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Immobilization of xylanase enzyme on poly-(HEMA-co-GMA) cryogel

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ABSTRACT

In this study, a polyethyleneimine (PEI) coated poly-(HEMA-co-GMA), hybrid cryogel column (HCC), was designed. HCC was synthesized via polymerization of gel-former factors at minus temperatures. The characterization experiments of the HCC were conducted through SEM, and FTIR experiments. At the end of the experimental periods, there was no significant decrease in the performance of the HCC. Then HCC used as a novel support for xylanase immobilization for the first time. The successful immobilization of xylanase was confirmed by FT-IR, while biochemical properties and stability of the PHG/PI-Xyl were evaluated in terms of optimum pH, optimum temperature, thermostability, storage stability, reusability, and kinetic parameters. The optimum activities for both free and immobilized enzymes were recorded at pH 6.0, while the optimum temperature for free was 55 °C, and for PHG/PI-Xyl was 60 °C. PHG/PI-Xyl displayed remarkable thermal stability for 180 min at 60 °C, with 53.55%, and for free Xyl 32.05% from the initial activity. Meanwhile, it retained up to 49% and 69 % for free and immobilized xylanase of original activities after 4 weeks of storage at room temperature. PHG/PI-Xyl retained about 58% of its original activity after 10 consecutive reuses, while Km for the free Xyl and PHG/PI-Xyl were calculated 4.05 mg/mL and 2.62 mg/mL, whereas Vmax 133.33 U/mL and 188.68 U/mL, respectively. As envisioned, this study suggests a promising way to solve the problems of high price and poor operational stability of the enzyme during biocatalytic.

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1. Introduction

Cryogels are biomaterials that are in a subcategory of hydrogels and have perfect physical features [1,2]. Cryogels have 3D elastic interconnected macropores, spongy morphologies and good mechanical robustness [3]. They are extremely versatile tools when converted to desired purposes [4] such as purification/adsorption of biomolecules [5-8], and removal of some pollutants [9-11]. Increasing the existing properties while designing cryogels will further increase their potential use in different fields [4]. The weak side of cryogels is their low adsorption capacity. This is due to the low surface area of the super macropores in the matrix [12-14]. Improving the binding capacity in some processes based on cryogels is of great importance [15].

After cellulose, xylan, a straight homopolymer made of D-xylose monomers joined by 1,4-glycosyl linkages, is regarded as the second most prevalent biomass that is naturally renewable [16, 17]. In light of the plant source, glucuronopyranosyl, 4-o-methyl-d-glucopyranosyl, α-l-arabinofuranosyl acetyl. As well as, connected to feruloyl and coumaroyl components of lignin, are present to variable degrees [18]. For a variety of commercial and industrial uses, xylanolytic enzymes use to convert xylan into shorter sugar residues is important [19]. Xylanase is one of these xylanolytic enzymes that are essential for xylan breakdown. Xylanase (EC 3.2.1.8), a hydrolytic enzyme employed in depolymerizing xylan, its used in a variety of industrial techniques, including whitening paper pulp with enzymes, juice clarification, oil extraction from plants, texture improvement in bakeries, bioconversion of agricultural wastes, bioscouring in textiles, and enhancing the animal feed digestibility [20, 21]. Bacteria, fungi, actinomycetes, and yeast have all been observed to produce xylanase [22]. With the use of commercially available substrates (xylan) or agricultural waste products (wheat bran, wheat straw, maize cob, sugar cane bagasse, etc.) as production substrates, xylanase can be produced both in submerged fermentation and in solid-state fermentation [23, 24]. To rise the brightness of the pulp, the paper and pulp companies utilized huge amounts of chlorine compounds, which resulted in the formation of harmful dioxins in the effluents. While on the other hand, pre-bleaching pulp with xylanase can preserve the brightness of the pulp while reducing chlorine usage by

20–30% [24]. For pulp bleaching, xylanase is recommended because it is most active at high temperatures and alkaline pH levels. Nevertheless, during industrial applications, free enzyme encounters challenges such as sensitivity and structural instability, as well as a failure to recover the active form of the enzyme from the reaction mixture for repeated use [25]. Enzyme immobilization offers a number of benefits, including the ability to process continuously and reuse the enzyme. At the industrial level, immobilized enzymes are preferable because they can be recycled and which lead to reduce production costs.

