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Immobilisation of *Candida rugosa* Lipase on Aminated Polyethylene/Polypropylene Microfibrous Sheet Modified with Oxirane Group

Hazimah Akbar Tajudin[1], Ma'an F. Alkhatib[2*], Mohamed Elwathig Saeed Mirghani[2], Hamzah Mohd Salleh[1] and Mohamed Mahmoud Nasef[3]

[1]*International Institute for Halal Research & Training(INHART), International Islamic University Malaysia, 50728, Kuala Lumpur, Malaysia*

[2]*Department of Biotechnology Engineering, International Islamic University Malaysia, 50728, Kuala Lumpur, Malaysia*

[3]*Chemical Engineering Department, Universiti Tecknologi Petronas, 32610, Seri Iskandar, Perak, Malaysia*

An active microfibrous substrate containing aminated brush obtained by radiation-induced grafting of glycidyl methacrylate (GMA) onto a polyethylene/polypropylene (PE/PP) microfibrous sheet followed by amination reaction was prepared and used for immobilisation of *Candida rugosa* lipase under various conditions. The aminated microfibrous sheet was characterised by Fourier-transform infrared spectroscopy (FTIR-ATR) and field emission scanning electron microscope (FESEM). The amine group density on the aminated microfibrous sheet was found to be 3.33mmol/g. Response surface methodology (RSM) was applied to model and optimise the immobilisation conditions including immobilisation time (2-6 h), medium pH (pH 7-9) and enzyme/support ratio (5.0-9.0mg/cm^2). The model generated from RSM was significantly correlated with the studied parameters for the residual activity of the immobilised lipase. The optimum values for immobilisation time, medium pH, and enzyme/support ratio were found to be 4.24h, pH 8, and 8.51mg/cm^2 respectively. The enzymatic activity using *p*-nitrophenyl palmitate (*p*NPP) as substrate was 1.4588U/cm^2 under optimum conditions. The pH endurance, storage, and thermal stability of the immobilised lipase were remarkably enhanced. The immobilised lipase can be readily recovered and more than 50% of its activity was retained following 10 cycles. The results of this study suggested that the aminated microfibrous sheet of PE/PP grafted with poly(GMA) is a promising polymer support for enzyme immobilisation with high potential for broad biocatalytic applications.

Keywords: radiation induced grafting, oxirane group, enzyme immobilisation, *Candida rugosa* lipase, biocatalytic reactions

I. INTRODUCTION

Enzymes are biocatalysts that play crucial roles in the development of bioprocesses and sustainable technologies and thus, rising an interest in many industries (Nandy, 2016). Of all, lipase (triacylglycerol ester hydrolase, EC 3.1.1.3) has found to have the utmost use in industrial applications (Andualema and Gessesse, 2012). This is due to its remarkable catalytic properties including high competency, specificity, selectivity, and its capability to work in mild reaction conditions. Not only that, the utilisation of lipase

*corresponding author: maan@iium.edu.my

provides an environmentally friendly catalyst as a green solution for industrial processes (Tran and Balkus, 2011). Thus, lipase has been widely used for esterification, processing of fats and oils, food processing, ester hydrolysis, and transesterification in oleochemical industry, detergents, degreasing formulations, pharmaceuticals, and cosmetics (Yuce-dursun *et al.*, 2016; Zhang *et al.*, 2015). However, like other enzymes, the application of soluble lipase is limited due to some drawbacks such as non-reusability, separation difficulty from products, prohibitive cost, and low stability over wide ranges of industrial settings including pH, temperature, and solvent (Ma *et al.*, 2016; Kuo *et al.*, 2012). This situation resulted in an increase of industrial demand for lipase itself as well as for the development of immobilised lipase with enhanced stability and reusability through an immobilisation approach.

Immobilisation of enzymes on carriers offers a significant cost-benefit effect by facilitating enzyme recycling and enabling improvements in stability (Alkhatib *et al.*, 2012). Microbial source enzymes are commonly preferred over others because of their outstanding catalytic versatility, further upgrading potential and rapid growth on inexpensive feedstocks. Among lipase enzymes, lipase derived from yeast called *Candida rugosa* is one of the most well-studied and best characterised species. *C. rugosa* lipase is the source of seven isoforms of extracellular lipase with different substrate specificity and high applicability in the biotransformation of products in various industries (Trbojević Ivić *et al.*, 2016; Lasoń and Ogonowski, 2010).

Support materials and methods for enzyme immobilisation are being continuously developed to enhance the properties of soluble enzymes. The performances of the immobilised enzymes rely firmly on the nature of the functional groups and the type of the support materials (Goto *et al.*, 2007). The enzyme immobilisation support materials should also be biologically safe, chemically stable, mechanically intact, non-toxic, eco-friendly, and highly potential for protein binding (Trbojević Ivić *et al.*, 2016). The greatest advantage of synthetic polymers as support materials is that the monomers build the polymeric chain can be selected as it strongly affects the solubility, stability, porosity and mechanical properties of the polymer (Zdarta *et al.*, 2018).

Various methods for enzyme immobilisation including covalent binding, microencapsulation, adsorption, and entrapment have been used on many supports/substrates such as epoxy modified silica (Yuce-dursun *et al.*, 2016), sol-gel hybrid coating film (Rueda *et al.*, 2015), octyl-agarose beads (Rueda *et al.*, 2015), polyethyleneimine, glutaraldehyde-agarose, sepabeads, agarose, octyl-agarose, glyoxyl-agarose (Zhang *et al.*, 2015) and epoxy-activated microspheres (Zhang *et al.*, 2015). Of them all, covalent enzyme immobilisation is the most interesting technique to enhance the stability and reusability by improving the structure rigidity and reducing the protein unfolding (Chiou and Wu, 2004). In this technique, the immobilisation frequently starts with a surface alteration or activation step (Chiou and Wu, 2004). The extent of the effectiveness of this technique with regard to stability, activity, selectivity, and specificity of the enzyme function depends on the proper environment and suitability of the used matrix (Venkatanagaraju and Divakar, 2017). It is important to understand the 3-dimensional structure in designing and structuring lipases

for specified purposes so that the active site is not interrupted for reaction (Dwevedi, 2016). Moreover, enzyme immobilisation was found to be affected by factors such as immobilisation time, enzyme concentration, pH medium, substrate amount, and temperature (Alkhatib *et al.*, 2012). In this regard, experimental design is an important tool to study the interactive effects of the experimental factors with a minimal number of experiments, therefore reducing the cost. Response Surface Methodology (RSM) provides the technique to investigate the interactions of two or more factors to acquire the optimal values of the significant parameters under investigation and to predict the response (Nasef *et al.*, 2016).

