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Immobilization of Glucoamylase on Polypropylene Fibers Modified by Radiation Induced Graft Copolymerization

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Glucoamylase (GA) had been covalently immobilized on polypropylene grafted poly-acrylamide (PP-g-P(AAm)) and polypropylene grafted poly-acrylic acid (PP-g-P(AAc)) fibers by the use of carbodiimide (CDI) as a coupling agent. The polymeric support for enzyme immobilization was prepared using radiation that induced graft copolymerization of acrylamide (AAm) and acrylic acid (AAc) monomers, individually, onto polypropylene fibers followed by chemical treatments. Effects of pH and temperature on the relative activity, as well as storage stability and reusability of the immobilized enzyme, were studied. The kinetic effect of immobilization was also studied to find that, the K_m values for glucoamylase immobilized on PP-g-P(AAc) and PP-g-P(AAm) fibers are 3.4 and 5.88, respectively, i.e., higher than that for free glucoamylase of K_m 1.6. An enhancement in the thermal stability of the glucoamylase is observed upon immobilization. The optimum temperature for the immobilized enzyme on both PP-g-P(AAc) and PP-g-P(AAm) was found to be shifted 5°C higher than that of the free enzyme at 55°C. Investigation of reusability of the immobilized enzyme showed that after 10 cycles the immobilized enzyme retained 60% and 45% of its original activity for PP-g-P(AAc) and PP-g-P(AAm) carriers, respectively.

Keywords: glucoamylase; immobilization; radiation; grafting

1 Introduction

Radiation-induced graft copolymerization started in the 1950's and continued to be a subject of intensive research with the objective of obtaining modified materials for various applications and accepted by industry. Applying this technique enables one to change polymer wettability, adhesion, printability, metalization, anti-fog properties, antistatic properties, and biocompatibility. The range of applications of such radiation-modified materials is being expanded to the synthesis of biomaterials, environmental applications, etc (1–3). The versatile nature of this technique is attributed to the ability to control the degree of grafting by proper selection of irradiation conditions (4). Also, it has potential in modifying the chemical and physical properties of pre-existing polymeric materials without altering their inherent properties. Thus, it is of particular interest for achieving the desired properties since various commercial polymers are available in different forms (films, resins and fibers) can be used as grafting substrates (5). Polypropylene (PP) was the first synthetic stereo-regular polymer to achieve industrial importance and it is presently the fastest growing fiber for technical end-uses where high tensile strength coupled with low-cost are essential features (6). However, such a polymer has to be activated prior to the graft polymerization because it has no active side chain or end-groups. In this procedure, plasma grafting is an effective method, as well as irradiation by UV or gamma and electron beam (7–9). In practice, the pre-irradiation method has been given much attention because of its simplicity, little homopolymer formation, and the grafting can be carried out at any time away from the irradiation sources (10).

Pre-irradiation grafting of acrylic acid (AAc) or acrylamide (AAm) onto different polymer substrates has been investigated earlier (11–19). Polymeric material is irradiated in air, so that the macroradicals formed were converted to peroxides and hydroperoxides. The advantage of this process was that the intermediate polymer peroxide could be kept at room temperature for long periods of time before performing the final grafting reaction. When the irradiated polymer is heated in the presence of the monomer, such peroxides or hydroperoxides decompose to give $PO^{\bullet}$ radicals that survive as active sites for the grafting reaction.

Immobilization of enzymes is now a widely used approach for obtaining reusable enzymes. It is generally

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carried out by adsorption, covalent coupling to solid matrices, entrapment in polymeric substances like polyacrylamide, and encapsulation (20, 21). The use of commercial enzymes without appropriate immobilization led not only to the waste, but also the limited reuse of enzymes. In spite of these well-known disadvantages, the immobilization of α-amylase and glucoamylase has not been performed frequently because they act on macromolecular substrates such as starch and dextrin inside the carriers (22–24). When these enzymes are used to produce glucose (dextrose), they are allowed to react at 55°C for 2–4 days. Hydrolysis time could be effectively reduced if thermally stable forms of the glucoamylase were available. Often, immobilization results in improvement of enzyme stability under process conditions. Immobilization also allows the use of enzymes in various reactor designs like packed or fluidized beds (25). The disadvantage of enzyme immobilization is that it results in an activity decrease and diffusion limitation of substrate, intermediate and product (26).

Glucoamylase (amyloglucosidase, EC 3.2.1.3) catalyze the hydrolysis of α-1,4 and α-1,6 glucosidic linkages to release β-D glucose from non-reducing ends of starch and non-related poly- and oligosaccharides. These enzymes are widely used in the manufacture of glucose and fructose syrup, as well as in industrial saccharification (27, 28). Glucoamylases are originally of fungal origin usually occurring in multiple forms (29). Fungal GAs have two domains, namely a catalytic domain and a starch-binding domain. The two domains are connected by an O-glycosylated polypeptide linker located at the N-terminus and has a confirmed role in protecting the enzyme form proteolysis (30). The starch-binding domain of GA plays an active role in hydrolyzing row starch and support the enzyme adsorption to the cell wall where local increase of enzyme concentration may result in enhanced glucose flow to the cell (31, 32). Although GAs from most sources are unstable at higher temperatures, so development of a thermostable GA capable of performing industrial saccharification at elevated temperatures, would thus be of significant importance to the starch processing industry. Immobilization of GA has been studied using various supports, such as active carbon (33), polymer (26, 34) and inorganic materials (35). Immobilization of amylases on, mainly, water insoluble carriers, seems to be the most promising way to obtain more stable and reusable forms of enzymes thus; it is not surprising that starch-degrading enzymes have been studied extensively in immobilized forms (36–38). Immobilized GA on polyurethane polymers is to have observed greater thermostability and improved stability under various denaturation conditions (39).

In the present work, the attention was focused on immobilization of glucoamylase on polypropylene grafted fibers as carriers *via* various anchor groups, as well as characterization of the immobilized enzyme-carrier. In a comparative study, pre-irradiation grafting of acrylic acid (AAc) and acrylamide (AAm) individually onto PP fibers were investigated to achieve suitable support for glucoamylase immobilization to be used for starch hydrolysis.

2 Experimental

2.1 Materials

Polypropylene fiber (PP) is produced by Misr Fiber, a part of Oriental Wavers Company (10th of Ramadan City; El Nasagoon El-Sharkyoun). Acrylamide (AAm) was purchased from Merck and Acrylic acid (AAc) of purity 99% was purchased from Aldrich. Amyloglucosidase (EC 3.2.1.3) from *Rhizopus sp*., 21, 10U/g, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, Brilliant Blue G Dye content approx.; 95%, Glucose Assay Reagent, Glucose standard, Protein Standard (BSA) 1 mg/mL, Starch from corn, Sodium phosphate dibasic, Sodium phosphate monobasic all were purchased from Sigma Chemical Co. USA. The other chemicals, such as solvents and inorganic salts, were reagent grade and used without further purification.

