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Preparation and Characterisation of Cyclodextrin Glucanotransferase Enzyme Immobilised in Electrospun Nanofibrous Membrane

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Abstract: An industrial enzyme, Cyclodextrin glucanotransferase (CGTase), was immobilised in polyvinyl alcohol (PVA) nanofiber (average diameter around 200 nm) membrane via co-electrospinning of the CGTase/PVA mixture followed with glutaraldehyde vapour phase cross-linking. Addition of enzyme with concentration ranging from 1.5 to 7.5 % to the PVA solution (8 wt%) caused significant changes to the liquid jet behaviours which consequently affected the nanofiber structures and sizes. Incorporation of CGTase in the PVA membrane was confirmed by Raman spectroscopic analysis. The Raman spectra also showed no structural changes occurred to the enzyme after subjected to the electrostatic spinning and cross-linking reaction. The immobilised enzyme showed excellent catalytic efficiency with up to 3.6 times higher enzyme loading, 25 % higher activity and good reusability in comparison with CGTase/PVA film made up from the same starting solution (control).

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Introduction

Cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19) is an industrial enzyme that primarily catalyses the transglycosylation (cyclisation) of starch to produce cyclodextrins (CDs), a closed ring structured molecules composed of six or more glucose units. Steric arrangement of these glucose units formed a hollow truncated cone shaped molecule with a hydrophilic surface to promote solubilisation and a hydrophobic cavity that enables them to form specific inclusion complexes with various compounds (Li et al., 2007). This unique feature promotes numerous applications of CDs in the development of food products, chemical industries, agriculture, pharmaceutical, textile, and environmental protection (Del Valle, 2004). However, enzymatic production of CDs is often hampered by the relatively high price and low stability of the biocatalyst (Martín et al., 2002), difficulty of enzyme recovery and high operational costs for product purification (Sardar & Ahmad, 2015). Immobilisation of enzyme using a solid support is well recognized as a promising approach to overcome such shortcomings.

Enzyme immobilisation represents the integration of an enzyme onto/into a support material (Fang et al., 2011). Enzymes in immobilised form are more robust and stable towards any changes in the environmental conditions (i.e temperature, pH) in comparison with the free enzyme (Homaei et al., 2013). More importantly, immobilisation allows simplification of the product purification process (Jochems et al., 2011), which provides the opportunity for scaling up and enables the enzyme to be reused and applied in a...
continuous process (Hwang and Gu, 2013). Despite these advantages, extending the use of immobilised enzymes to various fields and applications requires further improvement and a better understanding of the current techniques to enhance their catalytic efficiency.

To date, many types of materials have been designed for enzyme support or carrier. Properties of these support materials, particularly the size and structure, play an important role in determining the immobilised enzyme performance. Reduction of the support geometric size can improve the catalytic efficiency by providing high surface area for enzyme attachment (Sardar and Ahmad, 2015). In this sense, growing interest is being shown in integrating enzymes with nanostructured materials, particularly nanofibers.

Electrospinning is a process in which electrostatic force is applied to a polymer solution to generate fibers with diameters ranging from several microns down to smaller than 100 nm (Sill and von Recum, 2008). Nanofibers produced through electrospinning offers several outstanding features for enzyme immobilisation: a) size, morphology and composition can be control to meet the requirements of enzyme support, b) ease of functionalisation for various purposes, c) high porosity and interconnectivity to reduce mass transfer limitation throughout the meshes and d) the fibers can be collected in the form of membrane to facilitate the biocatalyst recovery and reuse, which would allow them to be applied in an enzymatic membrane bioreactor (Agarwal et al., 2008; Herricks et al., 2005; Kim & Kim, 2017; Tran & Balkus, 2012).

