Bioresource Technology 115 (2012) 152–157

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Electrospun polyacrylonitrile nanofibrous membranes for chitosanase immobilization and its application in selective production of chitooligosaccharides

Sujata Sinha [a,*], Sanjay R. Dhakate [b], Pankaj Kumar [b], R.B. Mathur [b], Pushplata Tripathi [c], Subhash Chand [a,*]

[a] Department of Biochemical Engineering and Biotechnology, Indian Institute of Technology, Hauz Khas, New Delhi 110016, India
[b] Physics and Engineering of Carbon, Division of Materials Physics and Engineering, National Physical Laboratory (CSIR), Dr. K.S. Krishnan Marg, New Delhi 110012, India
[c] School of Sciences, Indira Gandhi National Open University, Maidan Garhi, New Delhi 110068, India

ARTICLE INFO

Article history:
Available online 2 December 2011

Keywords:
Nanofibers
Immobilization
Chitosan
Chitooligosaccharide

ABSTRACT

Polyacrylonitrile nanofibrous membranes (PANNFM) were prepared by electrospinning from 10 wt.% of PAN solution and its surface was modified by amidination reaction. A new chitosan degrading enzyme from *Aspergillus* sp. was covalently immobilized on PANNFM. Immobilization efficiency of 80% was achieved by activating PANNFM surface for 30 min followed by 2 h treatment with enzyme solution. The optimum temperature and pH for immobilized enzyme were 50 °C and 5.8, respectively. The immobilized chitosanase retained >70% activity after ten repeated batch reaction and could be stored up to 60 days at 4 °C with minor loss in activity. Chitosan hydrolysis using different substrates were studied using immobilized chitosanase in batch conditions. Continuous selective production of chitooligosaccharides (dimer to hexamer) by changing the temperature was achieved by PANNFM-chitosanase.

1. Introduction

Chitosanases are group of hydrolytic enzymes which act on chitosan and can be of great industrial application due to enormous availability of chitin and chitosan in nature (Somashekar and Joseph, 1996). Chitosan is deacetylated chitin; most abundant polymer after cellulose and due to its biological properties and biocompatibility (Kim, 2010) has great potential to be applied in different areas like agricultural, industrial, biomedical, etc. Poor solubility of chitosan makes it difficult to be used at large scale. Interestingly, low molecular weight chitosan (LMWC) and chitooligosaccharides (COS) are readily soluble in water due to free amino group in D-glucosamine (Jeon et al., 2000) and show excellent biological activities (Kim and Rajapakse, 2005). Monomers of chitosan (D-glucosamine and N-acetyl D-glucosamine) has biomedical applications like arthritis treatment, dentistry, wound healing, etc. and has been studied as food supplement (Kajimoto et al., 1998). Chitosan can be depolymerized either by chemically or enzymatically. However, chemical method is avoided because of low yield, toxicity, pollution, high cost and non fitness for human consumption. Use of chitosan degrading enzyme is limited at industrial scale due to its high cost and limited availability (Kim and Rajapakse, 2005). Chitosanase enzymes have been found in large number of microbes including bacteria and fungi (Somashekar and Joseph, 1996) which can be used in production of COS, but most of these enzymes have low substrate specificity and enzyme is inducible in nature (Shimosaka et al., 1995). In order to find a novel microbial chitosanase, we screened microbes from soil sample rich in fish waste and isolated partially purified enzyme was immobilized on electrospun polyacrylonitrile nanofibrous membranes (PANNFM). Enzyme immobilization improves reusability and has other advantages like scale up, ease in recycling, continuous operation and product purification. Performance of immobilized enzyme generally depends on choice of matrix and method of immobilization. One dimensional nanofibers have extremely high surface area to volume ratio and excellently interconnected pore structure. The interconnectivity of electrospun supports circumvent the mass transfer limitations and have been used as immobilization matrix for a number of enzymes (Wang et al., 2009). NFMs from natural polymers are generally less stable chemically and mechanically than those from synthetic polymers. PAN is a polymer with good stability and mechanical properties (Kim et al., 2005). Derivatives of PAN have also been used for enzyme immobilization with an aim to introduce functional groups into the polymer backbone due to the inertness and hydrophobicity of acrylonitrile monomer (Ye et al., 2006). In this study, PANNFM was used for immobilization of chitosanase after activation of surface by amidination reaction and were studied for residual activity, reusability, optimum pH and temperature. PANNFM-chitosanase was used for batch hydrolysis of different chitosan substrates and selective production of glucosamine and COS was achieved.

* Corresponding authors. Tel.: +91 11 2659 1004; fax: +91 11 2658 2282.
E-mail addresses: sinha738@gmail.com (S. Sinha), subhashc@dbeb.iitd.ac.in, subhashc46@hotmail.com (S. Chand).

0960-8524/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biortech.2011.11.101

2. Methods

2.1. Materials

Commercial chitosan from crab shell, average molecular weight 290 kDa, 93% N-deacetylated (DAC); were kind gift from Marine Chemicals, Chennai, India. Chitosan from shrimp shell (>75% deacetylated) and PAN powder were procured from Sigma–Aldrich, Germany and N,N-Dimethylformamide (DMF) was purchased from Fisher Scientific, India. Glucosamine hydrochloride and Chitin were purchased from Hi-media. All other chemicals were procured from SRL, Mumbai, India and were of analytical grade.

2.2. Preparation of PANNFM by electrospinning

The PAN solution (10 wt.%) was prepared in DMF by mixing it in multi frequency ultrasonic bath (Life Care Equipment Pvt. Ltd., Mumbai, India) for one hour at 50 °C. Continuous stirring for 15–20 h was done to get uniformly mixed solution and was spun using electrospinning unit (ESPIN-NANO). Conditions for electrospinning were as follows: needle orifice: 0.55 mm, rotating cylindrical collector diameter: 30 mm, voltage applied: 5–20 kV, rotational speed of the collector: 2000 rpm, distance between tip to collector: 20 cm and flow rate of solution 0.2 ml/h which was maintained using computer control program.