In this study, the cryogel of poly-(HEMA-co-GMA)] coated with polyethyleneimine (PEI) hybrid cryogel column (HCC), a new kind of functional, low-cost was synthesized. Xylanase enzyme immobilization on PHG/PI-Xyl cryogel column was carried out by the interactions with the amino groups of PEI. The morphological structural of PHG/PI was investigated with Scanning Electron Microscopy (SEM), while the synthesized PHG/PI and enzyme-immobilized PHG/PI-Xyl were investigated by Fourier transform infrared (FT-IR). Biochemical parameters including optimum pH and temperature, thermal and storage stability, reusability, and kinetics (Km and Vmax) of free immobilized xylanase on PHG/PI cryogel column were investigated. The originality of this study to break down xylan into tiny sugar is further enhanced by the requirement for more work to translate the given approach to an industrial scale. The current study, to the best of our knowledge, the first attempt at immobilizing xylanase on a PHG/PI cryogel column for application in xylan degradation.

2. Materials and methods

2.1. Materials

HEMA (2-Hydroxyethyl methacrylate) was provided by Fluka A.G (Buchs, Switzerland). MBAAm *(N,N'*-methylene-bis-acrylamide), TEMED *(N,N,N',N'*-Tetramethylethylene-diamine) and APS (ammonium persulfate), xylanase and beechwood xylan were purchased from Sigma–Aldrich Chemical Co. Other chemicals were purchased at reagent grade from Merck AG (Darmstadt, Germany).

2.2. Synthesis of poly-(HEMA-co-GMA) cryogel column

HEMA (174 µL) as monomer and MMBAm (10 mg) as the cross-linker were stirred within 1 mL of deionized water for cryo-polymerization technique. Then 20 µL of glycidyl methacrylate (GMA) was added to this prepared solution as a co-monomer. The mixture was then poured into a 2.5 mL plastic syringe. It was exposed to nitrogen gas for about 2 minutes to remove dissolved oxygen. After that, 100 µL (10% (w/v) APS as free radical generator and 20 µL TEMED as catalyst was added to the resulting mixture and the mixture was left at − 14°C for 24 hours. After 24 hours, the synthesized poly(HEMA-co-GMA) column was brought to room temperature for dissolution and washed with water, then with water-ethanol mixture to remove impurities.

2.3. PEI coating of poly-(HEMA-co-GMA) cryogel column

PEI molecules were immobilized on the prepared cryogel over reactive glycidyl groups in GMA. For this purpose, 15 mL of GMA (10%, w/v, pH 10.6) solution was prepared due to the viscosity of PEI. Then, the obtained poly (HEMA-co-GMA) cryogel was taken into the prepared solution (4 hours, 50 °C, 100 rpm) for the interaction between reactive groups of GMA and PEI. After 4 hours, the product of PHG/PI cryogel was repeatedly washed with distilled water to remove the unreacted PEI molecules.

2.4. Xylanase immobilization on PHG/PI

100 mg *PHG/PI* were drooped in acetate buffer solution (50 mM, pH 6.0), then 5 mL xylanase enzyme solution was added to the PHG/PI cryogel column with continuous shaking for 24 h at 4°C. After 24 h, the *PHG/PI* cryogel column were removed from the solution and washed repeatedly three times with acetate buffer (50 mM, pH 6.0) to ensure the removal of unbound xylanase. Finally, the immobilized enzyme (*PHG/PI-Xyl*) was kept at +4 °C for future use (Fig. 1).

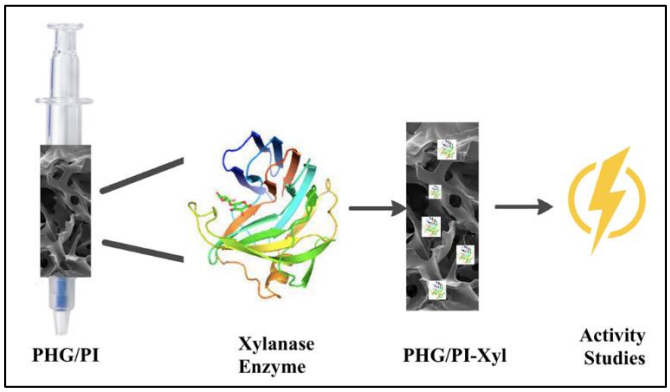

Figure 1. *Schematic illustration immobilization of xylanase on PHG/PI and activity study.*