The utilisation of electrospun nanofibers for enzyme immobilisation have been achieved mainly through physical adsorption and covalent binding. Both of these methods allow the enzyme to "only" attach on the outer surface of the support, creating monolayer coverage of enzyme which might limit the loading capacity. To encounter this issue, a simple method which involves the encapsulation of an enzyme by co-electrospinning of enzyme and polymer solution mixture has been introduced (Dai et al., 2013; Fazel et al., 2016; Sakai et al., 2008). Enzymes such as lipase (Sakai et al., 2008; Sawada et al., 2012), laccase (Dai et al., 2013), horseradish peroxidase (HRP) (Fazel et al., 2016) and acetylcholinesterase (Moradzadeghan et al., 2010) have been successfully immobilised in polymer membranes with a remarkable improvement in enzyme loading efficiency. Their catalytic performances were also remained upon immobilisation, confirming that either the electric charge applied during the electrospinning process or the interference of polymer materials had minimal or no negative effects on the enzyme structure and catalytic function.

In spite of the simplicity of the co-electrospinning strategy and the excellent features possessed by the electrospun polymeric nanofibrous membranes, to this extent, very few reports concerning nanofibers as a support for enzyme immobilisation have been published. Previously, the authors have shown that CGTase could be successfully immobilised into a nanofibrous polyvinyl alcohol (PVA) membrane (Saalalah et al., 2016) by co-electrospinning of CGTase and PVA mixture, which allow the enzyme to attach and encapsulated in the nanofibers. The PVA solution is rich with hydroxyl groups that enable the formation of secondary bonding with the enzyme molecules. Therefore, by mixing the enzyme with PVA, the complex 3-dimensional structure and strong molecular interactions between the enzyme molecules could be interrupted (Wu, Yuan, & Sheng, 2005), which allow the formation of CGTase/PVA nanofibers by electrospinning. However, the previous study only focused on the feasibility of immobilising the CGTase enzyme in the PVA nanofiber at a low enzyme concentration (1 % v/v) and the structural changes that might occur during the immobilisation process were not comprehensively considered. Moreover, although several studies related to enzyme immobilisation using co-electrospinning approach have been done in the past, to our knowledge, no studies have been conducted to investigate the effect of addition of enzyme to the polymer solution on the behaviour of the electrified jet. This information is of interest to gain a thorough understanding and better control of the electrospinning process which affect the properties and performance of the immobilised enzyme/nanofiber.

In the present study, the effect of varying the CGTase enzyme concentration from 1.5 to 7.5% v/v on the electrospinning process and the nanofibrous membrane morphology and size were investigated. Efficiency of the immobilised CGTase/PVA nanofiber was investigated by analysing the enzyme loading, activity and reusability. The CGTase/PVA film made up from the same electrospinning solution was used as a control.
Materials and Methods

Materials

Polyvinyl alcohol (PVA) (85000-124000 Da, 99 % degree of hydrolysis), Glutaraldehyde (GA) solution (25 % in water), Bradford reagent, and bovine serum albumin (BSA, 67000 Da) were supplied by Sigma-Aldrich, USA. CGTase (EC 2.4.1.19) from Bacillus macerans was purchased from Amano Enzyme, Inc., Japan. α-cyclodextrin (α-CD) was purchased from Acros Organics, USA. Soluble starch and hydrochloric acid (32 %w/v) were provided by Merck, Germany. All other chemicals used were of analytical reagent grade.

Preparation and characterisation of CGTase/PVA solution

A PVA solution with a concentration of 8 % w/v was prepared by dissolving PVA powder in acetate buffer (100 mM, pH 6) under mild stirring at 90 °C. The solution was allowed to cool to room temperature before mixing with CGTase to obtain a CGTase/PVA mixture with 1.5 to 7.5 % v/v CGTase concentration. Prior to electrospinning, properties of the CGTase/PVA solutions which include the viscosity and conductivity were analysed using a Cannon-Fenske viscometer (Cannon Instrument Company, USA) and conductivity meter (Mettler Toledo, Switzerland), respectively, to study its effect on the electrospinning and fiber formation. Properties of the samples were checked regularly to ensure that the prepared solutions had constant properties prior to electrospinning.