Radiation-induced graft polymerisation is one of the most effective techniques for introducing functional groups to different polymer substrates of various morphologies without altering their inherent properties using various monomers (Nasef and Güven, 2012). Thus, this technique has been used for the preparation of various functionalised polymers for separation and purification purposes and is suitable for many chemical and biological processes (Nasef and Güven, 2012). Monomers such as glycidyl methacrylate (GMA), which is highly versatile in allowing further chemical modifications of the obtained PGMA grafted substrates such as polyethylene/polypropylene (PE/PP) microfibrous sheets, led to active polymers suitable for variety of biological systems such as protein extraction, DNA carriers, peptide separation and drug delivery systems (Barsbay *et al.*, 2016; Pino-Ramos *et al.*, 2016; Benaglia *et al.*, 2013). Studies involving immobilisation of various enzymes such as invertase (Danisman *et al.*, 2004), laccase (Kumar *et al.*, 2016), horseradish peroxidase (Kumar *et al.*, 2016) on epoxy-functionalised supports has also been investigated on various occasions (Pérez *et al.*, 2006).

Lipases have been immobilised on poly(glycidyl methacrylate) grafted PE/PP of different physical forms such as hollow fibre membranes (Abrol *et al.*, 2007), beads (Karagoz *et al.*, 2010), and nanocomposites (Nasef *et al.*, 2016). However, applying functionalised PE/PP microfibrous substrates, a cheap and an abundant polyolefin, is likely to impart various desirable properties to lipase immobilisation including a high feed diffusion rate and fast kinetics compared to conventional substrates. This is due to the large void volumes and surface areas as well as the chemical inertness of PE/PP microfibrous sheets.

The objective of this study was to prepare functionalised PE/PP microfibrous sheets by radiation-induced grafting of GMA followed by amination with diethylamine (DEA) and subsequent alkalisation to immobilise *C. rugosa* lipase under optimum conditions determined by RSM. The properties of aminated PGMA-PE/PP sheets were evaluated at various stages. The properties of the immobilised lipase including optimum pH, residual activity, thermal and pH stability, storage stability, and reusability were evaluated.

II. MATERIALS AND METHODS

A. Materials

A PE/PP nonwoven sheet (Teijin Ltd., Japan) was used as the microfibrous substrate. GMA (purity¿99.5, Fluka) was used as the graft-

ing monomer without further purification. Diethylamine (DEA) (Fluka) and ethanol were used as received. Deionised (DI) water (18MΩ) was obtained from a NANOpure DIamond^{TM} water purifier. Lipase was derived from *C. rugosa* (L1754-Type VII, 2265 700U/mg solid). One unit (U) of lipase activity was defined as the amount of enzyme that liberated 1.0 μmol of *p*-nitrophenol per minute under the assay conditions. Polyoxyethylene sorbitan monolaurate (Tween-20) surfactant, *p*-Nitrophenyl Palmitate (*p*-NPP), *p*-Nitrophenol (*p*-NP), bovine serum albumin (BSA) and all other chemicals were purchased from Sigma-Aldrich Co. and used as received. Unless otherwise noted, all reagents were of analytical grade.

B. Preparation of the grafted PE/PP-based fibrous substrate

Preparation of a poly(GMA) grafted PE/PP microfibrous matrix was carried out by radiation-induced grafting of GMA using a pre-irradiation method reported elsewhere (Nasef *et al.*, 2016). The electron-beam (EB)-irradiated sheet (irradiation conditions: 2MeV, 3mA at 28^{o}C and 60kGy at 10kGy/pass) was brought into contact with N_2 flushed GMA emulsion containing 7% GMA and 0.5% Tween-20 in DI water for 1 h at 60^{o}C in the grafting reactor. After the reaction, the poly(GMA) grafted PE/PP sheet was taken out and rinsed in DI water for 8 h followed by multiple washes using methanol to eliminate the leftover homopolymer and monomer before being vacuum dried. The sample weight was determined by comparing it with the initial weight and the degree of grafting (G%) in the samples was calculated based on the weight increase in the grafted samples using equation [1]:

$$G(\%) = \frac{m_g - m_o}{m_g} \times 100 \qquad (1)$$

where, m_g and m_o are the weights of the grafted and original PE/PP microfibrous sheet respectively.

C. Pre-treatment of the Poly(GMA) grafted PE/PP microfibrous sheet polymer

The poly(GMA) grafted PE/PP microfibrous sheet was pre-treated with diethyamine/ethanol (50:50, v/v) under constant agitation of 140rpm at 40^{o}C for 4 h. After amination, the aminated sheet was removed and washed several times with ethanol to eliminate any unreacted diethylamine. The sample was vacuum dried for 2 h at a drying temperature of 80^{o}C. The initial and final weights of the polymer were determined, and the amine group density was determined using equation [2]:

$$Amine\ group\ density\left(\frac{mmol}{g}\right) = \frac{W_a - W_g}{W_g} \times \frac{1000}{73.14} \qquad (2)$$

where, W_a and W_g are the weights of the final aminated and initial grafted samples respectively. The denominator value of 73.14 is the molecular weight of diethylamine. The dried aminated sample was alkalised by immersing in 1M NaOH solution overnight to increase the alkalinity followed by washing with distilled water until pH 7 was reached, followed by drying. The steps from the modification of the PE/PP substrate to the pre-treatment process are shown in Figure 1.