2.5. Xylanase activity

The method described by Bailey et al. was used to determine the activity of xylanase by the increase in the formation of xylose [26], beechwood xylan was used as the substrate after employing a slightly modified. The xylanase activity was determined by the increase in production rate of xylose under the recommended conditions and DNS (3,5-dinitrosalicylicacid) was used as color indicator. Beechwood

xylan was dissolved in asetat buffer to create a xylan solution (1%, w/v) in (50 mM, pH 6.0). 1 mL of the substrate was added to 5 mg of cryogel containing xylanase, then the reaction mixture was incubated with heated to 50 °C for 10 minutes (under shaking). Then 1 mL of DNS was added and the reaction mixture was immediately cooled in cold water, after DNS addations the reaction mixture was boiled for 5 min at 100 °C. Furthermore, by using a UV-VIS spectrophotometer, the absorbance of complex between released reduce sugars and DNS reagent was determined at 540 nm. One unit (U) of xylanase activity was defined as the amount of enzyme catalyzed in the release of 1 μmol of reducing sugar as xylose equivalent per minute under the specified assay conditions.

2.6. Effect of pH and temperature

The effect of pH on free xyl and PHG/PI-Xyl was investigated by dissolved xylan substrate in different buffers, the buffer pH changed from 3.0 to 10.0, while the effect of temperature on the free xyl and PHG/PI-Xyl activity was investigated at different temperatures scales from 30 °C to 70 °C. The highest activity was accepted as 100% and the results were converted to relative activities. The data with the highest activity was determined as the optimum value.

2.7. Thermal and storage stability

To examine the thermal stability of the PHG/PI-Xyl, free and immobilized xylanase were incubated at 60 °C at various times (from 0 to 180 min). At the end of each incubation period, a standard enzyme assay was conducted to determine the amount of remaining enzyme activity. The activity observed for 0 min was taken as control 100%.

The storage stability of the PHG/PI-Xyl and free xylanase enzyme were examined by storing them at 25 °C for four weeks. The Enzyme activity was evaluated on the first day and considered as control, 100%, and subsequent activities were transformed to relative activity.

2.8. Reusability

To determine the reusability of the PHG/PI-Xyl, the activity was measured under optimum conditions for 10 cycles. After each measurement, the cryogel was separate from the supernatant then DNS reagent was added. Further, the activity was restarted by adding a fresh substrate to the immobilized enzyme. The first measured enzyme activity was accepted as 100% and this process was repeated for 10 successive reuses.

2.9. Values of Km & Vmax

To estimate the kinetic parameters (*Km & Vmax*), the activity of the PHG/PI-Xyl was measured at different xylan concentrations (1-5 %) under standard assay conditions. *Km* and *Vmax* values were estimated via the Lineweaver-Burk plot.

2.10. Characterization studies

The functional groups of *HCC* and enzyme bind to *HCC* were characterized by Fourier Transform Infrared Spectrometer (FTIR 8000 Series, Shimadzu, Japan). The surface morphology of cryogel was tested by using scanning electron microscopy (SEM, EVO LS 10 ZEISS 5600 SEM, Tokyo, Japan). For this aim, the samples were swollen in deionized water. Next, the samples were taken in to absolute (98%) ethanol to change the water with alcohol molecules in the pore structures. After this procedure, the alcohol is diffused. The samples were put inside an oven (60 °C) to eliminate the alcohol molecules from the samples without damaging their morphologies. The dehydrated cryogels were coated with gold-palladium (40:60). Afterward, they were taken to the SEM device for image acquisition.

3. Results and discussion

3.1. Characterization studies

The FTIR spectra of PHG/PI and PHG/PI-Xyl shows Fig.2. These spectra indicates that the broad peak about 3100-3200/cm was because of stretching bands of –OH and N–H groups. C–H stretching stretching stretching band was monitored at 2950/cm. In addition, a peak at 1717/cm of the stretching vibration of the C=O group was determined in the HEMA and GMA ester [1, 27]. After enzyme immobilization, the FTIR spectrum of the hybrid cryogel showed two new peaks at around 1550/cm and 1660/cm. These peaks can be attributed to amide I (vibration of C=O) amide II (a combination of C–N stretching and N–H vibration in the protein backbone), respectively [28]. Moreover, a small decrease in the asymmetric stretching of the epoxy group was also observed at about 900/cm with the immobilization of the enzyme. These results showed that the enzyme was successfully immobilized on the surface of the cryogel.

