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Preparation and characterization of UV-curable polymeric support for covalent immobilization of xylanase enzyme

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ABSTRACT

The hydroxyl group of poly(ethylene glycol) monoacrylate (PEGMA) was activated by 1,1′-carbonyldiimidazole (CDI) and then a xylanase enzyme was immobilized to amine active PEGMA. UV-curable polymeric support formulation was prepared by mixing the xylanase bonded PEGMA, aliphatic polyester, 2-hydroxyethyl methacrylate (HEMA), poly(ethylene glycol) diacrylate (PEGDA) and photoinitiator. After UV irradiation, the enzymatic activity of the polymeric matrix was evaluated and compared with the corresponding free enzyme. By immobilization, the temperature resistance of the enzyme was improved and showed maximum activity at 60 °C. pH dependent activities of the free and immobilized enzymes were also investigated, and it was found that the pH of maximum activity for the free enzyme was 6.0, while for the optimal pH of the immobilized enzyme was 6.5. The immobilized enzyme retained 75% of its activity after 33 runs. The morphology of the polymeric support was characterized by scanning electron microscopy (SEM) and energy dispersive spectroscopy (EDS) coupled with SEM was used to explore the chemical composition. The results have confirmed the evidence of enzyme in the structure of the polymeric material.

1. Introduction

Enzymes, compared with conventional chemical catalysts, are more attractive biocatalyst as they are highly effective under ambient conditions and exhibit a number of features that make them beneficial [1,2]. However, the main drawback of the enzymes is indeed their short lifetime. There are several techniques available that may permit to improve the enzyme features, including many areas of science that have suffered impressive developments in the last years: microbiology, protein engineering, chemistry of proteins, etc. [3]. However, some apparently older fashioned techniques, as immobilization, have been revealed in the last times as a very powerful tool to improve almost all enzyme properties, if properly designed [4–9]. A variety of chemical and physical methods have been developed for immobilizing enzymes [10]. The most stable enzyme-support material is, indeed, formed by covalent bonds between reactive functional groups of the support and enzyme molecules. It is important to consider all the functional groups on support material and enzyme molecule. In principle, amino ($-NH_2$) from lysine residues, carboxylic acid (–COOH) from aspartic and glutamic residues, hydroxyl (–OH) from serine and tyrosine residues and cysteine groups on enzyme molecules can be utilized for covalent binding. Therefore, the reactive functional groups on the support material are very essential for coupling.

Synthetic polymers are widely used as enzyme immobilization carriers because they show perfect mechanical stability; they are less susceptible to bacterial attacks and easily prepared in desired geometrical configurations [11]. And also, for improving biocompatibility of these polymeric materials, bio-friendly components can be introduced into the polymeric chain via chemical modification [12]. However, the major barrier of the most polymeric materials is the lack of highly reactive functional groups on the surface to undergo direct coupling of enzymes [10,11]. By simple coupling strategies, different reactive groups of the supporting material (–OH, $-NH_2$ and –COOH) can be chemically activated. 1,1′-Carbonyldiimidazole, a carbonylating agent, converts the free hydroxyl group of the support material into imidazolyl-carbamate groups. The imidazolyl-carbamate groups react easily with amine containing ligand forming relatively stable N-alkyl carbamates [13–16].

In the present work, polyethylene glycol monoacrylate [PEGMA] having a free hydroxyl group, activated with 1,1′-carbonyldiimidazole and then reacted with the amino group of the xylanase enzyme. The xylanase can hydrolyze β-1,4-glycosidic linkages of the xylan backbone to produce xylooligosaccharides and xylose [17–19]. Xylan is a hemicellulosic polysaccharide, which constitutes 20–35% dry weight of wood and agricultural wastes [18]. In paper and pulp industries xylanase enzyme is used for biobleaching and bioprocessing of pulps. And also in food indus-

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try, xylooligosaccharides are used as sweeteners and components of prebiotic products.

The immobilization of xylanase was successfully accomplished and polymeric support material was prepared by mixing the commercial polyester acrylate, poly(2-hydroxyethyl methacrylate), poly(ethylene glycol) diacrylate and xylanase attached PEGMA. Polymerization process was performed under UV lamp for 3 min. The enzymatic activity of xylanase was evaluated to determine the effect of the immobilization process. Scanning electron microscopy (SEM) was used to characterize the surface of the support material.

