



Immobilisation of cyclodextrin glucanotransferase into polyvinyl alcohol (PVA) nanofibres via electrospinning



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ARTICLE INFO

Article history:

Received 5 January 2016

Received in revised form 7 March 2016

Accepted 7 March 2016

Available online 9 March 2016

Keywords:

Nanofibres

Immobilisation

CGTase

Electrospinning

Cyclodextrins

ABSTRACT

Immobilisation of cyclodextrin glucanotransferase (CGTase) on nanofibres was demonstrated. CGTase solution (1% v/v) and PVA (8 wt%) solution were mixed followed by electrospinning (−9 kV, 3 h). CGTase/PVA nanofibres with an average diameter of 176 ± 46 nm were successfully produced. The nanofibres that consist of immobilised CGTase were crosslinked with glutaraldehyde vapour. A CGTase/PVA film made up from the same mixture and treated the same way was used as a control experiment. The immobilised CGTase on nanofibres showed superior performance with nearly a 2.5 fold higher enzyme loading and 31% higher enzyme activity in comparison with the film.

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1. Introduction

Cyclodextrin glucanotransferase (CGTase) is an industrially important enzyme that catalyses the transglycosylation reaction of starch and linear oligosaccharides to form ring structured molecules, called cyclodextrins (CDs) [1]. CDs possess the unique property of having a hydrophilic outer surface and a hydrophobic cavity which enables them to form an inclusion complex with guest molecules [2]. Due to this feature, they are widely used in industrial and research applications. The use of CDs has increased by about 20–30% per annum, mainly in food and pharmaceutical products [3].

Generally, free or soluble CGTase are used for the industrial production of CDs [4]. However, the main drawback in the industrial application of free enzymes is their relatively high price and low stability [5]. Immobilising the enzyme by fixing to a solid support will allow the enzyme to be recovered and reused, thus reducing the amount of enzyme required, and simplifying product

purification, which makes the enzymatic processes more economically feasible [6–8]. To this end, several CGTase have been immobilised using different supports and immobilisation methods [3,9].

The trend of enzyme immobilisation has now shifted towards the utilisation of nanostructured materials as the enzyme support, which includes nanoporous materials, nanoparticles, and nanofibres [10]. In comparison with the conventional supports, nanostructured materials offer the intrinsic characteristic of an extremely high surface area to volume ratio which is desirable for improvement of the immobilisation efficiency. Immobilised CGTase on nanoporous silica and nanoparticles show a remarkable improvement in terms of enzyme loading and activity recovery [11,12]. However, despite the advantages, some of the problems associated with nanoporous media are such as limited enzyme-substrate interaction due to the enzyme confinement on its inner surface and the complex recovery procedures of the nanoparticles [10]. In this regard, increasing interest has been shown in incorporating enzymes into nanofibrous materials, particularly electrospun nanofibres [6,13–15].

Electrospinning involves electrical atomisation of a viscous liquid that is capable of producing fibres with a diameter ranging

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from the micro to nanometre scale. With high porosity and interconnectivity, the electrospun nanofibres can form a membrane which can be easily recovered, reused and could reduce the mass transfer limitation [16,17]. These unique characteristics enable the application of electrospun nanofibres in diverse fields such as controlled release of compounds in drug delivery, templates or scaffolds in tissue engineering, reinforcement for composite materials and membranes for water filtration systems [6,8]. In biocatalysis, an enzyme immobilised on a nanofibrous membrane allows repeated usage of the enzyme and simultaneous biocatalytic reaction and enzyme-product separation, which are the main requirements for the application of an immobilised enzyme in a membrane-bioreactor [10,13].

In this study, the immobilisation of CGTase into a nanofibrous PVA membrane *via* electrospinning of CGTase and a PVA solution was demonstrated. To the knowledge of the authors, this is the first application of nanofibres as a support for CGTase. The performance of the immobilised enzyme was examined in terms of enzyme loading and catalytic activity with CGTase/PVA nanofibres. In addition, the CGTase/PVA film that was produced from the same solution was used as a control.