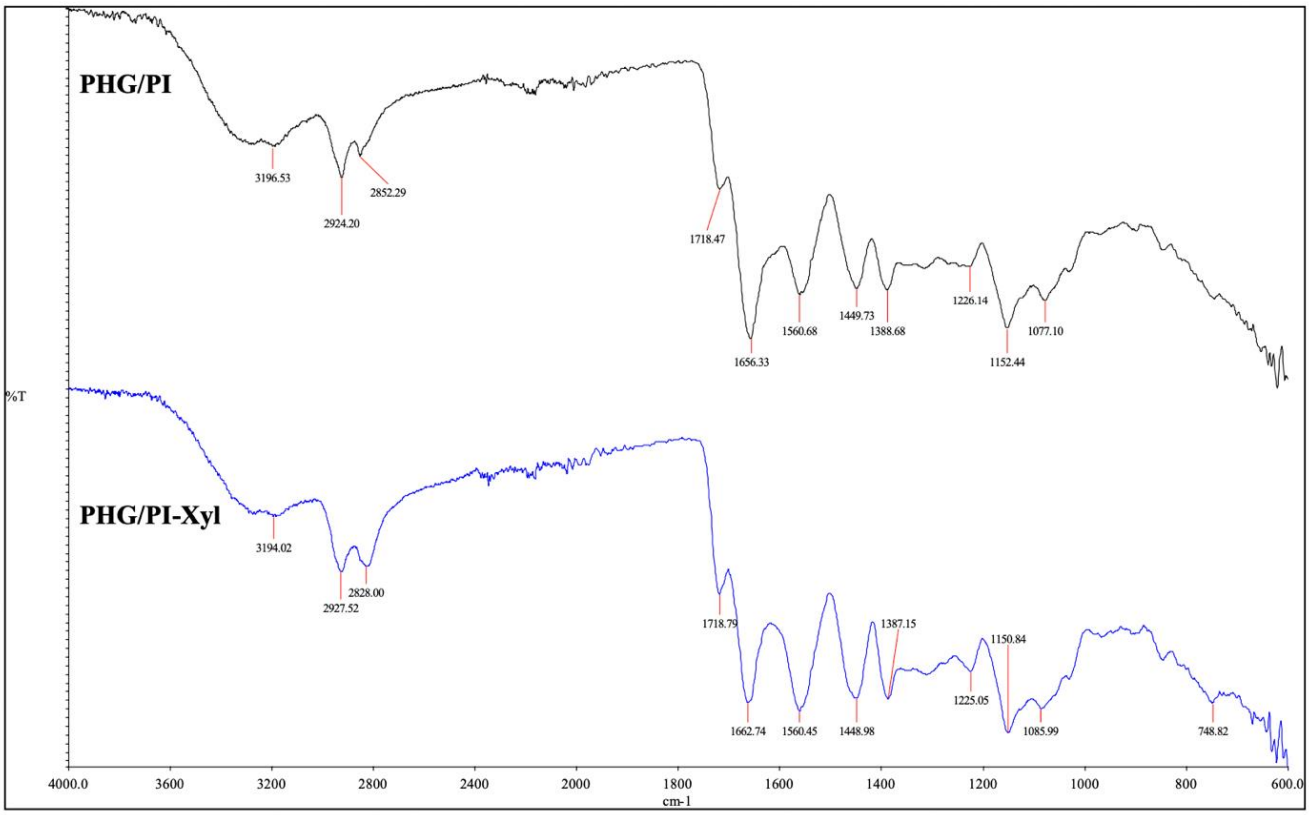

Figure 2. *FTIR spectra of PHG/PI and PHG/PI-Xyl.*

The SEM photos with magnified in different sizes of the PHG/PI cryogel column was shown in Fig.3. Integrated pores were observed in the PHG/PI cryogel structure. Thus, this structure created a high surface area for high immobilization performance. The reusability of HCC cryogel column showed very important performance. All stages were repeated 10 times with the same cryogel column, and a important changing wasn't observed in cryogel column structure.

