LIPASE IMMOBILIZATION ON FIBERS GRAFTED WITH POLYGLYCIDYL METHACHRYLATE

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ABSTRACT: Lipase enzyme originated from wheat germ was immobilized on nylon -6- grafted with polyglycidyl methachrylate (PGMA). The immobilization of enzyme experiments were designed and studied using face centred central composite design (FCCCD) under response surface methodology (RSM). Prior to immobilization, the polymer was activated with diethyl amine/ethanol to introduce an amine functional group to facilitate covalent bonding with the enzyme. The immobilized and free enzymes were characterized for effect of temperature and pH on enzyme activity, stability, storage and reusability as well as kinetics studies. ANOVA revealed that optimum lipase activity of 0.287 U/ml was achieved at immobilization time of 5 h, pH of 6 and 1.0 mg/ml for enzyme concentration. The optimum temperatures and pH for immobilized and free enzymes were 45 °C and 35 °C, and 8 and 7, respectively. The immobilized enzyme showed higher stability compared to free enzyme. The immobilized enzyme retained 18% of its activity after being recycled 8 times. In a storage stability test, immobilized lipase was able to retain 70% of its activity after being stored for 30 days, while free enzyme activity dropped to 15 % after 20 days of storage.

***ABSTRAK*:**Enzim Lipase telah dihasilkan daripada mikroorganisma pegun gandum di atas nilon -6- dan digraf bersama poliglisidel methakrilet (PGMA). Enzim pegun ini direka dan dikaji secara eksperimen menggunakan reka bentuk campuran pusat pada permukaan (FCCCD) di bawah kaedah tindak balas permukaan (RSM). Sebelum menjadi pegun, polimer ini telah diaktifkan dengan dietil amine/ethanol bagi menghasilkan kumpulan fungsi amine bagi membantu ikatan kovalen atom pada enzim. Enzim pegun dan bebas ini telah dikategorikan mengikut kesan enzim ke atas suhu, aktiviti enzim ke atas kesan pH, kestabilan, keboleh-simpanan dan keboleh-gunaan balik, serta ujian tindak balas kinetik. ANOVA membuktikan bahawa aktiviti optimum enzim lipase ini adalah sebanyak 0.287 U/ml telah terhasil selama 5 jam pegun, pada pH 6 dan kepekatan enzim sebanyak 1.0 mg/ml. Suhu dan pH optimum, pada enzim pegun dan enzim bebas ini adalah pada 45 °C dan 35 °C, dan pH 8 dan 7, masing-masing. Enzim pegun ini menunjukkan lebih stabil daripada enzim bebas. Enzim pegun dilihat kekal 18% daripada aktivitinya selepas 8 kali ulangan. Melalui ujian kestabilan simpanan, enzim lipase pegun dapat mengekalkan 70%

daripada aktivinya selepas disimpan selama 30 hari, manakala aktiviti enzim bebas telah menurun kepada 15% selepas 20 hari dalam simpanan.

KEYWORDS: *immobilization; enzyme; lipase; optimization; FCCCD; RSM; fibers; nylon; stability; reusability; kinetics study*

1. INTRODUCTION

Lipases (triacylglycerol ester hydrolase, EC 3.1.1.3) play an important role in industry. Their capability to break triacylglycerols down to fatty acids and glycerol make them the most promising in biodiesel production using transesterification of oils. Lipase is also used in food and flavour making, drug synthesis, and perfumery and cosmetics [1]. Due to the shortcomings of free enzymes, such as having low stability, non-reusability and difficulty in separation from the products, immobilization technology is introduced to overcome these drawbacks. Immobilization of enzymes is the most frequently used method to convey the needed characteristics of conventional heterogeneous catalysts into biological catalysts [2]. Various mechanisms are applied to immobilize enzymes such as covalent binding physical adsorption, ionic interaction, or entrapment [3]. Numerous benefits are conceivable through immobilization including improved stability, reusability, ease in separation of final products, greater control in catalysis [4], considerable cost benefits for industrial processes [5], and the ability to stop the reaction quickly by removing the enzyme from the reaction solution [6].