2. Materials and methods

2.1. Materials

Polyvinyl alcohol (PVA) with an average molecular weight of 85 000–124 000 Da and a 99% degree of hydrolysis was obtained from Sigma Aldrich, USA. CGTase (EC 2.4.1.19) from *Bacillus macerans* was procured from Amano Enzyme, Inc., Japan. α -CD was provided by Acros Organics, USA. The Bradford reagent, bovine serum albumin (BSA, 67 000 Da) and glutaraldehyde (GA) solution (25% (w/v) in water) were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Soluble starch and hydrochloric acid (32% (w/v)) were provided by Merck, Germany. All the other chemicals used were of analytical reagent grade.

2.2. Methods

2.2.1. Preparation of PVA/CGTase solution

PVA powder was dissolved in a phosphate buffer (100 mM, pH 6) at 90 °C under mild stirring to create a PVA solution of 8% (w/v) concentration. The solution was then allowed to cool at room temperature before mixing with CGTase solution to produce a CGTase/PVA mixture with 1% (v/v) enzyme concentration.

2.2.2. Electrospinning of CGTase/PVA solution

The freshly prepared CGTase/PVA solution was fed into a 10 ml plastic syringe fitted with a 16 G stainless steel capillary (length \times OD \times ID = 80 \times 1.61 \times 1.25 mm). The electrospinning was performed by applying a high electric field (-9 kV) to the capillary for a total of 3 h and with a flow rate of 0.5 ml/h. The electrospinning modes were observed using a digital camera equipped with a macro lens. The PVA/CGTase nanofibres were collected using a rotating collector which was covered with aluminium foil (grounded) and placed at 20 cm away from the capillary tip. The nanofibres obtained were then dried overnight in a desiccator before detaching from the aluminium foil and stored at 4 °C before further reactions and analyses. A schematic diagram for the method of CGTase/PVA nanofibre production and the electrospinning system is presented in Fig. 1.

2.2.3. Vapour phase glutaraldehyde (GA) crosslinking

The membrane was subjected to glutaraldehyde vapour phase crosslinking to prevent the dissolution of the hydrophilic PVA as well as to improve the enzyme-PVA interaction. The crosslinking was conducted for 2.5 h at ambient temperature in the presence of hydrochloric acid (HCl) as a catalyst with a 3:1 ratio of GA:HCl, as previously described by Shaikh et al. [18].

2.2.4. Characterisation of PVA/CGTase membrane

The morphology of the PVA/CGTase membrane was observed using a Scanning Electron Microscope (SEM) (JSM 6510, JEOL,

