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Laccase Immobilisation on Poly(ethylene) Terephthalate Grafted with Maleic Anhydride (PET-g-MAH) Nanofiber Mat

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A comparative study was carried out on three different enzyme immobilisation methods which were physical adsorptions (PA), covalent bonding (CV) and covalent bonding of cross-linked enzyme aggregates (CL) for laccase immobilisation on Poly(ethylene) Terephthalate (PET) grafted with Maleic Anhydride (MAH) nanofiber mats. The chemically inert PET was successfully grafted with MAH at temperature between 40 – 45 °C and used as the carrier to immobilised laccase in the form of electrospun nanofiber mats. The peaks from carbonyl (C=O) and alkene (C=C) groups appeared on the spectral subtraction between PET and PET-g-MAH nanofiber mats. These groups might be the potential group to form covalent bond between the amine groups of laccase enzyme. Laccase immobilised on the PET-g-MAH nanofiber mats using CL methods using glutaraldehyde as crosslinker gave the best performance with the highest enzyme loading and immobilisation yield which were 40.88 µg/mg and 48.37 %. On top of that, the immobilised laccase on PET-g-MAH nanofiber mats also managed to retain 69.01 % of its initial activity after 10 repeated cycles of 2, 2-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) oxidations. These results demonstrate that PET-g-MAH nanofiber is a good material to be considered as laccase carrier.

1. Introduction

Laccase is an enzyme with a good potential to be used as biocatalyst as it has the ability to catalyse the oxidation of a wide variety of substrate (Madhavi and Lele, 2009). However, the application of enzyme is usually limited by its stability and reusability. Laccase immobilisation on carrier is foreseen as the solution to solve this problem (Fernández-fernánde et al, 2013). On the other hand, the remarkable properties of the nanomaterial such as small size, high surface area and large surface area to volume ratio have grabbed the attention of the researchers in recent times to use nanomaterials as the carriers for the enzyme immobilisation. Nanomaterial not only offers remarkable properties for the immobilised enzyme, but it offers higher enzyme loading, enhances the mechanical strength and also minimizes the diffusional problems (Ahmad and Sardar, 2015).
Among the nanomaterial, electrospun nanofiber has become a spotlight to various researcher because it can be modified easily by using a different combination of polymer or by adding another type of nanomaterial in the system (Wang et al., 2009). It has been used in different types of application such as material for biomaterial field (Benatti et al., 2016), dye absorbent (Zarrini et al., 2017) and so on. Nanofiber has a great potential to be used as the carrier for enzyme immobilisation which can be obtained by using an electro-spinning process. There are various methods reported on immobilisation of enzyme on nanofiber mats using different methods such as glucose oxidase entrapment in graphene modified polyvinyl alcohol nanofiber mats (Wu et al, 2014), laccase covalent bonding with polyacrylonitrile/polyviniylidene fluoride mixed with nano-copper nanofibers (Xu et al., 2017) and crosslinked lysozyme aggregates on electrospun chitosan nanofibers (Park et al., 2013).

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Poly(ethylene) terephthalate (PET) is an inexpensive polymer which is most widely used in the synthetic material world. Regardless of the price, the structure of PET as nanofiber mats had been proved to have good physical and mechanical properties (Veleirinhho et al., 2008). These would be additions features of PET nanofibers mats to be used as a carrier for enzyme immobilisation; owing to its large surface area. Unfortunately, PET is also a hydrophobic and chemically inert polymer which limits its potential to be used as an excellent enzyme carrier. Previously, several researchers have successfully immobilized enzyme on PET fiber by modifying its surface. For instances, horseradish peroxidase was successfully immobilised on PET fiber grafted with acrylamide (Temoçin and Yiğitoğlu, 2009). Besides, Silva et al., (2015) had managed to immobilise trypsin on the mixture of PET and polylactic acid nanofiber without any enzyme leaching during the activity reaction. However, the surface of the modified PET nanofiber needed to be activated prior to covalently bonded with the enzyme through the immobilisation process.
In this study, PET was modified using maleic anhydride (MAH) through a grafting reaction before the electro-spinning process. MAH had been reported successfully grafted onto various types of polymers and capable of enhancing the properties of the polymer (Rzayev and Zakir, 2011). Besides that, MAH has the ability to self-initiate without the presence of the initiator (Deng and Yang, 2001). Therefore, PET-g-MAH solution was used to fabricate the nanofiber which laccase can form a direct covalent bond with PET-g-MAH nanofiber mats without surface activation. The aims of this study were to graft MAH onto PET and investigated the most suitable methods for laccase to immobilise on PET based nanofiber mats. Therefore, the spectral subtraction method using OMNIC software was used to determine the successful grafting reaction of MAH with PET. Two parameters studied were the immobilisation yield and the protein loading at three different immobilisation methods which were physical adsorption on PET nanofiber mats (PA), covalent bonding on PET-g-MAH nanofiber mats (CV) and covalent bonding of cross-linked laccase aggregate on PET-g-MAH nanofiber mats (CL) using glutaraldehyde as crosslinker.