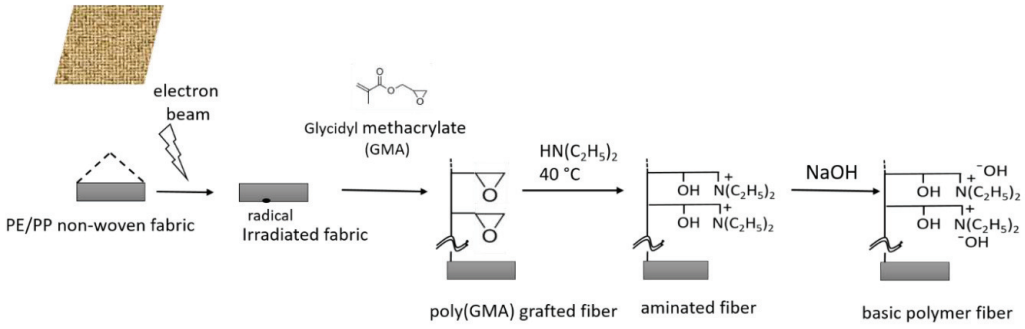

Figure 1. Preparation of poly(GMA) grafted PE/PP microfibrous sheet

D. Immobilising *C. rugosa* lipase on activated poly(GMA) grafted PE/PP microfibrous sheet

C. rugosa lipase was immobilised covalently on the aminated poly(GMA) grafted PE/PP microfibrous sheet. A sample area of 1cm^2 was added to a 3ml solution of 50mM phosphate buffer (different pH) consisting of 5.0-9.0mg of lipase. The reaction mixture was incubated with continuous agitation of 180rpm at a temperature of 37^{o}C. The time for incubation was varied accordingly between 2-6 h. The loaded substrate was rinsed in a phosphate buffer prior to storage until determination of the lipase residual activity.

E. Characterisation of activated polymer

The incorporation of poly(GMA) and DEA was investigated using Fourier infrared spectroscopy coupled with attenuated total reflectance (FTIR-ATR) (Thermo Fischer Scientific) with 4cm^{-1} resolution at a transmission mode in a range of frequency at 500-4000cm^{-1}. Morphological changes accompanied the applied modification and the amination of PE/PP sheets was observed using a field emission scanning electron microscope (JSM-6700F, JEOL, USA) at 20kV accelerating voltage on gold sputter-coated samples.

F. Experiment design and data analysis

In optimising the immobilisation conditions, response surface methodology (RSM) was applied to maximise the residual lipase activity of the immobilised enzymes. Three parameters: immobilisation time (x_1), medium pH (x_2) and the enzyme/support ratio (x_3) were chosen as the independent variables for lipase immobilisation conditions whereas enzyme residual activity was the dependent variable (response). The 3-factor and 3-level central composite design (CCD) was chosen to obtain the optimum values of these parameters and their interactions. The parameters with ranges of values taken under study were 2-6 h for x_1, 7-9 for x_2, and 5.0-9.0mg/cm^2 for x_3. Overall, there were 20 experimental runs and each was carried out in triplicate. The response results of the residual activity were evaluated using Design Expert and SPSS softwares. All experiments were carried concurrently to reduce the effect of experimental error in the observed reaction.

G. Enzyme activity test

Determination of the lipase residual activity assay was carried out spectrophotometrically following the method described by Yang *et al.* (2016) using substrate of *p*-nitrophenyl palmitate (*p*NPP). The hydrolysis of *p*NPP by lipase to produce *p*-nitrophenol was detected at 410nm. A sample area of $1cm^2$ poly(GMA) grafted PE/PP microfibrous sheet containing immobilised lipase was transferred into a reaction medium containing $100\mu l$ of 0.4% (w/v) *p*NPP substrate in ethanol and 1.88ml of 50mM phosphate buffer solution at pH 7. The activity was allowed to take place at 45^oC for 15 min. The reaction was stopped instantly after 15 min by immersing in 100^oC water bath for 5 min. To check for any spontaneous hydrolysis of substrate used, a reaction medium without enzyme was employed to act as a control. The mean of three parallel experiments were calculated to determine each response value for the immobilised enzyme residual activity.

H. Finding optimal temperature and pH for lipase activity

The optimum temperature for the immobilised lipase and its soluble counterpart were determined by measuring their relative activity after incubation with substrate. The immobilised and free lipases were immersed in 50mM phosphate buffer (pH 7) while the temperature was varied in the range of 25-65^oC. In contrast, the optimum pH was determined by immersing both immobilised and free lipase at different pH (6-11) buffer under optimum temperature.

I. Properties of lipase enzymes on thermal, pH and storage stability and reusability

In evaluating the thermal stability of immobilised and free lipase, both forms were kept in a 50mM substrate-free phosphate buffer solution for 30 min, at varying temperature of 25-65^oC under optimum pH. The samples were taken and cooled to room temperature after the incubation period before being added to the *p*NPP substrate. Enzyme assays were conducted at optimum conditions. In the pH stability test, both immobilised and free enzymes were incubated for 30 min in different substrate-free pH buffers (6-11) after which the lipase was transferred to fresh buffer with optimum pH with the presence of substrate and the activity was evaluated at an optimum temperature as described above.

The ability of the immobilised lipase to be reused was evaluated by recovering the tested sample from the reaction medium by spatula and washing several times with phosphate buffer (pH 7). This is to eliminate any residue or unreacted substrate on the fibrous sheet. Then, the sample was once again introduced into a new reaction medium having the same experimental conditions. The same immobilised lipase sample was reused up to 12 times under the same procedure. The first activity cycle was considered as 100% in comparison with the relative activity of others to determine the reusability of immobilised lipase residual activity. To determine lipase storage stability, immobilised and free lipases were respectively kept in 50mM phosphate buffer (pH 7) at 4^oC for up to 30 days. Aliquots were periodically extracted for activity determination on day 1, 3, 5, 10, 20 and 30. To determine the residual activity, the activity assay of day 1 was

set as 100%.