Among many supports, immobilization on polymer support has gained more interest. Polymer supports have good mechanical properties, can be produced in mass quantities and can be easily modified for immobilization with maintained stability [7,8]. Several studies showed that lipase enzymes have been immobilized on different types of support including chitosan beads [9], Electrospun cellulose nanofiber membrane [10] Sepabeads® [11], ferric silica nanocomposite [12] and octyl and cyanogen bromide (CNBr) agarose beads [13].

Radiation-induced graft polymerization (RIGP) is a useful method to functionalize various polymer materials [14]. Nylon-6, which is also known as polycaprolactam and polyamide 6, possesses excellent mechanical and physical properties and has become the most widely used synthetic polymer for fibres [15]. Introducing functional groups on the Nylon-6 polymer surface with RIG can introduce new chemical characteristics to the polymer while its main properties remain unchanged [16] making it promising candidate for enzyme immobilization.

In this study, lipase enzyme was immobilized onto the nylon-6 grafted polyglycidyl methachrylate (PGMA) with covalent linkage. Three conditions, namely, reaction pH, reaction time, and enzyme/support ratio were optimized using response surface methodology (RSM) and face centred central composite design (FCCCD). The optimum temperature and pH, thermal stability, storage stability and reusability, as well as kinetic parameters were determined.

2. MATERIALS AND METHODS

2.1 Material

Nylon-6 polymer was purchased from Reliance Sdn. Bhd. GMA, polyoxyethylene sorbitanmonolaurate or Tween-20 and diethyl amine (DEA) were purchased from Sigma-Aldrich (USA). Lipase (solid form, 5-15 units/mg protein) from wheat germ was also procured from Sigma-Aldrich (USA). All reagents used were of analytical grade or higher

and used as procured.

2.2 Synthesis and Activation of Polymer

Nylon-6-grafted with PGMA was synthesized by radiation-induced emulsion graft copolymerization. Typically, nylon-6 samples were placed in a PE zipper bags which were purged using purified N2, sealed and irradiated using an electron beam accelerator (EPS 3000) to a total dose of 10 kGy. The deoxygenated grafting solution was transferred to an ampoule containing irradiated polymer. PGMA grafted onto nylon-6 with grafting yield of 280% was obtained using grafting solution containing a mixture of 5 wt% of GMA and 0.5 wt% of Tween 20 in DI water with reaction time 3 hours at 40 ºC. Nylon-6-grafted with PGMA was introduced with the amine group where the PGMA-fiber was treated with diethyl amine/ethanol solution (1:1, v/v) at 40 °C for 4 hours with 100 rpm agitation. The aminated fiber was taken out of the solution using forceps and washed several times with absolute ethanol then dried for 2 hours at 60 °C in oven. After the drying process, the amine group density was calculated by Eq. (1) [14]:

$$Amine\ group\ density\ \left(\frac{mmole}{gram} - \text{adsorption}\right) = \left[\frac{(Wf-Wg)}{Wf}\right] \times \left(\frac{1000}{\text{MW}}\right) \quad (1)$$

where W_g and W_f are the weights of nylon-6 grafted PGMA before and after amination and MW is the molecular weight of diethyl amine. The aminated fiber then was soaked in hydrochloric acid solution (1M) followed by sodium hydroxide solution (1M) overnight. Then, the aminated fiber underwent oven drying for 2 hours at 60 °C and was stored in a desiccator to avoid moisture.

2.3 Statistical Optimization for Lipase Immobilization

Statistical optimization is a practical approach for studying several factors associated with the production process. This method is commonly used in reducing the number of experiments and for quick screening of the experimental field. Statistical evaluation of the data from an appropriately designed set of experiments can demonstrate the form of interaction between the parameters. In this study, Design Expert software (version 7.0.0) has been chosen to analyse the process parameters that influence the immobilization of lipase.

