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Immobilization of α-amylase onto cyclic carbonate bearing hybrid material

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ABSTRACT

Under ambient conditions, 4-((3-trimethoxysilyl)propoxy)methyl)1,3-dioxolan-2-one was synthesized from 3-glycidyloxypropyl trimethoxysilane. Methacrylate and cyclic carbonate functional hybrid matrix was prepared by sol-gel method. α-Amylase was covalently bounded onto the matrix *via* cyclic carbonate functionality. Immobilization yield was found as 34.4 ± 2.6 mg per gram of hybrid matrix. The maximum activity was observed at pH 6.5. Immobilization did not change the pH-dependency of the enzyme. The immobilized enzyme had a higher activity at elevated temperature (50–80° C) than the free one. Immobilized enzyme exhibited 80% activity after 20 runs and 69% after 30 runs. Free enzyme lost its activity completely within 15 days. Immobilized enzyme lost only 30% of its activity in 25 days. V_{max} values for the free and immobilized enzymes were calculated as 58×10^{-3} and 5.2×10^{-3} mg/ml min^{-1}, respectively.

1. Introduction

Enzymes are biocatalysts, having some excellent properties such as high activity, selectivity and specificity that make them advantageous compared to chemical ones (Mateo, Palomo, Fernandez-Lorente, Guisan, & Fernandez-Lafuente, 2007). However, many of the enzymes are stable only under mild experimental and environmental conditions and their application procedure is unfortunately restricted due to their short half lives *in vivo* (Bajpai & Bhanu, 2003; Reshmi, Sanjay, & Sugunan, 2006).

Enzyme immobilization is preferred to enhance enzyme stability, multiple and repetitive use and to remove the enzyme from the reaction mixture easily (Hasirci, Aksoy, & Tumturk, 2006; Pandya, Jasra, Newalkar, & Bhatt, 2005; Altun & Cetinus, 2007). Immobilization can be achieved by two different ways; chemical and physical methods (Tumturk, Aksoy, & Hasırcı, 2000; Vidinha et al., 2006). In chemical method, enzyme and support material bind each other by covalent bonds, on the other hand in the physical method, there are weak interactions between enzyme and support. Covalent binding has some advantages such as increasing the thermal and storage stability of the enzyme, prevention of leakage and decreasing diffusion problems of the substrates and products in the reaction medium (Hasirci et al., 2006). Support materials used in enzyme immobilization are classified as inorganic supports, synthetic polymers and natural macromolecules (Ye, Xu, Che, Wu, & Seta, 2005). Generally, the main demand of a support is to elicit high chemical and mechanical stability, a hydrophilic nature and also non-toxic behavior. Polymeric materials are suitable candidates due to their reactive functional groups, good mechanical properties, ease of preparation method and applicability to introduce bio-friendly components for improving biocompatibility (Rebros, Rosenberg, Mlichova, & Kristofikova, 2007; Sankalia, Mashru, Sankalia, & Sutariya, 2007).

Bryjak (2003) reported the immobilization of amylase by using the copolymers of butyl acrylate / pentaerythrite triacrylate or butyl acrylate / ethylene glycol dimethacrylate that were modified with anchor groups: -OH, –COOH and $–NH_2$. These anchor groups were then activated by glutaraldehyde, divinyl sulfone and carbodiimide procedures, respectively. Amongst, the glutaraldehyde method was found to give the highest activity.

Kahraman, Ogan, Kayaman Apohan, and Gungor (2006a), Kahraman, Bayramoglu, Apohan, and Güngör (2007) investigated the α -amylase immobilization on a UV curable substrate mainly composed of acrylated epoxidized soybean resin. For enzyme immobilization, entrapment and covalent binding methods were investigated. Upon immobilization by the two methods the catalytic properties of the enzyme were not considerably changed as compared to non-immobilized form, only the pH profile was broadened for the immobilized enzyme. Immobilized enzyme prepared by the binding method possesses relatively higher activity compared with those obtained by the entrapment method.