III. RESULTS AND DISCUSSION

A. Preparation of poly(GMA) grafted PE/PP microfibrous sheet polymer with amino group

Grafting of GMA led to the incorporation of covalently bonded epoxy rings originating from the grafted poly(GMA) on the surfaces of the PE/PP microfibrous sheet, which allowed further ring opening modification through amination. The features of FTIR spectra shown in Figure 2 provide evidence of the activation involving incorporation of poly(GMA) to the PE/PP microfibrous sheet and subsequent amination of the grafted sheet. Compared to a pristine PE/PP sheet having typical features of CH_2 and CH_3 bands in the region of 2800-3100cm^{-1}, the poly(GMA) grafted sheet showed additional strong bands at 906 and 844cm^{-1}, representing the epoxy group. This was coupled with a carbonyl group peak at 1724cm^{-1}. After amination, the peaks representing the epoxy group disappeared with parallel appearance of a –OH broad peak at 3500cm^{-1} resulted from the ring opening reaction. The remaining carbonyl group peak at 1724cm^{-1} proved that the epoxy ring opening took place by amination. The FTIR results of poly(GMA) grafted and aminated PE/PP sheets in this study are in a complete agreement with literature obtained by Nasef *et al.* (2014). The amine group density of the activated polymer is calculated to be 3.33mmol/g. After immobilisation, the alkyl group peak at 1153cm^{-1} showed a reduction in intensity in which is used for covalent attachment for the enzyme to be immobilised on the polymer. This showed that covalent attachment forms a peptide bond between enzyme and substrate releasing alkyl group from the polymer.

FESEM images provided further evidence of grafting of GMA amination and subsequent lipase immobilisation as indicated by the difference in the fibre diameters (Figure 3). The pristine PE/PP showed fibres with diameters in the range of 12∼15μm (Figure 3(A)) compared to 25∼33μm diameter in poly(GMA) grafted PE/PP microfibrous sheet shown in Figure 3 (B). The aminated poly(GMA) grafted PE/PP microfibrous sheet shows similar morphology with minor increase in fibre diameter (Figure 3(C)). The diameter of lipase immobilised poly(GMA) grafted PE/PP fibres further increased and formed some spots as shown in Figure 3 (D) indicating the attachment of lipase between polymer networks. It can be concluded that the procedure employed in this study i.e. grafting and functionalisation were successful in modifying the fibrous structure of PE/PP permanently with poly(GMA) offering epoxy rings that were aminated prior to hosting the lipase enzyme.

The use of synthetic polymer allows the control of the length of the matrix support-enzyme spacers for improved enzyme activity (Zdarta *et al.*, 2018). This result is in accordance to the study by Goto *et al.* (2007) which showed that the pre-treatment of polymer with diethylamine groups has allowed the expansion of polymer due to mutual electrostatic repulsion for multilayer enzyme immobilisation and space for enzyme activity reactions to occur effectively. This can be shown from the observation of swelling that has occurred within the aminated poly-

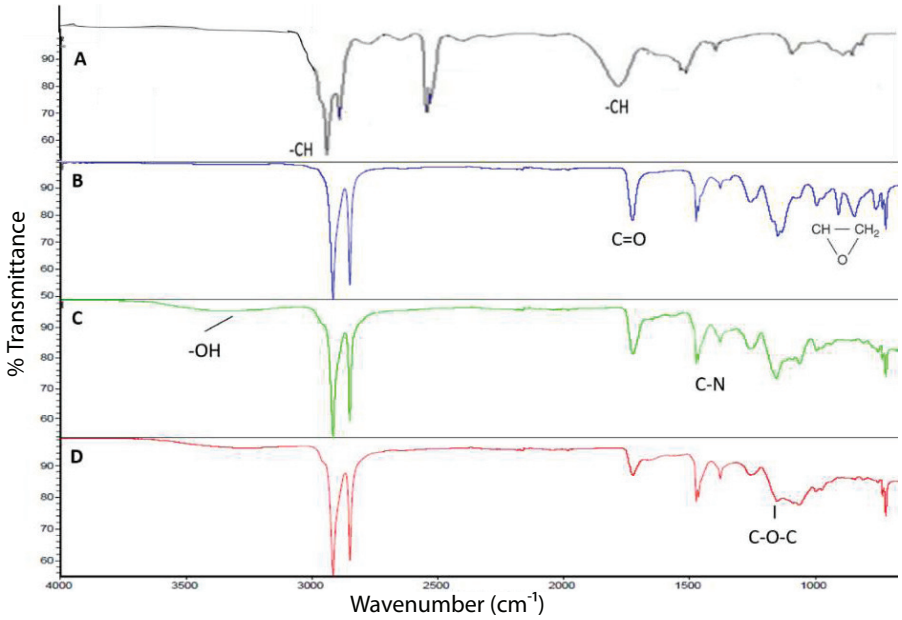

Figure 2. FTIR spectra of: (A) pristine PE/PP (B) poly(GMA) grafted PE/PP and (C) aminated poly(GMA) grafted PE/PP (D) lipase immobilisation on poly(GMA) grafted PE/PP microfibrous sheets