2.2 Post Irradiation Grafting Method

The PP fibers were exposed to γ-rays from ^{60}Co source at dose rate of 3.2 kGy/h, in the presence of air to a total dose of 5∼100 kGy. The grafting reaction was conducted in a Pyrex bottle. Water as a solvent was added first, followed by the addition of ferrous ions in the form of Mohr's salt (ammonium ferrous sulfate) for acrylic acid grafting and $FeSO_4$ for acrylamide grafting, then sulfuric acid and monomers were added. The irradiated PP fibers were immersed in the monomer solution and purged by bubbling argon for 20 min. The grafting reaction was carried out by placing the bottles in a water bath set at the relevant temperature. After the grafting reaction is completed, the samples were washed thoroughly with hot distilled water, and then soaked overnight in distilled water to eliminate the residual monomer and homopolymers. Further extraction of homopolymers, which may be occluded in the grafted fibers was through an autoclaving solution at 127°C. The fibers were then dried in vacuum oven for 24 h at 50–60°C and weighed. The degree of grafting was calculated as follows:

$$\text{Degree of grafting } (\%) = [(\text{Wg} - \text{Wo})/\text{Wo}] \times 100$$

Where Wo and Wg represent the weights of the initial and grafted fibers, respectively.

2.3 Immobilization of Glucoamylase onto PP-g-P(AAc) and PP-g-P(AAm) Fibers

Two different methods were utilized for the immobilization of glucoamylase on PP-g-P(AAc) and PP-g-P(AAm) fibers as previously described (40, 41).

In the first method, carbodiimide (CDI) was used as a coupling agent for the immobilization of GA. The

condensation reaction of the amino group of the support with the carboxyl groups of enzyme in the presence of carbodiimide Diagram 1. The amino group containing supports provide a method of binding enzyme *via* their carboxyl groups. In this method, the support and condensing agent are added simultaneously to the enzyme solution. During the condensation reaction, amide bonds are formed between amino groups of the support and carboxyl group of the enzyme. PP-g-P(AAm) fibers 0.1 g was equilibrated in phosphate buffer (50 mM, pH 7.5) for 2 h. It was then transferred to the same fresh buffer (20 mL) containing glucoamylase (2.0 mg mL^{-1}) and carbodiimide (10 mg). The reaction was carried out at 4°C for 18 h, with continuous stirring. The enzyme-immobilized fibers were washed with NaCl solution (20 mL, 0.5 M) and then with phosphate buffer (50 mM, pH 7.0).

In the second method, glucoamylase immobilization used is similar to that reported for immobilization of other enzymes such as invertase (42) glucose oxidase (43) and trypsin (31). For covalent immobilization of glucoamylase onto PP-g-P(AAc) fibers, the COOH groups of the grafted AAc polymer were preactivated for 1 h with carbodiimide at 30°C in PBS (0.1 M, pH 7.4) containing 5 mg mL^{-1} of carbodiimide. The polymer fibers were then transferred to the PBS (0.1 M, pH 7.4) containing glucoamylase at a concentration of 5 mg/mL, Diagram 2.

2.4 Determination of Immobilization Efficiency

The amount of protein in the enzyme solution and in the wash solutions was determined by using Coomassie Brilliant Blue as previously described (44) using BSA as a standard. The amount of bound enzyme was calculated as follows:

$$q = [(C_i - C_f) \cdot V]/W$$

Where q is the amount of bound enzyme onto the fibers (mg g^{-1}), C_i and C_f are the initial and final concentrations of the enzyme in the reaction medium (mg g^{-1}), V is the volume of the reaction medium (mL), and W is the weight of the fibers (g).

2.5 Determination of Glucoamylase Activity

Activities of free and immobilized glucoamylase were determined with the starch-iodine colorimetric method as previously described (45). Assay solutions containing free or immobilized enzyme in phosphate buffer (PB) (0.05 mg enzyme or 0.1 g fibers in 2.5 mL PB) were added to the starch solution (3 mL, 2% w/v) at 55°C. From this solution, 0.1 mL aliquots were taken every 5 min and added to a solution which consists of stopping reagent HCl (5 mL, 0.1 mol/L), iodine solution (5 mL) and PB (0.9 mL). The absorbance due to the formed blue color was measured at 600 nm. (UV-VIS Spectrophotometer (Perkin Elme)). Starch concentration was determined from the calibration curve and used in the calculation of enzyme activity. One unit of glucoamylase activity was defined as the amount of enzyme which hydrolyzes 1 mg of starch in 1 min at 55°C and pH 4.5.

2.6 Determination of Glucose Concentration

Glucose is phosphorylated by adenosine triphosphate (ATP) in the reaction catalyzed by hexokinase. Glucose-6-phosphate is then oxidized to 6-phosphogluconate in the presence of nicotinamide adenine dinucleotide (NAD) in a reaction catalyzed by glucose-6-phosphate dehydrogenase. During this oxidation, an equimolar amount of NAD^+ is reduced to NADH. The resulting increase in UV absorbance at 340 nm is directly proportional to glucose concentration according to the method previously described (46).

2.7 Determination of Km and V_{max} Values

In order to investigate a possibility that the hydrolysis of starch is retarded by the diffusional resistance of the substrates, the most common method is to compare Michael's

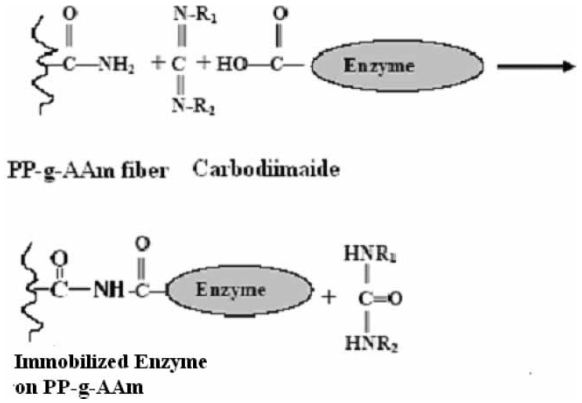

Diagram 1. The reaction for coupling of glucoamylase on PP-g-P(AAm).

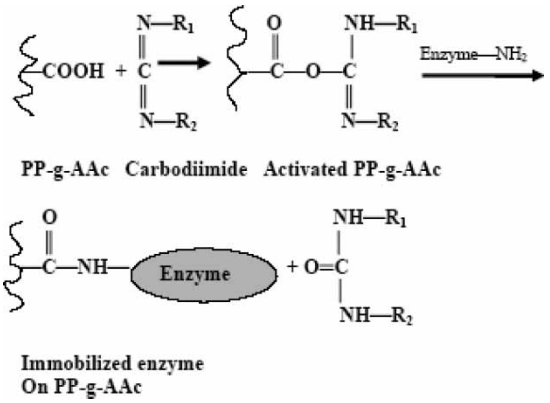

Diagram 2. The reaction for coupling of glucoamylase on PP-g-P(AAc).

constant, K_m of immobilized enzyme obtained from the Lineweaver-Burk plot with that of a free enzyme. According to the method previously described (47), K_m and V_{max} values were determined by measuring the initial rates of the reaction of glucoamylase with the starch solution at different concentrations (1–5% w/v).

