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Silver Nano Particles Modified Chitosan Beads: An Improved Stability of Cellulase by Immobilization

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Abstract:

Cellulase refers to a family of enzymes which act in concert to hydrolyze cellulose. Cellulases are widely distributed throughout the biosphere and are most manifest in fungal and microbial organisms. Immobilization of biomolecules onto insoluble supports is an important tool for the fabrication of a diverse range of functional materials or devices. It provides many distinct advantages including enhanced stability, easy separation from reaction mixture. In recent years, nano-sized silver nano particles have received increasing attention in various fields, including biomedical and environmental applications, due to their small size, high specific surface area, and low toxicity. The chitosan beads were embedded with Silver nano particle and cellulase was immobilized on modified chitosan beads. As a result of this modification, the immobilized cellulase beads showed better pH stability than free enzyme at neutral pH range. The activity yield of the immobilized cellulase was found to be as 83%, and it was found no change of the optimum temperature after immobilization.

Keywords: cellulase, immobilization, silver nano particles, chitosan beads.

Introduction

Cellulase is an enzyme complex which breaks down cellulose to beta-glucose. It is produced mainly by symbiotic bacteria in the ruminating chambers of herbivores[1]. Aside from ruminants, most animals (including humans) do not produce cellulase, and are therefore unable to use most of the energy contained in plant material [2]. Cellulase refers to a family of enzymes which act in concert to hydrolyze cellulose. Cellulases are widely distributed throughout the biosphere and are most manifest in fungal and microbial organisms. Three general types of enzymes make up the cellulase enzyme complex[2][3]. Endocellulase breaks internal bonds to disrupt the

crystalline structure of cellulose and expose individual cellulase polysaccharide chains[4]. Exocellulase cleaves 2-4 units from the ends of the exposed chains produced by endocellulase, resulting in the tetrasaccharides or disaccharide such as cellobiose [5].Cellobiase or beta-glucosidase hydrolyses the endocellulase product into individual monosaccharides[5][6].

The number of applications of immobilized enzymes is increasing steadily[7]. Occasionally, however, experimental investigations have produced unexpected results such as a significant reduction or even an increase in activity compared with soluble enzymes[8]. Chitosan, a poly (d-glucosamine), is obtained from chitin by deacetylating its acetamide groups with a strong alkaline solution. The objective of this study is to prepare silver nanoparticles embedded in chitosan, which is a natural polymer, using microwave irradiation for the immobilization of cellulase enzyme[9-12]. After a reduction process assisted by microwave irradiation cellulase will be immobilize with silver nano particles embedded chitosan beads and kinetic behaviour of cellulase will be studied by spectrophotometric method[10-14].

2.Materials & Methods:

2.1 chemicals

Commercial acid cellulase obtained from Aspergillus Niger was a gifted sample from advanced enzyme technologies Thane, Mumbai, India. Maleic anhydride AR grade was obtained from M/S Sisco Research Laboratories, Mumbai, India. 3, 5-dinitrosalicylic acid (DNSA) AR Grade was obtained from M/S Spectrochem, Mumbai, India. Chitosan flakes, tripolyphosphate, epichlorohydrin were purchased from Sigma Chem. Co. (St. Louis, MO, USA);Other reagents were purchased from M/S S.D. Fine Chemicals, Mumbai, India and were of analytical grade.

2.2 Preparation of cross-linked chitosan beads:

Chitosan solution was prepared by dissolving 1.0 g chitosan flakes into 50 ml of 2% (v/v) acetic acid solution. The solution was dropped through a needle into a gently shaken tripolyphosphate(TPP) solution. The TPP solution (2%, w/v) was prepared by dissolving sodium TPP (5 g) in 250 ml of distilled water and its pH value was adjusted to pH 8.2 by 1N HCl. The chitosan solution was dropped into the TPP solution and the gelled spheres formed instantaneously. After 4 h of hardening, the beads were separated from the tripolyphosphate solution by filtration.

Five grams of wet beads were put into a flask that contains 12.5 ml sodium hydroxide (pH 10.0) and cross-linking agent, epichlorohydrin (0.04 M) was added and then stirred at 50 °C for 6 h. The cross-linked chitosan beads were then washed intensively with distilled water to remove any unreacted epichlorohydrin.

2.3 Synthesis of chitosan-based silver nanoparticles composites solution:

1.0 ml of 20.0 mm $AgNO_3$ solution and 100 mL of chitosan solution (prepared by adding 3.0 g of chitosan in 100 mL of 2% (v/v) acetic acid) were mixed and stirred until homogenous. The mixture was subjected to 12 short burst of microwave irradiation using a microwave oven at frequency of 2.45 GHz at power output of 200

W. Each short burst of irradiation lasted for 1 min. The mixture was cooled (35-40 sec at room temperature) between each irradiation. The ramp/cool cycle was repeated 12 times. The reduction of Ag^+ ions was monitored by sampling an aliquot (2 ml) of the solution after 3, 5, 7, 9 and 12 cycles and measuring the UV-Vis spectra of the solution.

