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Glucosamine Production Using Immobilized Chitosanase on PAN nanofibres

Sujata Sinha[1], Rohit Kumar[1], Sanjay R Dhakate[2], Subhash Chand[1+]

[1]Department of Biochemical Engineering and Biotechnology, Indian Institute of Technology, Delhi-110016 (India)

[2] Physics and Engineering of Carbon, Division of Materials Physics and Engineering, National Physical Laboratory, CSIR, Dr. K. S. Krishnan Marg, New Delhi-110012 (India)

Abstract. Crude chitosanase enzyme from *Aspergillus* sp. was immobilized on electrospun PAN nanofibres (7.5 wt %) using glutaraldehyde as cross linking agent. Protein loading was quantitative after 30 min treatment with glutaraldehyde and activity retention of the immobilized enzyme was 70%. Immobilization conditions and characterization of the immobilized enzymes were carried out. Glucosamine from different chitosan substrates using immobilized enzyme was produced. Yield of glucosamine from crab shell and shrimp shell chitosan was found to be as high as 70% and 40% respectively.

Keywords: Chitosanase, Immobilization, glucosamine, PAN, Nanofibres

1. Introduction

Glucosamine produced by hydrolysis of chitosan has attracted attention due to its therapeutic activity in osteoarthritis and has been evaluated as food supplement [1]. It also has applications in promoting wound healing, bone regeneration, and antibacterial effect in dentistry [2]. Acetylated form of glucosamine is sweeter in taste and fit for oral administration. Sulfate and hydrochloric salt of glucosamine has already been commercialized. Chitosan may be hydrolyzed with acids at high temperatures to form glucosamine but this method is of low yield, high cost, generates a lot of acidic waste which leads to environmental pollution and not fit for human use. Thus, enzymatic method is preferable for production of glucosamine [3].

Exo chitosanases or exo-β-d-glucosaminidases (GlcN-ase), cleaves glucosamine residue continuously from non reducing end of the chitosan. Very few chitosan degrading enzymes have been shown to have only exo activity and not available commercially. Some of these enzymes have also been shown to have transglycosylation activity [4] and could be used for synthesis of designer oligosaccharides to be used in food industry.

Enzyme immobilization has been a popular strategy for most large scale applications due to the ease in recycling, storage, continuous operation and product purification. Poor biocatalytic efficiency of immobilized enzymes, however, often limits the development of large scale bioprocessing to compete with traditional chemical processes. Improvement of biocatalytic efficiency can be achieved by manipulating the structure of carrier material for enzyme immobilization. Immobilization of Chitosan degrading enzyme has been reported on few surfaces like on agar gel [5], chitin [6] and amylase coated nanoparticle [7], however no reports are there of immobilization of chitosan degrading enzyme on naofibres. Nanofibres, as support for enzyme immobilization excel not only due to its high surface area to volume ratio but they show less mass

[1+] Corresponding author. Tel: +91-26591004, fax: +91-11-6582282
E-mail address: subhashc@dbeb.iitd.ac.in, subhashc46@hotmail.com

transfer resistance and their recycling is easier as compared to nanoparticles [8]. Polyacrylonitrile (PAN) is a polymer with good stability and mechanical properties as compared to natural polymer. PAN derivatives 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 [9]. However, it is desirable to have a method for direct conjugation of enzyme molecules onto the surfaces of PAN nanofibers as synthesis of PAN derivatives demand complicated steps. In this study, PAN nanofibers were used directly for chitosanase immobilization using glutaraldehyde (GA) as cross linking agent, glucosamine production with different chitosan substrates using immobilized enzymes were carried out and yield was compared.

2. Materials and methods

Electrospun PAN nanofibres membrane (PANNFM) (average diameter: 250 nm) were gift from National Physical Lab (NPL), New Delhi, India. 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) were procured from Sigma-Aldrich. Glucosamine standard was procured from Hi-media. All other chemicals were procured from SRL, Mumbai, India and were of analytical grade. Chitosan of different degree of deacetylation were prepared by method followed by Zhou et al, 2003 [10].