2. Experimental

2.1. Materials

Poly(ethylene glycol) monoacrylate [PEGMA] (M_n = 375) and poly(ethylene glycol) diacrylate [PEGDA] (M_n = 575) were purchased from Aldrich Chemical Co. Polyester acrylate was supplied from Sartomer. Xylanase from *Trichoderma viride*, 1,1′-carbonyldiimidazole (CDI) and 2-hydroxyl ethyl methacrylate (HEMA) were purchased from Fluka. The photoinitiator 1-hydroxy-cyclohexyl-phenyl-ketone was purchased from Ciba Speciality Chemicals. Xylan from Birchwood, 3,5-dinitrosalicylic acid (DNSA), bovine serum albumin (BSA) were all obtained from Sigma–Aldrich. All other chemicals were of analytical grade and were purchased from Merck AG. Freshly double distilled water was used throughout.

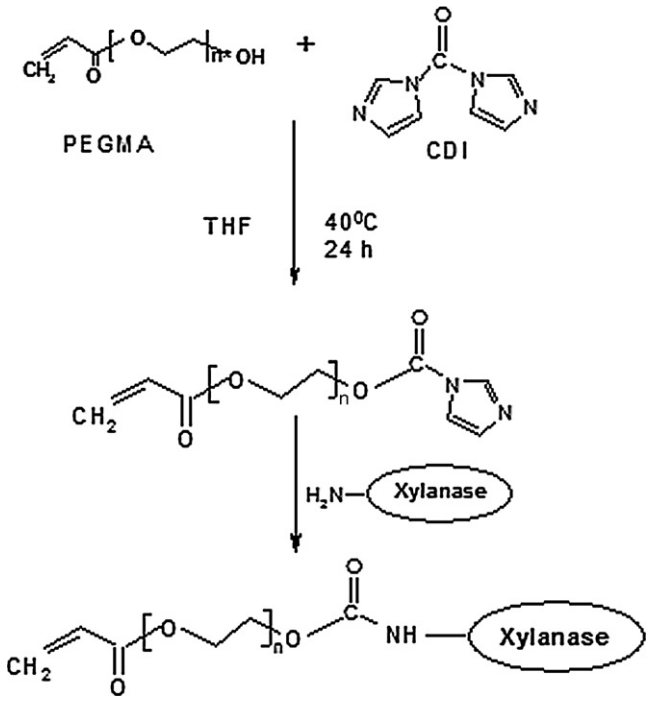
2.2. Activation of PEGMA and immobilization of xylanase

PEGMA activation was prepared according to our previous study [20]. PEGMA (26.6 mmol) was transferred into a three-neck round-bottomed flask filled with tetrahydrofuran (THF) and purged with nitrogen atmosphere. CDI (29.3 mmol) was added to the reaction flask and stirred magnetically for 24 h. The reaction temperature was 40 °C. THF was distilled off in a rotary evaporator and the activated PEGMA was stored at 4 °C until use. The hydroxyl group of the PEGMA was reacted with CDI and the final product has an amine reactive imidazolyl-carbamate group. For the immobilization of the enzyme, activated PEGMA was soaked into xylanase solution (NaCl solution of xylanase 0.6 g/5 ml) under stirring for 24 h at room temperature (pH 6.1). A representation of this immobilization process is shown in Scheme 1.

After the immobilization process completed, the mixture was extracted with dichloromethane for three times. The washing solution was kept for measuring the amount of covalently bound enzymes on the support. The protein concentration within the extract was measured using Coomassie Brillant Blue reagent as described by Bradford assay [21]. A calibration curve prepared with BSA solution of known concentration was used in the calculation of protein in the enzyme and wash solution. From the results of protein recovery, the amount of immobilized enzyme per weight of material was calculated.

2.3. Preparation of polymeric support material

Polymeric support containing xylanase, was prepared by mixing xylanase bounded PEGMA (10 wt.%), polyester acrylate (30 wt.%), HEMA (50 wt.%), PEGDA (10 wt.%) and the photoinitiator (3% of total weight). The prepared formulation was then transferred in a round shaped Teflon® mold (R = 4 mm). After 180 s irradiation under UV-lamp, 1 mm thick polymeric support was obtained. All the prepared polymeric support was immersed in a large excess of distilled water for a day to remove the unreacted monomers and residual initiator and then, were dried in a vacuum oven at 30 °C.