A 3-factor and 3-level face centered central composite design (FCCCD) under RSM was employed in this study. The effect of immobilization time (x_1), immobilization pH (x_2), and enzyme/support ratio (x_3) on lipase activity was evaluated at immobilization time of 2-10 h; pH of 6-8; and an enzyme concentration of 0.4-1.0 mg/ml.

Analysis of Variance (ANOVA) results were used to analyse the statistical data. This analysis included the Fischer's F-test, p-Value (which is used to indicate statistical significance for each of the coefficients tested and is also important to understand the pattern of the mutual interactions between the parameters), and the coefficient of determination R^2 (that measures the fit of the regression model). A value of R^2 closer to 1 indicates there are outstanding correlations between the independent variables. The α- level was set at 0.05 intervals, a p-value which is less than this value indicates that the model terms are significant.

2.4 Immobilization of Lipase Enzyme

Immobilization of lipase onto polymer support was carried out by immersing 20 mg of activated polymer in various concentrations of lipase (0.4 mg/ml, 0.7 mg/ml and 1.0 mg/ml) in a phosphate buffer solution (PBS) (50 mM, pH 7) and the mixture was incubated at 28-37 °C with 100 rpm agitation for different times (2, 6, 12 h). Protein concentration in final solution was measured by Bradford protein assay [17]. The amount of lipase immobilized was calculated based on the difference in lipase initial and final concentrations. The recovery efficiency (RE) was defined as "the percentage of enzymes on the polymer by detecting the protein concentration before and after immobilization" [18]. The RE value is calculated according to Eq. (2):

$$RE = \frac{Cp,o - Cp,r}{Cp,o} \times 100\% \quad (2)$$

where, $C_{p,o}$ is the protein concentration of added lipase (mg protein/mL); $C_{p,r}$ is the protein concentration of lipase in the solution and washed water after immobilization (mg protein/mL).

2.5 Measurement of lipase activity

The activity of lipase enzyme was found by employing *p*-nitrophenyl palmitate (*p*-NPP) as a substrate. Stock solution consisted of 28 mg of dissolved *p*-NPP in 100 ml of Triton X-100 and 1.7 ml of 1% sodium dodecyl sulfate while stirring. Then the solution mixture was heated on a hot plate until the solution become clear and no turbidity was seen. To start the enzymatic reaction, 1 mL of *p*-NPP stock solution was incubated with 1 mL of 0.1 M Tris-HCl (pH 8.2) and 20 mg immobilized enzyme at 37°C for 30 min. Then, 1 mL NaOH (1 M) was added to stop the enzyme reaction. The molar extinction coefficient for *p*-nitrophenol is 15,000 $M^{-1}cm^{-1}$ at 311 nm. Manifestly, one unit (U) of enzyme activity is defined as the amount of enzyme that liberates 1 mmol *p*-nitrophenol per minute under assay condition.

2.6 Effect of Temperature and pH on Enzyme Activity

The immobilized enzyme (20 mg matrix) was placed in phosphate buffer (0.1 M, pH 7) and assayed for residual lipase activity at different temperatures (25 - 65°C). For pH, the same amount of enzyme was assayed in 0.1 M PBS at pH range of (5-9).

2.7 Stability Profile of Immobilized Lipase

The pH stability of immobilized lipase was studied by incubating the enzyme for 30 minutes without addition of substrate in buffers of varying pH in the range of (5-12). After the incubation period, the residual enzyme activity was evaluated. Residual activities were calculated as the ratio of the activity of immobilized enzyme after incubation to the activity at the optimum reaction pH.

Thermal stability of immobilized lipase was tested by incubating the immobilised enzyme at varying temperatures in the range of 20–60 °C for 30 minutes at pH 7 and determining the activity at its optimum reaction temperature. Relative residual activity was measured by setting the highest activity at 100%.

2.8 Reusability of Immobilized Lipase

Immobilized lipase was washed thrice with PBS (50 mM, pH 7) to remove residual substrate. Enzyme activity was assayed at 45 °C for up to eight times. The residual activity was determined by taking the first activity cycle as 100%.