2.8 pH Measurement

The pH of the solutions that used for the treatment experiments was measured using pH meter (Sper Scientific, Ltd., Scottsdale, Arizona).

2.9 Determination of Protein Concentration

Protein was determined by the use of Coomassie Blue G Dye (44). The Coomassie Brilliant Blue reagent was a solution containing 100 mg Coomassie Brilliant Blue, 50 mL ethanol of purity 95% and 100 mL H_3PO_4 of concentration 85% dissolved in water and diluted to 1000 mL.

2.10 Swelling Measurements

The clean dried grafted fibers of known weight were immersed in distilled water at room temperature $\sim 25^{\circ}C$ until the equilibrium was reached (24 h in most cases). The fibers were removed, blotted quickly with absorbent paper and then weighed. The swelling percent was calculated as follows:

$$\text{Swelling } (\%) = [(Ws - Wg)/Wg] \times 100$$

Where Wg and Ws represent the weights of dry and wet grafted fibers, respectively.

2.11 FTIR Spectroscopy

Analysis by IR spectrophotometry was carried out using Mattson 1000 (Unicam Ltd., England) FTIR.

2.12 Spectrophotometric Measurement

Analysis by UV spectrophtometry was carried out using a Perkin-Elmer Lambda 40 in the range from 190 to 900°A.

3 Results and Discussion

3.1 Effect of Reaction Time on the Degree of Grafting

Figure 1 shows the degree of grafting-time curves for the grafting of AAc and AAm, individually, onto PP fibers. It can be seen that, for AAc the degree of grafting increased slowly at the initial time and then went up fast giving a maximum after 3 h, then a sudden decrease with a further increase in reaction time is observed. However, for AAm, the degree of grafting increases with increasing the reaction time, until 4 h. It is also observed that, after 2 h of the reaction, the graft yield is about 800% for AAc and 200% for AAm, although, the concentration of AAc is 20 wt.% and AAm is 30 wt.% in the reaction mixture. The increase in the graft addon with the reaction time is due to the increase in the number and length of graft chains. The addition of sulfuric acid and appropriate concentration of Fe^{2+} can greatly enhance the graft yield. It is assumed that sulfuric acid accelerates the decomposition of hydroperoxide in the presence of Fe^{2+} to form $PO^{\bullet}$ radicals which can initiate grafting reaction (48). For AAc grafting, it is possible that after 3 h of the reaction, the concentration of PAAc becomes so large that a water-soluble polymer is formed leading to a sudden decrease in the graft yield.

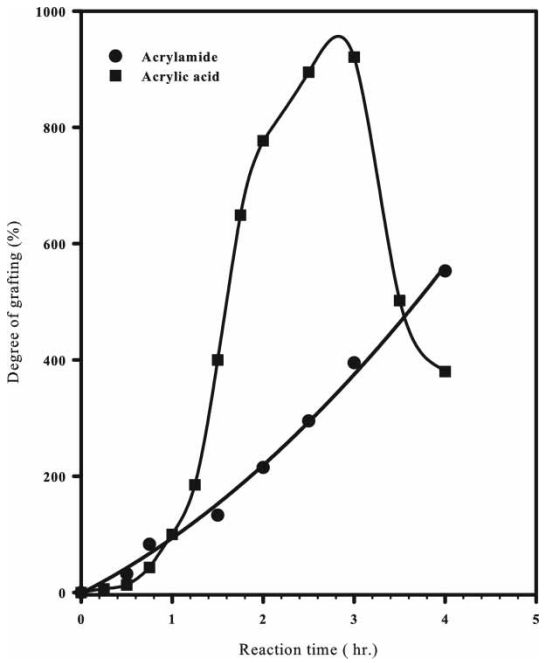

Fig. 1. Degree of grafting as a function of reaction time. AAc conc.: 20 wt.%, reaction temp.: 70°C, H_2SO_4 conc.: 0.2 M, Mohr's salt conc.: 5×10^{-4} M, Irradiation dose: 20 kGy. AAm conc.: 30 wt.%, reaction temp.: 80°C, H_2SO_4 conc.: 0.1 M, $FeSO_4$ conc.: 0.001 M, Irradiation dose: 20 kGy.

3.2 Influence of Storage Time for the Irradiated Polypropylene Fibers

The irradiated polypropylene fibers were stored at room temperature before grafting with AAc or AAm. The variation of the grafting yield with storage time is shown in Figure 2. The results indicated that the grafting yield depends mainly on the radical yield of monomers. It is assumed that the grafting reaction was initiated by both trapped radicals and peroxides. It seems that the amount of trapped radicals formed by irradiation decreased with storage time as a result the grafting activity of PP fibers decreased. However, these radicals survived long enough for initiation of grafting sites. The peroxides which were produced by gamma

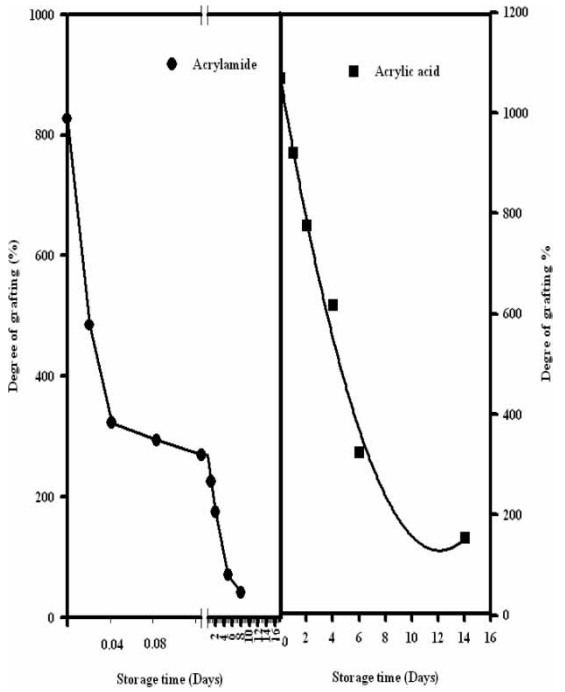

Fig. 2. Effect of storage time of irradiated fibers on the grafting process. AAc conc.: 20 wt.%, reaction temp.: 70°C, H_2SO_4 conc.: 0.2 M, Mohr's salt conc.: 5×10^{-4} M. Irradiation dose 20 kGy, reaction time: 2 h. AAm conc.: 30 wt.% reaction temp.: 80°C, H_2SO_4 conc.: 5×10^{-3} M, $FeSO_4$ conc. 5×10^{-4} M. Irradiation dose 20 kGy, reaction time: 2 h.

irradiation are stable at room temperature, while the trapped radicals may decay due to recombination and be partly transferred to the stable species such as POOR or POOH.