Formation of CGTase/PVA membrane by electrospinning

The CGTase/PVA membrane was fabricated using the similar electrospinning system described in Saallah et al. (2016) with slight modifications on the experimental conditions. The system consisted of a high voltage supply, a stainless steel capillary (16 G, length × OD × ID = 80 × 1.61 × 1.25 mm) connected to a syringe pump and a grounded rotating collector covered with aluminium foil (Fig. 1(A)). The electrospinning modes were observed using a digital camera (Nikon, D 90) equipped with a macro lens (Nikon, AF Micro-Nikkor 105 mm f/2.8 D). Table 1 summarises the parameters used for the fabrication of the CGTase/PVA membrane. The membrane obtained was then dried overnight in a desiccator before being detached and subjected to glutaraldehyde vapour phase crosslinking vapour for 2.5 hours at an ambient temperature in the presence of hydrochloric acid (HCl) as a catalyst (3:1 ratio of GA: HCl).

Scanning electron microscopy (SEM) analysis

A Scanning Electron Microscope (JSM 6510, JEOL, Japan) was used to observe the morphology of the immobilised enzymes. The analysis was performed using an accelerating voltage of 10 kV. All samples were sputter-coated with gold (JFC 1200, JEOL, Japan) under vacuum before the SEM observations. The images obtained from the SEM analysis were processed using ImageJ software (NIH, Bethesda) to obtain the average size and distribution of at least 120 fibers.

Raman spectroscopy analysis

The effect of the immobilisation process on the enzyme functional groups was investigated by using a Raman spectrometer (Nicolet Almega XR, Thermo Scientific Corp., USA). The analysis was performed at room temperature with a 532 nm laser as the excitation source. The time duration and the number of exposures were set at 1 s and 10 times, respectively while the laser power was set at 15 mW to avoid destroying the target structure. The data was collected and analysed by OMNIC software (Thermo Scientific Corp., USA).

Determination of the amount of bound enzyme

The enzyme loading was analysed by using a Bradford assay (Ivanova, 2010). Bradford reagent was prepared by diluting a Coomassie Blue G-250 reagent (Sigma-Aldrich, USA) with distilled water at a ratio of 1:5. In this assay, the enzyme-loaded support (before cross-linking) was incubated in 200 μL phosphate buffer for 12 h at 4 °C followed by the addition 1800 μL of Bradford reagent to the solution and gentle mixing

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Experimental conditions</th>
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<tbody>
<tr>
<td>Electrospinning parameters</td>
<td>Ambient conditions</td>
</tr>
<tr>
<td>Voltage</td>
<td>-9 kV</td>
</tr>
<tr>
<td>Flow rate</td>
<td>0.2 ml/hr</td>
</tr>
<tr>
<td>Tip to collector distance</td>
<td>10 cm</td>
</tr>
<tr>
<td>Duration</td>
<td>3 h</td>
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(3 min) to allow binding of the protein. The absorbance was measured at 595 nm using a UV-VIS spectrometer (MSV-370, Jasco, Japan). The enzyme loading efficiency was determined by taking the ratio of the amount of protein (mg) in the membrane to the whole membrane mass (g). Bovine serum albumin (BSA) was used as the standard.

**Enzyme activity analysis**

The CGTase activity was measured based on the production of α-CD as previously described in (Higuti et al., 2004). The α-CD concentration was assayed by the decrease in absorbance at 506 nm caused by the formation of the methyl orange α-CD complex. The process involved the reaction of immobilised enzyme with 1 mL soluble starch solution (1 wt% in 100 mM phosphate buffer at pH 6) at 60 °C for 5 minutes. Then, the mixture was placed in chilled water followed by the addition of 0.1 mL of HCl (1.2 M) to stop the enzymatic reaction. A volume of 2 mL of methyl orange (0.035 mM) was then added to the reaction solution. The mixtures were maintained at room temperature for 15 minutes. Absorbance was measured using a UV-VIS spectrometer. One unit of the enzyme activity was defined as the amount of enzyme that produced 1 μM of α-CD per minute under the assay conditions.