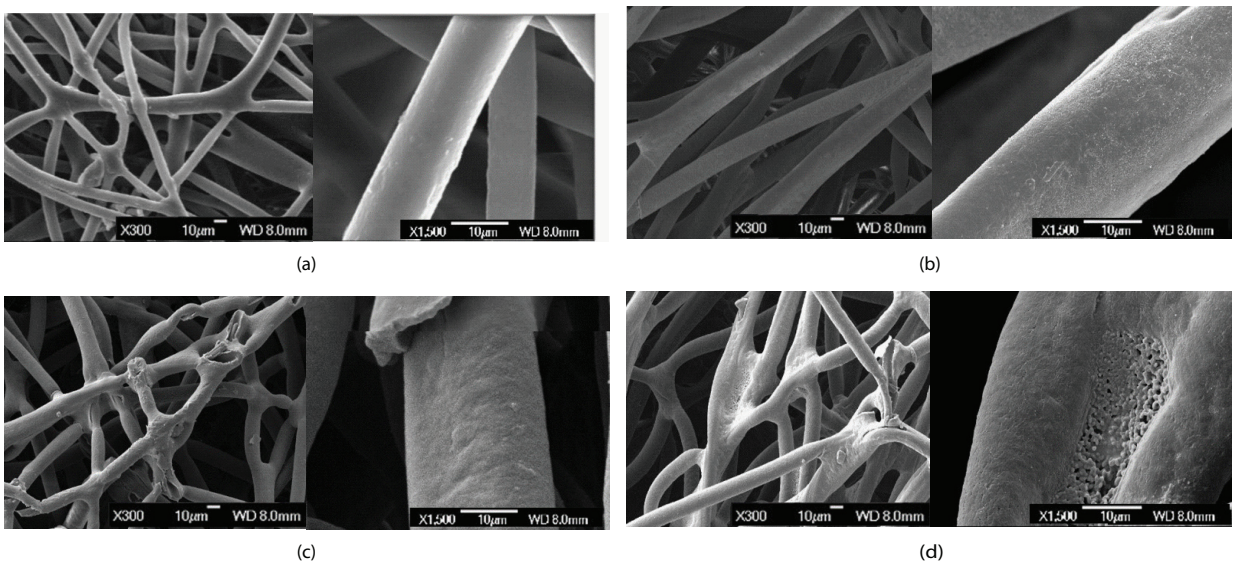

Figure 3. FESEM images of pristine PE/PP (A), poly(GMA) grafted PE/PP (B), aminated poly(GMA) grafted PE/PP (C), and lipase immobilised poly(GMA) grafted PE/PP (D) microfibrous sheets at magnification of 300x and 1500x, respectively

mer and improved enzyme activity after the pretreatment. Besides, the hydrophilicity of chemically modified polymer allows better solubility of enzyme in the solution. The aminated polymer provides excellent mechanical strength, chemical and thermal stability and spacious surface

area for enzyme immobilisation by covalent attachment. The hydrophilic surface illustrates good resistance to non-specific bonding between biomolecules and the membrane surfaces (Zhu and Sun, 2012).

The use of grafted polymer served the same function as a spacer arm in providing further extension for efficient enzyme immobilisation. Various studies conducted previously, had modified the polymer prior to immobilisation to increase enzyme loading, give efficient bonding, and improve enzyme activity. Modifications provided more area for the immobilised lipase to be accessed by the lipid substrate. Lipase attached directly to the polymer surface tends to lose its active conformation in its attempt to maximise contact with the surface, which consequently gives low activity (Bayramoğlu, 2005). The use of synthetic polymer for enzyme immobilisation by covalent binding allows improved enzyme activity and stability.

B. Optimisation of lipase immobilisation on poly(GMA) grafted PE/PP microfibrous sheet

The nature of the experimental result and optimum values for the variables under study were determined by applying face-centred central composite design (FCCCD) with RSM. For the optimisation, the independent variables chosen were immobilisation time, medium pH, and enzyme/support ratio (Table 1) to maximise the response value of the immobilised lipase by determining the residual activity. The influences of variables on the result were calculated by the Design Expert software v.7.1 (Stat-Ease Inc. Minneapolis).

The 'analysis of variance' (ANOVA) test for the response surface quadratic model is displayed in Table 2. The established F-value of the model, which is 11.76, indicated that the model was significant. The chance that this F-value was generated due to noise is only 3%. The probability value (Prob>F=0.0003), which was very low, proved that the model and its term were very significant and sufficient to explain the actual relationship between the significant parameters and the response. Moreover, the insignificant 'lack of fit' with F-value=3.28 was highly desirable and related to the pure error ($p>0.05$). The value demonstrated the precision and reliability of the designed model for good reproducibility. The determination coefficient (R^2) indicated the accuracy of the model with the value of 0.9136. This indicated that 91.36% of the variables matched the response. A coefficient of determination value near to 1 showed that the experimental data were defined and well represented by the model established.

Post hoc comparisons using Tukey test indicated that the mean score for the time between 2 to 4 h and 4 to 6 h were significantly different. However, mean score for the time between 2 to 6 h was not significantly different. The mean score for pH showed pH 7 to pH 8 and pH 8 to pH 9 were significantly different while pH 7 to pH 9 was not significantly. Factor enzyme/support ratio showed mean score for all groups of 5.0, 7.0 and 9.0mg/cm^2 were significantly different to one another.

Table 1. Responses of optimisation of enzyme immobilisation by RSM

Treatment no.	Factor			Enzyme residual activity (U) per cm^2
	Time (h), x_1	pH, x_2	Enzyme/support ratio (mg/cm^2), x_3	
1	0(4)	-1(7)	0(7.0)	0.4838
2	1(6)	-1(7)	-1(5.0)	0.2766
3	-1(2)	1(9)	1(9.0)	0.5452
4	-1(2)	0(8)	0(7.0)	0.6771
5	1(6)	0(8)	0(7.0)	0.8109
6	0(4)	0(8)	0(7.0)	0.9933
7	0(4)	0(8)	1(9.0)	0.9220
8	0(4)	1(9)	0(7.0)	0.6761
9	0(4)	0(8)	0(7.0)	0.9150
10	-1(2)	-1(7)	-1(5.0)	0.4491
11	1(6)	-1(7)	1(9.0)	0.6320
12	0(4)	0(8)	0(7.0)	0.9676
13	0(4)	0(8)	0(7.0)	0.9933
14	-1(2)	1(9)	-1(5.0)	0.3638
15	1(6)	1(9)	1(9.0)	0.5071
16	-1(2)	-1(7)	1(9.0)	0.5205
17	0(4)	0(8)	-1(5.0)	0.6543
18	1(6)	1(9)	-1(5.0)	0.3886
19	0(4)	0(8)	0(7.0)	0.9319
20	0(4)	0(8)	0(7.0)	0.8248