2.9 Storage Study

Immobilized lipase and free lipase were stored in PBS (50 mM, pH 7) at 4 °C for up to 30 days. The remaining activity was measured on a weekly basis.

2.10 Kinetics Study

Kinetic parameters of immobilized and free lipase were performed in phosphate buffer (50 mM, pH 7) at 45 °C. To evaluate the value of kinetic parameters which are K_M and V_{max}, the concentration of *p*-NPP substrate was varied in the range (0.1-3.0 mM). A few plots were utilized to determine K_M and V_{max} of the enzyme. The best fitted plot was selected from the highest R^2 value.

3. RESULTS AND DISCUSSION

3.1 Optimization of Lipase Immobilization

The optimization of the lipase immobilisation on nylon-6 polymer was conducted using response surface methodology (RSM) under face centred central composite design (FCCCD). The agitation was set up at 100 rpm and temperature of 37°C. The highest immobilized lipase activity was 0.222 U/mL obtained at pH 7, 6 hrs immobilization time, and enzyme concentration of 0.70 g/mL. The lowest activity of 0.004 U/mL was at pH 7, 6 hrs immobilization time, and enzyme concentration of 0.40 mg/mL.

The effect of each factor and its interactions were calculated using a Design Expert program (version 7.0.0, Stat–Ease Inc., USA). As the data was fitted with several models and consequently investigated using analysis of variance (ANOVA), the results showed that the quadratic polynomial model is the most acceptable to describe immobilization of lipase. The mathematical prediction model for immobilization of lipase was derived (Eq. (3)) under ANOVA test as below

$$Y = -1.00 + 0.052x_1 - 0.19x_2 + 0.37x_3 - 0.18x_1x_2 - 0.42x_1x_3 - 0.12x_2x_3 - 0.52x_2^2 - 0.27x_3^2 \quad (3)$$

where x_1 is immobilization pH, x_2 is the immobilization time and x_3 is the enzyme concentration.

ANOVA results are shown in Table 1. The model F-value of 11.60 implies the model is significant. There is only a 0.02% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.05 indicate model terms are significant. Meanwhile, lack of fit value (0.1088) was insignificant with respect to pure error. This indicated a good and reliable model. Thus, it could be used as a target response. The coefficient of determination (R^2=0.894) also indicated good correlation between the independent variables.

Figure 1 shows the 3D plot of the interaction between enzyme concentration and time. It can be observed that there is increase in enzyme activity with concentration; however, the highest enzyme activity is attained at medium time. At longer immobilization time the activity decreases substantially.

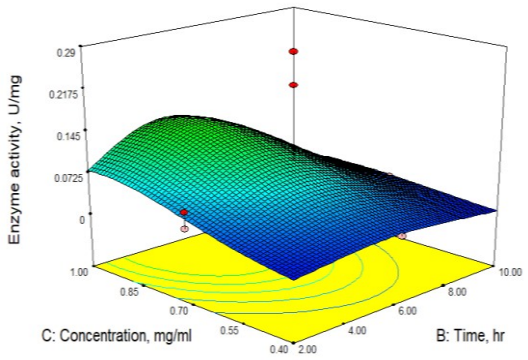

Fig. 1: 3D surface plot of enzyme concentration and time vs enzyme activity

Table 1 Analysis of variance (ANOVA) for immobilized enzyme

Source	F Value	p-value Prob > F	Remarks
Model	11.6	0.0002	significant
A-pH	0.3	0.6216	
B-Time	2.5	0.1436	
C-Concen.	9.0	0.0120	
AB	1.5	0.2519	
AC	7.9	0.0168	
BC	0.8	0.3774	
B^2	24.9	0.0004	
C^2	6.8	0.0244	
Lack of Fit	2.9	0.1088	not significant
R^2	0.89		

3.2 Model Validation for Immobilized Lipase Optimization

The accuracy of the model was validated under the optimal conditions obtained from FCCCD. The response (enzyme activity) was set at maximum. The validation test was performed by choosing three random runs according to Table 2. Based on the validation test, since the model is significant, lack of fit is insignificant and percentage error is low, it could be concluded that the model was reliable and reproducible.

