UNIVERSITI PUTRA MALAYSIA

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By

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September 2014

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Faculty : Engineering

There are many types of nanostructured materials that have been used for enzyme immobilization which include nanoporous, nanoparticles and nanofibers. The use of nanofibers as support material is favorable owing to their high porosity and interconnectivity and can be easily recovered and reuse. In this study, cyclodextrin glucanotransferase (CGTase) enzyme was successfully immobilised on PVA nanofibers via post-spinning and simultaneous electrospraying and electrospinning. In the post-spinning method, the PVA solution was electrospun to produce nanofibrous membrane at first and followed by the electrospraying of CGTase particles onto the membrane. The latter method involved the simultaneous electrospraying of CGTase solution and electrospinning of PVA solution conducted at opposite polarity. Before the immobilisation step, the transformation of CGTase from solution to solid particles via electrospraying in Taylor cone-jet mode was studied to obtain fine and monodispersed particles that can be attached uniformly on the PVA membrane. The CGTase functional groups and activity were preserved during the process as confirmed by FTIR and enzyme activity analysis. The Columbic fission that occurred during electrospraying has changed the enzyme morphology from clusters into a single particle as observed by Scanning Electron Microscope (SEM) and effectively reduced the average enzyme particle size from 200 ± 117 nm to 75 ± 34 nm when the spraying tip to the collector distance was increased from 10 cm to 25 cm. The enzyme particles collected at the longest distance demonstrated the highest enzyme activity. The microstructure of electrosprayed CGTase immobilised on PVA nanofibers was observed using SEM and the effectiveness of the two immobilisation approaches was compared in terms of enzyme loading, enzyme activity and reusability with enzyme concentration ranging from 1 to 7.5% v/v. Post-spinning deposition produced nanofibers with denser particles deposited on its surface, while uniform distribution of particles within the nanofibers was observed when simultaneous electrospraying and electrospinning was applied. Higher enzyme loading efficiency was obtained by using the simultaneous method with maximum value of 14 mg/g compared to 9 mg/g for the post-spinning method. The enzyme activity analysis showed that up to 17% higher enzyme activity could be achieved through the simultaneous method in comparison to the post-spinning. Vapour phase crosslinking that was applied to the CGTase/PVA membranes to facilitate the enzyme reusability did not cause

significant losses to the immobilised enzyme activity. The membranes produced via both the post-spinning and simultaneous method exhibited almost similar trend of reusability with up to 50% of the initial enzyme activity retained after the fifth cycle of the enzymatic reaction. The results indicate that the electrospraying and and electrospinning hybrid method is a promising approach for enzyme immobilisation.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk Ijazah Sarjana Sains