Scheme 1. Schematic presentation of chemical reaction between a hydroxyl group of PEGMA and 1,1′-carbonyldiimidazole resulting in formation of reactive imidazolyl-carbamate group. The imidazolyl-carbamate group reacts with the amino group of the xylanase enzyme forming a urethane like bond.

2.4. Effects on enzyme activity

The effect of pH on free and immobilized xylanase was measured by incubating 10 μL (5 mg/mL = 16.385 U) of the enzyme and 490 μL of buffer solutions in the range of pH of 4.0–7.0, containing 1% (w/v) birchwood xylan. The mixture was incubated in a water bath at 50 °C for 15 min. The buffer solutions were: 10 mM acetate buffer for pH 4.0, 5.0, 5.5, 6.0 and 10 mM phosphate buffer for pH 6.5 and 7.0. Xylanase activity was assayed using Birchwood xylan 1% solution as the substrate, as described by Bailey et al. [22], and the amount of reducing sugars released was determined by dinitrosalicylic acid (DNSA) method [23] using xylose as a standard. One unit of enzyme activity (IU) was defined as the quantity of enzyme forming 1 μmol of reducing sugars in 1 min.

The temperature of maximum activity of free (pH 6.0) and immobilized xylanase (pH 6.5) was determined by carrying out the standard assay procedure at different temperatures (15–80 °C).

2.5. Re-use of immobilized xylanase

490 μL of 2.5% xylan in 10 mM phosphate buffer (pH 6.5) was added to the immobilized enzyme and incubated for 15 min at 60 °C, under constant shaking for each cycle. At the end of the reaction, immobilized enzyme was taken and washed with distilled water and then added a substrate solution to start a new cycle. The supernatant was assayed for reducing sugar [23].

2.6. Effect of storage time of immobilized xylanase

The storage time of immobilized xylanase was determined by carrying out the pH 6.0 and 4 °C at different times (1–40 days). The residual activities were calculated as a percentage of initial activity. Sodium azide was added to the immobilized xylanase against to any bacterial contamination.

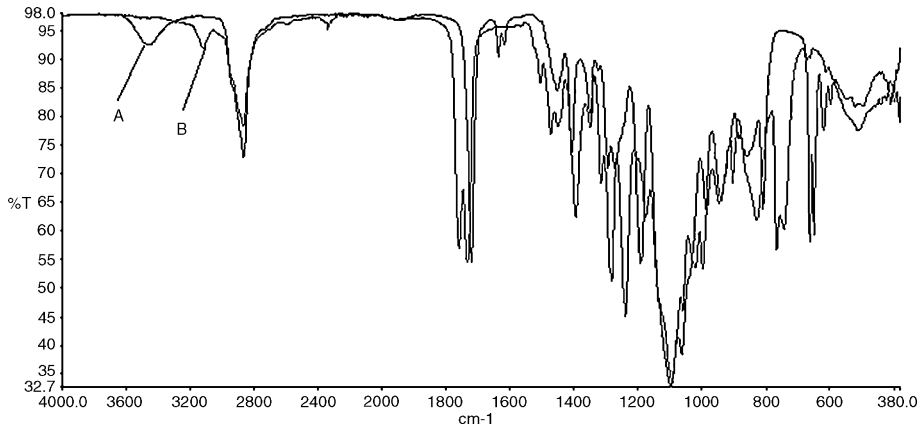


Fig. 1. ATR-FTIR spectrum of PEGMA and imidazolyl-carbamate functionalized PEGMA (A: PEGMA) (B: amine active PEGMA).

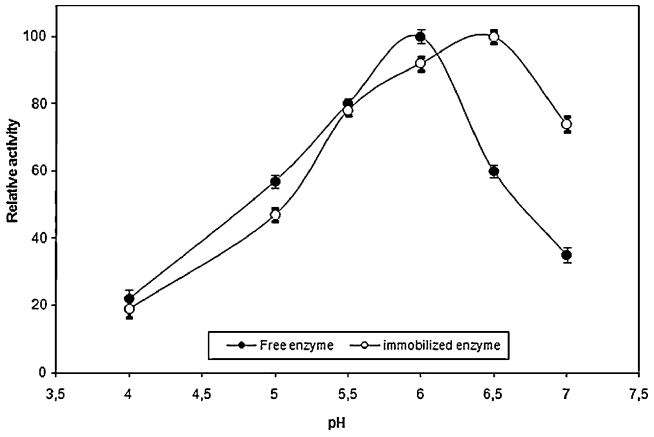


Fig. 2. Effect of pH on activity free and immobilized xylanase enzyme.