2.4 Preparation of silver-impregnated chitosan beads:

The brownish mixture was dropped through a seven-gauge needle into 2.0 M sodium hydroxide solution and the gelled spheres formed instantaneously. The formed chitosan-silver nanoparticle beads (Fig1.) were kept in the sodium hydroxide solution for 24 h and washed with distilled water until the washing solution became neutral.

2.5 Preparation of cross-linked chitosan- silver nanoparticle with microwave irradiation:

Cross-linked chitosan beads with and without silver nanoparticles embedded were prepared using epichlorohydrin (ECH) as the cross-linking agent using microwave irradiation method. Epichlorohydrin solution of 0.04 M containing 0.067 M sodium hydroxide was prepared (pH = 10). Freshly prepared, 20 g of wet chitosan-silver nanoparticle composite beads or 20 g of wet pure chitosan beads were put into a flask with 100 ml of the epichlorohydrin solution and stirred to allow proper mixing. The mixture was subjected to short bursts of microwave irradiation using a microwave oven at frequency of 2.45 GHz at power output of 200 W. Each short burst of irradiation lasted for 30sec the mixture was cooled between each irradiation. This ramp/cool cycle was repeated 12 times. After the 12th cycle, the cross-linked chitosan beads were filtered and washed extensively with distilled water to remove any unreacted epichlorohydrin and air dried. The newly formed cross-linked chitosan embedded silver nanoparticles beads were ground and sieved to a uniform size (<250 μm) before use. These were known as cross-linked chitosan-silver nanoparticle composite micro-beads or pure cross-linked chitosan micro-beads.

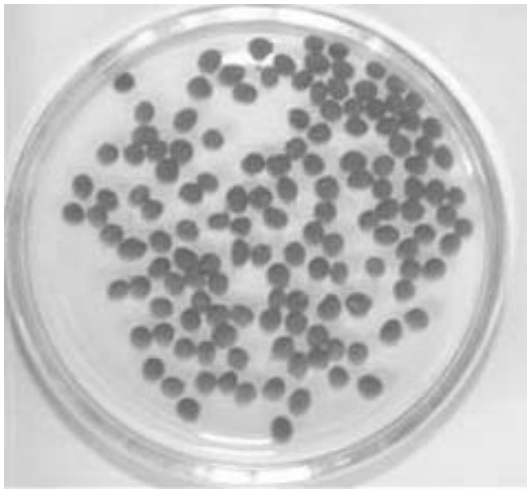


Fig. 1: Cross-linked Chitosan-silver Nanoparticle with Microwave Irradiation

2.6 Swelling test of chitosan and cross-linked chitosan beads:

Chitosan and cross-linked chitosan beads were tested with regard to their solubility in each of 5% (v/v) acetic acid, distilled water and 0.10 M sodium hydroxide solution by adding 0.10 g of chitosan and cross-linked chitosan beads in each of the dilute acid, distilled water and dilute alkaline solutions for a period of 24 h with stirring.

The swelling studies of chitosan and cross-linked chitosan beads were carried out in distilled water at room temperature for a period of 24 h. The percentages of swelling of these beads were calculated by using the Eq. 1:

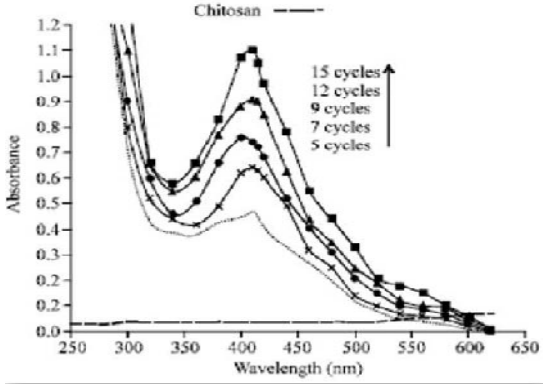
$$percentage\ of\ swelling = \frac{Ws-W}{W} \times 100\% \qquad \textbf{e.q.-------1}$$

Where, W_s is the weight of swollen beads (g) and W is the weight of dry beads (g).

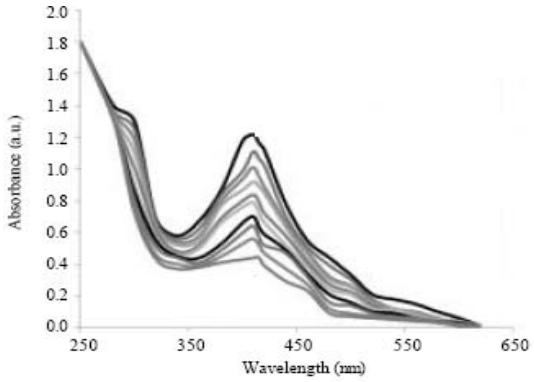
2.7 kinetic study:

Kinetic study of cellulase and modified cellulase after immobilization with chitosan beads and silver nanoparticle embedded chitosan beads were studied.