3.3 Effect of Monomer Concentration on the Degree of Grafting

The dependence of the grafting degree on AAm and AAc concentration was investigated and shown in Figure 3. There is an induction period and no significant increase in the grafting yield was observed up to 20 wt.% of AAm and 10 wt.% of AAc, above which the graft yield increases sharply. The results suggested that there is an optimum composition of the diluent and monomer at which time the effect of chain transfer is more pronounced. At low monomer concentration, percentage of grafting was found to be dependent only on the amount of trapped radicals in PP-fibers that seem to be relatively stable beside the high termination rate for such a dilute solution of monomer. With increasing monomer concentration, the gel effect may be reached and the mobility of growing chains decreases, as a result the termination reaction of growing chain is depressed. The leveling off in the grafting yield of AAm beyond 25% is at least partly caused by a decrease of monomer concentration in the polymer matrix (15). As in all similar systems, grafting starts at the surface of the polymer and then proceeds inwards by progressive diffusion of the monomer

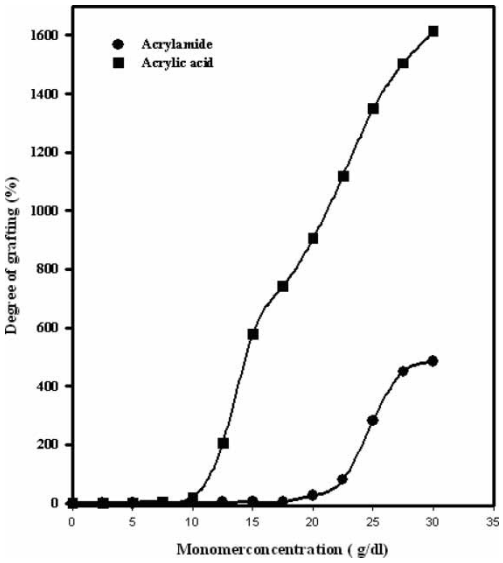

Fig. 3. Effect of monomer concentration on the grafting process. A) AAc: reaction temp.: 70°C, H_2SO_4 conc.: 0.2 M, Mohr's salt conc. 5×10^{-4} M; B) AAm: reaction temp.: 80°C, H_2SO_4 conc.: 5×10^{-3} M, $FeSO_4$ conc. 5×10^{-4} M, irradiation dose 20 kGy, reaction time: 2 h.

through the grafted zones. At any stage of grafting, monomer diffusion is controlled by the swelling of the grafted polymer.

3.4 Swelling Behavior

Water content is very important for the practical application of the prepared carrier. The grafted fibers may swell in water and have hydrophilic properties due to the introduction of hydrophilic groups; carboxyl and amide. Figure 4 shows the water uptake percent for PP grafted fibers as a function of degree of grafting. It can be seen that the water uptake percent increases with increasing the degree of grafting for both AAc and AAm grafted fibers. However, at a given degree of grafting, the PP-g-P(AAm) fibers posses much higher water uptake than that of the PP-g-P(AAc) ones.

The water uptake increases with increasing grafting yield because of the increase in the hydrophilicity of such graft copolymers with the increase in the number of carboxyl and amide groups, which are hydrophilic in nature. The contribution of the decrease in crystallinity of the fibers in regulating the water penetration is an important factor influencing the water uptake in addition to the increase of hydrophilicity. With increasing the grafting level, i.e., decreasing the crystallinity of the fibers, water molecules can easily penetrate to the non-crystalline region. It is reasonable to conclude that the swelling behavior of the grafted fibers is dependent mainly on the number and form of the hydrophilic groups that were introduced into the graft copolymers.

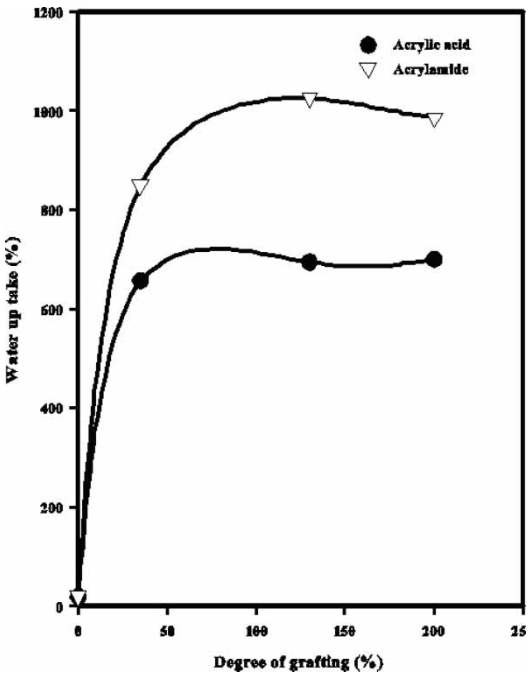

Fig. 4. Effect of fibers functional groups on water uptake, for PP-g-AAc and PP-g-AAm, preirradiated by gamma rays.

3.5 Effect of Degree of Grafting on Glucoamylase Immobilization

The amount of immobilized GA on PP-g-P(AAc) and PP-g-P(AAm) were expressed as the weight of immobilized glucoamylase per gram grafted fibers. Figure 5 shows the amounts of immobilized enzyme as a function of degree of grafting. It can be seen that, the higher the concentration of anchor groups, the higher the amount of bound protein, thereafter, it tends to level off. It is also obvious that, the amount of bound glucoamylase was lower for the AAm-grafted carrier than that for AAc-grafted ones. The results suggest that the higher the concentration of anchor groups, the higher the probability of multipoint attachment of proteins to the carrier. At high percentage of grafting >600 it is possible to reach the conditions of overloaded carrier surface. Alternatively, the strong affinity of enzyme for the matrix and the low affinity or even repulsion between immobilized enzyme molecules at the polymer carrier and soluble enzyme limits the extent that soluble enzyme reaches and adsorbs to the internal portions of the polymer carrier.

3.6 Effect of Degree of Grafting on the Relative Activity of Glucoamylase

Figure 6 represents the relative activity of GA immobilized on PP-g-P(AAc) and PP-g-P(AAm) fibers of different degree of grafting. It is observed that the relative activity of immobilized enzyme increases sharply with increasing degree of grafting up to 100%, and then a gradual increase in activity is observed. This means that, the increase of graft percentage. i.e., increase in the number and length of graft chains were accompanied by a decline of activity. It could be noticed that, for AAc-grafted fibers, the activity of immobilized enzyme reached 95% when the percentage of grafting was 100%. Meanwhile, the activity of enzyme immobilized AAm-grafted fiber at the same degree of grafting was almost 60%. The lower activity for enzyme immobilized AAm–grafted carrier than for the AAc grafted ones may be due to the lower amount of glucoamylase immobilized on AAm-grafted fibers. The data suggest that the increase in activity must be associated with an increase in the amount of immobilized enzyme. However, a further increase in the graft percentage may be lead to steric hindrance between the large molecules and the enzyme active sites. It is reported that, at low graft concentration, the observed enzyme activities increase with increasing surface-graft concentration (49).