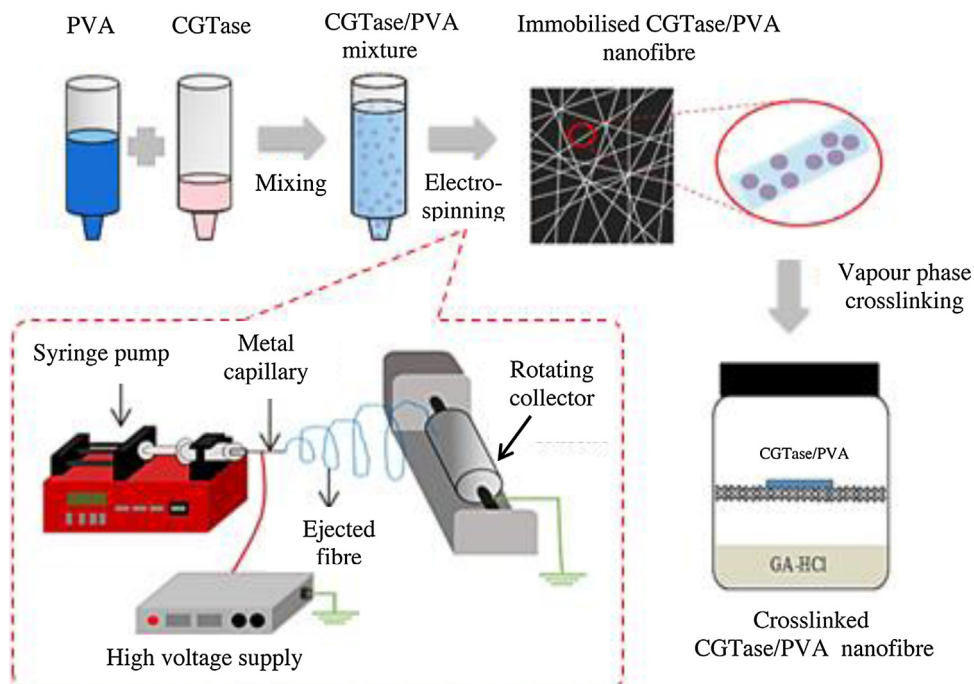


Fig. 1. Schematic of the method used to produce CGTase/PVA nanofibres.

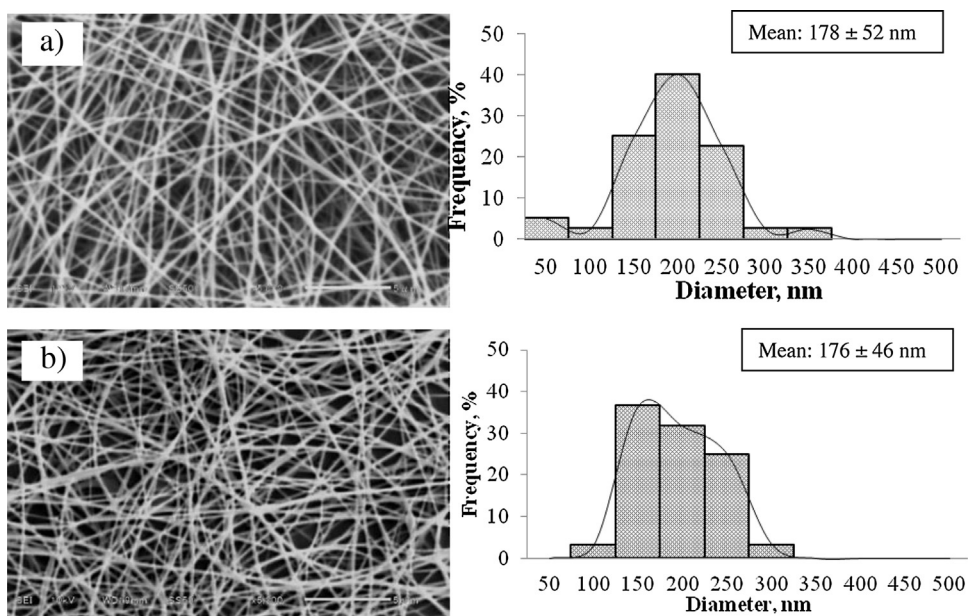


Fig. 2. SEM images and size distribution of electrospun nanofibrous membrane: (a) PVA; (b) CGTase/PVA.

Tokyo). The sample was cut into 0.5 cm x 0.5 cm pieces and mounted on stubs using carbon tape. The analysis was performed using an accelerating voltage of 10 kV. Prior to the analysis, the sample was sputter-coated with gold (JFC 1200, JEOL, Tokyo) under vacuum to avoid a charging effect during the SEM observations. The sizes of at least 60 fibres were determined using ImageJ software (NIH, Bethesda) and the value presented as the average.