Cyclic carbonates can react with amine groups to yield hydroxyl urethanes. There are several studies in literature about cyclic carbonate groups containing polymeric support for enzyme immobilization (Chen, Van der Does, & Bantjes, 1993a; Chen, Van der Does, & Bantjes, 1993b; Dean, 2003).

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Chen et al. (1993b) prepared microspheric hydrogel beads *via* cross-linking poly(vinylene carbonate) with Jeffamines. The beads were used as a matrix for the immobilization of the alkaline phosphatase.

The aim of this study was to prepare a new enzyme carrier having cyclic carbonate groups by using sol-gel technique. Therefore, 3-(methacryloyloxy)propyltrimethoxysilane (MEMO) and prehydrolyzed tetraethylorthosilicate (TEOS) were mixed with 4-((3-trimethoxysilyl)propoxy)methyl)1,3-dioxolan-2-one (CPS) and after condensation an inorganic matrix (CTM) bearing cyclic carbonate groups was obtained. α -Amylase enzyme was covalently bounded onto cyclic carbonate groups. To gain an organic-inorganic hybrid support for enzyme immobilization, UV curing process was achieved by mixing enzyme-bounded matrix with commercial acrylate resins. Enzyme activity of free and immobilized α -amylase was examined at different pH (5.0–8.0) and temperature (30–80° C). The storage stability and reusability of the covalently immobilized enzyme was studied and analyzed.

2. Materials and methods

2.1. Materials and characterization

1-Hydroxy-cyclohexyl-phenyl-ketone (Irgacure 184) was supplied by Ciba Specialty Chemicals. Tetraethylorthosilicate (TEOS), 3-(methacryloyloxy)propyltrimethoxysilane (MEMO) and *p*-toluenesulfonic acid (PTSA) were purchased from Merck. 2-Hydroxyethyl methacrylate (HEMA) was obtained from Fluka Chemie. The Photomer®4028 (Epoxy acrylate) and Photomer®4072 (three acrylate) were provided by Cognis. N-Vinyl-2-pyrrolidone (NVP) was provided by ISP Turkey. 3-Glycidyloxypropyl trimethoxysilane (GPTMS), tetrabutylammonium bromide (TBAB), α -amylase (1,4-α -D-Glucan-glucanohydrolase; EC 3.2.1.1, Type VI-B from porcine pancreas, extra pure 35 U/mg), starch, maltose and 3,5-dinitrosalicylic acid (DNSA), bovine serum albumin (BSA) were all obtained from Sigma-Aldrich. Coomassie®Brilliant Blue G-250 was obtained from BIO-RAD. All other chemicals were of analytical grade and were purchased from Merck AG. Carbon dioxide was used after passage through a silica gel column. Freshly double distilled water was used throughout.

FT-IR spectra were recorded on Shimadzu 8300 FT-IR spectrometer. ^{13}C-NMR ($CDCl_3$) spectrum was obtained by using Varian model T-60 NMR spectrometer operated at 200 MHz. The solid state cross-polarization (CP)/magic-angle spinning (MAS) NMR spectra was recorded using a Varian Unity Inova spectrometer operated at 500 MHz. SEM (Scanning Electron Microscope) imaging was performed on a JEOL-JSM-5919LV, after applying a gold coating under reduced pressure.

2.2. Preparation of cyclic carbonate and methacrylate functional multicomponent inorganic matrix (CTM)

Initially, the synthesis of 4-((3-trimethoxysilyl)propoxy)-methyl)1,3-dioxolan-2-one (CPS) was carried out according to literature (Turunc et al., 2008). A representative procedure is as follows. The GPTMS were reacted with CO_2 at 110° C in the presence of TBAB for 24 h. The reactor was then cooled to room temperature, the excess CO_2 was vented out, and the catalyst was separated by centrifugation. FT-IR spectrum of synthesized CPS is given in Fig. 1.