IMOBILISASI *CYCLODEXTRIN GLUCANOTRANSFERASE* PADA GENTIAN NANO POLIVINIL ALKOHOL TERELEKTORPUTAR

Oleh

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Terdapat pelbagai jenis bahan berstruktur nano yang telah digunakan untuk imobilisasi enzim termasuk nanoporos,nanopartikel dan nanogentian. Penggunaan nanogentian sebagai bahan sokongan adalah lebih digalakkan kerana nanogentian mempunyai keporosan dan jaringan yang tinggi dan mudah diasingkan dan diguna semula. Dalam kajian ini, enzim *Cyclodextrin glucanotransferase* (CGTase) telah berjaya diimobilisasikan pada gentian nano polivinil alkohol (PVA) melalui dua kaedah hibrid elektrosemburan dan elektroputaran iaitu pasca-putaran, dan elektosemburan dan elektroputaran serentak. Dalam kaedah pasca-putaran, larutan PVA terlebih dahulu dielektroputar untuk menghasilkan membran bergentian nano, diikuti dengan elektrosemburan zarah CGTase pada membran tersebut. Kaedah yang seterusnya pula melibatkan elektrosemburan larutan CGTase dan elektroputaran larutan PVA dijalankan serentak. Sebelum langkah imobilisasi, transformasi CGTase daripada larutan kepada zarah pepejal melalui elektrosemburan dalam mod kon-jet Taylor dikaji untuk mendapatkan zarah yang halus dan monodispersi yang boleh melekat dengan sekata pada membran PVA. Kumpulan berfungsi dan aktiviti CGTase dapat dipelihara melalui kaedah ini sepertimana yang disahkan oleh analisis FTIR dan aktiviti enzim. Pemecahan Columbic yang berlaku semasa elektrosemburan telah mengubah morfologi enzim daripada berkelompok kepada partikel tunggal seperti yang diperhatikan melalui Mikroskop Elektron Payaran (SEM) dan berkesan mengurangkan saiz purata zarah enzim daripada 200 ± 117 nm kepada 75 ± 34 nm apabila jarak daripada hujung alat penyembur kepada alat pengumpul ditingkatkan daripada 10 cm kepada 25 cm. Zarah enzim yang dikutip pada jarak yang paling jauh menunjukkan aktiviti enzim yang paling tinggi. Struktur mikro CGTase yang telah diimobilisasi pada nanogentian PVA diperhatikan dengan menggunakan SEM dan keberkesanan kedua-dua kaedah imobilisasi ini dibandingkan dari segi muatan enzim, aktiviti enzim dan kebolehan diguna semula dengan kepekatan enzim antara 1 hingga 7.5% v/v. Kaedah pasca-putaran menghasilkan nanogentian dengan partikel yang padat melekat pada permukaannya, manakala partikel tersebar secara sekata dapat dilihat dengan menggunakan kaedah serentak. Kecekapan muatan enzim yang lebih tinggi telah diperolehi dengan menggunakan kaedah serentak dengan nilai maksimum 14 mg/g berbanding 9 mg/g bagi kaedah pasca-putaran. Analisis aktiviti enzim menunjukkan bahawa aktiviti enzim sehingga 17% lebih tinggi boleh dicapai melalui kaedah serentak berbanding

dengan saingannya. Paut-silang fasa wap yang telah diaplikasikan terhadap membran CGTase/PVA untuk membolehkan penggunaan semula enzim tidak menyebabkan kehilangan yang besar kepada aktiviti enzim yang telah diimobilisasi. Membran yang dihasilkan melalui kedua-dua kaedah pasca-putaran dan kaedah serentak menunjukkan trend yang serupa dari segi kebolehan diguna semula di mana 50% daripada aktiviti enzim yang asal dapat dikekalkan selepas lima kali melalui tindak balas enzim. Secara keseluruhannya, kaedah hibrid elektrosemburan dan elektroputaran adalah satu pendekatan yang sangat berpotensi untuk imobilisasi enzim.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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This is to confirm that:

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LIST OF ABBREVIATIONS

% v/v	Percent volume per volume
% w/v	Percent weight per volume
D	Distance
Da	Dalton
d_d	Droplet diameter
E_c	Applied field at capillary tip
E_{co}	Onset electric field for corona initiation
E_{on}	Onset electric field
E_{on}	Onset electric field
I	Current
K	Conductivity
Q	Flow rate
q	Droplet charge
Q_R	Rayleigh charge limit
r	Droplet radius
r_c	Capillary radius
V	Droplet velocity
V_c	Applied voltage
wt %	Percent by weight
X	Fissility
γ	Surface tension
ε_o	Electrical permittivity of vacuum
ε_r	Relative permittivity
η	Viscosity
θ	Half angle of cone

ρ	Liquid density
HEMDA	Hexamethylenediamine
MgO	Magnesium oxide
n.d	Not declared
PCL	Polycaprolactone
PEG	Polyethylene glycol
PEI	Polyethyleneiminne
PEO	Polyethylene oxide
PHB	Poly(3-hydroxybutyrate)
PLA	Polylactide
PLACL	Poly-L-lactic acid-co-e- caprolactone
PLGA	Poly(lactic-co-glycolic acid) (PLGA)
PS	Polystyrene
PVA	Polyvinyl alcohol
PVP	Poly(vinylpyrrolidone)
PZC	Point of zero charge
SEM	Scanning Electron Microscope
THF	Tetrahydrofuran
TiO_2	Titanium dioxide
USD	United States dollar
UV-VIS	Ultraviolet-Visible
ZnO	Zinc oxide
α-CD	Alpha cyclodextrin
α-CT	Aplha-chymotryspin
β-CD	Beta cyclodextrin
γ-CD	Gamma cyclodextrin

CHAPTER 1

INTRODUCTION

This chapter covers the overview and problem statements, objectives, scope, and significance of the study.