2.2.5. Enzyme loading analysis

The amount of enzyme loaded on the PVA was determined using colorimetric analysis following the modified Bradford assay, as described by Ivanova (2010) [3] with slight modification [15]. The enzyme loaded support (before crosslinking) was incubated in 200 μ l of phosphate buffer for 12 h at 4 °C. Then, 1800 μ l of Bradford reagent was added into the solution. The mixture was gently mixed for 3 min to allow binding of the protein and the absorbance was measured at 595 nm. Bovine serum albumin (BSA) was used as a standard. The enzyme loading efficiency was determined by using the following equation:

Enzyme loading efficiency

$$= \frac{\text{Amount of protein in the membrane (mg)}}{\text{Whole membrane (g)}}$$

2.2.6. Enzyme activity analysis

CGTase activity was measured as α -CD formation activity, as previously described in [19] with slight modifications [20]. Briefly, the immobilised enzyme was incubated with 1 ml of 1% (w/v) soluble starch solution prepared in a phosphate buffer (100 mM, pH 6) at 60 °C for 5 min. The reaction was stopped by immediate cooling in chilled water, followed by the addition of 0.1 ml of HCl (1.2 M) and 2 ml of methyl orange (0.035 mM). The mixtures were maintained at room temperature for 15 min. Absorbance was measured at 506 nm using a UV-vis spectrometer (Ultrospec 3100 Pro, GE Healthcare Sdn Bhd., Kuala Lumpur). One unit of enzyme activity was defined as the amount of enzyme that produced 1 μ M of α -CD per min under the assay conditions.

3. Results and discussion

3.1. Characteristics of the electrospun nanofibrous membrane

Electrospinning of PVA solution produced smooth and uniform fibres with an average size of 178 ± 52 nm. The CGTase solution could not be electrospun alone to produce fibres due to its complex molecular structure and strong inter and intra-molecular bonding among the enzyme molecules [21]. When mixed with PVA solution, it is possible for the enzyme to be electrospun because of the capability of PVA to form secondary bonding with the enzyme molecules, so that the complex structure and strong bonding among the enzyme molecules could be disrupted [15,16]. As shown in Fig. 2, the addition of the enzyme into the PVA solution did not cause a significant change in the nanofibre morphology. Although the fibre size distribution was slightly different, the average fibre size was not affected, suggesting that the CGTase/PVA mixture could sustain the electrospinning process and was successfully transformed into a CGTase/PVA nanofibrous membrane.

3.2. Enzyme loading efficiency and enzyme activity

The loading efficiency and activity of the immobilised CGTase in the electrospun nanofibres is presented in Fig. 3. CGTase/PVA film prepared from the same solution as in Section 2.2.2 was used as the control experiment. The nanofibres exhibited a high enzyme loading efficiency, which was almost 2.5 fold higher (3.86 ± 0.35 mg/g) than the film (1.63 ± 0.17 mg/g), and with a 31% higher activity. The high enzyme loading obtained for the nanofibres was mainly attributed to the three dimensional structure of the support in combination with its high interconnectivity and porosity [15] as previously shown in Fig. 2, which provided a high surface area for the enzyme attachment. The result obtained here confirmed the good immobilisation efficiency of the nanofibre support. Besides the high enzyme loading, the high enzyme activity achieved by the CGTase/PVA nanofibres was contributed by the minimal diffusion resistance as the substrate

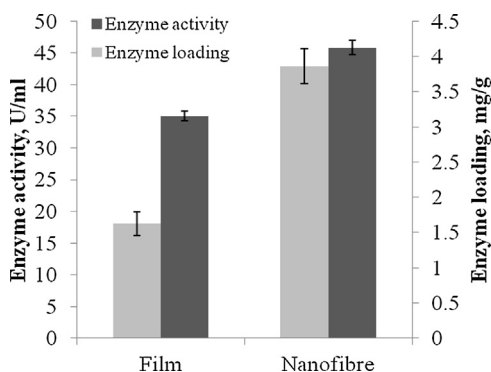


Fig. 3. Enzyme loading and activity analysis of CGTase immobilised in PVA nanofibre and PVA film.

and the product could move freely within the porous nanofibre network [22], thus improving the enzyme-substrate interaction.