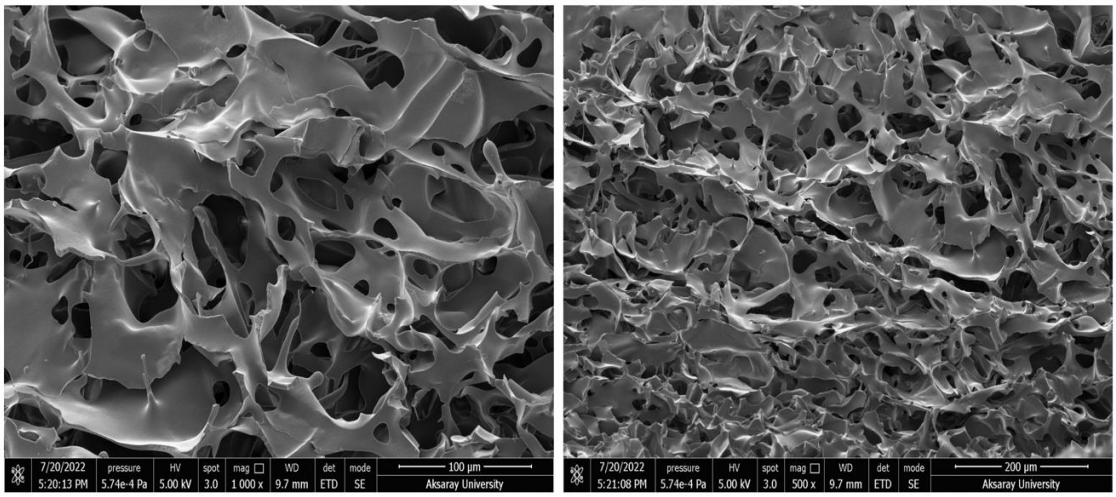

Figure 3. *SEM images of PHG/PI.*

3.2. Effect of change in pH and temperature on activity

The activity with varying pH is a significant parameter in the applications of enzymes immobilization. The activity of the

free and immobilized xylanase was monitored for the pH values ranging from 3.0 to 10.0. According to observed data (Fig. 4A), the maximal activity of the free *Xyl* and immobilized PHG/PI-Xyl were founded at pH 6.0. However, the residual activity of the PHG/PI-Xyl started to decrease above pH 6.0. For instance, only 64% of the PHG/PI-Xyl remained active at pH 10.0, while free xylanase enzyme was founded at 52 % at pH 10.0. It is observed that the PHG/PI-Xyl exhibited high relative activity in the pH ranges from 3.0–9.0. This excellent performance of the PHG/PI-Xyl could be due to the interactions between the xylanase and the PHG/PI cryogel column. In addition, compared to the free enzyme, the activity of the immobilized enzyme was generally higher across the majority of pH ranges. In our earlier work, they determined the optimum pH of the xylanase enzyme which was founded as pH 6.0 [29]. Similarly, in our other study, the optimum pH for free and immobilized xylanase was found at pH 7.0 [30]. The possible reason for this slight shift in pH due to the charge of the support materials that they used. According to the pH stability tests, the immobilized xylanase was marginally more stable than the loose enzyme. The higher pH stability was probably advantageous for the immobilized enzyme to be used in the industrial application.