1.1 Overview and problem statements

In the drive towards sustainable and environmental friendly technology, a highly efficient biocatalyst known as an enzyme has been utilized extensively for a wide range of applications (Wang & Hsieh, 2008). These include the synthesis of pharmaceutical products, food processing, fabrication of biosensors, biofuel and bioremediation (Brena, González-Pombo, & Batista-Viera, 2013; Hwang & Gu, 2013). The growing interest in utilising enzymes in industrial processes has resulted in the rapid growth of the global enzyme business which was worth about 7 billion USD in 2013 with a growth rate of 6.7 % (Gupta, Rajput, Sharma, & Gupta, 2013; Jochems, Satyawali, Diels, & Dejonghe, 2011).

Enzymes offer many benefits in comparison with the conventional inorganic catalyst. Notable among them are their high selectivity, reduced side reactions and mild reaction conditions (Sheldon, 2007). Despite the advantages offered, enzymes however are generally unstable and difficult to recover and reuse, which limits their efficiency in industrial applications (Iyer & Ananthanarayan, 2008; Liese & Hilterhaus, 2013). These drawbacks could be overcomed by immobilising enzyme by fixing it to a solid support (Sheldon & van Pelt, 2013).

The performance of the immobilised enzyme strongly is affected by the properties of the support materials such as size and structure. Reduction of the support geometric size provide extremely high surface area for enzyme attachment which resulted in high enzyme loading and effectively improve the catalytic efficiency (Kim, Grate & Wang, 2006). In this regard, increasing interest is being shown in incorporating enzymes into nanostructured materials which include nanoporous, nanoparticles and nanofibers.

Immobilisation of cyclodextrin glucanotransferase (CGTase) into nanostructures has been achieved by using nanoparticles and nanoporous. CGTase responsible for catalyzing transglycosylation reaction of starch into cyclodextrins (CDs), a ring structured molecules that have been used in numerous applications due to their unique feature of having both the hydrophobic internal cavity and hydrophilic surface. In comparison to the conventional support materials that have been applied for CGTase such as agarose, eupergit c and alginate, the immobilisation of CGTase using magnetic nanoparticles and nanoporous silica has shown significant improvement in enzyme stability and enzyme loading (Ibrahim *et al.*, 2014; Ivanova, 2010a). However, despite the advantages, some of the drawbacks of the nanoparticles and nanoporous materials are difficult to overcome.

The major problem associated with nanoporous media is the confinement of the enzyme on its inner surface which limits the enzyme-substrate interaction and reduces the catalytic efficiency (Kim, Grate, & Wang, 2006). In the case of nanoparticles, their dispersion in the reaction solution and the associated complex recovery procedure is a daunting task. Magnetic nanoparticles can be easily recovered due to their magnetic properties but the fabrication and activation procedures before the immobilisation steps are more complex (Kim, Jia, & Wang, 2006).

Electrospun nanofibers could be an excellent candidate to be used as an enzyme support for several reasons. First, various types of polymer including both natural and synthetic polymers can be processed into nanofibers with different characteristics to meet the requirements of enzyme support. Secondly, the high porosity and interconnectivity of the nanofibers could reduce the mass transfer limitation through the meshes. Next, the non-woven nanofibers meshes can be easily recovered and reused which would allow them to be applied in an enzymatic membrane bioreactor (Herricks *et al.*, 2005).

Immobilisation of enzymes using nanofibers support has been achieved mainly through a surface attachment and encapsulation method. The attachmnent method generally uses hydrophobic polymers such as polystyrene and polysulphone which requires several modification steps to increase their biocompatibility for enzyme attachment (Yunrong, 2010). Encapsulation of enzymes within the nanofibers structure can be realised through co-electrospinning of the enzyme and a water soluble polymer followed by the crosslinking method (Dror, Kuhn, Avrahami, & Zussman, 2008). This method requires only a very simple process and high enzyme loading can be achieved. However, the limited accessibility of the substrate to the enzyme and the difficulty of controlling the fibre structure with the addition of an enzyme remains a chief issue.