3.3. The effect of crosslinking on the enzyme activity

Glutaraldehyde (GA) has been widely used in enzyme immobilisation due to its high reactivity towards the amine group of the protein and hydroxyl group of the carrier materials [23]. However, a severe loss in enzyme activity is usually observed after crosslinking with glutaraldehyde solution [18]. Several researchers have reported the successful application of vapour phase glutaraldehyde crosslinking on enzymes such as glucose oxidase [24-*arunas*], sarcosine oxidase [25], cellulase [15] and laccase [26] with a retained activity of up to 83.7%. In this study, glutaraldehyde crosslinking of CGTase at the vapour phase was conducted after the immobilisation and its effect on the enzyme activity is presented in Fig. 4. The crosslinking process did not have a great effect on the enzyme activity as the enzyme could retain up to 95% and 84% of its initial enzyme activity for the nanofibres and film, respectively. The high activity retention obtained here may be attributed to the formation of a protective layer of PVA in which the enzyme was protected from the external surroundings, as only a small amount of the GA vapour could pass through the PVA layer [26]. In addition, immobilised CGTase in the nanofibre support showed higher activity retention compared to the film which might be attributed to the additional protection provided by the nanofibre network and the high porosity of the membrane which allowed the substrate to diffuse to/off the membrane easily for maximal enzyme-substrate interaction [17,26].

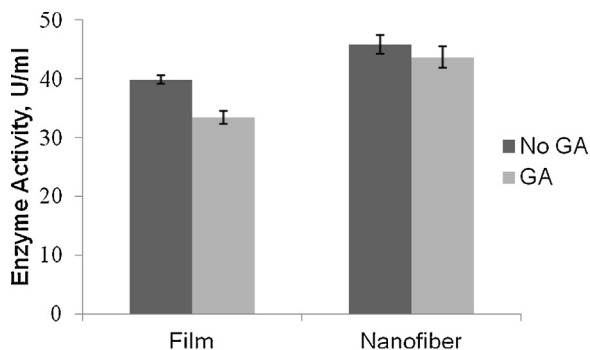


Fig. 4. Effect of vapour phase GA crosslinking reaction on CGTase activity.

4. Conclusion

The electrospinning technique has effectively immobilised the CGTase enzyme into the PVA nanofibrous membrane without affecting the fibre structure and size. The nanofibres produced show excellent immobilisation efficiency with a 2.5 fold higher enzyme loading and almost 31% higher enzyme activity in comparison with the film (control). The vapour phase crosslinking reaction applied to the PVA/CGTase membranes caused no remarkable effect on the enzyme activity. The findings of the present study suggest that nanofibres are a potential support for CGTase immobilisation. The method employed could be extended by the use of a higher enzyme concentration to further improve the overall enzymatic performance. Furthermore, this simple and versatile method can be applied to other types of biologically active substances such as cells and proteins to improve their stability and expand their potential applications especially as biosensors, for bioremediation, biofuel cells and large scale biocatalysis operation.

Acknowledgements

This research was supported by the Putra grants GP-IPS, Universiti Putra Malaysia, (UPM/700-2/GP-IPS/2015/9465200), Science fund, JSPS Kakenhi No. 26420761 (IWL) and a Research Entity Initiative (REI) Grant by Universiti Teknologi MARA (UiTM) (Project no. 600-RMI/DANA/5/3/REI (2/2013)). S. Saallah holds a scholarship from the Ministry of Higher Education (MOHE), Malaysia under the SLAB scheme. The authors acknowledge the input from Dr Motoyuki Iijima, Mr Pramujo Widiatmoko, Mr K. Kusdianto and Mr Keisuke Naito from the Tokyo University of Agriculture and Technology for their assistance in the experimental analysis.

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