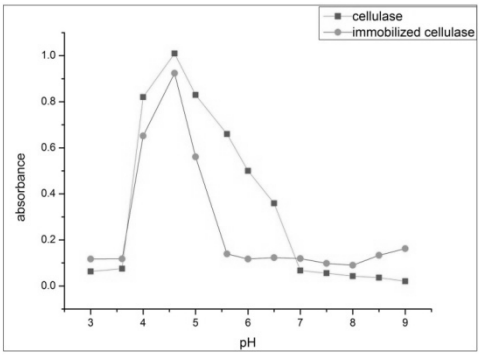
Enzyme assay were done of native cellulase enzyme, and immobilized cellulase enzyme with chitosan beads and silver nanoparticle embedded chitosan beads with spectrophotometric method at 540 nm.



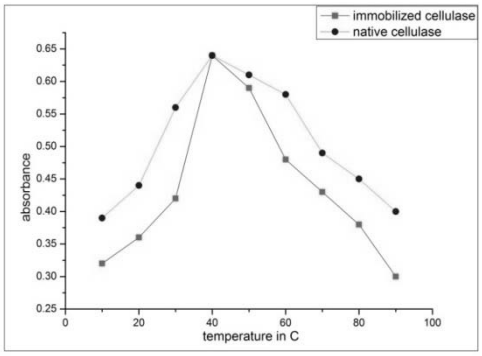
Graph 1: UV-Vis Spectra Recorded As a Function of Number of Cycles of Microwave Irradiation



Graph 2: Time Dependent UV-Visible Spectra



Graph 3: pH Stability of Immobilized Modified Cellulase



Graph 4: Optimum Temperature of Modified Immobilized Cellulase

3.Result and discussion:

3.1.chitosan study

Chitosan beads have porous structure. However, immobilization of cellulase on the surface of chitosan beads rather than in beads is important for overcoming of diffusion limitations because cellulose which is the substrate of cellulase is a macromolecule.

3.2.chitosan beads

3.2.1.chitosan solubility test

It was observed that chitosan beads were soluble in 5% acetic acid (v/v), but insoluble in both 0.10 M NaOH solution and distilled water. However after cross linking with epichlorohydrin, the cross-linked chitosan was found to be insoluble in either acidic or alkaline medium, as well as distilled water. It is well known that the high hydrophilicity of chitosan beads or raw chitosan are due to primary amine groups, which makes chitosan easily soluble in dilute acetic or formic acid solutions to yield a hydrogel in water. Therefore, the cross-linking treatment of chitosan reinforces its chemical stability in organic acidic media, making it useful for the removal of chemical pollutants from wastewaters of acidic nature.

3.2.2 chitosan swollen test

The swelling behaviour of chitosan improved greatly after cross-linking. It was observed that non-cross-linked chitosan beads had 37.5 and 32.6% swelling when allowed to remain in distilled water and 0.1 M NaOH solution respectively for 24 h at room temperature. However, the swelling for cross-linked chitosan beads was only 15.3% in distilled water and 11.8% in NaOH under similar conditions. From the results obtained, cross-linking modification does not only increase the surface area and reinforce the chemical strength of the chitosan beads but also reduces the swelling of the beads. These more rigid and chemical stable cross-linked chitosan beads are less prone to swelling and are better suited for use in an adsorption column.

3.2.3.temperature and pH study:

The native cellulase had a pH optimum at about 4.0 and the hydrolytic activity decreased sharply toward higher pH. The pH optima of the immobilized enzyme were shifted to neutral pH. This phenomenon might be caused by the polyanionic micro-environment surrounding the enzyme molecules.

The temperature dependence of the enzyme activity was studied in the temperature range of 10–90°C. Optimum temperature of the free enzyme and the modified enzyme beads found to be 40 °C. The modified beads showed better activity at higher temperatures than free enzyme after 1 h incubation.

The storage stability of an enzyme is of significant importance for scheduling its application in a particular reaction. The immobilized cellulase showed good storage and operational stability in comparison with native enzyme.

4.conclusions:

Silver nano particle modified chitosan beads were developed in order to improve the activity of immobilized cellulase on chitosan beads. The silver nano particle were introduced onto the chitosan beads after activation with epichlorohydrin with microwave irradiation and confirmed by UV-Vis spectrophotometer. *Cellulase* was immobilized onto the SNP embedded chitosan beads and its activities were assayed. The immobilized cellulase on silver nano particles embedded chitosan beads showed optimal conditions at 40°C and pH 4.5 and a higher thermal stability than the free cellulase. The reusability of the immobilized cellulase was tested and it was confirmed that the washing procedure was important. The immobilized cellulase showed good storage stability and kinetic properties as well. We also confirmed that the silver nano particles embedded chitosan beads were a good support for the immobilization of cellulase and that they could be applied for immobilization of other useful enzymes too.

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