Considering the advantages of nanofibers as an immobilisation support and the limitations of the current immobilisation techniques, in this study the immobilisation of CGTase enzyme on PVA nanofibers through an electrospraying and electrospinning hybrid method is demonstrated. Electrospinning enables the formation of fibres with diameter ranging from micro to nano-metre scale from a viscous liquid by applying electrical forces on the liquid, while electrospraying involves electrical atomisation of a non-viscose liquid into fine solid particles by subjecting the liquid to high voltage (Jaworek *et al.*, 2009). The argument put forward here is that this hybrid method could allow deposition of CGTase particles on the nanofibers without affecting the fibres properties. The electrosprayed particles attached to the nanofibers surface could provide maximal enzyme substrate interaction. An overview of the research is shown in **Figure 1.1**.

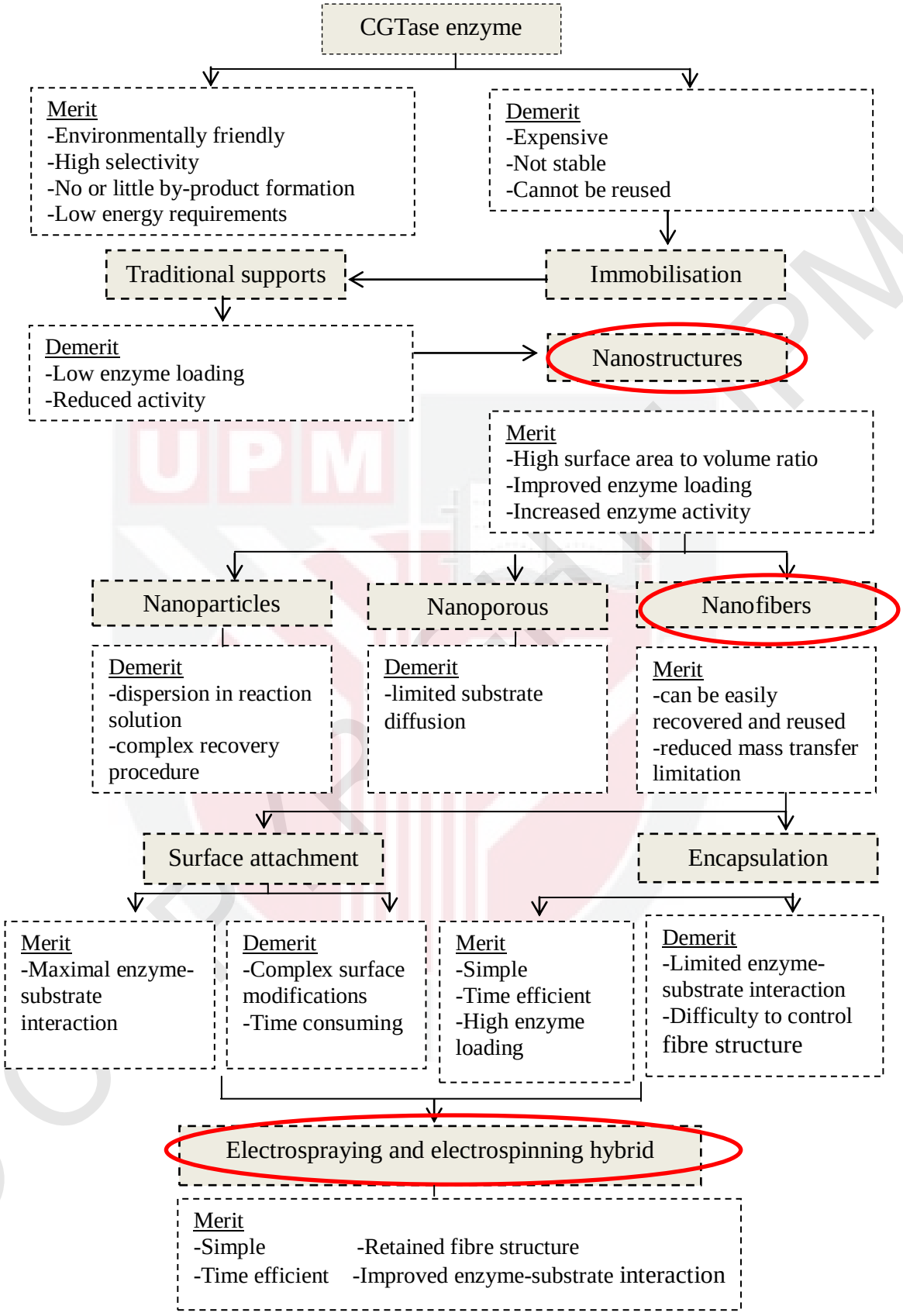

Figure 1.1: Research overview

1.2 Objectives of the study

The aim of this work is to evaluate the use of nanofibers as a support for immobilising the CGTase enzyme by using the electrospraying and electrospinning hybrid method. The objectives of this study are:

1. To study the transformation of CGTase enzyme from solution to fine and monodispersed solid particles via electrospraying.
2. To immobilize CGTase on polyvinyl alcohol (PVA) nanofibers via post spinning and simultaneous electrospraying and electrospinning methods.
3. To evaluate the immobilized enzyme microstructure, loading efficiency, catalytic activity and reusability.

1.3 Scope of the study

The purpose of this study is to develop an immobilised enzyme-nanofibrous membrane by combining the electrospraying and electrospinning approach. CGTase from *Bacillus macerans* is chosen as a model enzyme while polyvinyl alcohol (PVA) nanofibers are selected as an immobilisation matrix.

During the electrospraying of CGTase solution, the spraying tip to collector distance is varied in the range of 10 – 25 cm to examine its effect on the enzyme particle structure and size. Scanning electron microscope (SEM) was used to observe the morphology of the CGTase particles. The SEM images were analyzed by using image processing software to determine the particle size and distribution. Fourier Transform Infra-red (FTIR) spectrometer was used to identify the changes in the enzyme functional group before and after the electrospraying.

