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Octadecanoic acid/silica particles synthesis for enzyme immobilization: Characterization and evaluation of biocatalytic activity

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Abstract

Water insoluble enzyme particles (WIEP) were prepared by first anchoring the octadecanoic acid over porous silica particles (OAS) followed by diastase enzyme binding. These particles were characterized using FT-IR, X-ray crystallography and X-ray photoelectron spectroscopy (XPS) and evaluated for starch hydrolysis activity. The results indicated that the enzyme binding to OAS particles was dependent on pH and temperature of the medium. Maximum enzyme binding (>48%) to OAS particles was observed at pH 3 and at 30 °C, while highest biocatalytic activity of WIEP was observed at pH 4 and at 40 °C. The storage stability study of water insoluble enzyme particles was evaluated for more than 70 days without any enzyme activity loss.

Keywords: Enzyme; Immobilization; Silica; Enzyme activity

1. Introduction

Immobilization of enzymes on solid supports is an important target of biotechnology [1–4]. Up to now, a great variety of systems designed for the purpose have been introduced and discussed. Supports suitable for technical applications should maintain a high level of enzyme activity while preventing a possible leaching out during the reaction. The techniques used to immobilize the enzyme is one of the key factors for developing reliable biosensors, thus new immobilization schemes and novel materials that can improve the analytical capacities of sensor devices are highly desired [5]. However, the synthesis of biocompatible surfaces having biological components adsorptivity is an interesting challenge for development of micron or sub-micron scale technologies. Recent trends in this context suggested the increasing interest in development of large-pore nanoscale mesoporous silica particles as immobilization supports. This is mainly because, mesoporous silica has been one of the most attractive targets for various groups to achieve the efficiency of mesopore usage and rapid mass transfer and also has potential application in catalysis, adsorption, separation as well as inclusion chemistry [6–10].

In past decades, gold nanoparticles (AuNPs) had attracted a continuous interest due to their unusual properties in electronics, optics [11,12] especially in biotechnology and nanotechnology fields involving biosensors [13,14], DNA hybridization [15–17], and biocatalysts [18]. Many methods of AuNPs' modification with alkanethiols provided different functional gold surfaces for facile combinations with biomolecules [19–21]. However, utilization of functionalized AuNPs is a cost-intensive and multistep preparation process. Therefore, scientific efforts have been focused towards biosilica as an immobilization matrix [22]. Literature reports on preparation of nanoscale mesoporous silica particle and subsequent enzyme adsorption studies revealed the ultrafast enzyme adsorption and the amount of enzyme immobilized to be much more in large-pore nanoscale mesoporous silica particles than the conventional mesoporous silica (SBA-15) [23]. Recently, we reported a novel synthesis of ferric impregnated silica nanoparticles for the binding of a biocatalyst [24]. In the present study an effort has been made to understand the importance of organic-silica particles as an immobilization matrix material in biotransformations, where octadecanoic acid

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anchored silica (OAS) particles were developed by first anchoring of octadecanoic acid over porous silica particles followed by binding the diastase enzyme (EC 3.2.1.1), which is known to catalyze the hydrolysis of starch into maltose. These water insoluble enzyme particles (WIEP) were characterized using different analytical tools and evaluated for their biocatalytic potential.

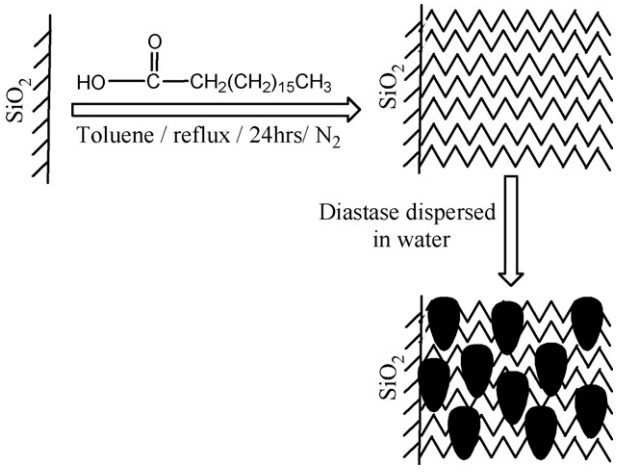
2. Experimental

2.1. Materials and chemicals

Silica particles (100–200 mesh) and octadecanoic acid were obtained from Merck India whereas soluble starch, 3,5-dinitrosalicylic acid, diastase and other chemicals were purchased from Hi-Media, India. Commercial grade toluene and chloroform were dried and distilled prior to use. The results reported in this paper were average values of triplicate experiments.