Then, TEOS was prehydrolyzed by the following procedure. 3 g (14.4 mmol) TEOS, 0.77 ml (43.2 mmol) water and 0.028 g *p*-toluenesulfonic acid (catalyst) in 1.63 ml ethanol were mixed at 5° C. The water/silicone ratio is calculated as r = 3. The mixture was allowed to warm to room temperature and stirred overnight. Prehydrolyzed TEOS and 3-(methacryloyloxy)propyltrimethoxysilane and CPS were mixed over night at room temperature to yield hybrid matrix (CTM). The synthesis of CTM is illustrated in Fig. 2.

2.3. Enzyme Immobilization

For the immobilization of the α -amylase enzyme, 5 g CTM was added into the enzyme solution (0.4 g α -amylase in 50 ml ethanol) and the immobilization process was carried out at 25° C for 12 h in a shaking water bath. Enzyme bounded CTM was filtered and physically bound enzyme was removed by washing the support three times with phosphate buffer (100 ml 0.02 M sodium phosphate buffer, pH = 6.9 with 0.006 M NaCl). The washing solution was kept for measuring the amount of covalently bound enzyme on the support. The enzyme protein concentration within the extract was measured using Coomassie Brillant Blue reagent as described by Bradford (Bradford, 1976). 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 bound protein per weight of material was calculated. Enzyme immobilization experiments were re-

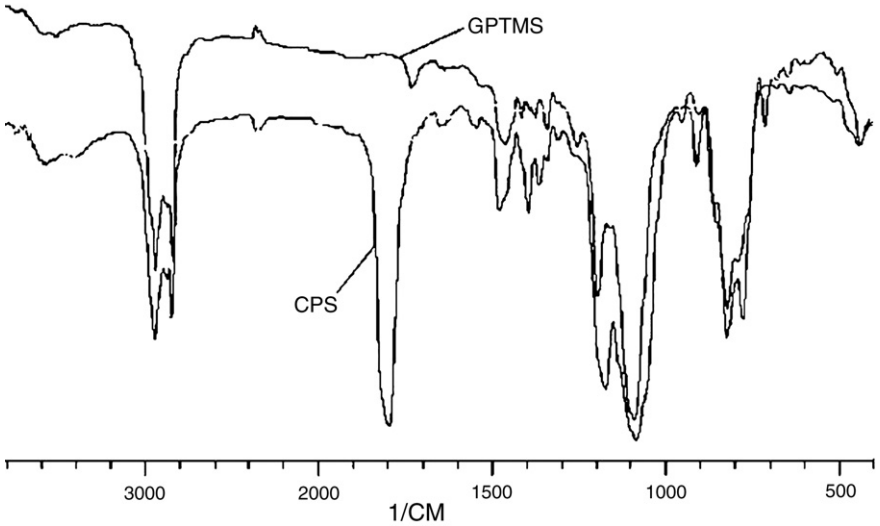

Fig. 1. FT-IR spectra of CPS and GPTMS.