**Reusability**

The recycling stability of the immobilised CGTase was examined by measuring the activity repeatedly. The CGTase/PVA mixture with 7.5% enzyme concentration is chosen as a representative. The immobilised enzyme was incubated with soluble starch in 100 mM phosphate buffer (pH 6) at 60 °C for 5 minutes, followed by the separation of immobilised enzyme from the reaction product. The activity was measured in the supernatant using the method described in the previous section. Before starting a new cycle, the immobilised CGTase was washed thoroughly with the buffer and was re-suspended in a freshly prepared substrate solution.

**Results and discussion**

**Solution properties**

During electrostatic processing of a solution containing biomolecules, electrochemical reactions inside the capillary might occur which causes dramatic changes in the pH of the solution, as previously reported (Morozov, 2009). The presence of buffering compounds in the spinning solution will prevent or minimised the pH changes. In our previous study, the samples were prepared in phosphate buffer. However, through our preliminary experiment on the hydrodynamic behaviour of the enzyme in different buffers, we found that the enzyme in phosphate buffer (1% v/v) exist as aggregates with average size of 825 nm while in acetic buffer, the aggregate size is almost four times smaller (225 nm), suggesting that the enzyme have better stability in acetic buffer than in phosphate buffer (results not shown). Therefore, in the present study, the CGTase/PVA solutions were prepared in acetic buffer.

The viscosity and conductivity of the aqueous CGTase and 8 wt% PVA in acetic buffer solutions as well as their mixtures were examined to investigate their effects on electrospinning and fiber formation. The 8 wt% PVA in acetic buffer solution has a viscosity of 616.47 cP while the CGTase solution has a much lower viscosity (28.32 cP), which indicates that the intermolecular hydrogen bonding between PVA molecules was much stronger than the interactions between the CGTase molecules (Wang & Hsieh, 2008). Addition of enzyme to the PVA solution at concentration of 1.5% to 7.5% reduces the solution viscosity from 579.26 cP to 454.25 cP. Opposing observation was found for the solution conductivity in which the conductivity increases with the increase of enzyme concentration in the PVA solution. The CGTase solution has almost 4 times higher conductivity than the PVA solution. Therefore, higher concentration of enzyme presence in the solution will eventually increases the solution conductivity.

<table>
<thead>
<tr>
<th>Sample</th>
<th>PVA concentration (wt %)</th>
<th>CGTase concentration (% v/v)</th>
<th>Viscosity (cP)</th>
<th>Conductivity (mS/cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8</td>
<td>0</td>
<td>616.47</td>
<td>8.46</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>1.5</td>
<td>579.26</td>
<td>9.41</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>2.5</td>
<td>555.89</td>
<td>10.64</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>5.0</td>
<td>502.10</td>
<td>12.07</td>
</tr>
<tr>
<td>5</td>
<td>8</td>
<td>7.5</td>
<td>454.25</td>
<td>13.59</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>100</td>
<td>28.32</td>
<td>35.6</td>
</tr>
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</table>
Properties of the samples are summarised in Table 2.

**Electrospinning of the CGTase/PVA**

The mechanism of fiber production by electrospinning generally involved several processes which included liquid jet initiation, elongation, whipping, thinning and drying (Fig. 1(B)) (Huang et al., 2003). As the strength of the electric field applied to the liquid overcomes its surface tension, govern by the repulsive forces between the like charges and attractive forces between the oppositely charged liquid and the collector, the liquid meniscus changes from a rounded to a cone shaped (Taylor cone). A liquid jet was eventually ejected from the tip of the cone and travels towards the collector. While the fiber jet is accelerated towards the collector, it undergoes several processes before being deposited. Initially, the jet travels in a straight path. Due to the strong repulsive forces between the like charges in the polymer jet, the jet path was disrupted, resulted in bending instability which promotes the jet thinning and drying process (D. Li & Xia, 2004).