2.2. OAS particles synthesis

OAS particles were synthesized from porous silica particles and ocatdecanoic acid, a biocompatible long-chain fatty acid. A typical synthesis was performed by degassing and dissolving 5 g of octadecanoic acid in dry toluene (15 mL) in a schlenk tube under nitrogen atmosphere. In a separate schlenk tube, 15 g of activated (silica heated to 100 °C under vacuum for 12 h) silica particles were degassed with nitrogen gas. The above solution was then transferred through canula to the silica particle Schlenk tube under nitrogen atmosphere. The above mixture was stirred at the reflux temperature for 24 h and then the OAS particles were filtered off and washed with excess of toluene and chloroform several times to remove free octadecanoic acid. The OAS particles were collected and dried in air and then under vacuum for 12 h at room temperature. The resultant OAS particles were used as immobilization matrix in preparation of WIEP. The schematic representation of OAS particle preparation and enzyme binding is represented in Scheme 1.



Scheme 1. Schematic representation of water insoluble enzyme particle preparation.

2.3. Enzyme binding with OAS particles

Commercially available diastase enzyme was used in this investigation. Hundred milligrams of enzyme was dissolved in 1 mL of distilled water and to this 100 mg of OAS particles was added under nitrogen atmosphere. This mixture was incubated at 30 °C for 30 min under constant mixing environment. The contents were then centrifuged at 10,000 rpm for 5 min at 30 °C. The unbound enzyme from OAS particles was removed by washing with sterilized distilled water till the supernatant was free of protein and/or enzyme activity. The WIEP were then dried using vacuum evaporator and used for characterization and evaluation of enzyme activity. The maximum enzyme binding was optimized by performing the temperature-dependent experiments at different pH environments.

2.4. Enzyme activity measurement

The enzyme activity was measured according to [25] by estimating the maltose produced during starch hydrolysis using 3,5-dinitrosalicylic acid as a coupling reagent. The reaction mixture containing 1.0 mL of 1% soluble starch in 0.01 M phosphate buffer (pH 5.0) and 10 mg of WIEP was incubated at 30 °C for 5 min. The reaction was stopped by adding 2.0 mL of 1% 3,5-dinitrosalicylic acid solution followed by heating in a boiling water bath for 5 min. The solution was then cooled to room temperature and the volume was made up to 10 mL with distilled water. The absorbance of the reaction mixture was determined at 540 nm in a UV–Vis spectrophotometer. Temperature effect on enzyme was studied by performing the reaction at different temperatures (30–80 °C). For this the free enzyme and WIPE particles were incubated at respective temperature for 30 min for stabilization and enzyme activity was measured. One unit of the enzyme was defined as the amount of enzyme capable of producing 1 μM of reducing sugar (as maltose) from 1% soluble starch as substrate in 1 min at 40 °C unless otherwise mentioned.

2.5. Calculation of K_m, V_{max} and activation energy values

Reaction kinetics for WIEP and free enzyme were estimated by measuring the catalytic activity at different substrate concentration ranging from 0.5 to 3.0% at 40 °C. K_m and V_{max} values were calculated by representing the enzyme activity in Lineweaver–Burk equation [26].

2.6. Protein estimation

The protein content was estimated according to method of Lowry et al. [27].

2.7. Fourier transform infrared spectroscopy (FT-IR) analysis

FT-IR studies were conducted using Thermo Nicolet Nexus 670 Spectrometer. De-moisturized samples before and after enzyme binding (1–2 mg) were homogenized with 100 mg of

dry KBr and made into pellets. These pellets were analyzed for transmittance in the range of 4000–400 cm^{-1}.

2.8. X-ray photoelectron spectroscopy

X-ray photoelectron spectroscopy (XPS) measurements were obtained on a KRATOS-AXIS 165 instrument equipped with dual aluminum–magnesium anodes using Mg Kα radiation. The X-ray power supplied was 15 kV and 5 mA. The pressure of the analysis chamber during the scan was 10^{-9} Torr. The peak positions were based on calibration with respect to the 'C 1s' peak at 284.6 eV. The obtained XPS spectra were fitted using a nonlinear square method with the convolution of Lorentzian and Gaussian functions after the polynomial background subtraction from the raw data.

2.9. X-ray diffraction (XRD) measurement

XRD measurement of the octadecanoic acid anchored on silica (OAS) particles before and after binding with enzyme (WIEP) mounted onto quartz substrate was done on D/8 BRUKER AXS operating at 40 kV at a current of 30 mA with Cu kα radiation.

3. Results and discussion

3.1. Characterization of OAS particles and WIEP

3.1.1. FT-IR study

Fig. 1 shows the FT-IR spectra recorded for preheated porous silica (Curve 1), OAS particles (Curve 2) and octadecanoic acid (Curve 3). The spectra indicated mainly two differences; one in shortening of OH vibration band noticed at 3361 cm^{-1} after anchoring the octadecanoic acid (Curve 2) with porous silica (Curve 1) and second is presence of methylene antisymmetic (2850 cm^{-1}) and symmetric (2920 cm^{-1}) vibration of hydrocarbon chain of octadecanoic acid on OAS particles spectra (Curve 2) suggesting anchoring of octadecanoic acid on porous silica particles was successfully performed. This is further confirmed based on the fact that, OAS spectra were obtained after removal of free octadecanoic acid from OAS particles.

