 4°C for the further use. β-Glucosidase was supplied by the lab of biochemical engineering in Nanjing Forestry University.

Glutaraldehyde solution (25%), chitosan, acetic acid and other reagents were used without any other purification.

Preparation of DEChN. DEChN was prepared following the methods described in detail in the previous paper.[8]. In short, the purified α-chitin was deacetylated in 33% (w/w) NaOH solution at 90°C for 4 h. The final partially deacetylated chitin with the degree of deacetylation of 28% was washed with deionized water and stored at 4°C. For the preparation of chitin nanofibers (DEChN), the partially deacetylated chitin was suspended in deionized water at the ratio of 0.4 g : 100 ml, then the pH of the suspension was adjusted to about 3 using acetic acid under constant stirring, then this obtained suspension was homogenized and treated with ultrasonication, after centrifugation, the chitin nano-dispersion was prepared successfully.

Preparation of CS and DEChN/CS composite beads. Chitosan (CS, 2 g) were dissolved in 100 ml acetic acid (0.5 %) solution. This CS solution was then centrifuged (5000 r/min) to remove gas and undissolved CS.

CS solution and DEChN dispersion were blended together in a beaker with constant stirring. The mass ratio of DEChN and CS was 0:10 1:10 1:7.5 1:5, respectively, the yielded blendings were concentrated by heating at 90°C to reach the total mass concentration of 2%. By using a syringe, the obtained blendings were sprayed drop-wise at a constant rate into a neutralizing solution containing 5 M NaOH and 95% ethanol in a volume ratio of 5:1. The formed beads were left in solution for 12 h. Thereafter, the beads were washed with deionized water until the washing supernatant was neutral, and stored in deionized water at 4°C. The composite beads prepared thereof were named as CS, DEChN(1)/CS(10), DEChN(1)/CS(7.5), and DEChN(1)/CS(5), respectively.

Immobilization of β-glucosidase. The beads (1 g, dry weight) with different mass ratio of CS and DEChN and the β-glucosidase (original enzyme activity was 39 IU) were incubated in the phosphate buffer (pH 5.0) at 4°C for 12 h followed by crosslinking with 1% glutaraldehyde at room temperature for 2 hours with constant agitating. Finally, the enzyme-loaded beads were washed with phosphate buffer solution (pH 5.0), and stored in the same buffer at 4°C for the further use.

Assay the activity of free and immobilized β-glucosidase. The activity of β-glucosidase was determined as p-nitrophenyl β-D-glucopyranoside (pNPG). The enzyme solution (0.1 ml) was added in a test tube and preheating for 5 minutes in a water bath at 50°C, and then reacted for 10 minutes with the substrates (pNPG). The sodium carbonate solution (1M, 2 ml) was added to terminate the reaction followed by adding 10 ml of deionized water. The absorbance of the reaction solution was measured at 400 nm.[9]

The activity of immobilized β-glucosidase was measured as the same procedure as above described. For each test, the three enzyme-loaded beads were added to 0.9 ml pNPG (5 mmol/L) and reacted for 10 minutes in a water bath at 50°C. And the total immobilized enzyme activity was calculated according to the number of immobilized beads. The enzyme immobilization efficiency was expressed as a percentage of immobilized enzyme activity relative to the highest enzyme activity.

Instrumental analysis

Viscosity measurements. The viscosity measurements of the CS solution and the DEChN/CS blendings were conducted by using RS60000 (HAAKER, German) equipped with a Kegel C35/1° Til cone plate C35/1° Til. Measurements were made using a small sample adapter on solutions (0.25 ml) at 25°C.

Determination of intrinsic viscosity and molecular weight. Dilute solutions were used to determine the intrinsic viscosity, the 5% DMAC/LiCl solution was prepared by weighing the salt rapidly and adding the solvent required. Salt concentrations are given as g of LiCl per 100 g of binary (LiCl and DMAC) solution. And the concentration of chitin was given as g of chitin per 100 g of ternary solution. Viscosities were determined with a suspended-level Ubbelohde viscometer at 25°C having flow times for the solvent of > 150 s. The molecular weight of the purified crab shell α-chitin used in this study, Mv, was calculated to be 2.9 x 10^5 by using the Mark-Houwink equation[10]. And the commercial chitosan were with medium molecular weight (1.9-3.7 x 10^5).