Initially, it was thought that the high charge density caused the jet to split into several fibers, hence reducing the fiber size before being collected. However, high speed photographic observation revealed that the jet was actually whipped rapidly in a spiral motion instead of splitting. Behaviour of the electrified jet during electrospinning was influenced by a number of parameters including the processing parameters, polymer and solvent properties as has been discussed intensively by Reneker and Yarin, (2008). To study the effect of addition of enzyme with various concentration on the jet behaviour, a mixture of 8 wt% PVA solution and enzyme solution with a concentration ranging from 1.5% to 7.5 %v/v was subjected to electrospinning to produce a CGTase/ PVA nanofibrous membrane. The 8 wt% PVA solution in acetate buffer (without addition of enzyme) was used as a control. During the electrospinning process, the jet behaviour was captured with a digital camera (Fig. 2).

As can be seen from the photograph in Fig. 2 (a) and Fig. 2 (b), it is evident that the jet was initially travelled in a straight path and after reaching a certain distance, the jet experienced bending instability and started to whip in a spiral motion until deposited on the collector. However, increasing the enzyme concentration in the polymer solution caused significant changes in the jet behaviour. At a low enzyme concentration (1.5%), the jet travel following the same pattern as in the electrospinning of PVA solution without the addition of an enzyme (control). The formation of droplets along the jet could be seen when the enzyme concentration was increased to 2.5 %. A further increase in the enzyme concentration resulted in the jet splaying into multiple jets with the appearance of more droplets.

![Fig. 1](image1.png)

**Fig. 1** (A) Setup of the electrospinning system and (B) Mechanism of fiber formation during electrospinning

![Fig. 2](image2.png)

**Fig. 2** Jet break-up mechanism observed during the electrospinning of PVA solution with enzyme concentration ranging from a) 0% (PVA only), b) 1.5%, c) 2.5%, d) 5.0% and e) 7.5%.
The result obtained indicated that the addition of enzyme above 1.5% disrupted the stability of the PVA solution to undergo electrospinning, which can be correlated to the decreases of the solution viscosity with addition of enzyme. Viscosity of the solution which is affected by the polymer chain entanglements plays a critical role in controlling the electrospinning process. In low viscosity solution, the density of chain entanglements is low and the ejected jet has insufficient viscoelastic forces to withstand the electrical forces. Therefore, the jet broke-up into several jets and accompanied with droplets. For solution with sufficiently high viscosity, the chain entanglement in the polymer solution created a sufficient viscoelastic forces that allow the jet to stretch and elongate. Moreover, additions of enzyme to the polymer solution also change the solution conductivity. Angammana (2011) reported that the probability of ejecting multi-jets increases with the increases in solution conductivity which might be attributed to the enhancement of the local field at the surface of the fluid.

**Characteristics of CGTase/PVA nanofiber**

The morphology, size distribution, and the average diameter of electrospun nanofibrous membranes with different enzyme concentrations are presented in Fig. 3. Electrospinning of 8 wt% PVA solution produced smooth fibers with an average diameter of 200 ± 38 nm. However, as can be seen from the SEM images, the addition of an enzyme in the PVA solution caused significant morphological changes to the fibers, which supported the previous findings concerning the changes of the jet behaviour as explained in the previous section.

A mixture of 1.5% CGTase in PVA solution could sustain the electrospinning with continuous fiber formation. The diameter of the fiber also reduced slightly to 195 ± 46 nm. By increasing the amount of CGTase to 2.5% in the electrospinning solution, larger fibers were produced and caused the formation of beads. Further increasing the amount of CGTase disrupted the electrospinning process which produced a mixture of fibers with interconnected beads. Electrospinning of 7.5% CGTase in PVA solution could eliminate the appearance of beads but the fibers become flattened. Interestingly, the formation of beads along the fibers with increasing enzyme concentration was in agreement with the trends observed by Zander (2013). As the enzyme concentration in the polymer solution increased, the solution viscosity decreased and the bead shape changed from ellipse to round. In addition, the spacing between the beads was also affected.