2. Experimental procedure

2.1 Materials

PET pellet was purchased from ALBA PET SDN. BHD. Johor, Malaysia, MAH pellets and BCA protein assay kit were purchased from MERCK, Laccase was obtained from *Trametes versicolor* (enzyme activity ≥ 0.5 U/mg), Trifluroacetic acid (TFA), Dicholoromethane (DCM), Gluteraldehyde, 2, 2-azino-bis 3-ethylbenzothiazoline-6- sulfonic acid (ABTS) were purchased from Sigma-Aldrich. All other chemicals used in this study were purchased either from Sigma-Aldrich, MERCK, QREC and Wako Chemical.

2.2 Grafting of PET

The dope solution of PET was prepared by dissolving the pellets in the mixture of TFA and DCM solvents at a ratio 4:1 until fully dissolved (Veleirinhho et al., 2008). The mixture was stirred until the pellets were completely dissolved in the solvent. Then, MAH was added to the mixture at 4:1 (PET:MAH) ratio and was heated at temperature range between 40 °C to 50 °C for 30 minutes to initiate the grafting. The solution was left overnight before the electro-spinning.

2.3 Preparation of PET grafted MAH nanofiber mats

Three different concentrations of 10 % (w/v), 20 % (w/v) and 30 % (w/v) PET solution were spinned using elctro-spinning. The process was conducted at room temperature with voltage of 15-16 kV and flow rate of 1.0 mL/hr. The fibers were collected after 4 h on an aluminium foil-covered rotating drum with rotation speed of 300 rpm which is located 18 cm away from the needle tip. After the fabrication completed, the mat was washed using acetone to remove all the unreacted MAH and dried at room temperature overnight (Silva et al., 2015). All laccase immobilisation experiments were conducted using 1.5 x 1.5 cm PET-g-MAH square nanofiber mats weight in the range of 4-7 mg.

2.4 Laccase immobilisation procedures

The PET nanofiber mat was used for PA method and PET-g-MAH nanofiber mat was used for CV and CL method. The mat was placed in a vial containing 1 mg/ml of laccase solution (Xu et al., 2017) in pH 4.0 sodium acetate buffer. Later, the vial was placed inside incubator shaker at 25 °C under 250 rpm shaking for 6 h. The immobilisations of PA and CV methods were completed at this stage while the CL method was continued by adding 0.5 % (v/v) of glutaraldehyde solution (Silva et al., 2015) into the vial at 20 °C under 250 rpm shaking for 1 h. The mat was washed several times using pH 4.0 sodium acetate buffer to remove the unattached enzyme on the nanofiber mats before calculating the enzyme loading and the activity.

2.5 Enzyme loading

The enzyme loading (EL) on the nanofiber mats was determined by the enzyme concentrations before and after immobilisation. The enzyme concentrations were measured by using BCA protein assay as stated at Eq(1).

$$Enzyme\ Loading(\%) = \frac{P_i - P_f}{P_i}\ X\ 100\ \% \quad (1)$$

where, P_i is the enzyme amount subjected to be immobilised and P_f is the enzyme amount in the solution after immobilisation.