BET analysis. The BET of the CS and the DEChN/CS composite beads was determined by using TriStar 3020 (Micromeritics, USA). Before adsorption measurements, the samples were out-gassed under vacuum at least 2 days at 333 K.

Determination of XRD. X-Ray diffraction pattern (XRD) was performed on an Ultima IV (Ultima IV, Japan) at the voltage of
40 KV with 30 mA, and the 2θ angle was scanned from 5° to 35°.

**Determination of FT-IR spectra.** FT-IR spectra of the samples recorded from 400 to 4000 cm\(^{-1}\) using KBr pellets at room temperature were obtained by Nicolet Antaris FT-NIR. Lyophilization process of the freeze-dried beads at -80°C was done before analyzing.

**TGA analysis.** The thermogravimetric analysis (TGA) was performed by HCT-1/2 (HENVEN-HJ, China) at a heating rate of 10 °C/min in N\(_2\) atmosphere over a temperature of 25-700°C.

**Morphology observation.** The morphologies of the CS and the DEChN/CS composite beads were analyzed by the scanning electron microscope (SEM) on JEOL-JSM 7600F (JEOL, Tokyo, Japan). The samples were coated with gold before the examination.

**Results and discussion**

**Characterization of the CS solution and the DEChN/CS blendings.**

Fig. 1a shows the photographs of the pure CS solution and the DEChN/CS blendings with different ratios. The DEChN had the width of 6-7 nm and length of 200-800 nm as shown in Fig. 1b. All of the solution and the blendings were transparent with high light transmittance (Fig.1c) and stable stood without any precipitation. Originally, the partially deacetylated chitin nanofibers (DEChN) can be dispersed in water at pH 3-4 to form a stable and transparent aqueous nano-fiber dispersions. Here, instead of the acidic water, the chitosan solution with pH around 3 might act as the dispersing media for chitin nanofibers to form the stable and transparent DEChN/CS blendings. The phenomenon also indicated that there was no optical behavior typically caused by aggregation of any bi- and multimolecular species within the tested concentration range and the interaction between CS and DEChN was not covalent grafting, but only simple mixing or physically blending.[10]

The viscosities of the pure CS solution and the DEChN/CS blendings that with the same total concentration of 2% (w/v) were shown in Fig. 1d. All of the DEChN/CS blendings had the viscosities as high as that of the pure CS solution even with lower CS content. The viscosity of a polymer solution is the characteristics of its intermolecular interactions between polymer chains. The high viscosity of chitosan solution is partly due to the strong hydrogen bonding between NH\(_2\) and OH groups of chitosan polymer chains.[1] However, within DEChN/CS blendings, the degree of deacetylation of the DEChN was much lower than that of the CS, and therefore with lower average amounts of NH\(_2\) groups, it came to a conjecture that the similar rheological properties of the DEChN/CS blendings to that of the CS solution[11] might be caused by the remarkably long lengths of the DEChN.

The similar UV-vis absorption spectra and the rheological properties of the CS solution and the DEChN/CS blendings indicated that they might have the similar fluid-to-gel (beads) processing properties.

**Characterization of the CS and the DEChN/CS composite beads.**

The gel beads with average diameter of 3 mm were successfully prepared from both the CS solution and the DEChN/CS blendings as shown in Fig. 2a. The FT-IR spectra, X-ray diffraction pattern and TG analysis were applied to characterize some of the physicochemical and thermal properties of the beads.

Fig. 1. The photographs (a), UV-vis light transmittances (c) and the viscosities (d) of the CS solution and the DEChN/CS blendings, and the TEM picture of DEChN (b). (for all samples, the total concentration was 2% (w/v)).