The support to immobilise the CGTase particles was prepared by electrospinning of the PVA solution with concentration ranging from 6 to 10 wt%. The jet behaviour during the electrospinning was captured with a digital camera while the fibers morphology and size was analyzed using SEM and ImageJ software, respectively.
The CGTase particles were immobilised on the electrospun PVA nanofibers through post-spinning, and simultaneous electrospraying and electropinning. The deposition of the CGTase particles on the PVA nanofibers was characterized by using SEM. The performance of the CGTase immobilised via the post-spinning and simultaneous electrospraying and electrospinning were analyzed and compared in terms of enzyme loading efficiency, catalytic efficiency and reusability. The analyses were conducted by using UV-VIS spectrophotometer. The enzyme loading efficiency was measured using the Bradford method to determine the amount of protein that can be attached to the support. The catalytic efficiency was analysed based on the amount of the α-CD produced after the enzymatic reaction of CGTase and starch. The reusability of the immobilised enzyme was determined by measuring the activity repeatedly after subsequent batch reaction of α-CD production.

Overall, the study was conducted to investigate the feasibility of electrospraying and electrospinning to immobilise the CGTase enzyme. No process optimization involved. The ranges of parameters used were selected based on the range suggested by previous researchers. During the work, measurement of solution viscosity and

surface tension were unable to be performed. The value for viscosity and surface tension reported in the thesis especially for calculation part was obtained from the literature. Besides that, during electrospraying and electrospinning, wastage of materials that affect the yield might be occurred. However, this wastage is not considered in the present work.

1.4 Significance of the study

In this research, a new method of CGTase immobilisation on nanofibers support is demonstrated. It is expected that this new method will provide a new commercially viable route for the immobilisation and stabilisation of CGTase and other type of enzymes and biomolecules such as proteins and cells. In addition, based on the unique advantages of immobilised CGTase, it is further expected that the resultant nanostructured biocatalyst will facilitate new and expanded uses of CGTase enzymes in bioprocess applications such as bioconversions and biosensors.

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