C. Interactions of immobilisation factors and response

Surface response plots of the quadratic polynomial model were used to interpret the effects of the immobilisation conditions on the response of enzyme residual activity. One variable was kept fixed while the interactions of the other two variables were analysed. The fitted response function was represented by the 3D response surface and 2D contour plots. Figure 4 (a) displays the influences of immobilisation time, medium pH, and their mutual interaction on the enzyme activity at an enzyme/support ratio of $7.0mg/cm^2$. As the value increased, the immobilised lipase residual activity increased (from 0.48 to $0.87U/cm^2$ poly(GMA) polymer) and the highest activity was achieved at the central point of the plot. The red colour gradient indicated the optimum level of time and pH

Table 2. ANOVA for RSM FCCD model

	SS	Df	MS	F-value	P-Value	
Model	0.92	9	0.10	11.76	0.0003	Significant
Residual	0.087	10	8.72E-003			
Lack of Fit	0.067	5	0.013	3.28	0.1019	Not significant
Pure Error	0.020	5	4.074E-003			
Cor Total	1.01	19				

that were between the range of pH 7.5 – 8.5 and time of 3 – 5 h. Although immobilisation may be performed at neutral pH in many cases, incubation at alkaline pH values, where the reactivity of the protein nucleophiles may be improved, was convenient to have a high enzyme-support reaction (Kuo *et al.*, 2012). Figure 4 (b) displays the effects of the immobilisation time, the enzyme/support ratio and their mutual interaction on the enzyme activity at pH 7. For an immobilisation time between 2 and 6 h, the residual activity showed a gradual increase (0.65 to 0.99U/cm^2 poly(GMA) polymer) as the enzyme/support ratio increased from 5.0 to 9.0mg/cm^2. The combined result of pH and the enzyme/support ratio (Figure 4 (c)) on the immobilised lipase residual activity at a constant immobilisation time of 4 h increased the residual activity (over 0.8575U /cm^2 poly(GMA) polymer) starting from pH 8 and enzyme/support ratio from 7.0 – 9.0mg/cm^2.

The results showed that the residual activity of immobilised lipase increased with enzyme/support ratio at average pH and time. However, when pH increased beyond 8, the residual activity dropped off. Suitable mild alkalinity conditions and higher enzyme/support ratios needed to be applied to increase the enzyme attachment competency on the poly(GMA) grafted PE/PP microfibrous sheet, thereby allowing the higher activity. Two theories can be used to describe this occurrence. First, sufficient enzyme/support ratio was necessary for the enzyme to immobilise on the polymer and increasing that ratio gave better chances for the immobilisation to occur. Second, a slightly alkaline condition allowed for the best bond between the enzyme and the membrane. Higher pH medium affected the ionic condition for the enzyme immobilisation to the polymer. The suitable time was crucial in providing better immobilisation effect. The extension of time caused the enzyme on the polymer to be saturated and thus, the active site of enzyme activity was blocked, decreasing the lipase activity (Yuce-dursun *et al.*, 2016). Besides, saturation limitation with a deficient amount of substrate decreased the chance for the enzyme activity to take place.

Time, pH, and enzyme/support ratio had a significant effect on the immobilisation of lipase and thus affected the residual activity. Sufficient time and suitable pH with high enzyme/support ratio were required for an optimum condition for enzyme immobilisation in giving the highest residual activity.

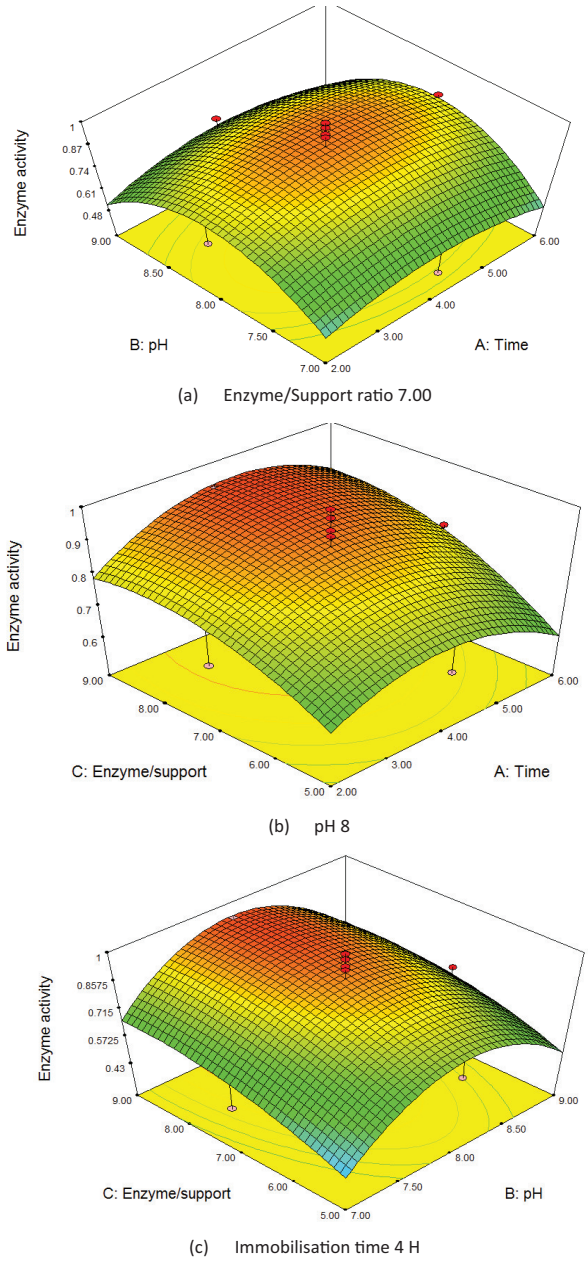

Figure 4. 3D surface plots showing the effects of immobilisation factors and their interaction. (a) pH and time (b) enzyme/support ration and time (c) enzyme/support ration and pH

D. Achieving optimum condition for immobilisation

The optimum immobilisation conditions were established using the statistical analysis of the Design Expert software that calculated the estimated maximum response from the results. The estimated immobilised lipase activity (response; Y) was figured following the immobilisation model, as shown in Table 3. According to the analysis, the optimum immobilisation conditions, which depended on the highest residual activity, could be obtained at an immobilisation time of 4.24 h, a medium pH of 8, and an

enzyme/support ratio of 8.51mg/cm^2 with the maximum experimental activity of 1.46U/cm^2.