Fig. 2. Pictures(a), FT-IR(b), XRD (c) and TGA (d) of the CS beads and the DEChN/CS composite beads.
The spectra of all samples in the FT-IR spectra (Fig. 2b) included bands at approximately 3361, 2874, 1653, 1591, 1420, 1376, 1319, 1150, 1067, 1030, 897, 663 and 570 cm\(^{-1}\) that were related to the structure of chitosan\(^{[12]}\). Though the spectra of the CS beads and the DEChN/CS composite beads were similar to each other as a while, they still showed the differences in the absorption intensities. As expected, the absorption bands of the amide I and amide II groups increased as the content of DEChN increased, while the peak of amino groups (-NH\(_2\), 1591 cm\(^{-1}\)) decreased correspondingly\(^{[13]}\).

X-Ray diffraction patterns of the CS and the DEChN/CS beads were shown in Fig. 2c. The presence of the two peaks at 10.6° and 20° was in agreement with the characteristic diffractogram of the original chitosan\(^{[14]}\). Compared to the spectra of CS, the peak at 9.4° in the spectra of DEChN/CS was similar to the spectra of chitin extracted from shrimp shell\(^{[15]}\), which also indicated the presence of DEChN in the composite beads.

The thermal degradation of the CS and the DEChN/CS composite beads started around 25°C in N\(_2\) atmosphere at a heating rate of 10°C/min (Fig. 2d). All of the curves showed similar trends. The first stage ranging between 50-100°C might correspond to the loss of water, the second stage of weight loss started at 260°C and continued up to 400°C during which there was 50% weight loss due to the chemical degradation, the results corresponded well to the reported thermal degradation of the chitosan nanofibers\(^{[16,17]}\). The filling of the small amount of chitin nanofibers did not affect much the thermal degradability of the chitosan beads.

Fig. 3 showed the G' and G'' modulus of the CS beads and the DEChN/CS composite beads at the frequency of 1 Hz (25°C). For all beads, the loss modulus (G'') was higher than the storage modulus (G'), which was a classic gel character. It can be also observed that the higher chitin nanofibers content (e.g. DEChN(1)/CS(5) and DEChN(1)/CS(7.5)), the higher G' and G'' modulus, which indicated that the mechanical property of the composite beads was improved by the filling of the DEChN.

Morphology observations of the CS and the DEChN/CS composite beads.

The morphology of the CS beads and the DEChN/CS composite beads were observed by SEM. Fig. 4a shows the surface morphology of the beads. In contrast to the sheetlike network with a little big pores on the surface of the CS beads, all of the DEChN/CS composite beads showed fibrous network with a wealth of pores. According to the cross-section diagram shown in Fig. 4b, both of sheetlike structure and fibrous network were found in the CS beads, as the percentage of the DEChN increases, the less sheetlike section and the richer fibrous network were appeared. Such structures promote the internal cross-linking and the porosity of the beads. Such a porous configuration indicated that the filling of the DEChN into the CS solution could undergo dramatic changes on morphologic structure during the beads formation.

Fig. 3. Rheological measurements of the storage modulus G' and the loss modulus G'' of the CS beads and the DEChN/CS composite beads.

Fig. 4. Scanning electron micrographs of the CS beads and the DEChN/CS composite beads of the surface (a) and the cross-section (b) diagram.
As shown in Fig. 5a, the CS and the DEChN/CS composite beads both exhibited type III nitrogen adsorption-desorption isotherm, the type III isotherm exhibited the prominent adsorption at high relative pressures (P/P₀), indicating the macropore adsorption. The BET specific surface areas for the CS, DEChN(1)/CS(10), DEChN(1)/CS(7.5), DEChN(1)/CS(5) composite beads were 46.47 m²/g, 172.14 m²/g, 166.95 m²/g and 229.71 m²/g, respectively. The more DEChN content, the bigger specific area of the beads, which indicated the decrease of the aggregation of the regenerated chitosan polymers by the filling of the chitin nanofibers. And the pore size distribution (Fig. 5b) calculated from the BJH method clearly showed that comparing to the CS beads, the DEChN/CS composite beads had a broad pore size ranging from 20-60 nm, which was corresponding well to the normal enzyme size (ranging at the level of several hundreds of angstroms). The morphologic characters and the pore size distribution of the DEChN/CS composite beads, together with the improved mechanical strength (indicated by rheological measurements in Fig. 3), were supposed to be able to improve the enzyme loading, diffusing, and also the enzyme immobilization[19].