Table 2 Validation test on optimization of immobilized lipase

Parameters			Immobilized enzyme activity (U/mL)		
pH	Time (h)	Enzyme concen (mg/mL)	Pred	Exper	Error (%)
7	4.94	0.90	0.145	0.133	9.49
7	4.99	0.90	0.146	0.130	12.00
7	5.03	0.90	0.145	0.125	15.87

3.3 Optimum Temperature and pH of Immobilized Lipase Activity Assay

The optimum temperature for free lipase was at 35 °C whereas for immobilised lipase, the maximum activity was at 45 °C as shown in Fig. 2. The optimum temperature profile of

the immobilized lipase was broader compared to the free lipase. This may be due to the decrease in conformational mobility of immobilized lipase because of the covalent bonding between the enzyme and polymer [19]. The free lipase lost almost 60% of its activity when exposed to temperatures above 35 °C. This low residual activity may be attributed to a significant amount of enzyme being denatured when exposed to elevated temperature [20]. Free lipase is denatured when heated at above 45 °C [21]. Other studies supported improved thermostability of the immobilized lipase as compared to free enzyme including [10] and [21].

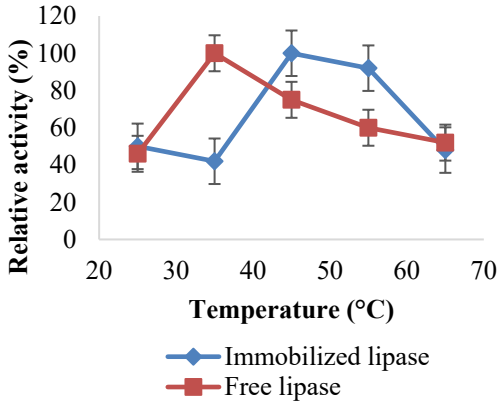

Fig. 2: Optimum temperatures for both immobilized and free lipase.

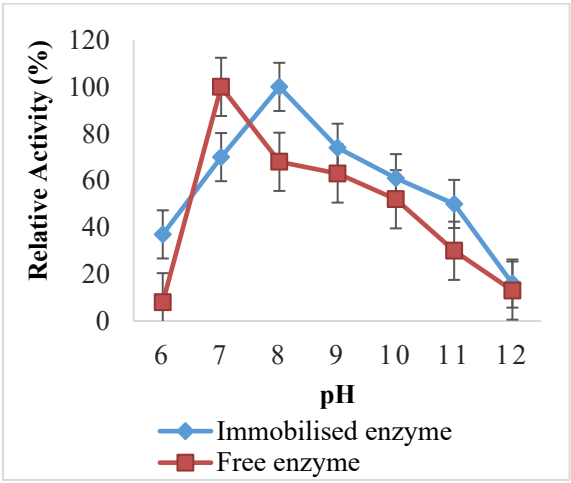

Fig. 3: Optimum pH for both immobilized and free lipase.

The optimum pH for free lipase was at pH 7, as shown in Fig. 3. This may result from the ionization states of several groups on amino acid chains that result in suitable conformation of lipase molecules [22]. After immobilization, the maximum activity was observed at pH 8. This alkaline shift could be an effect from the alteration in the microenvironment of the enzyme caused by immobilization on the support system [17].

3.4 Thermal Stability of Immobilized Lipase

Figure 4 shows the thermal stability of immobilized and free lipase. It can be seen that when increasing the temperature to 55 °C, the immobilized enzyme retained 74% of its activity compared to free lipase, which dropped to 18% residual activity. This result indicated that the immobilized enzyme was more stable than its free counterpart at higher

temperatures. Zhu and Sun [19] proposed that enhanced thermal stability was a result of covalent attachment, which limited conformational change and disallowed the denaturation process of the immobilized lipase at higher temperature. For free enzyme, high temperature might interrupt the globular structure of the proteins thus leading to the denaturation [23].