E. Characterisation of immobilised lipase

1. *Optimum temperature for immobilised lipase residual activity*

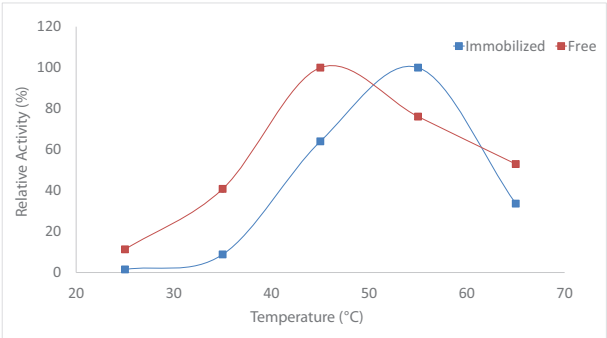

Figure 5. Effect of temperature on enzyme activity for immobilised and free lipase

Figure 5 shows the highest relative activity on the hydrolysis of lipase on *p*-NPP obtained at 45^{o}C for free lipase and 55^{o}C for immobilised lipase at pH 7. The immobilised lipase showed higher optimum temperature in contrast to the free enzyme, which lost more than 30% of its activity when incubated above 45^{o}C. The conformational mobility of immobilised lipase was restricted due to the covalent bond that existed on the matrix and thus required higher kinetic energy to attain suitable conformation for catalytic reaction. While substrate conversion continued at high temperature, enzyme denaturation appeared to be shifted to an even higher temperature where lipase was denatured. In the study of Yuce-Dursun (Yuce-dursun *et al.*, 2016), in which *C. rugosa* lipase was covalently immobilised on hybrid epoxy-silica polymer films, the immobilised enzyme showed improved thermostability over the free enzyme. Other studies supported improved thermostability on immobilised lipase, including Huang *et al.* (2011) and Zhu and Sun (2012). The results appear to support the notion that immobilised enzyme via covalent attachment to a matrix demonstrated increased resistance to heat.

2. *Optimum pH for immobilised lipase residual activity*

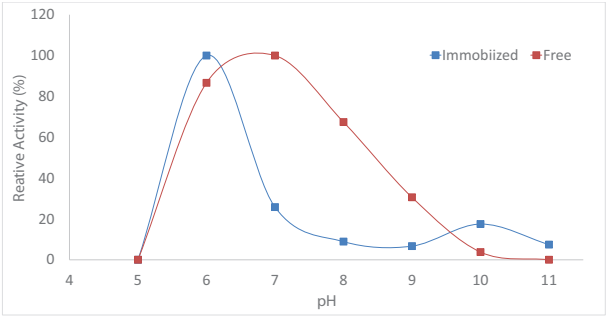

Figure 6. Effect of pH on enzyme activity for immobilised and free lipase

The optimum pH for free lipase was at pH 7. After immobilisation, the optimum activity was slightly shifted to the acidic region of pH 6 (Figure 6). This condition was due to the proton exchange properties of the immobilisation polymer that produced a different microenvironment characteristic around the enzymes than the bulk solution (Tastan *et al.*, 2011). The polycationic polymer surface of grafted poly(GMA) causes its microenvironment pH value to be higher than its bulk pH. The shift occurred due to the covalent attachment on the surface of the support which limited the transition of lipase conformation against the change of pH. A study by Yucedursun *et al.* (2016) reported similar result for optimum pH of free and immobilised *C. rugosa*

Table 3. Estimated and experimental maximum (lipase residual activity)

Validation	Time (h), X_1	pH, X_2	Enzyme/Support Ratio (mg/cm^2), X_3	Estimated Activity (U/cm^2)	Experimental Activity (U/cm^2)
1	4.39	7.77	8.40	0.93	1.41
2	4.12	8.15	8.97	0.93	1.44
3	4.24	8.00	8.51	0.94	1.46
4	4.54	8.12	8.41	0.94	0.99

lipase by covalent bonding on epoxy-silica polymer films at 7.0 and 6.0, respectively. Ren *et al.* (2011) in his study revealed that the reasons for a shift toward the acidic regions are due to the covalent attachment on the surface of the support which limits the transition of lipase conformation against the change of pH. The presence of the amine groups on the surface of polymer near the active sites of lipase molecules that could diminish the concentration of protons. Therefore, both the notions support the shift result of the optimum pH found for the grafted-PGMA polymer.

3. Stability of the immobilised lipase

Temperature and pH stability, reusability and storage life were the essential aspects for the commercial application of biocatalyst. Figure 7 (a) shows the effect of temperature on the free and immobilised lipase that was tested for their relative activity at each optimum pH and temperature. Below 35^{o}C, the activity of both immobilised and free lipase was not easily affected. However, the relative activity of the free lipase showed a significant drop above 35^{o}C and it decreased about 32% of the initial activity at 65^{o}C. In contrast, immobilised lipase decreased less and more slowly than the free lipase and still retained up to 50% of activity at 65^{o}C. This was because the presence of covalent linkage in immobilised lipase improved thermal stability, thereby reducing protein denaturation at higher temperature (Huang *et al.*, 2011). Furthermore, the fibrous mesh helped prevent denaturation as well (Huang *et al.*, 2011). A study by (Trbojević Ivić *et al.*, 2016; Zhu and Sun, 2012) show similar result where they found the residual activity of the immobilised and free lipase remained to be 73% and 54% respectively after 20 min of incubation at 60^{o}C.