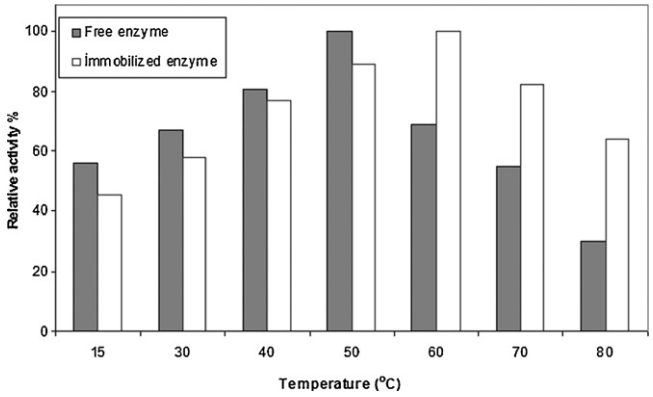


Fig. 3. Relative enzyme activity as a function of temperature for free and immobilized xylanase.

2.7. Physical characterization

SEM cross-section image from the polymeric support material was acquired at acceleration voltage of 10 kV by using Philips XL30SEM FEG. Before imaging, the material was soaked in liquid nitrogen to enable a regular break just with simple bending.

3. Results and discussion

Xylan is a generic term used to describe a variety of highly complex polysaccharides that are found in the plant cell walls and some algae. Xylan backbone is released from lignocellulosic matrices with determining by (i) pulping under controlled acidic conditions such as in the sulfite process, (ii) aqueous-steam treatments such as 'steam explosion', (iii) organosolve process such as water/ethanol. For all these treatments, xylan rich oligomers are soluble in water and can be conveniently post-hydrolyzed to monomers by xylanase [24]. Thus, the immobilization of xylanase is very essential for ease of application and stabilization purpose.

3.1. Characterization studies

Our strategy was, firstly, to prepare an oligomer bearing reactive imidazolyl-carbamate group that can form covalent bonds with the amine groups of the xylanase. Scheme 1 presents the reaction occurred between PEGMA and 1,1′-carbonyldiimidazole. The hydroxyl group of the PEGMA was converted to imidazolyl-carbamate. The reaction was followed by ATR-FTIR and the spectrum was given in Fig. 1. It is clearly seen that the –OH stretching band of PEGMA at 3400 cm^{-1} was replaced with the imide band of N-acylimide terminated PEGMA. Two different carbonyl groups, one from the acrylate and the other from the imidazole groups caused a split in the band at 1750 cm^{-1}. The spectrum proved that the chemical modification was carried out successfully. In the case of immobilization, the amine groups of the xylanase were coupled with imidazolyl-carbamate groups of PEGMA under ambient conditions. The bond formed between the polymer, and enzyme is a urethane-like group exhibiting high chemical stability. The amount of immobilized enzymes per weight of polymer was found 201 mg/g polymeric support.

The next step of the investigation includes the preparation of polymeric support. The xylanase bounded PEGMA containing for-

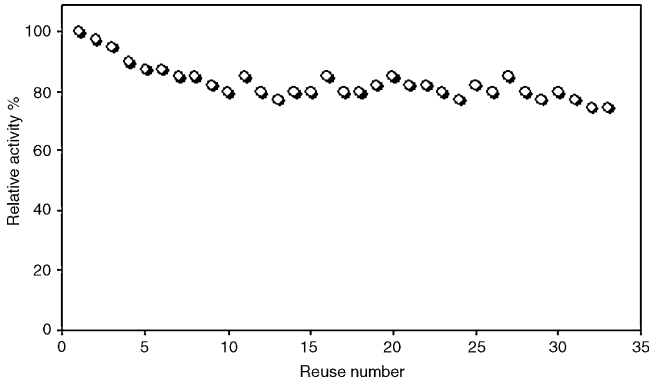


Fig. 4. Reusability of immobilized xylanase.