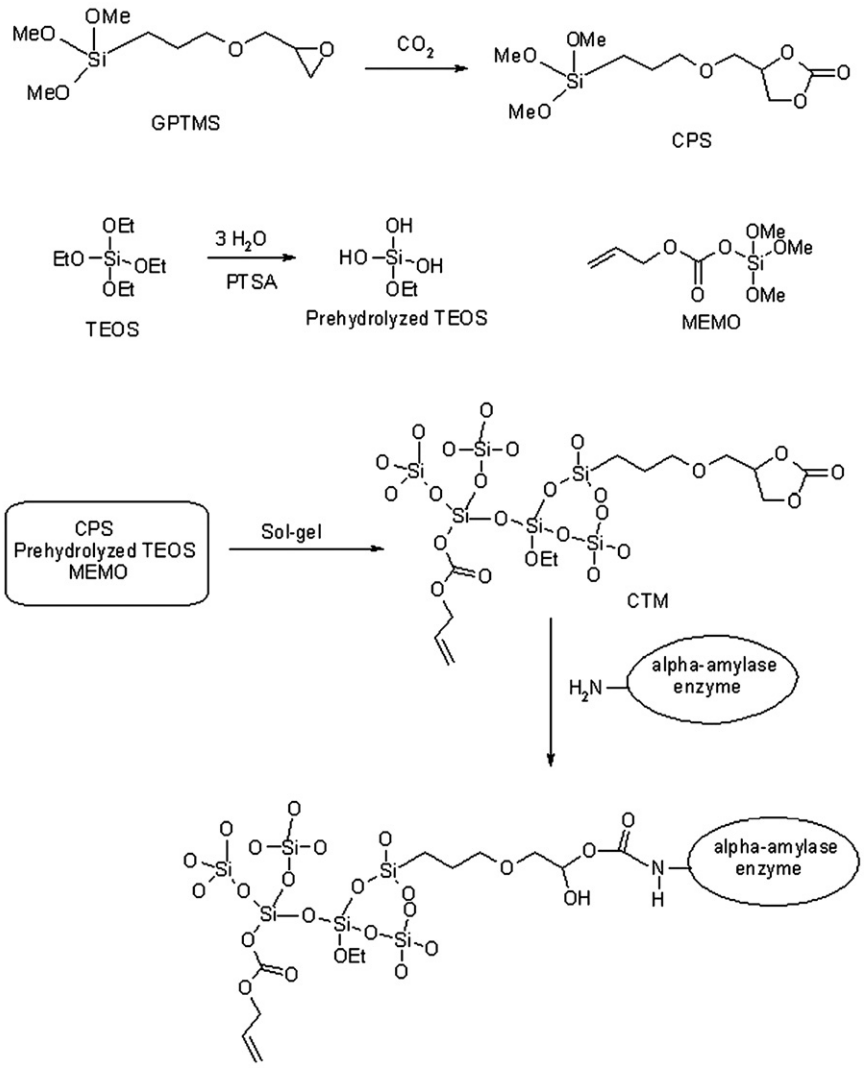

Fig. 2. The reaction scheme of CPS and enzyme bound CTM.

peated four times and the average immobilization value was found as 34.4 ± 2.6 mg per gram of CTM. A representation of this immobilization process is shown in Fig. 2.

2.4. Preparation of immobilized enzyme containing hybrid films

Hybrid films containing immobilized enzyme were prepared by mixing Photomer 4072 (triacrylate), Photomer 4028 (Epoxy acrylate), HEMA, NVP, CTM and the photoinitiator Irgacure-184. The prepared formulation was then transferred into a round shaped Teflon™ mold (R = 4 mm). In order to prevent the inhibiting effect of oxygen, the mixture in the mold was covered by a transparent 25 μm thick Teflon™ film before irradiation with a high pressure UV-lamp (OSRAM, 300 W), a quartz glass plate was placed over. The Teflon film was also used to obtain a smooth surface. After 200 s irradiation under UV-lamp, 1 mm thick free films were obtained.

2.5. Activity Assays of free and immobilized α -amylase

The activities of free and immobilized α -amylase were determined by the assay suggested by Bernfield (1951). Briefly 1 wt% starch solution was prepared by dissolving soluble starch in 100 ml 0.02 M sodium phosphate buffer, pH = 6.9 with 0.006 M NaCl. A known amount of hybrid films containing immobilized amylase enzyme was placed into a test vial. Then 0.5 ml starch solution was added and the system was incubated in a water bath with constant shaking at 25 °C for exactly 5 min. After that the polymeric films were taken out from the vial. The reaction was stopped by adding 1 ml of 3,5-dinitrosalicylic acid reagent. Incubation was performed in a boiling water bath for 5 min. After dilution with distilled water, the amount of reduced sugar (maltose) produced was determined spectrophotometrically at 540 nm. In each set of experiments, a standard curve was prepared with maltose solutions of different concentrations. An enzyme activity unit (IU) was defined as the amount of enzyme liberating 1 μmol maltose per minute under the assay conditions. The immobilized enzyme had to be washed with distilled water before re-measuring its activity in successive used cycles. Each result is an average of three or four separate experiments. Activity of the enzyme is calculated from the following equation:

$$\text{Activity}(\text{IUmg}^{-1}) = \frac{\text{released maltose}(\mu\text{mol})}{\text{amount of } \alpha\text{-amylase}(\text{mg}) \times \text{min}}$$

The effect of temperature on enzyme activity was studied in the range of 30–80° C. The effect of pH on enzyme activity was studied in the range of pH 5.0–8.0

The results of dependence of pH, temperature, storage and used up capacity are presented in a normalized form with the highest value of each set being assigned the value of 100% activity.