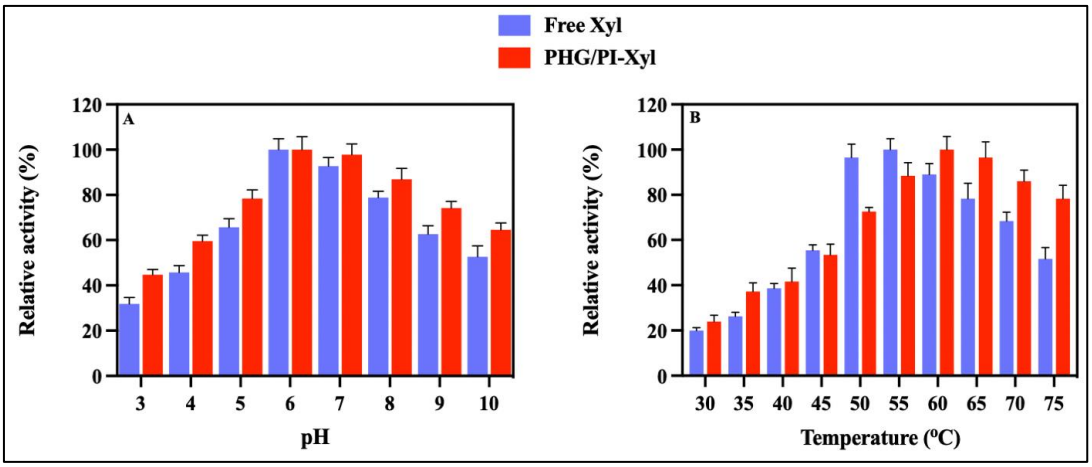

Figure 4. *The effect of different pH (A) and temperature values (B) on the activity of free and immobilized xylanase.*

The enzyme activity at various temperatures is also important for practical applications. Therefore, the activities of the free and immobilized xylanase enzyme were measured at various temperatures at pH 6.0 in order to examine the temperature dependence. The optimum temperature of free Xyl and PHG/PI-Xyl immobilized enzymes were measured to be 55 and 60 °C, respectively (Fig. 4B). In addition, the PHG/PI-Xyl maintained 95% and free Xyl maintained 78% of its activity at 65 °C. And the relative activity of free Xyl was only 51% while PHG/PI-Xyl was 78 % at 75 °C. The results indicated that the microenvironment of the support may have protected the enzyme from inappropriate changes at high temperature. Hence, the xylanase had better tolerance to high temperature. In prior investigations, a similar outcome of optimum temperature for immobilized enzymes and a very close result for free enzyme has been observed. However, depending on the type of interaction between the enzyme and the matrix as well as the matrix, the extent of displacement differed [31]. As a result of the intermolecular connections that form between the enzyme molecules and the matrix support, the structure of the enzyme molecule is made stiff and is therefore less susceptible to the denaturing effects of temperature [32].

3.3. Thermal and storage stability

The thermal stability study of free Xyl and PHG/PI-Xyl immobilized enzymes were carried out by measuring its activity at 60 °C and different incubation times (0, 10, 30, 60, 120, and 180 min) and were compared (Fig. 5A). The free Xyl and PHG/PI-Xyl showed 76.35%, and 90.48% of the relative activity at 30 min. On the other hand, after 60 min of incubation, the free Xyl and PHG/PI-Xyl preserved 61.13%, and 80.98% of their initial activity, respectively, while at the end of 180 min free Xyl and PHG/PI-Xyl preserved 32.05% and 53.55 % of its original activity, respectively. These findings revealed that the cryogel column provides a suitable microenvironment for the immobilized xylanase and provides protection against environmental factors such as high temperature that may reduce the activity. After immobilization, xylanase was adsorbed to the PHG/PI cryogel column, which inhibited changed in conformation and reduced molecular mobility due to hydrogen bonds, ionic, electrostatic and non-covalent interactions between the enzyme and carrier matrix. Overall, the thermal stability of xylanase enhanced after immobilization on the cryogel. The results obtained are comparable or even better than those reported in the literature using different supports [30].

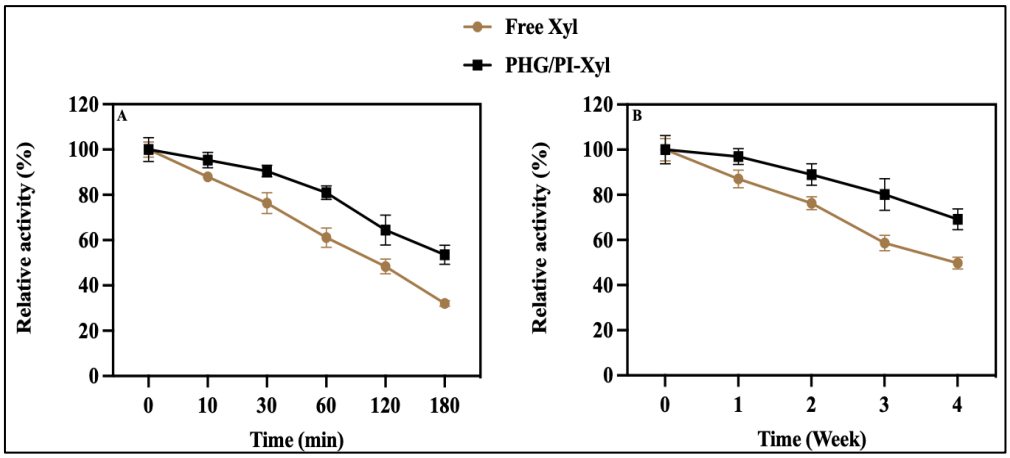

Figure 5. *Thermal stability (A) storage stability (B) of free and immobilized xylanase.*