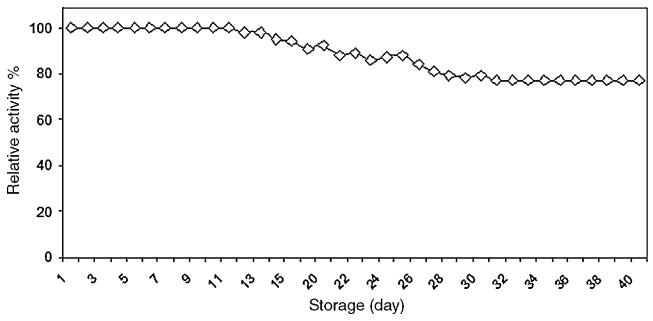


Fig. 5. Effect of storage time on activity immobilized xylanase.

mulation was exposed to UV light. After 180 s irradiation a tacky material was produced. The gel percentage of the material was found 95%. In addition, the swelling behavior of the materials was evaluated and found 46 ± 2.7%.

3.2. Influence of pH on activity and stability of free and immobilized xylanase

Even though the immobilized enzyme gains more stable form than free one, unfortunately, both free and immobilized enzymes influenced by the environmental parameters. Thus, studies were carried out at various pH and temperature in order to understand the optimum conditions.

pH is one of the important parameters influence the enzyme activity in aqueous solutions. The effect of pH for free and immobilized enzyme was investigated within pH 4.0 and 7.0 at 50 °C during 15 min incubation periods. The relative activity as a function of pH is depicted in Fig. 2. As shown, the pH of maximum activity for free xylanase was found to be 6.0. Meanwhile, the activity of the immobilized enzyme was changed, and it was found 6.5. This change means that the immobilized form is more protected than the free form [19]. At pH 4.0 and 5.0, the relative activities of free and immobilized enzymes are close to each other. This trend continues during pH 6, but as the pH increases to pH 6.5 the free enzyme lost its activity in which the immobilized one showed the maximum activity. Even though covalent binding provided a good activity at pH 6.5, it was not stable at pH 7.0 and lost 25% of its activity. To sum up, the general pH profile of the immobilized enzyme is indeed more stable than the free one and its activity is linearly increased as the pH increased to 6.5.

3.3. pH of maximum activity and temperature of maximum activity

Fig. 3 gives the variation of activity with temperature for free and immobilized xylanase. The thermal inactivation assay for free enzyme was found to be 50 °C, whereas it was shifted to 60 °C for the immobilized enzyme. This result indicated that the immobilized xylanase could resist denaturation at elevated temperatures.

3.4. Re-usage

The main advantage of the immobilized enzymes over free enzymes is their improved stability for repeated usage and storage. The ability of the immobilized enzyme to remain active was tested by recording the changes in activity after repeated washes.