2.6. Determination of K_m and V_{max} values

For the determination of kinetic constants, the initial rates of the reaction of the enzyme with starch solution using different concentration of the substrate were determined (0.25–2.5 mg/ml).

3. Results and discussion

3.1. Characterization of support

3-Glycidyloxypropyl-trimethoxysilane (GPTMS) was converted to [4-((3-trimethoxysilyl)propoxy)methyl)1,3-dioxolan-2-one] (CPS) containing five membered cyclic carbonates by reaction with carbon dioxide. The extent of the reaction was followed using FTIR spectroscopy by focusing on the appearance of a new peak at 1789 cm^{-1} due to the carbonyl of the cyclic carbonate moieties (Fig. 1). It is similar to that given for the reaction of oxiranes with carbon dioxide (Turunc et al., 2008; Rokicki & Wojciechowski, 1990). The ^{13}C NMR spectrum shows the carbonyl carbon signal of synthesized cyclic carbonates (CPS) at 155 ppm (Pawel, Magdalena, & Rokicki, 2007). This evidence demonstrates that the CPS was prepared successfully.

The ^{29}Si CP/MAS NMR spectrum of the multicomponent matrix (CTM) shows mainly five kinds of signals were observed at −43 ppm (R–Si, $(OCH_2CH_3)_3$), T0, −54 ppm (R–Si, $(OSi)_2(OH)$) or (R–Si, $(OSi)_2(OCH_2CH_3)$), T2, −79 ppm ($Si(OH)(OCH_3)_2$) Q0, −89 ppm ($Si(OSi)(OCH_3)_y(OH)_{3-y}$, Q1, and −100 ppm ($Si(OSi)_3(OCH_3)$) or ($Si(OSi)_3$ (OH)), Q3. T2 peak was dominant. The signals at −43, −54, −79, −89, and −100 ppm for T0, T2, Q0, Q1 and Q3, respectively. Si-NMR results are similar with the data reported in the literature (Kahraman, Kuğu, Menceloğlu, Kayaman-Apohan, & Güngör, 2006b; Van Nieuwenhuyse, Bounor-Legare, Boisson, Cassagnau, & Michel, 2008).

Covalent coupling of alpha-amylase directly onto CTM was carried out by the reaction of the cyclic carbonate groups of CTM with the amine functionalities of the enzyme, as shown in Fig. 2. Coupling reaction was monitored by FTIR measurement. The peak intensity of the cyclic carbonate groups at 1789 cm^{-1} was diminished. The carbamate groups of enzyme bounded CTM (-O-CO-NH-Enzyme) into appeared at 1724 cm^{-1} (Van Nieuwenhuyse et al., 2008). From these results it is concluded that alpha-amylase was covalently bound onto CTM successfully.

Surface morphology of the enzyme bounded CTM and its hybrid film are shown in Fig. 3. We can see from SEM image (Fig. 3) the enzyme bounded CTM shows glassy, rigid and disordered particulate shape. SEM–EDS were used to identify the chemical composition on the particle surface. EDS analysis revealed that the particles are composed of 29% C, 1% N, 44% O and 26% Si. This result demonstrates that hybrid network was formed successfully and enzyme was attached on the backbone homogeneously.

3.2. Immobilization efficiency

The amount of the covalently bound enzyme was found as 34.4 ± 2.6 mg per gram of CTM. There are several studies including α-amylase immobilization in which the binding capacity of the support materials is labile due to the characteristic properties of the prepared materials. In our previous study (Kahraman et al., 2007), the coupling capacity of the α-amylase immobilized amine functionalized glass beads was reported as 25.2 mg per gram of glass support. Hasirci et al. (2006) studied poly(dimer acid-co-alkyl polyamine) particles that were activated by CDI, EDA and HMDA, respectively. The amount of bound enzyme was found as 7.6, 6.5 and 39.3 mg per gram of each particle orderly.