2.6 Laccase activity assay

Laccase activity was measured using method published previously (Kumar et al., 2014) with slight modification. It was conducted by monitoring the oxidation of 1 mM ABTS in pH 4 sodium acetate buffer at 420 nm. The reactions took place for 3 minutes in the cuvette containing 1.35 mL of 1 mM ABTS solution, 1.5 mL of 0.1 M sodium acetate buffer and 0.15 mL of laccase solution. The starting time was taken as soon as laccase solution was added into the cuvette. The activity was calculated using Eq(2).

$$U = \frac{\Delta A\ X\ V\ X\ 10^6}{\varepsilon\ X\ L\ X\ t} \quad (2)$$

where, ΔA is the change in absorbance at 420 nm after reaction, V is the reaction volume, ε is molar extinction coefficient ($M^{-1}\ cm^{-1}$), L is the optical path, and t is the reaction time. One unit of enzyme activity (U) is defined as the amount of enzyme requires to oxidised 1 μmol of ABTS per min. The immobilisation yield is measured using the formula (Sheldon and van Pelt, 2013) as shown in Eq (3).

$$IY\ (\%) = \frac{U_I - U_F}{U_I}\ X\ 100 \quad (3)$$

where U_I is the activity of the laccase solution before the immobilisation and U_F is the activity of the laccase solution after the immobilisation.

2.7 Fourier Transform Infrared Spectroscopy (FTIR)

The qualitative information on the functional group was determined and analysed by using a Perkin Elmer 1600 FTIR spectrophotometer. The data was analysed at spectral range of 4000 – 300 cm^{-1}. The results obtained were tabulated using OMNIC software and compared using spectral subtraction method.

2.8 Scanning Electron Microscopy (SEM)

The morphologies of the nanofiber mats surface were analysed by utilizing SEMEDAX, XL40: PW6822/10, SEM analyser. The micro images were taken at 1.5K magnifications.

2.9 Reusability test

The method with the highest immobilisation yield and protein loading was chosen for the reusability test. The test was conducted by measuring the relative activity of the immobilised laccase for 10 cycles of ABTS oxidations and the initial activity was considered as 100 %. The PET-g-MAH nanofiber mats were rinsed with acetate buffer at the end of each test to remove unreacted substrates before the next cycle.

3. Results and discussion

3.1 Grafting of PET with MAH

The grafting reaction was taking placed in liquid phase of PET solution with temperature between 40 °C to 50 °C. MAH was chosen as the monomer because of its ability to self initiate without the presence of initiator (Deng and Yang, 2001), hydrophilic property and the presence of the reactive group such as C=C and C=O to anchor the N-H group of enzyme for immobilisation. As shown in Fig. 1, the FTIR spectrum of MAH on PET-g-MAH nanofiber mat was barely noticeable which may be causes by the higher ratio of PET than MAH. Similar results reported by Tamizifar and Sun (2018) when grafting vinyl monomers onto PET fibers surface using controlled radical graft polymerization. However, the success of grafting can be confirmed by using spectral subtraction method from OMNIC software. Two peaks appeared at 1,734 and 1,719 cm^{-1}, which referred to the C=O stretching of maleic structure on spectral subtraction. Moreover, the peak corresponding to C=C and =C-H of the maleic structure have also appeared at 1,671 and 1,022 cm^{-1}. Maleic was grafted onto PET surface using the C-O bond. Hence, peaks from C-O stretches were appeared at 1,341 and 1,272 cm^{-1}.