The above presented data indicated that the effectiveness of immobilization of enzymes on the substrate of large molecules is mostly affected by a proper selection of protein concentration used in the coupling mixture rather than by a carrier structure. As a consequence of such a relation, three kinds of responses in the immobilized enzyme activity could be noticed. Firstly, the overloading of the carrier's surface may cause steric hindrance of a large starch

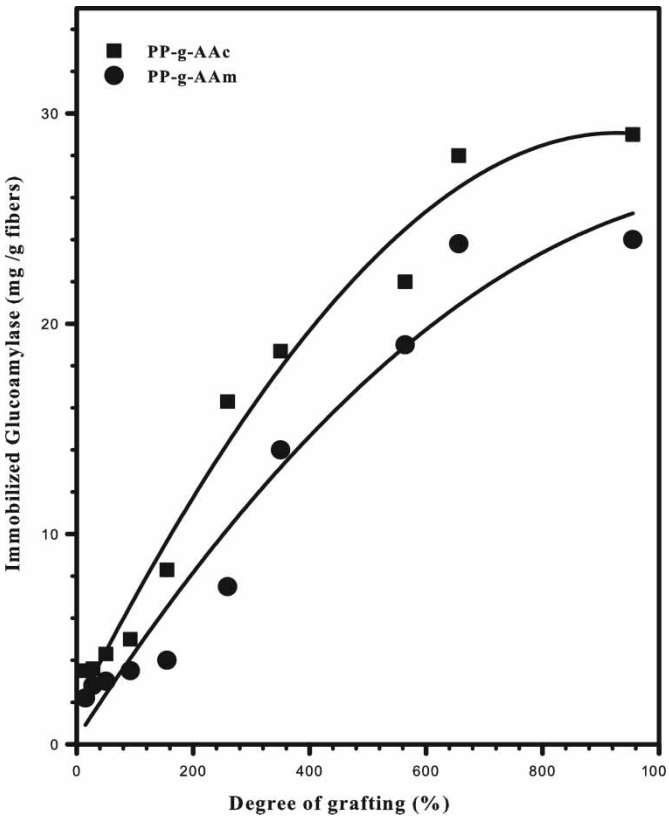

Fig. 5. Effect of degree of grafting on the immobilized amount of glucoamylase at pH; 4.5 and temperature; 55°C. Initial glucoamylase concentration: 2 mg/mL.

molecule and enzyme active site, leading to lowering in the enzyme activity. Secondly, the decrease of activity, as a result of changes in the enzyme structure by multipoint covalent modification, could be visible. Thirdly, the greater the number of linkages between protein and carrier's surface, the more stable preparation should be obtained (50).

3.7 FTIR Spectroscopy

Figure 7 shows the IR spectra of PP-blank, PP-g-P(AAc), PP-g-P(AAc) activated with carbodiimide for the covalent bonding with enzyme and enzyme immobilized on PP-g-P(AAc) fibers. New absorption bands related to the acid groups of PAAc are observed after the grafting process at 1650 cm^{-1} and 3200 cm^{-1}. The absence of the band around 3200 cm^{-1} and the gradual decrease in intensity of the band at 1650 cm^{-1} after carbodimmide activation indicated that the free acid groups are decreased. At the same time, there is a new characteristic band observed at 2360 cm^{-1} which was assigned to (N═C═N) of carbodimmide. A similar resonance band was observed for the carbodiimide units in the crystalline phase of silicon dicarbodiimide (51). The spectra of PP-g-P(AAc), after enzyme immobilization, shows a broad absorption band around 3270 cm^{-1} related to NH stretching and complete disappearance of the band at 1650 cm^{-1} of the acid groups. A relevant point is the almost complete disappearance of the CH_2 stretching vibration doubler of PP (2).

3.8 Hydrolysis of Starch by the Immobilized Enzyme

The free and immobilized enzyme were incubated at 55°C in 100 mL of 5% starch solution in 50 mM acetate buffer, at pH 4.5 under gentle shaking (100 rpm) for a different incubation time. After 15 min of incubation, the fibers were retrieved, saccharification was determined at regular intervals of time for 5 h by measuring the residual starch and the increase in reducing sugar content. The starch content was estimated by an iodine method (43), while, the reducing sugars were assayed in terms of glucose by hexokinase method (44). Several workers have demonstrated the synergistic effect of the glucoamylase on starch degradation (42).

Figure 8 represents the breakdown of starch (a) and the rate of glucose formation (b) for the free glucoamylase and immobilized systems. The free enzyme started immediately, revealing the disappearance of almost 85% of starch at the end of the first 30 min of incubation. The remaining starch after 2 h of incubation indicated overall liquefaction efficiencies of almost zero % for free enzyme. However, it was 15% and 40% for glucoamylase immobilized on PP-g-P(AAc) and PP-g-P(AAm), respectively, after 4 h of incubation. The data obtained demonstrated that, for the free enzyme, the rate of glucose formation i.e., rate of starch hydrolysis, was very fast during the first 30 min of incubation then it decreased gradually with incubation time attaining total yields of 480 mg/mL after 2 h of incubation. For the immobilized enzyme, the rate of transformation was slow compared to that of the free enzyme. The rate of transformation for enzyme immobilized on AAc-grafted fiber was higher than that of enzyme immobilized on AAm-grafted fiber and the total yield was 300 mg/mL and 218 mg/mL for AAc and AAm-grafted fibers, respectively. Such results reflect the

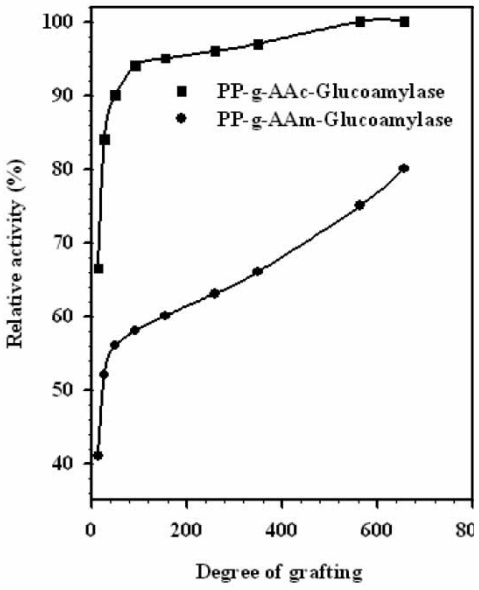

Fig. 6. Effect of degree of grafting on relative activity of the immobilized glucoamylase at pH, 4.5, temperature; 55°C and 5% (w/v) starch as substrate, glucoamylase concentration: 5 mg/g fiber.