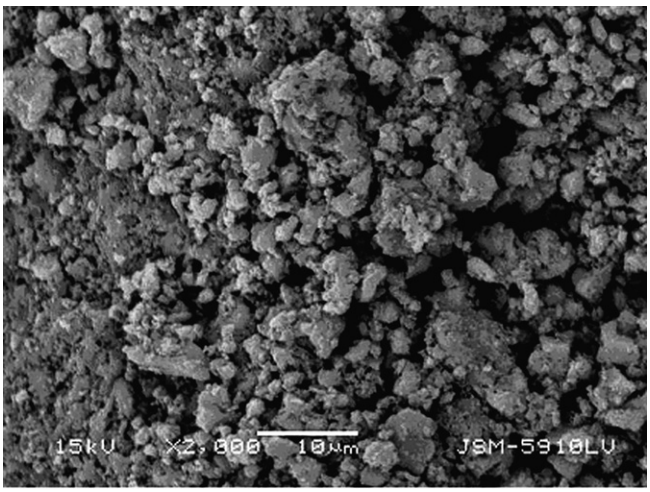

Fig. 3. SEM images of the enzyme bound CTM.

3.3. Effect of pH on activity

The effect of pH on free and immobilized α-amylase was investigated in buffer solutions in the range of pH 5.0–8.0 at 25° C (Fig. 4). Both free and immobilized enzymes are sensitive to the pH, however immobilization did not change the pH-response of the enzyme. The maximum activity was observed at pH 6.5. El-Batal, Atia, and Eid (2005) have studied the stabilization of α-amylase by using anionic surfactant. They compared the activity of soluble and immobilized enzyme as a function of pH and found that both the free and bounded α-amylase had optimum activity at the same pH.

3.4. Effect of temperature on activity

The activity of free and immobilized α-amylase was assayed at various temperatures (30–80 °C). As it can be seen in Fig. 5, maximum activities were observed at 30° C for both free and immobilized enzymes. One can see that at mild temperatures, the free enzyme exhibited higher stability in comparison to the immobilized one. However, there was a sharp difference in activity loss between free and immobilized enzyme, which was approximately 17% at 50° C. Barely, 50° C was a critical temperature for the free enzyme because after that temperature its activity retained 45%.

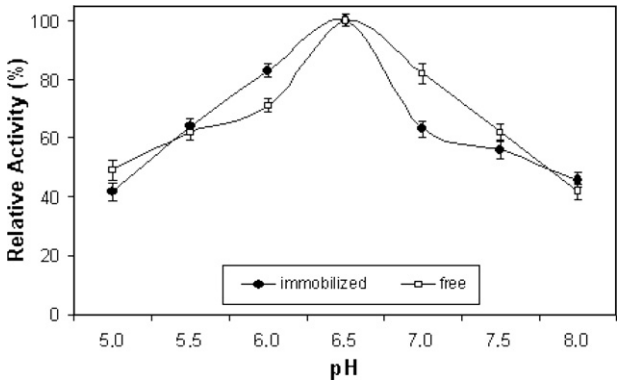

Fig. 4. Effect of pH on the relative activity of free and immobilized enzymes.

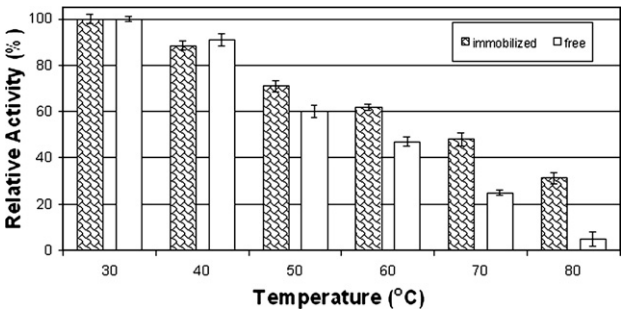

Fig. 5. Influence of temperature on percentage relative activity of free and immobilized α-amylase.