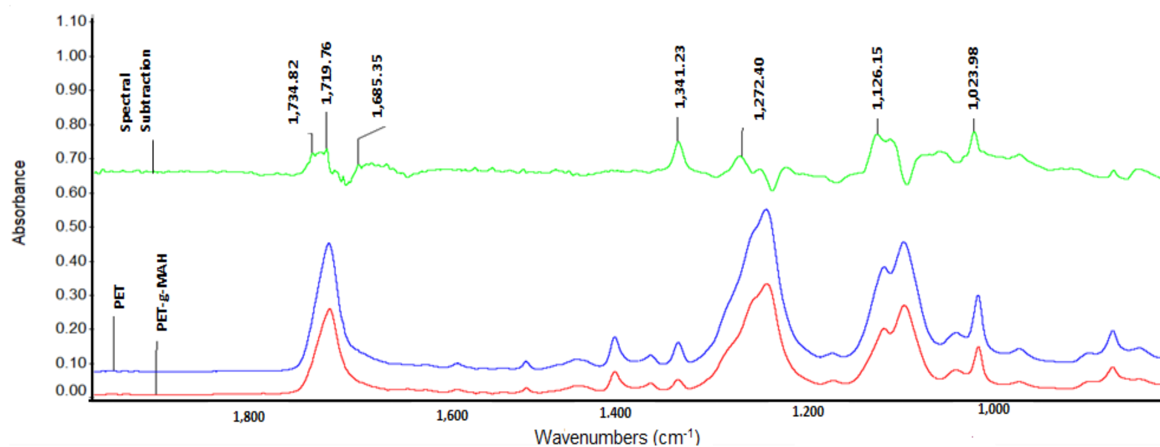

Figure 1: Fourier transform infrared spectroscopy (FTIR) spectra of the PET nanofiber mat, PET-g-MAH nanofiber mat and spectral subtraction of PET and PET-g-MAH nanofiber mats absorbance

3.2 Effect of different PET concentration on the average fiber diameter of the nanofiber

The average diameter of the nanofiber is hugely effects by the concentration of the polymer (Veleirinhho et al., 2008). Normally, the polymer concentration is linearly increased with the average diameter of the fiber. As can be seen in Fig. 2, when the PET concentration increased from 10 % to 30 % (w/v) the average diameter of the fiber was also increased about 20 times at 30 % (w/v). The average diameters of the fibers increased from 238 ± 53 nm at 10 % (w/v) to 844 ± 149 nm at 20 % (w/v) and continue to increase at 30 % (w/v) about 4870 ±1350 nm. This is due to the increasing in the PET solution viscosity and elasticity as the PET concentration increased. Since the entire electrospinning process depending on the electrostatic force, the viscosity of the polymer plays important role to counteract this external force. Higher viscosity will help the polymer to withstand the force and form fibers on the collector (Lee et al., 2016). Therefore, it was observed that during the spinning process of 20 % and 30 % (w/v) PET concentrations, the solutions were more stretchable than at 10 % (w/v) PET concentration. At 10 % (w/v) PET concentration, the formation of liquid droplets occurred instead of stretchable PET fiber, resulting in electrospraying. This situation happens in electrospinning if the viscosity and elasticity of the polymer use are too low. The polymer could not resist the force and causes the polymer to break out into beads which causes defect to the mats produced. Unlike at 20 % (w/v) and 30 % (w/v) the fiber were in uniform sized and orderly arranged. The diameter of the fiber is also increase as the viscosity increase because under the same electrostatic force, higher viscosity polymer will prevents the polymer jet from being stretched. Since the purpose of this study is to immobilise enzyme on the nanofiber mats, a uniform arrangement of fiber, bead free and lower average fiber diameters mats are considered in order to have an optimum surface area for the enzyme to attach on the nanofiber mats. As a result, 20 % (w/v) PET concentration was chosen as suitable concentration to be used for laccase immobilisation.

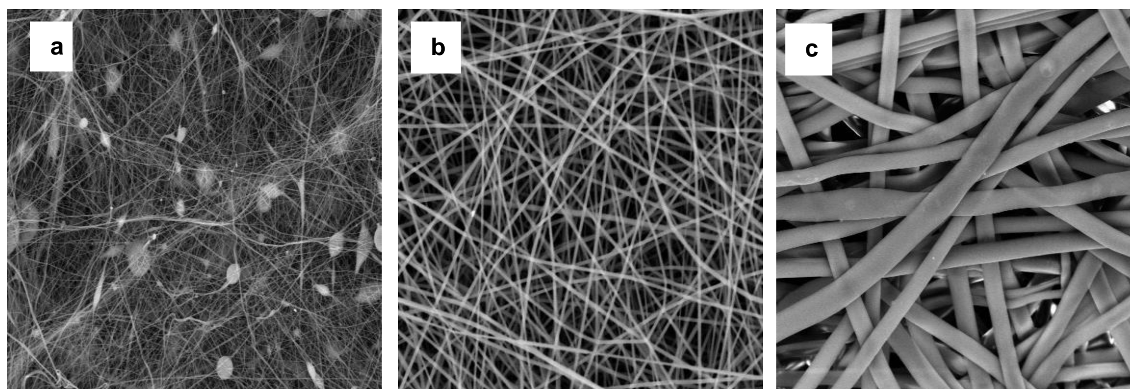

Figure 2: SEM image at x1500 magnification showing the morphology of the nanofiber at different PET concentration: (a) 10 % (w/v) (b) 20 % (w/v) (c) 30 % (w/v)