The important advantages of immobilized enzymes in industrial areas have better storage stability compared to free enzymes. The storage stability of the free and immobilized xylanase was determined by measuring activity every single week for 4 weeks at 25 °C. As seen in (Fig. 5B), at the end of the 2 weeks the retained enzyme activity for free Xyl and immobilized PHG/PI-Xyl 76% and 88%, respectively of initial activities. Nevertheless, the activity was still above 49% and 69 % for free and immobilized xylanase, respectively at the end of the 4 weeks. A significant improvement in the enzyme activity after immobilization on cryogel column was observed with increasing storage time. The satisfactory ability of the cryogel column in long-term storage was owing to multiple linkages between the xylanase and the cryogel was present, which prevented enzyme denaturation or leaching from the support. Taken together, it is reasonable to conclude that the PHG/PI-Xyl displayed enhanced storage stability.

3.4. Reusability

The reusability of immobilized xylanase is the key to cost-effectiveness for industrial applications. Reusability of the PHG/PI-Xyl was investigated by hydrolyzing of xylan for 10 consecutive cycles. The residual activity was found out to be 90.5 % after 3 cycles, 81.38% after 5 cycles, 69.78% after 8 cycles, and even after 10 cycles the residual activity was still above 58% from the initial activity as shown in (Fig. 6). The relative activity of the PHG/PI-Xyl gradually decreased through the reaction cycles due to due to partial exhaustion of xylanase or its leaching from the carrier. Nonetheless, since the xylanase activity was maintained above half of the initial activity up to 10 reuse cycles, it can be considered that a highly stable biocatalyst is obtained by the immobilization of xylanase on PHG/PI. On the other hand, this PHG/PI-Xyl appears to be much more reusable than its counterparts reported in the literature using other support. For instance, immobilized xylanase on a magnetic chitosan support retained about 22% of initial activity after 6 cycles [33], about 50% of residual activity was obtained after 4 cycles for immobilized covalent immobilization of xylanase from thermomyces lanuginosus on aminated superparamagnetic graphene oxide nanocomposite [34], and about 62% retained of its activity after six precipitations for immobilization of thermomyces lanuginosus SSBP xylanase using Eudragit S-100 [35]. Compared to these findings, immobilizing xylanase on PHG/PI could promising and bring a chance for xylanase to be used in industrial application and as biocatalysts in continuous reactors.

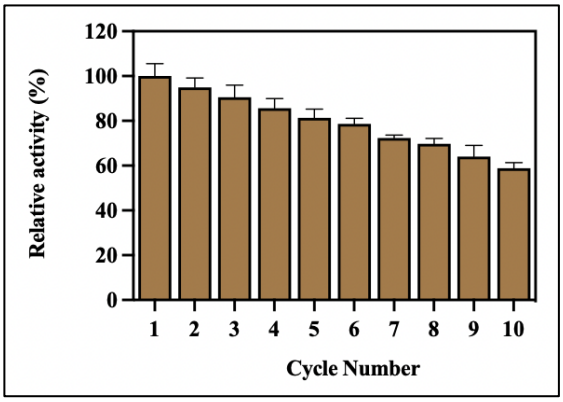

Figure 6: *The reusability of the PHG/PI-Xyl using xylan as the substrate.*

3.5. Values of Km and Vmax

The values of *Km* and *Vmax* from the Lineweaver–Burk plot were observed in (Fig. 7). Xylan was used to examine the relationship between substrate concentration and reaction rate of free and immobilized xylanase, different xylan concentrations of 0.5 –10 mg/mL were used and the enzyme activity assay has occurred at optimum condition. The *Km* value of the PHG/PI-Xyl was founded 2.62 mg/mL which