Finally at 80° C, the relative activity loss was calculated as approximately 70% for immobilized enzyme and 95% for the free one. The immobilized enzyme had a higher activity at high temperatures (50–80 °C) than the free one. It is well known that the activity of immobilized enzymes, especially in a covalently bound system, is more resistant against temperature than the free form (Hasirci et al., 2006) .

3.5. Reusability and storage stability

Enzymes are very sensitive biocatalysts against environmental conditions and may lose their activities quiet easily. Thus, it is meaningful to characterize their reusability and storage stability for preparative or industrial uses. Reusability experiments were performed at 25 °C. After incubation, immobilized enzyme containing support was removed from the reaction medium and washed twice with distilled water. Activity was determined in the same manner as in the enzyme assay. To determine the reusability of the immobilized enzyme, the activity of the same support sample was measured sequentially 30 times within a day. The activity found for each repetition was compared with the initial activity assuming it possesses 100% activity. The decrease in activity after repeated use was given in Fig. 6. It was measured that; immobilized enzyme exhibited 80% activity after 20 runs and 69% at 30th run.

In addition, the free and immobilized enzymes were stored in phosphate buffer (0.02 M pH 6.9) at 4 °C and their activities were tested for 25 days. Free enzyme lost its activity completely within 15 days. Immobilized enzyme lost only 30% of its activity at the end of 25 days. This result showed that by immobilization, the enzyme gained more stable character than the free one.

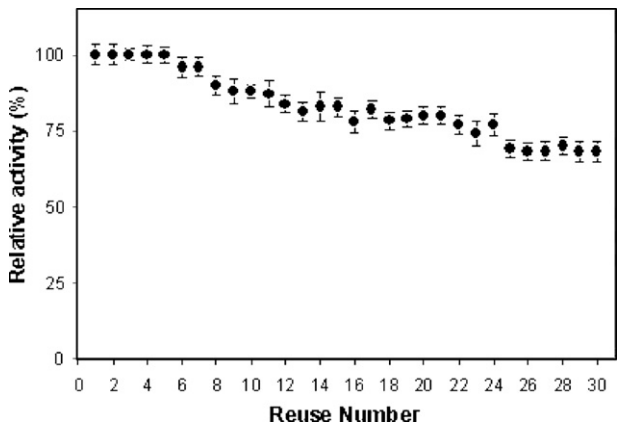

Fig. 6. Reuse capacity of the immobilized enzyme.

3.6. Kinetic studies

Catalytic properties of the free and immobilized enzymes were evaluated by using soluble starch as a substrate. The Michaelis-Menten constant K_m and maximum activity V_{max} of the free and immobilized enzymes were estimated at pH 6.9 and 25 °C. K_m values were found as 15.8 and 9.09 mg/ml for free and immobilized enzymes, orderly. The K_m value is known as the affinity of the enzymes to substrates (Cosulich, Russo, Pasquale, & Mariani 2000; Park, Haam, Jang, Ahn, & Kim, 2005) and the lower values of K_m emphasize the higher affinity between enzymes and substrates. The results have shown that the affinity of the α-amylase to its substrate was increased by immobilization. V_{max} values for the free and immobilized enzymes were calculated as 58×10^{-3} and 5.2×10^{-3} mg/ml min^{-1}, respectively.

4. Conclusion

Cyclic carbonate and methacrylate functional matrix (CTM) was prepared and characterized by FTIR, ^{13}C-NMR, ^{29}Si NMR. α-Amylase was covalently bounded onto the CTM. Organic-inorganic hybrid support was prepared by UV-curing technique successfully. Enzyme assays demonstrated that, the immobilized enzyme exhibited better thermo-stability than the free one at temperatures between 50–80 °C. The optimum pH of the immobilized enzyme did not change. The storage stability and re-usability improved by the immobilization of enzyme on to the hybrid support.

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