3.3 Effect of different immobilisation methods on the enzyme loading and immobilisation yield

Three different immobilisation methods were investigated in this study. The first method was the PA method which was direct immobilisation of laccase on PET nanofiber mats without grafting MAH on the surface. It was reported that laccase have high affinity towards the hydrophobic material (Irena et al., 2009). Therefore, laccase able to adsorb on the PET nanofiber mats surface at 17.26 µg/mg of enzyme loading and 26.02 % immobilisation yield. In the second method, the CV immobilisation method was applied onto the PET-g-MAH nanofibet mats. It was found that the value of the enzyme loading and immobilisation yield were slightly increased at 21.61 µg/mg and 38.94 %. The presence of MAH group on the nanofiber mats formed a covalent bond with the NH group of the enzyme molecules (Lee et al., 2010) . In order to enhance the amount of laccase loading on the PET-g-MAH nanofiber mats, about 0.5 % (v/v) glutaraldehyde concentration (Silva et al., 2015) was added onto the laccase solution to crosslink the laccase attached on the mats surface and the free laccase in the solution in CL method. The presence of glutaraldehyde had formed multipoint linkage between the enzyme as reported previously (Lee *et al.*, 2016), thus resulting in more enzyme to attach on the PET-g-MAH nanofiber mats. Therefore, the enzyme loading increased up to 40.88 µg/mg about 1.9 times higher than CV method while the immobilisation yield which was 48.37 %. Previously, reported by Maryšková et al. (2016) the immobilisation yield of immobilised laccase on Polyamide 6/chitosan naofiber mats at 50.9 % which was slightly higher than current study. Another study by Lee et al. (2016) reported about 34.82 µg/mg of lipase loading on methyl cellulose nanofiber mats. Based on previous studies reported, the immobilisation yield and enzyme loading are difficult to compare as there are various factors that need to be considered such as type of carrier, method of immobilisation, type of enzyme and substrate used in the reactions.

3.4 Reusability of the immobilised laccase on PET-g-MAH nanofiber mats

The reusability of the immobilised enzyme is one of the important aspects in order to determine the stability of the immobilised enzyme to be used as biocatalyst. After 10 cycles of ABTS oxidation the immobilised laccase on PET-g-MAH nanofiber mats still managed to retain 69.01 % of its initial activity. Several studies also reported decreased in activity after repeated cycles, this may due to the inactivation of enzyme after repeated cycles (Taheran et al., 2017). A remarkable result was reported recently after using optimum immobilised condition of lipase to immobilise it onto polyvinyl alcohol/alginate nanofiber mats, the immobilised lipase managed to retain about 90% of its initial activity after 10 repeated cycles (İspirli Doğaç *et al.*, 2017). The result of present study may be improved by optimizing the immobilised conditions in term of enzyme concentrations, pH, immobilised temperature and immobilisation time as the property of enzyme itself is sensitive to the change of the surrounding. However, the present study still proved that the immobilised laccase was stable enough to be used in the reaction as biocatalyst as it able to retain more than half of its initial activity even after 10 repeated cycles.

4. Conclusions

The PET was successfully modified by grafting MAH on the PET solutions and use as the carrier for laccase immobilisation in the form of nanofiber mats. The covalent bonding of cross-linked laccase aggregates using glutaraldehyde with PET-g-MAH nanofiber mats was the best method with the highest enzyme loading (40.88 µg/mg) and immobilisation yield (48.37 %). By using CL method, the immobilised laccase was able to maintain high activity even after 10 cycles of consecutive reactions with ABTS. It is proven that the laccase immobilised on PET-g-MAH has potential to be used as biocatalyst in the industries. However, the optimization of laccase immobilised conditions on PET-g-MAH nanofiber mats is recommended for future study in order to obtain higher enzyme loading and immobilisation yield.

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