**Immobilization of β-glucosidase.**

The crosslinking of glutaraldehyde was applied to the enzyme immobilization on the CS and DEChN/CS beads so as to further improve the immobilization efficiency. Fig. 6 was the FT-IR and XRD spectra of the CS beads and the DEChN/CS composite beads after crosslinking by glutaraldehyde. All of the FT-IR spectrum (Fig. 6a) showed the same peaks of 3423, 2929, 1629, 1382 and 1070 cm⁻¹, which were different from the non-crosslinking ones (Fig. 2a ). The XRD patterns were also changed as well after crosslinking as shown Fig. 6b. Moreover, it was found that the numerical values of G’ and G” of the glutaraldehyde crosslinked CS and DEChN/CS beads (Fig. 7) were much higher than that of the non-crosslinking ones. All of the evidences indicated the effective crosslinking of glutaraldehyde with the CS and DEChN/CS beads. This crosslinking may not only promote the mechanical property of the beads but also can improve the immobilization efficiency of β-glucosidase.

Fig. 5. Nitrogen adsorption/desorption isotherms (a) and pore size distribution (b) of the CS beads and the DEChN/CS composite beads.

Fig. 6. FT-IR(a), XRD (b) of the CS beads and the DEChN/CS composite beads after crosslinking with glutaraldehyde.

Fig. 7. Rheological measurements of the storage modulus G’ and the loss modulus G” of the CS beads and the DEChN/CS composite beads after crosslinking with glutaraldehyde.
Table 1 shows the immobilized and free enzyme activity of the β-glucosidase based on the different CS and DEChN/CS supports. The immobilized enzyme activity represents the amounts of the enzyme immobilized on the CS and DEChN/CS composite beads, while, the free enzyme activity represents the amounts of the non-immobilized enzyme detected in the washing aqueous phase. As shown in Table 1 and Fig. 8, for all beads, the total enzyme activities calculated by summarizing the immobilized and the free enzyme activities were quite close to the original added ones, which indicated the enzyme conservation during the immobilization process. And as obviously shown in Fig. 8, the CS beads showed the lowest immobilization efficiency (40%), while, as the percentage of the DEChN increases, the immobilization efficiency was promoted to as high as 67%.

**Table 1.** Immobilized and free (non-immobilized) enzyme activity of the β-glucosidase

<table>
<thead>
<tr>
<th>Samples (1g)</th>
<th>Immobilized enzyme (IU/g)</th>
<th>Free enzyme (IU/g)</th>
<th>Total enzyme (IU/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS</td>
<td>15.704</td>
<td>20.501</td>
<td>36.205</td>
</tr>
<tr>
<td>DEChN(1)/CS(10)</td>
<td>21.702</td>
<td>17.388</td>
<td>39.090</td>
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<tr>
<td>DEChN(1)/CS(7.5)</td>
<td>24.070</td>
<td>14.145</td>
<td>38.215</td>
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<tr>
<td>DEChN(1)/CS(5)</td>
<td>25.950</td>
<td>13.886</td>
<td>39.836</td>
</tr>
</tbody>
</table>

*: The amount of enzyme (β-glucosidase) originally added was 39 IU/g

Conclusions

The pure chitosan (CS) beads were commonly used as immobilization supporters of enzymes or cells. In this study, the chitin nano-fibers (DEChN) prepared from partially deacetylated α-chitins were firstly used as the fillers to form DEChN/CS composite beads towards to the application of enhanced immobilization supporters.

The partially deacetylated chin nanofibers(DEChN) reinforced DEChN/CS composite beads exhibited a considerable improvement for the immobilization of β-glucosidase. The immobilization efficiency improved from 40% (CS) to 67% (DEChN(1)/CS (5)). The BET analysis, SEM observations and the rheological measurements of the CS and DEChN/CS composite beads showed that the DEChN/CS composite beads had higher porosity and better mechanical properties as well, which was normally considered as the contradictory material natures. When compared with chitosan beads, DEChN/CS composite beads not only kept the good biological properties, but also promoted the inter structure and the mechanical strength, which further improved the immobilization efficiency.

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**References**