2.1. Preparation of partial purified chitosanase

Aspergillus sp. was stored on agar slant at 4 ^{0}C.The microorganism was scrapped off from the agar slant and washed out with sterilized water. Then 1 ml of spore suspension was inoculated into a 250 ml flask containing 50 ml of the medium and incubated on a rotary shaker at 200 rpm for 72 h at 32^{0}C. The composition of the medium (pH 6.8) was: chitosan 1 %(w/v), KH_2PO_4 (0.15g/l), K_2HPO_4 (0.35g/l), MgSo4.5H_2O (0.25g/l), FeSo4.7H_2O (0.005g/l), ZnSo4 (0.001g/l), Mncl2 (0.001g/l). Finally, the mycelium was removed from the medium by filtration. The chitosanase was salted out by adding 20-90% saturation ammonium sulphate, and the precipitates were collected by centrifugation at 9000 rpm for 25 min. The precipitates were dissolved in an appropriate volume of distilled water and dialyzed against acetate buffer (100 mM, 5.5) overnight at 4^{0}C. Finally the partial purified enzyme was lyophilized, stored and was used for immobilization studies.

2.2. Immobilization of Chitosanase enzyme

The PAN nanofiber (0.05 gm) was treated with different concentration of glutaraldehyde (GA) (1-15%) for different time intervals (5-60 minutes) followed by washing with acetate buffer. This GA treated NFM was treated with 10 ml of (1-10 mg/ml) lyophilized chitosanase enzyme solution in 0.1 M acetate buffer solution (pH 5.5). After the immobilization, the membrane was removed from the solution and washed several times with buffer to remove any unbound enzyme and stored after lyophilization. Adsorption of enzyme on PANNFM was done by treating the nanofibre surfaces directly with enzyme solution.

2.3. Properties of Immobilized chitosanase

Enzyme activity was measured over the temperature range of 20–90^{0}C and pH range of 2–9 to evaluate the effect of temperature and pH on the activities of free and immobilized chitosanase. To evaluate the reusability and stability, the Chitosanase-immobilized NFM, after each reaction was washed with Acetate buffer (100 mM, 5.5) and reintroduced into substrate solution to start the next batch of reaction. The storage stabilities of immobilized enzyme were determined by incubating the PANNFM- enz in Acetate buffer solution at 4^{0}C up to two months and assayed for residual activity at predetermined times. Thermal stability of free and immobilized enzyme was checked by incubating at different temperature (20-90^{0}c) for 15 minutes and assay was done to check residual activity.

2.4. Assay

0.1 g of immobilized enzyme was added to 3 ml 1% (w/v) chitosan solution and incubated at 50^0 C for 30 min. The reaction was stopped by adding 0.5 ml 1 N NaOH solution. The mixture was centrifuged at 8,000 rpm for 15 min. The concentration of reducing sugar was determined by as that in case of free enzyme by modified schales' method [11] using standard curve of Glucosamine Hcl. One unit was defined as the amount of enzyme that could produce l μmol reducing sugar in 1 min. Yield calculation of glucosamine after

60 minutes of hydrolysis was done by same method as that of Sashiwa et al, 2003 [3] for N-acetylglucosamine. Protein concentration was determined with Coomassie Brilliant Blue reagent following Bradford's method [12]. BSA was used as standard to construct the calibration curve of protein.

2.5. Analytical methods

The detail surface morphology was studied by scanning electron microscope (SEM, Zeiss-EVO, MA-10, variable UK).

2.6. Thin layer chromatography

0.05 g of nanofibres with immobilized chitosanase enzyme was incubated with chitosan (1 % w/v) in Hcl, as sodium salt interferes in TLC (pH 6.0) at 50^0C for different time interval. At appropriate time interval reaction mixtures were withdrawn to stop the enzymatic reaction. Absolute ethanol was added to the mixture to a concentration of 70% and insoluble chitosan was discarded by centrifugation. The supernatant was concentrated by a rotary vacuum evaporator and then subjected to TLC on a silica G 60 plate (Merck) and developed in a solvent system composed of n-butanol: acetic acid: water (50:25:25) (v/v/v). The TLC plate was stained by spraying with ninhydrin (0.5%) reagent and bands of reducing sugars were visualized by keeping the plate in an oven at 80^0 C for 10 minutes. Blank was kept for reference without adding enzyme to chitosan solution.