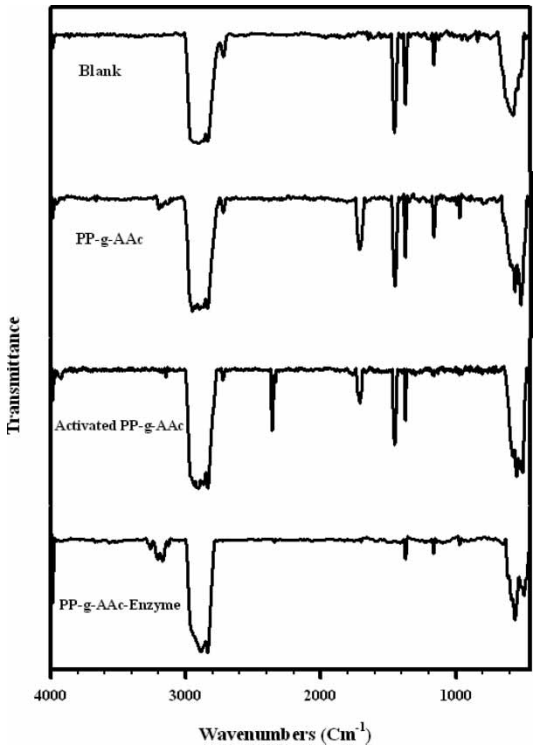

Fig. 7. FTIR spectra of PP-blank, PP-g-AAc, PP-grafted-activated and PP-grafted-immobilized glucoamylase.

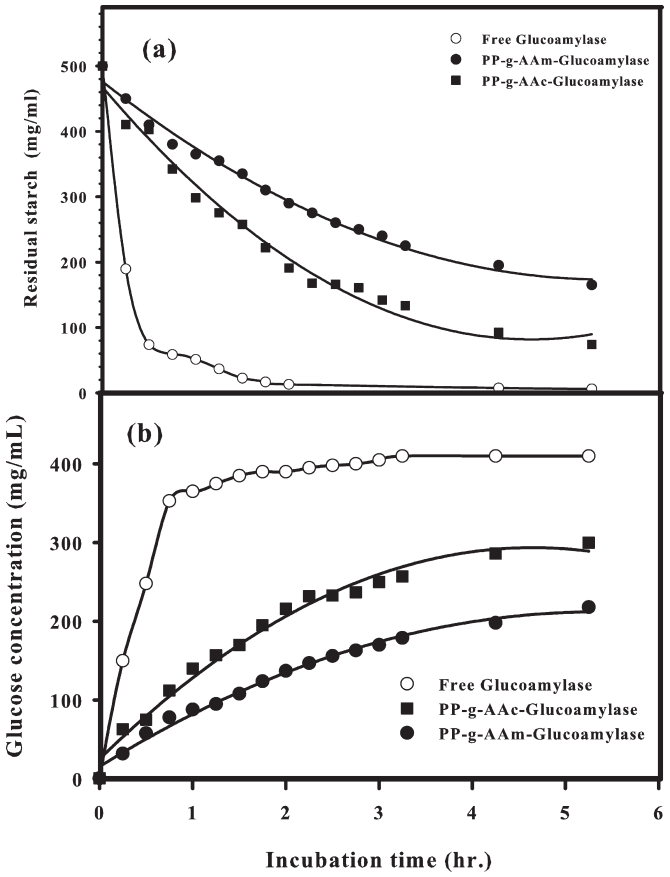

Fig. 8. (a) Starch hydrolysis as a function of incubation time; (b) Glucose production as a function of time. The free glucoamylase concentration 0.02 mg/ml, the equivalent immobilized glucoamylase concentration: 5 mg/g fibers. pH; 4.5, temperature; 55°C and 5% (w/v) starch as substrate.

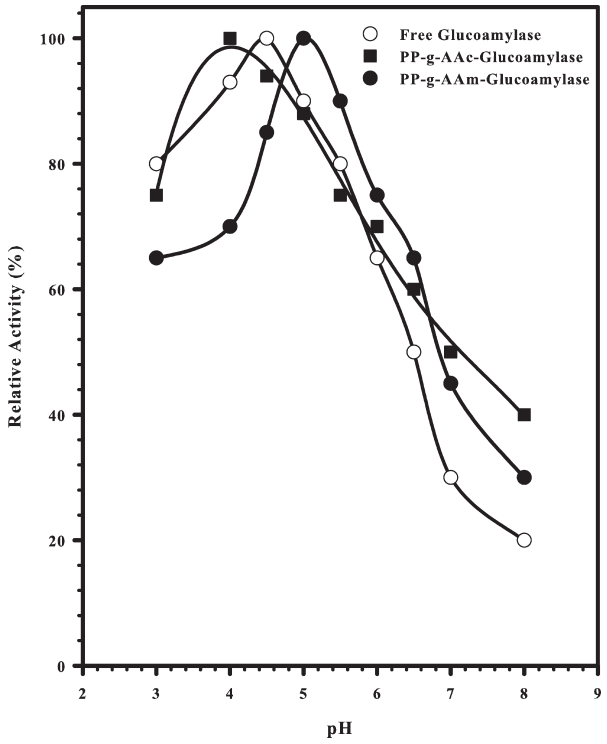

Fig. 9. pH profiles of the free and immobilized glucoamylase. The free glucoamylase concentration 0.02 mg/mL, the equivalent immobilized glucoamylase concentration: 5 mg/g fibers, temperature; 55°C and 5% (w/v) starch as substrate.

more stable preparation of enzyme immobilized on the AAc-grafted carrier beside the influence of diffusion of the substrate to the immobilized enzyme.

3.9 Effect of pH on the Enzyme Activity

The effect of pH on the activity of free and immobilized glucoamylase was studied in the pH range (3.0–8.0) and the results are shown in Figure 9. It is obvious that the immobilized enzyme had an optimum pH value which was lower or higher than that of the free enzyme, depending on type of the carrier. For the free glucoamylase, the pH value for optimum activity was found to be 4.5, while, it shifted by 0.5 pH units to the acidic region for the glucoamylase immobilized onto PP-g-P(AAc) fibers and 0.5 pH units to the alkaline region for glucoamylase immobilized on PP-g-P(AAm) fibers. Furthermore, the pH-dependent activity profile of the immobilized glucoamylase is considerably expanded in comparison to that of the free one. Thus, the activity of the enzyme becomes less sensitive to pH changes when it is immobilized. This result can be attributed to the stabilization of glucoamylase molecules resulting from a multi-point attachment of the enzyme molecules on the surface of the grafted fibers, as well as the diffusional limitations of the immobilized enzyme molecules.