Generally, electrospinning of solution with high viscosity produced thick fibers which were usually dried by the time they were collected (Zong et al., 2002) while the lower viscosity solution might reached the collector as “wet” fibers. Inadequate drying of the jet prior to reaching the collector usually resulted in beaded fibers. In some cases, incomplete drying also leads to the formation of flattened fibers attributed to the collapse of the already-dried skin layer by the “wet” core (Sill & von Recum, 2008; Supaphol, Suwantong, & Sangsanoth, 2012). The increment of fiber diameter with addition of enzyme might also be attributed to the poly (electrolyte) nature of enzyme while the formation of beaded fibers could be due to the accumulation of enzyme in those particular areas as reported by several researchers (Moreno-Cortez et al., 2015; Wu et al., 2005).

**Raman spectroscopy analysis**

Raman analysis was conducted to verify the
existence of the enzyme on the PVA membrane as well as to examine the effect of electrospinning and cross-linking on the enzyme functional groups. The spectrum of the PVA membrane showed a broad peak at 3000–3600 cm⁻¹ corresponding to the stretching vibration of PVA hydroxyl (O-H) group (Fig. 4 (a)). The sharp peak centred at 2920 cm⁻¹ referred to the stretching vibration of C-H from the alkyl group. The new peaks observed at 1653 cm⁻¹ and 1245 cm⁻¹ for the CGTase/PVA film and nanofibers belonged to the amide I and amide III groups of protein which confirmed the presence of CGTase in both the film and the nanofibers (Fig. 4 (b) and Fig. 4 (c)). The amide I was primarily characterised by the C-O stretching vibrations which were sensitive to the secondary structure of the protein backbone (~80 %) with a small contribution of N-H in-plane bending vibration while the amide III band was caused by the C-N stretching (Wen, 2007).

The CGTase/PVA nanofibrous membrane showed almost identical spectra with the CGTase/PVA film and the characteristic peaks of CGTase (amide I and amide III) existing in the spectra indicated that the electric field applied during electrospinning had no negative effect on the enzyme secondary structure. The vapour phase cross-linking reaction of the CGTase/PVA nanofibrous membrane with glutaraldehyde (GA) resulted in the disappearance of the peak at 1330 cm⁻¹ and 1100 cm⁻¹ (Fig. 4 d) which was attributed to the O-H deformation and the C O vibration bonds of the PVA secondary alcohols, showing that the membrane was successfully cross-linked with the GA (Prosanov and Matvenko, 2010). The spectra also confirmed that the enzyme functional groups were still preserved after the cross-linking process.

**Enzyme loading and activity analysis**

The efficiency of electrospun PVA nanofiber as a support for CGTase was measured in terms of enzyme loading and activity. A PVA film prepared from the same solution through a conventional casting method was used as control.

The result in Fig. 5 (a) showed that the enzyme loading increased along with the enzyme concentration for both types of support. However, for all ranges of enzyme concentration studied, the enzyme loading efficiency for nanofiber was always higher than the film due to the three-dimensional structure of the support which provided a high surface area for enzyme attachment. Further, the loading efficiency of immobilised enzyme on nanofiber showed a linear dependence with the enzyme concentration. In the case of the enzyme immobilised on the film, at a lower enzyme concentration, the

![Fig. 4](image1)  
**Fig. 4** Raman spectra of: a) PVA, b) CGTase/PVA film, c) CGTase/PVA nanofiber, and d) Crosslinked CGTase/PVA nanofiber. All the immobilised enzyme samples were prepared from 7.5% v/v CGTase enzyme in 8 wt% PVA solution.

![Fig. 5](image2)  
**Fig. 5** Enzyme loading (A) and enzyme activity (B) analysis of CGTase/PVA membranes with varied enzyme concentration. CGTase immobilised in PVA films were used as control.
enzyme loading increased linearly with enzyme concentration. However, further increases in the enzyme concentration showed insignificant changes in the enzyme loading efficiency which proved that the film was already saturated with enzymes, and thus prevented additional enzyme molecules from attaching to it.