was lower than the free xyl 4.05 mg/mL. This outcome means that the enzyme on the surface of PHG/PI-Xyl has more accessible potential active sites, thus increasing the affinity of xylanase to xylan substrate. In contrast, The *Vmax* of the PHG/PI-Xyl was 188.68 U/mL, which was higher than free xyl 133.33 U/mL. Increased *Vmax* of the PHG/PI-Xyl confirmed the enhanced biocatalytic activity of the enzyme after immobilization, which means a higher xylan hydrolysis rate than that free xyl. A study was reported for immobilized xylanase on a magnetic chitosan, according to the results of xylanase enzyme increased in *Vmax* value from 6.31 ± 0.63 to 11.6 ± 0.26 U/mL, and increased in *Km* concentration from 1.89 ± 0.46 to 3.15 ± 0.16 mg/mL after immobilization [33]. According to reports, the main factor causing high *Vmax* and lower *Km* could be immobilization, which prevents conformational change and results in limited substrate diffusion. In another study, for covalent immobilization of xylanase on graphene oxide nanocomposite, they obtained *Km* and *Vmax* values 55.0 mM and 1.7 mM/min for free xylanase and 83.0 mM and 2.5 mM/min for immobilization of xylanase, respectively [34]. As a result, xylanase immobilization into the PHG/PI provided more efficient catalytic capability than that of the free one.

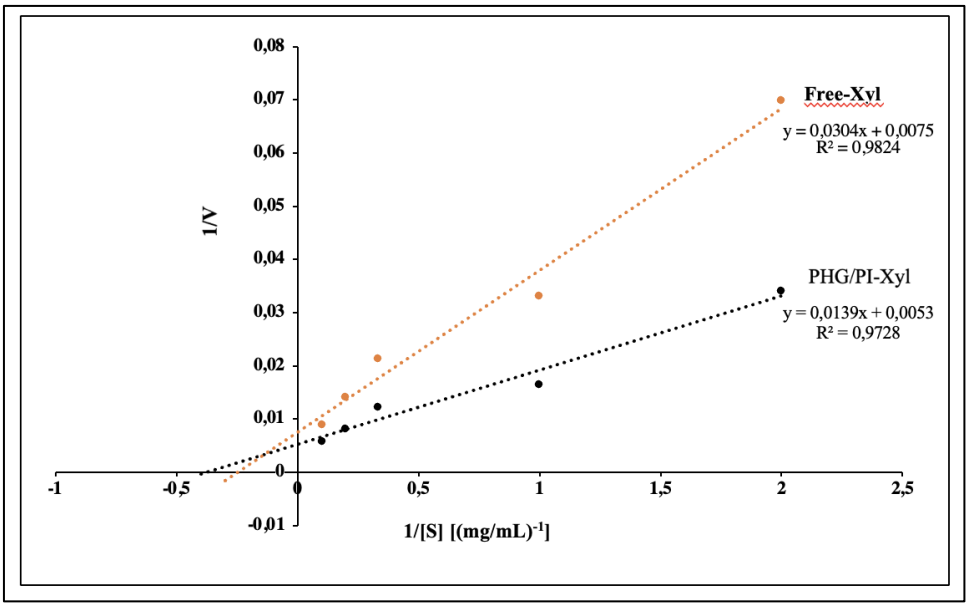

Figure 7. *The Lineweaver-Burk plot of the of free and immobilized xylanase.*

4. Conclusion

Herein, we demonstrated that PHG/PI cryogel column can be used as efficient as support materials for xylanase immobilization and its previously not reported in the literature. The materials were thoroughly characterized to investigate their structural and physicochemical properties before and after modification. Further, the successful immobilization of xylanase was verified by FT-IR. Significant enhancements of the xylanase stability after immobilization were observed. For instance, xylanase immobilized to the PHG/PI cryogel column structure exhibited broad tolerance against various pH and temperature conditions. The PHG/PI-Xyl showed good reusability as well as high thermal and storage stability. Moreover, the kinetic study reported a reduction in *Km* values with an accompanying rise in *Vmax* values, implying an increase in enzyme-substrate affinity. Taken together, improving the reuse of enzymes is a very important and targeted task since they contribute to the reduction of costs in industrial processes and the economic viability of the application

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