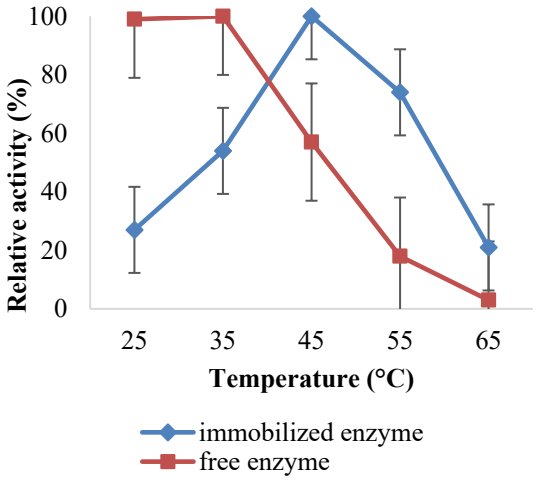

Fig. 4: Thermal stability of immobilized and free lipase.

3.5 pH Stability of Immobilized Lipase

Figure 5 compares pH stability of immobilized and free lipase. It can be clearly seen that immobilized lipase has better pH stability within the alkaline region, from pH 8 to pH 12. Pahujani et al. [24] reported that the immobilized lipase was fairly stable within pH 7.5 – 9.5. Lipase from a mutant strain of *Cornebacterium* sp. was immobilized and has been found stable at pH 8 [25]. According to [19], the multipoint attachment mechanism limits the undesired conformation against environment changes. This improves pH stability of the immobilized lipase.

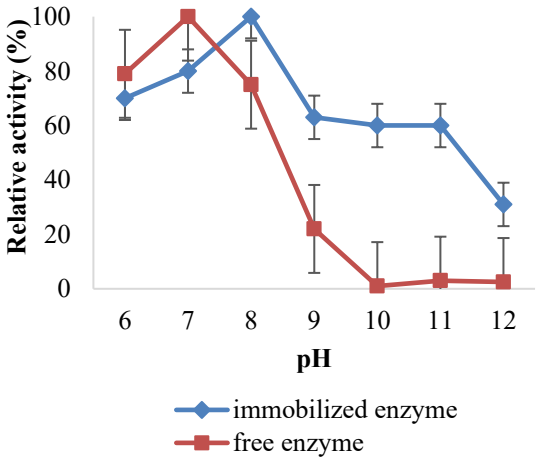

Fig. 5: pH stability of immobilized and free lipase.

3.6 Reusability of Immobilized Lipase

Figure 6 shows the effect of repeated use on the activity of immobilized lipase. In this study, the reusability test was conducted up to 8 cycles. The first cycle of this test was set as 100% and the residual activity of each cycle was calculated based on it. According to Fig. 6, immobilized lipase retained 78% of its residual activity up to 4 cycles. After 8 cycles, the

residual activity dropped to 18%. Previous study reported that lipase immobilized on CNBr-activated–Sepharose 4B retained full activity even after 13 cycles [26]. Huang et al. [22] reported that the remaining activity of lipase immobilized on the cellulose fibrous membrane dropped to 30% after 8 cycles. The activity of immobilized enzyme tends to decrease with repeated use. The loss of activity could be related to the inactivation of enzyme by continuous use and the leakage of protein from the polymer support. This can be improved by providing a stronger attachment mechanism in order to increase the recyclability.

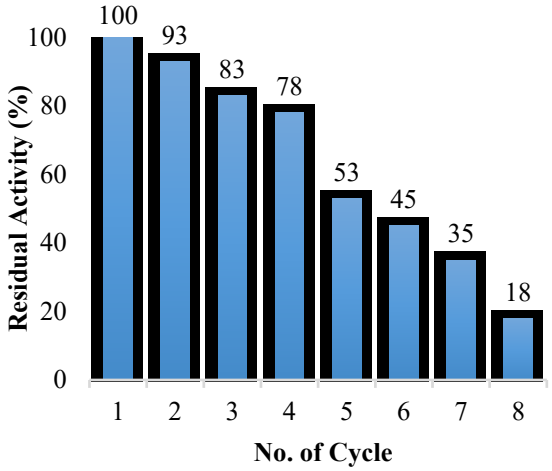

Fig. 6: Reusability of immobilized lipase.