A change in pH of the medium may give rise to denatured molecules. The shift in the value of pH for the immobilized glucoamylase on PP-g-AAc fibers is probably attributed to the changes in dissociation of AAc grafts with pH value, which in turn vary the extension of the graft chains, according to the pH of solution to control the enzyme activity. It was reported that at pH 4.0 the COOH groups of AAc polymer side chains may have no net charge, as a result; the electrostatic interactions between the support and the glucoamylase molecules is minimized (52). An optimum pH value of 4 is very close to the glucoamylase's isoelectrical point of pH 4.2 at which the enzyme is very active and stable. On the other hand, the shift in pH for the glucoamylase immobilized on PP-g-P(AAm) fibers to alkaline region possibly due to the secondary interactions (e.g. ionic and polar interactions, hydrogen bonding) between the enzyme and the polymeric matrix (22–24). The shift of PH lower or higher than the optimum PH value depends also on the type of the carrier, method of activation, method of immobilization, etc. (53).

3.10 Effect of Temperature on the Activity of Native and Immobilized Enzyme

The influence of temperature on the activity of free and immobilized glucoamylase are shown in Figure 10. The

optimum temperature for the immobilized enzyme was found to be 60°C, somewhat higher than that of the free enzyme (55°C), while the free glucoamylase activity showed a serious decrease above 55°C, which may be due to the predominance of thermal denaturation of the enzyme. It is also observed that at a given temperature below the optimum, the activity of the immobilized enzyme is higher than that of the free one. The results suggested that the immobilized glucoamylase became more stable against heat than the free enzyme. Such results might be supported by the existence of covalent bonds onto enzyme (24, 34).

3.11 Kinetic Effect of Immobilization

In order to study the influence of substrate on the catalytic activity of the immobilized enzymes, as well as to elucidate the kinetic effect of immobilization, the rates of the starch hydrolysis by the free and immobilized glucoamylase were determined. The starch concentration range was (1–5% w/v), at pH 4.5 and temperature of 55°C. Figure 11 shows the Lineweaver-Burk plots for the rates of starch hydrolysis by the free and immobilized glucoamylase.

The maximum reaction rate, V_{max}, and the apparent Michaelis constant, K_m, were determined from the linear regression of each plot in Figure 12, and presented in Table 1. It was found that the K_m values for the immobilized glucoamylase on the PP-g-P(AAc) and PP-g-P(AAm) fibers are approximately two and four-folds higher than that for the free glucoamylase, respectively. Such an increase in the apparent K_m values may be as a result of the conformational changes caused by the covalent immobilization or/and diffusion limitations arises from the AAc and AAm polymer side chains, and the spatial distribution of the glucoamylase molecules on the fiber surface, but at limited levels. In a similar study, it has been reported that, the apparent K_m values of glucoamylase immobilized directly in one step onto low-cost magnetic poly(styrene) particles, were found to be two and three times higher than that of the free enzyme (54).

The decrease in V_{max} value as a result of immobilization is considered to be associated with the increase in K_m value, since the lower the K_m value, the greater the affinity of the enzyme to the substrate. The change in affinity of the enzyme to its substrate is probably caused by structural changes in the enzyme due to the immobilization procedure and the lower accessibility of the substrate to active sites of the immobilized enzyme (55).

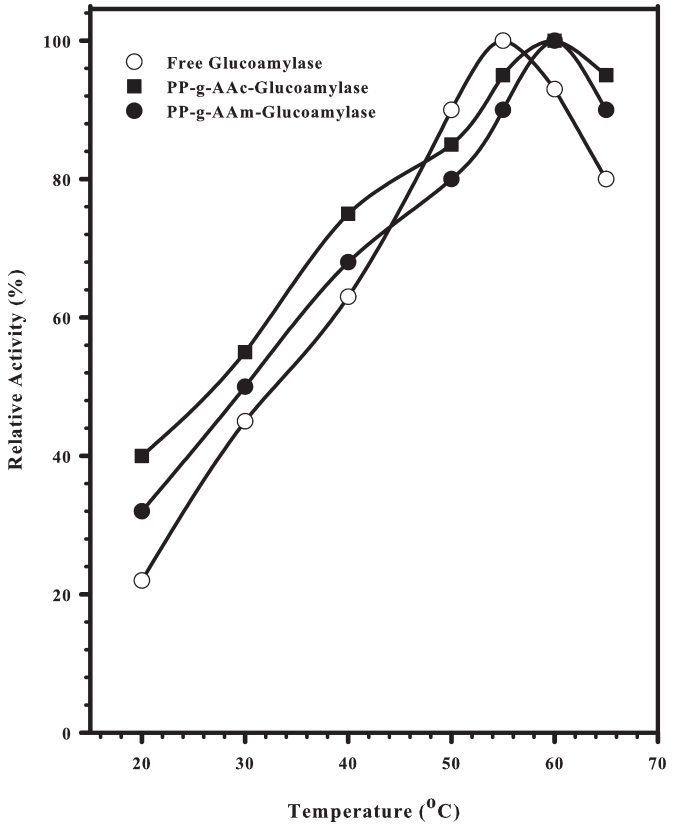

Fig. 10. Effect of temperature on the relative activity of free and immobilized glucoamylase. The free glucoamylase concentration 0.02 mg/ml, the equivalent immobilized glucoamylase concentration: 5 mg/g fibers, pH; 4.5, and 5% (w/v) starch as substrate.

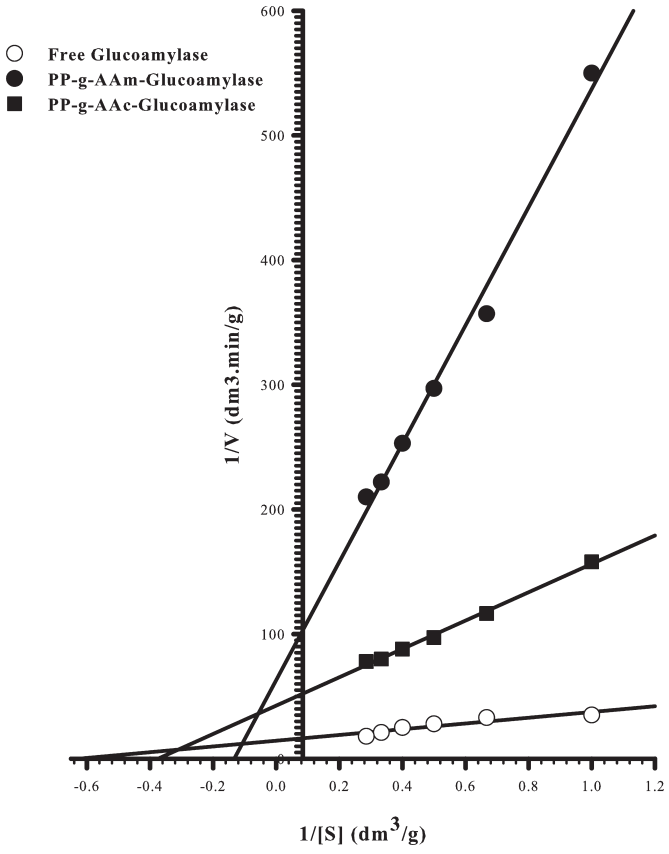

Fig. 11. Linweaver-Burk plots for the free and immobilized Glucoamylase on the PP-g-AAc and PP-g-AAm fibers. At pH; 4.5 and temperature; 55°C.