Fig. 5 (b) shows the result of the activity analysis of CGTase enzyme with different concentrations immobilised in PVA nanofiber and film. As expected, the activity of CGTase immobilised in both types of support increased as the enzyme concentration increased. The enzyme immobilised in the nanofiber showed higher activity than in the film which was mainly the result of high enzyme loading efficiency of the nanofiber as discussed above. However, by referring to the highest enzyme concentration (7.5%) although the enzyme loading can be increases up to 3.6 times by immobilising the enzyme in the nanofiber, the enzyme activity only increases by 25%, compared to the CGTase/PVA film. The diffusion limitation of the substrate and product from the immobilised enzyme has been reported as the main cause of low enzyme activity with higher enzyme loading. Furthermore, even though the enzyme was more likely to be exposed to the surface of the nanofibers and much less diffusion resistance could be expected with their high surface area and porosity compared to the film (Wu et al., 2005), using the co-electrospinning approach also caused the enzymes to be encapsulated inside the fiber as well as entrapped within the fiber layer. In other words, although high enzyme loading is achieved, not all the enzyme loaded to the nanofibers is exposed to the substrate to participate in the reaction, resulted in low activity (Moreno-Cortez et al., 2015).

Reusability

The ability of the immobilised enzyme to be used continuously is an important criterion to be taken into account for their practical application. The reusability analysis of the immobilised CGTase was carried out by measuring the activity repeatedly after successive washing. As seen in Fig. 6, the immobilised enzyme in nanofiber retained up to 70 % of its initial activity after it had been reused for three times and after that, the relative activity reduced gradually to 54 % at the 5th cycle. In contrast to nanofiber, the relative activity of immobilised enzyme in film reduced remarkably to about 40 % after the 3rd cycle and continued to decrease to 26 % after it has been reused for five times. A similar trend was also observed by other researchers when other types of enzyme were immobilised in PVA nanofiber (Fazel et al., 2016; Moradzadeh et al., 2010; Oktay et al., 2015).

The enzyme immobilised in nanofiber could retain the activity at a level two times higher than the film as a result of the high inter-connectivity of the nanofiber network. This network provided additional support for the enzyme. However, the continuous loss of activity observed in this study might be attributed to two possible reasons. Firstly, some of the enzyme molecules that were not successfully bonded with the support by the chemical cross-linking method were probably lost during the measurement process. Secondly, after subsequent reuse, the diameter of the fibers became larger due to the hydrophilicity of the PVA which reduced the effective surface area for the enzyme-substrate reaction (Fazel et al., 2016) as shown in the SEM image in Fig.6.

![Fig. 6](image)

Fig. 6 Relative activity of immobilised CGTase (7.5%) after used repeatedly. The inset showing SEM image of the CGTase/PVA nanofiber after reused for 9 times.

Conclusion

In conclusion, direct incorporation of enzyme into the polymer nanofiber has been successfully accomplished by co-electrospinning of CGTase/PVA mixture with remarkable improvement in enzyme loading, enzyme activity and reusability compared to the CGTase/PVA film. Addition of enzyme with various concentrations to the polymer solution has affected the solution properties (viscosity and conductivity) which resulted in various jet break-up patterns. Consequently, morphology of the nanofiber containing enzyme also changed from smooth to
beaded and flattened fiber, which affected the fiber size and the immobilised enzyme performances. By increasing the enzyme concentration in the polymer solution, the enzyme loading and activity also increased. The immobilised enzyme could sustain the interference of the polymer solution and high voltage applied during the processing, as well as the application of cross-linking reaction, suggesting that the immobilisation strategy employed in this study could improve the enzyme stability. From these findings, it is therefore suggested that this simple, versatile and non-destructive method can be potentially utilized for various application such as encapsulation of food nutrients and nutraceuticals, development of antimicrobial food packaging, fabrication of biosensors, and control release of drugs, among others.

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