3.7 Storage Stability of Immobilized Lipase

The stability of immobilized and free lipase was compared with respect to storage time. Both free and immobilized lipases were stored at 4 °C for 35 days then the activities of both enzymes were determined. The immobilized lipase was found to retain 70 % of its residual activity after 30 days of storage. On the other hand, after 20 days of storage at 4 °C, the activity of free lipase solution dropped to 15% of its original activity (Fig. 7). According to [19] free enzyme solution undergoes rapid reductions in catalytic activity during storage, making it difficult to recover the enzyme from the reaction mixture after catalytic reactions. This result shows the great potential of the polymer used in this study as a support for enzyme immobilization.

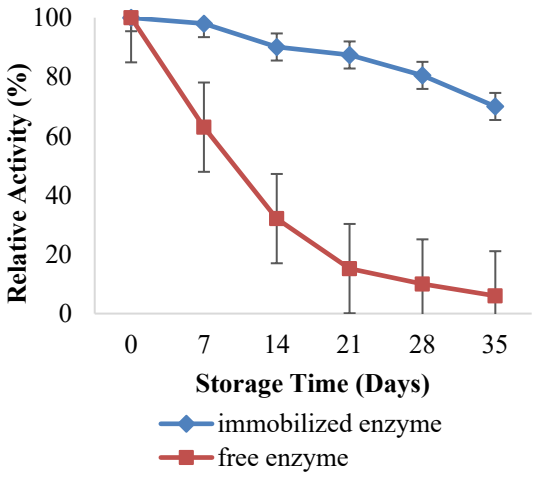

Fig. 7: Storage stability test of immobilized and free lipase.

3.8 Kinects Study

For the kinetics study of immobilized and free lipase enzyme, a Lineweaver-Burk Plot best fitted the data (Fig. 8). It was found that both V_{max} and K_M for immobilized lipase were reduced compared to the free enzymes. V_{max} and K_M values obtained were 0.012 mM/min and 7.250 mM for immobilized lipase, and 0.024 and 10.321 for free lipase, respectively. The lower value of K_M for immobilized lipase enzyme compared to free enzyme indicates a stronger and more efficient binding of substrate. V_{max}, on the other hand, exemplifies how quick the reaction is catalyzed by the enzyme. Lower V_{max} for the immobilized enzyme designates lower enzyme rate. This is commonly observed since the enzyme having lower activity after the immobilization process is caused by the lower accessibility of substrates towards the immobilized enzyme's active site [19].

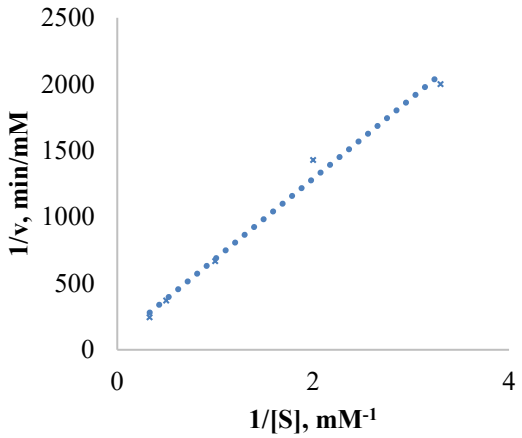

Fig. 8: Lineweaver–Burk Plot for immobilized lipase.

4. CONCLUSIONS

Lipase enzyme was successfully immobilized on nylon-6-grafted with PGMA. The optimum immobilization conditions were determined using RSM FCCCD. Immobilized enzyme showed higher stability at wide range of temperature and pH. Immobilized lipase retained 70% of its activity when stored for 35 days at 4°C, while free lipase lost 70% after 14 days. The immobilized lipase retained 78% of its activity after 4 cycles. This proposed immobilized enzyme-polymer system has potential in applications such as biodiesel production from oil.

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