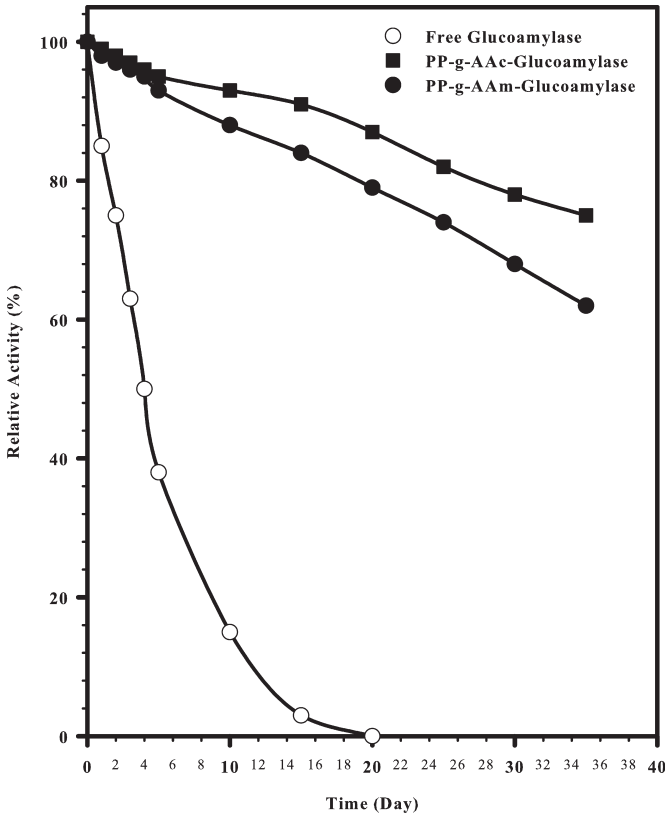

Fig. 12. The storage stability of the free and immobilized glucoamylase. Stored in the acetate buffer pH; 4.5, temperature; 4°C.

3.12 Storage Stability

The free and the immobilized glucoamylase were stored in acetate buffer solution (50 mM. of pH 4.5 at 4°C) then the activities were measured for a period of 35 days and the results are shown in Figure 12. The immobilized enzyme on both PP-g-P(AAc) and PP-g-P(AAm) fiber, lost about 25 and 38 percent of its activity, respectively during the tested storage period. Such a decrease in activity is explained as a time-dependent natural loss in enzyme activity.

3.13 Reusability of the Immobilized Enzyme

The reusability of immobilized enzyme system could make it advantageous than its free counterparts. The reusability of the immobilized enzyme was investigated as a batch system (Figure 13). It must be noted that at the end of each hydrolysis reaction, the reactor was washed thoroughly with a buffer solution for the next batch reaction. The activity of immobilized glucoamylase of the first batch was taken to be 100%. It was observed that, the retained activity of the immobilized enzyme decreased after 10 cycles to become 60% and 45% of its original activity for the PP-g-P(AAc) and PP-g-P(AAm) carriers, respectively.

Table 1. Kinetic parameters for the free and immobilized glucoamylase

	K_m	V_{max}: e-3	K_m/V_{max}
Free glucoamylase	1.6	71.4	23.02
PP-g-AAc-glucoamylase	3.4	25	113.7
PP-g-AAc-glucoamylase	5.88	16.6	474.3

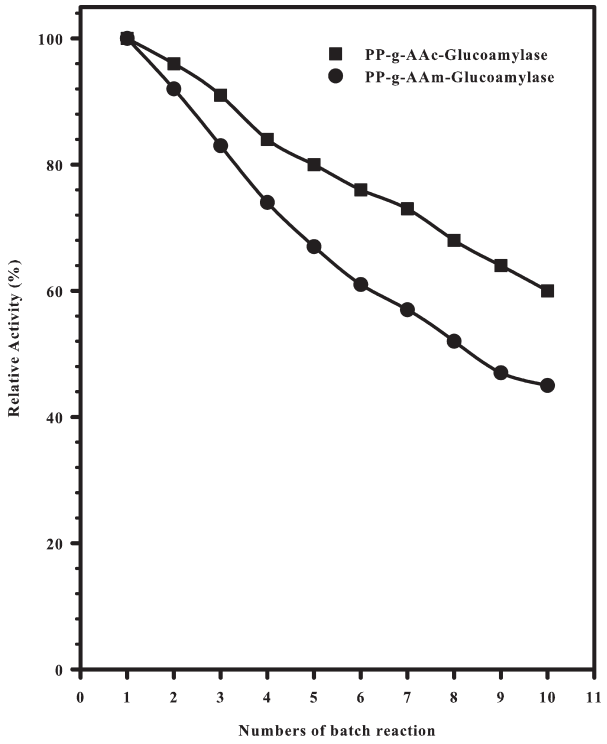

Fig. 13. Reusability of Glucoamylase-immobilized on PP-g-AAc and PP-g-AAm fibers. pH; 4.5, temperature; 55°C and 5% (w/v) starch as substrate.

4 Conclusions

Pre-irradiation grafting of acrylic acid (AAc) and acrylamide (AAm) individually onto PP fibers were investigated to achieve a suitable carrier for glucoamylase immobilization. It was found that, the higher the concentration of anchor groups, the higher the amount of bound protein, the higher the probability of multi-point attachment of proteins to the carrier. The maximum activity of the immobilized enzyme is obtained when the graft percentage was 100%. The enzyme activity reached 95% when it is immobilized onto AAc-grafted fibers; meanwhile, such activity is lowered to 60% for that immobilized on AAm-grafted fibers. The optimum pH for free glucoamylase is 4.5., such optimum is found to be shifted to pH 4.0 for the glucoamylase immobilized onto PP-g-AAc, while, it shifted to pH 5 for glucoamylase immobilized onto PP-g-AAm fibers. The immobilized glucoamylase displays significantly improved stability on both sides of the pH curve. Improvement of thermal stability of enzyme was achieved by immobilization. Since the optimum temperature for the immobilized enzyme was

found to be 60°C. i.e., which is higher than that of the free one (55°C). The maximum reaction rate, (V_{max}) and the apparent Michaelis constant, (K_m), were determined from the linear regression of the Lineweaver-Burk plots. The Km values for the immobilized glucoamylase on PP-g-AAc and PP-g-AAm fibers are approximately two and four-folds higher than that of the free glucoamylase, respectively.

It can be concluded that, the prepared synthetic carriers possessed good characteristics for enzyme immobilization via covalent bonding. Glucoamylase immobilized onto PP grafted acrylic acid fibers has higher activity than that immobilized onto the PP grafted acrylamide.

5 Acknowledgments

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