2.3. Screening and isolation of chitosanase producing microbial strain from soil

Chitosan minimal salt medium was used for screening and isolation. Soil samples, taken from marine waste dumping area in local market (Chittaranjan Park, New Delhi, India) was weighed, suspended in sterile water and serial dilutions were prepared. Plating was done on chitosan minimal salt agar medium. Composition of medium was as follows: pH 6.8, colloidal chitosan 1% (w/v), KH_2PO_4 (0.15 g/l), K_2HPO_4 (0.35 g/l), $MgSO_4{\cdot}5H_2O$ (0.25 g/l), $FeSO_4{\cdot}7H_2O$ (0.005 g/l), $ZnSO_4$ (0.001 g/l), $MnCl_2$ (0.001 g/l) and agar (2%) was added for making plates. Microbial colonies grown on the surface of agar plates were transferred to chitosan broth. Composition of chitosan broth medium was as follows (g/l): KH_2PO_4, 2.0; K_2HPO_4, 1.0; $MgSO_4{\cdot}7H_2O$, 0.5; NaCl, 0.5; $CaCl_2$, 0.1; yeast extract 0.5 and chitosan 15; the medium was adjusted to pH 6.0 with the help of acetic acid (1 N). Inoculated chitosan broth medium, after incubation for 6–7 days was centrifuged to remove microbial growth and supernatant was used for enzyme assay. Microbes showing good chitosanase (endo and exo) activities were selected and used for further enzyme production.

2.4. Preparation of partially purified chitosanase

Microbial cultures, stored on agar slants at 4 °C were scrapped off and washed out with sterile distilled water. One milliliter of microbial suspension was inoculated into a 250 ml flask containing 50 ml of the medium and incubated on a rotary shaker at 200 rpm for 3–4 days at 32 °C. The composition of the medium (pH 5.6) was same as that of chitosan broth. Finally, the cells of the culture broth were removed from the medium by centrifugation at 7500 rpm for 15 min at 4 °C and the supernatant was collected. The chitosanase was precipitated out by chilled acetone (80–90%) at 4 °C; precipitates were collected by centrifugation at 10,000 rpm for 20 min, washed repeatedly with sodium acetate buffer and dissolved in an appropriate volume of same buffer (100 mM, 5.5). This partially purified chitosanase enzyme was stored at 4 °C was used for immobilization.

2.5. Immobilization of chitosanase enzyme

All the immobilization steps were carried out by cutting the PANNFM into standard pieces and weighed. Adsorption of enzyme was done by adding the enzyme solution (10 ml of 1 mg/ml) to NFM kept in 100 ml flask for 2 h with mild shaking. NFM surface was activated by placing it in absolute ethanol and bubbling with 1 N solution of hydrogen chloride (6–7 ml) to produce the corresponding imidoester derivatives, a method followed by Li et al. (2007) for lipase immobilization on PAN. NFMs were washed with acetate buffer and were treated with chitosanase enzyme solution in 0.1 M acetate buffer solution (pH 5.5). Flask was put for shaking (100 rpm) at room temperature for 2 h. After the immobilization reaction, the membrane was removed from the solution and washed with acetate buffer several times to remove any unbound enzyme. NFM bound enzyme was lyophilized and was stored till further use.

2.6. Analytical methods

Surface morphology of NFM before and after immobilization was studied by scanning electron microscope (SEM, Zeiss-EVO, MA-10, variable UK). The electrospun PANNFM, amidinated or surface modified PANNFM, PANNFM-chitosanase were characterized by Fourier transform infra red spectrometer (FTIR Nicolet 5700). Small amounts of samples were separately mixed with KBr and prepared pellet was used for FTIR spectra.

2.7. Chitosanase assay

The activity of free and immobilized chitosanase was determined by 3,5-dinitrosalicylic acid (Miller, 1959) by measuring the rate of release of reducing sugar. Immobilized enzyme was weighed, added to 3 ml 1% chitosan solution and incubated at 37 °C for 30 min. The reaction was stopped by adding 0.5 ml 1 N NaOH solution in case of free enzyme while nanofibers were withdrawn to stop the reaction for immobilized enzyme. The withdrawn reaction mixture was centrifuged at 7000 rpm for 15 min to remove the chitosan and the concentration of reducing sugar was determined in supernatant. One unit of enzyme activity was defined as the amount of enzyme that could produce l μmol reducing sugar per min. The amount of protein was measured using the Bradford method (1976).

2.8. Properties of immobilized chitosanase

Immobilization efficiency was calculated by following formula:

$$\frac{\text{Specific activity of immobilized chitosanase enzyme (U/mg)}}{\text{Specific activity of soluble chitosanase enzyme (U/mg)}} \times 100$$

Activity and stability of free and immobilized enzyme was measured over the temperature range of 20–90 °C. Thermal stability of enzyme was checked by incubating PANNFM-chitosanase in acetate buffer at different temperatures for 30 min. pH optimum was determined by using buffer of different range for assay, glycine HCl (2.2–3.6); sodium acetate (3.6–5.6); sodium phosphate (5.8–8.5); and glycine NaOH (8.6–10.6). To evaluate the reusability, PANNFM-chitosanase, after each reaction was washed with acetate buffer (100 mM, 5.5) and introduced into substrate solution to start the next batch of reaction. This process was repeated up to 10 cycles. The storage stability of immobilized enzyme was determined by incubating the PANNFM-chitosanase in acetate buffer at