3. Results and discussion

3.1. Enzyme immobilization

Binding of enzyme (70%) on PANNFM was good in case of adsorption but reusability was very poor. Protein is washed out if, binding to the support is noncovalent in nature which leads to poor reusability. GA as cross linking agent for attaching enzyme on PANNFM showed good reusability (4-5 times) with minor loss in activity. Figure 1(a) shows the SEM micrograph of (7.5 wt %) PANNFM, (b, c) shows PAN surface treated with 10% GA and enzyme treated surface respectively. There was remarkable change in the surface morphology of nanofibres after enzyme treatment (from smooth to rough) which confirms enzyme binding on PANNFM. Immobilization of enzyme using GA is most frequent technique used worldwide [13] and has been used for amyloglucosidase enzyme on PAN [14] and for chitosanase immobilization on chitin [6]. 10% of GA concentration was found to be optimum for maximum protein binding (90%) per unit area of fibre (fig 2) while in case of chitin as support material it was 5% GA. GA treatment of PAN nanofibres were tried for different time interval of which 30 minutes seemed to be appropriate for maximum binding after which it didn't increase with time. 4.0 mg of chitosanase was optimum amount for maximum activity retention of chitosanase enzyme. Two hours treatment of PANNFM with enzyme led to maximum protein binding, while in case of chitin it took 24 hours. Optimum pH and temperature for free and immobilized enzymes were studied and compared. pH 4.0 and 50^0C temperature was optimum for immobilized enzymes while for free enzyme optimum was pH 6.0 and there was no change in optimum temperature. Acidic shift of immobilized enzyme has been reported mostly in case of support having cationic matrix [15]. Thermal stability of enzyme improved slightly (10-15%) above 50^0c which may be due to covalent bond formation between fibres and enzyme which provide stability.

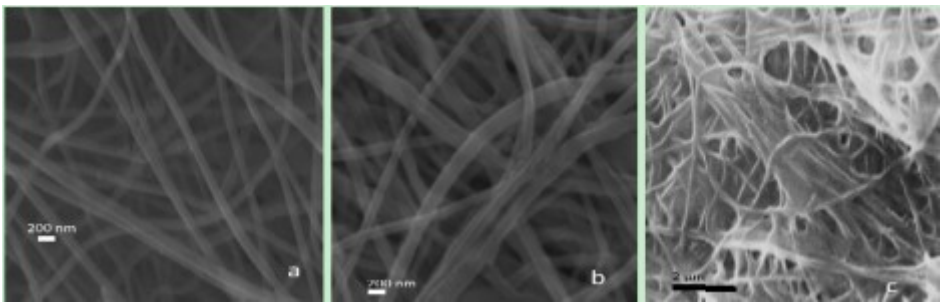

Figure 1: Scanning Electron Micrograph of PAN nanofibrous membrane (a) original nanofibrous membrane (7.5 wt. %) PAN (b) 10% GA treated PANNFM (c) chitosanase immobilized on GA treated NFM

3.2. Glucosamine production

Glucosamine production by enzymatic hydrolysis of chitosan and other substrates both with free and immobilized enzymes were carried out in batch set up and yield of glucosamine was compared. Yield of glucosamine in case of immobilized system was 70% that of free enzyme. This may be due to mass transfer limitations associated with PANNFM and inefficient mixing of substrate due to viscous nature of chitosan. Vigorous mixing of reaction mixture was avoided due to possibility of breakage of PANNFM. Lower yield in case of immobilized enzymes can't be ruled out but reusability, storage stability and ease of recycling are such points which can't be ignored as advantages associated with immobilized enzyme over free enzymes.

Immobilized enzyme could be stored for almost 50-60 days in lyophilized form with 20-30 % loss in activity. Different types of substrates i.e. chitosan from crab shell: degree of deacetylation (>60-90%) shrimp shell, chitin, chitosan trimer and glycol chitosan were tested for glucosamine production using immobilized enzymes and has been summarized in Table 1. TLC (figure 3) was done to find out, if enzyme is having endo activity and if chitooligosaccharides are being produced initially along with glucosamine, as it will reduce the yield of monomer i.e. glucosamine.