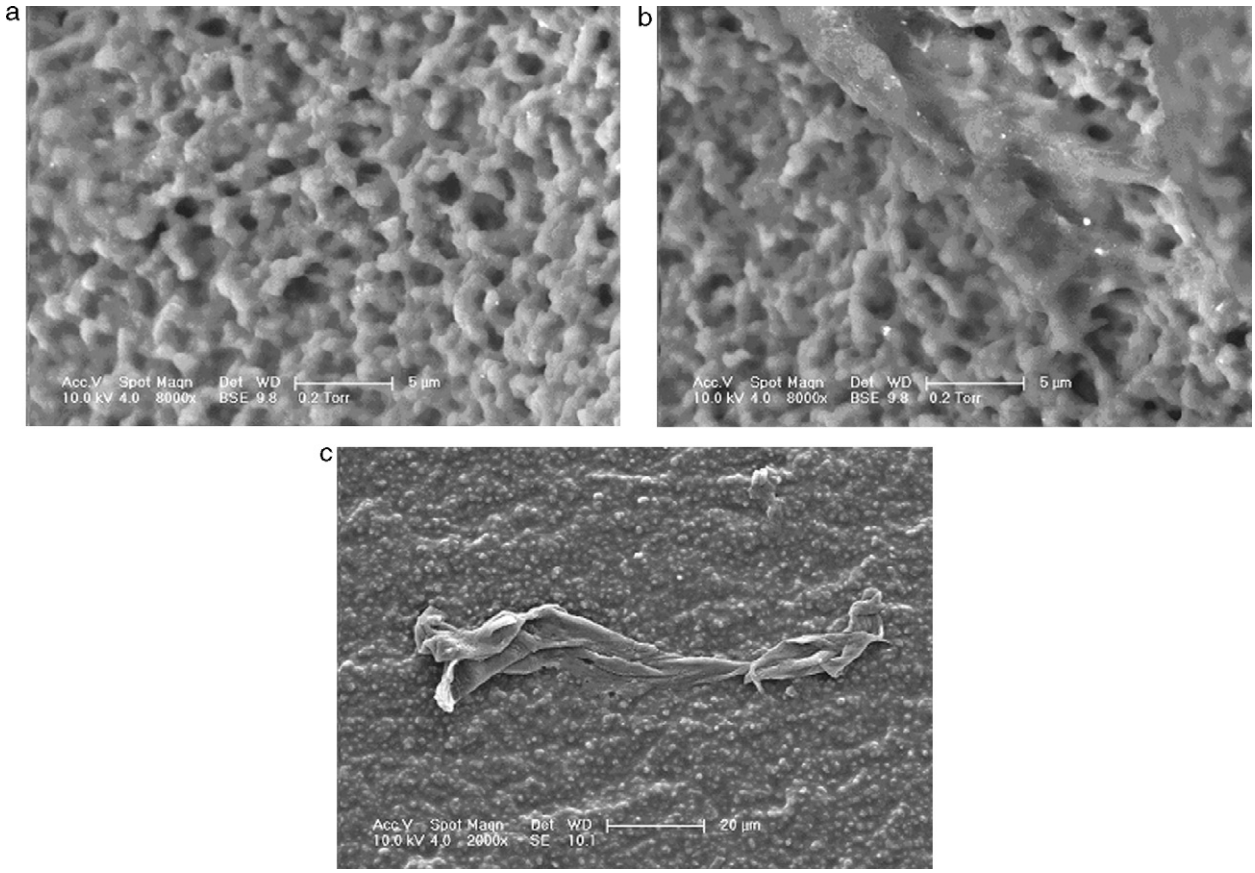


Fig. 6. (a) Scanning electron microscope (SEM) image of the polymeric support, the size of the pores was approximately 2.5 μm. (8000× magnification). (b) SEM image of the surface polymeric support represents a different part of the same materials surface (8000× magnification). (c) SEM image of the polymeric support: fibrillar ultrastructure (2000× magnification).

The activity found for each repetition was compared with the initial activity assuming it possesses 100% activity. 33 enzyme reaction cycles were done and the decrease in activity was given in Fig. 4. The residual activity of the immobilized xylanase was decreased with the increasing number of washes. At the end of 32nd cycle, the residual activity retained was approximately 75%.

3.5. Storage stability

The free and immobilized enzymes were stored in a phosphate buffer (0.02 M, pH 6.0) at 4 °C and their activities were tested for 40 days. Free enzyme lost about 19% of its activity within 30 days. This result was similar to the previous study done by Madamwar et al. [25–27]. According to their reports, at refrigeration temperature, no loss of activity was observed up to 2 weeks and after 4 weeks a marginal decrease (5–10%) was found. In Fig. 5, it was clearly seen that immobilized enzyme lost only 23% of its activity at the end of 40 days.

3.6. Physical characterization

Scanning electron microscopy (SEM) was used to observe the surface morphology of the immobilized enzyme containing support material. SEM images of the support were given in Fig. 6. As it can be seen from Fig. 6a, the surface of the polymeric support material exhibited a homogeneous porous structure and the size of the pores was approximately 2.5 μm. Fig. 6b represented a different part of the same materials surface. Independent from the porous structure, a prominent was observed on the surface. In addition, this prominent can be seen clearly in Fig. 6c and can be defined as a fibrillar ultrastructure. To determine the chemical composition of this ultrastructure SEM-EDS was obtained. The composition of the prominent included carbon (70.68%), nitrogen (13.23%) and oxygen (14.27%). The support material possesses carbon and oxygen atoms in their structure. However, the nitrogen atoms can be introduced to the structure through by adding the xylanase containing PEGMA. Therefore, the presence of the nitrogen mainly attributed to xylanase.

4. Conclusions

The immobilization of xylanase on a polymeric support has been obtained by covalently bonding the enzyme to the imidazolylcarbamate tethered poly(ethylene glycol) monoacrylate. The ATR-FTIR spectrum proved that the chemical modification was carried out successfully. The amount of immobilized enzymes per weight of polymer was found 201 mg/g support. The surface of the polymeric support material exhibited a homogeneous porous structure and the size of the pores was approximately 2.5 μm. The structural and functional characteristics of the new support were investigated. pH and temperature of maximum activity for the immobilized xylanase were pH 6.5 and 60 °C. Reuse studies demonstrated that the immobilized enzyme could reuse 32 times while retaining 75% of its activity. These results confirm that xylanase was successfully immobilized and gained more stable character compared to free one.

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