The effect of pH stability on the free and immobilised lipase, tested on its relative activity at optimum pH and temperature, is shown in Figure 7 (b). The activity of immobilised lipase on the poly(GMA) grafted PE/PP microfibrous sheet was retained throughout a wider pH range compared to free enzymes. Immobilised lipase was most stable at pH 9 and maintained better residual activity, exhibiting the lowest residual activity (∼48%) at pH 11. In contrast, free lipase was stable at pH 6 – 8, but was adversely affected once the pH was raised beyond this pH region. At pH 11, approximately only 12% resid-

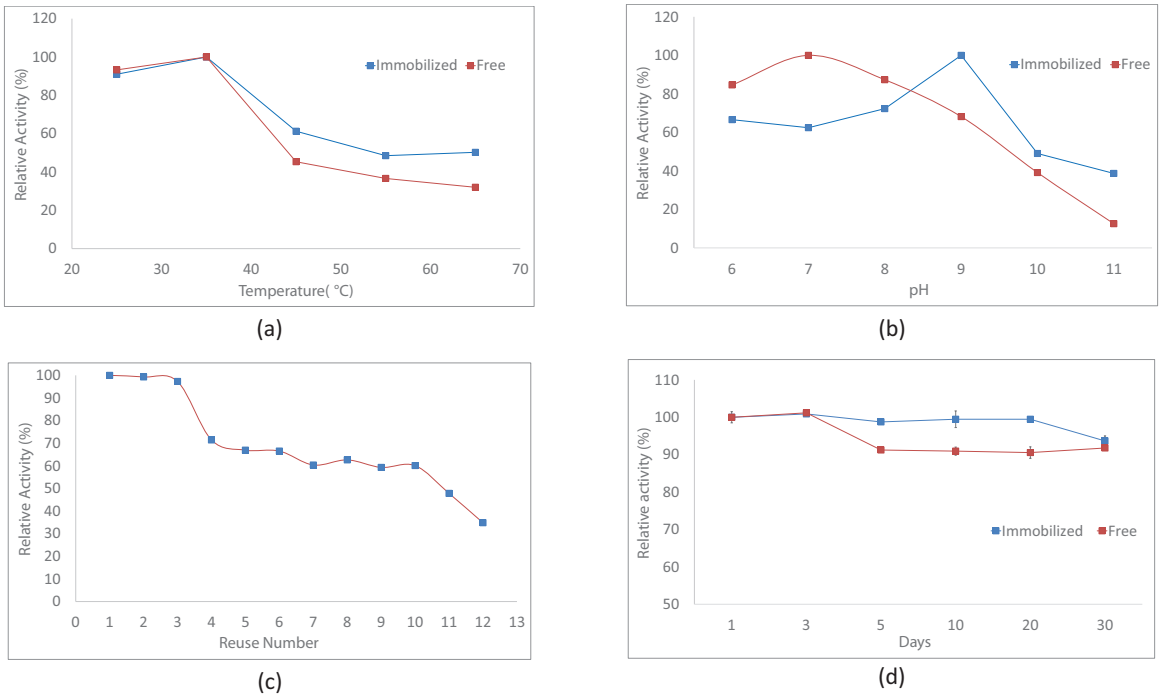

Figure 7. (a) Temperature stability and (b) pH stability of immobilised lipase over free enzymes; (c) reusability of immobilised lipase; (d) Storage stability

ual activity was retained. The improved stability of immobilised lipase is due to the multipoint attachment that physically restricted the mobility of amino acid residues against environmental changes and thus improved the pH stability of the immobilised lipase (Dwevedi, 2016). The increase in the stability performance of the immobilised lipase was due to the large surface area of the polymer which gave better geometrical congruence between the enzyme surface and high superficial density of the reactive group. The groups present were also very reactive, stable and showed minimal steric hindrances during reaction (Kuo *et al.*, 2012; Ren *et al.*, 2011). Several authors have shown a similar pattern of improved stability (Rivero and Palomo, 2016) proving that multipoint covalent bonding on the polymer support has been a very compelling approach to stabilise enzymes (Kuo *et al.*, 2012).

The most important benefit of immobilisation technology is the recycling of enzymes, which is extremely important for industrial applications. Figure 7(c) shows the activity of lipase immobilised on poly(GMA) grafted PE/PP microfibrous sheet after multiple cycles of separation and reuse. The immobilised lipase was seen to retain high activity on the first two cycles at 99% and 97% of the original activity, respectively. The residual activity dropped significantly on the fourth cycle onwards but still retained more than 60% for as many as ten cycles. However, the activity reduced below 50% after 10 cycles and retained only 34% of its initial activity at cycle number twelve. Enzymes that are attached by covalent bond are found to be very stable with higher reusability. It is possible that the activity was lost due to the inactivation of enzymes by continuous use and also to leakage of

the enzyme from the polymer by multiple separations, washes and soaking processes during the recycling reaction (Zhang *et al.*, 2015). A highly significant difference was observed in the relative activity between the immobilised and free lipases (Figure 7 (d)). The immobilised lipase retained its full activity until day 20 and dropped slightly on day 30 to 93% whereas the free enzyme drastically reduced its activity to 91% after the third day period. The immobilised lipase exhibited a higher storage stability than the free counterpart due to the presence of the attachment of a rigid covalent bond that prevented autodigestion and thermal denaturation (Dwevedi, 2016). The immobilising strategy involving the attachment between the enzyme subunit allowed further increase in its stability. However, the dissociation of the subunit caused by the inactivation over time led to the decrease of lipase activity towards the end of the experiment (Zdarta *et al.*, 2018).

IV. CONCLUSION

An interesting oxiran containing a fibrous substrate obtained by radiation-induced grafting of GMA onto a PE/PP microfibrous sheet, followed by amination and alkalisation was prepared and successfully used for the immobilisation of *C. rugosa* lipase. The lipase immobilisation conditions were carefully optimised using RSM. The optimum values for immobilisation time, medium pH, and enzyme/support ratio were found to be 4.24 h, pH 8, and 8.51mg/cm^2 respectively. The immobilised lipase showed an activity of 1.46U/cm^2 coupled with enhanced enzyme stability. Based on the results of this study, it can be concluded that radiation-induced grafting and subsequent functionalisation provide a highly efficient method for converting PE/PP microfibres into active substrates for enzyme immobilisation. RSM is an effective tool for optimisation of immobilisation conditions, leading to high enzyme activity and stability. Finally, the activated poly(GMA) grafted PE/PP microfibrous sheet showed strong potential for enzyme immobilisation for various biocatalytic reactions.

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