Production of N-acetyl D-glucosamine from chitin using free crude enzyme extract has been reported previously [16, 17] but yield per unit time (74% in 5days) was low as compared to this study. D-glucosamine production in batch hydrolysis using chitosanase immobilized on amylase coated magnetic nanoparticles and agar gel [7] disc have also been done. Chitosanase on magnetic nanoparticles was also producing oligomers of chitosan along with glucosamine, so it is not useful in the case of such systems where the oligomers are undesirable. Chitosanase immobilized on agar gel disc was mainly used for production of chitooligosaccharides and mass transfer limitation was associated with the system which could be easily removed with the help on nanofibres.

In conclusion, using crude enzyme attached on nanofibres for selective production of D-glucosamine is advantageous in comparison to free enzyme as they can be used for multiple batches of reaction and their removal is easy from the reaction mixture. These nanofibres attached enzyme can be stored for longer duration and there is reduced mass transfer limitation.

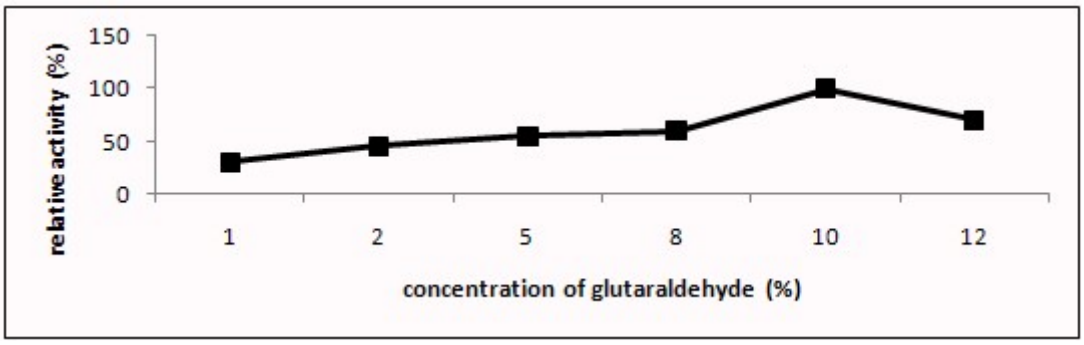

Figure 2: Effect of concentration of Glutaraldehyde on enzyme immobilization

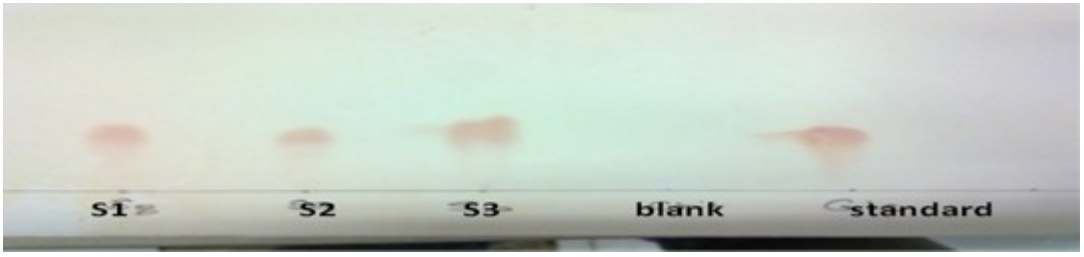

Figure 3: TLC photographs of Glucosamine production using immobilized enzymes (S1, S2, S3 are sample taken at different time (15 minutes, 30 minutes and 60 minutes) interval.

Substrate	Yield of glucosamine (%)	
	Free enzyme	**Immobilized enzyme**
1. Chitosan (DAC>90%)	100*	70
2. Chitosan (DAC>80%)	78	65
3. Chitosan (DAC>70%)	50	45
4. Chitosan (DAC>60%)	35	20
5. Chitosan (shrimp shell)	45	40
6. Chitosan trimer	90	78
7. chitin	20	nil
8. Colloidal chitosan	60	45
9. glycol chitosan	10	5

Table 1: yield of glucosamine by enzymatic hydrolysis (free and immobilized enzyme) of different type of chitosan, yield of glucosamine produced after hydrolysis of (DAC>90%) of chitosan by free enzyme was taken as 100%. Time of hydrolysis: 60 minutes, Yield (%) = [glucosamine produced (mmol)/ chitosan substrate (mmol)] x100

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5. References

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