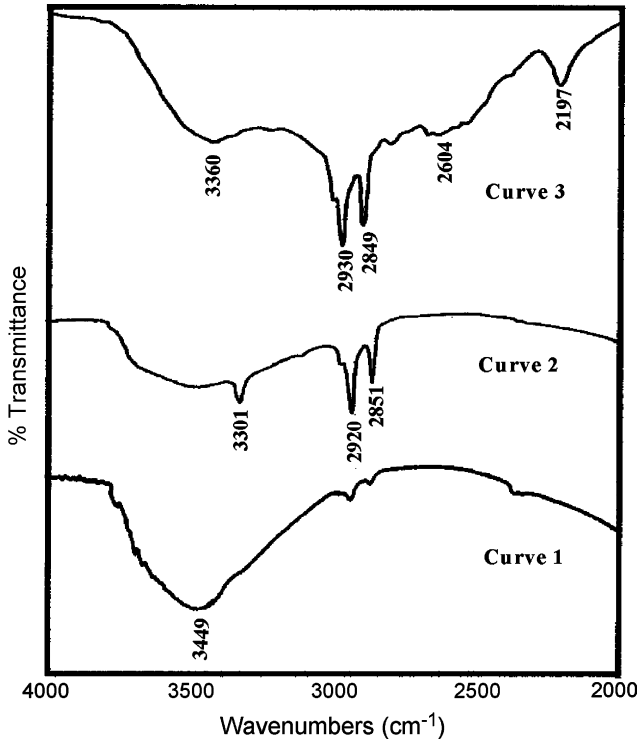


Fig. 1. FT-IR spectra of silica (Curve 1), OAS (Curve 2) and octadecanoic acid (Curve 3).

3.1.2. X-ray diffraction studies

X-ray powder diffraction patterns of OAS particles and WIEP hardly differ in the range $2\theta = 0.5^{\circ}$ (Fig. 2). The observed d_{003} basal spacing of the support that appeared at 3.97 Å (spectra A) slightly changed after the binding with enzyme (4.03 Å, spectra B), which indicates that the enzyme and octadecanoic acid are mainly located at the edge-on surface in the respective samples.

3.1.3. X-ray photoelectron spectroscopy analysis

XPS is a highly surface sensitive diagnostic tool for the assessment of the electronic state of elements. High resolution narrow scans of C 1s signals were used to obtain quantitative results for functional group compositions of modified silica template. However, the exact determination of the nature of the incorporated groups is not easy for many, as signals coming from CH, CN, CS or CO groups can be overlapped especially for WIEP sample. XPS survey scan for OAS particles show characteristic signals of carbon, silica and oxygen, whereas the survey scan for WIEP show nitrogen signal in addition to above signals which can be attributed to the peptide bonds present in the enzyme. Typical deconvolutions of the C 1s XPS spectra are shown in Fig. 3. The observed C 1s signals for OAS sample at 284.6, 286.1 and 287.9 eV are attributed to –C–.

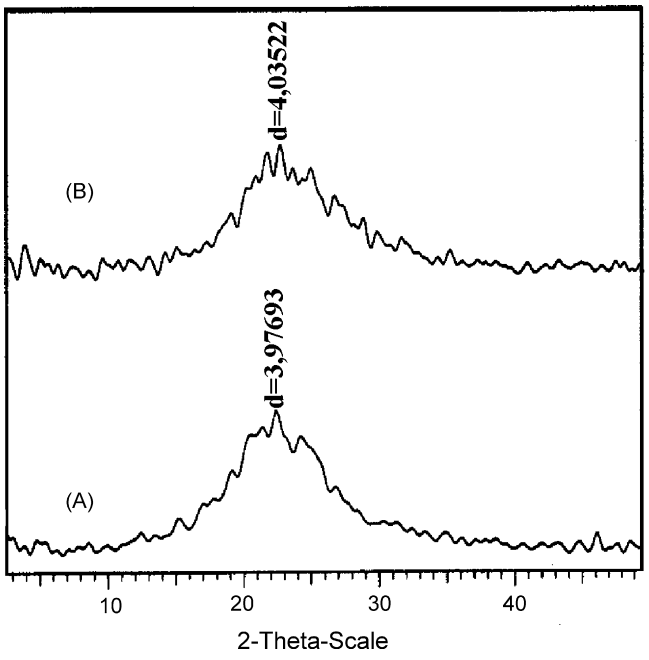


Fig. 2. X-ray diffraction graph of OAS particles (A) and WIEP (B).

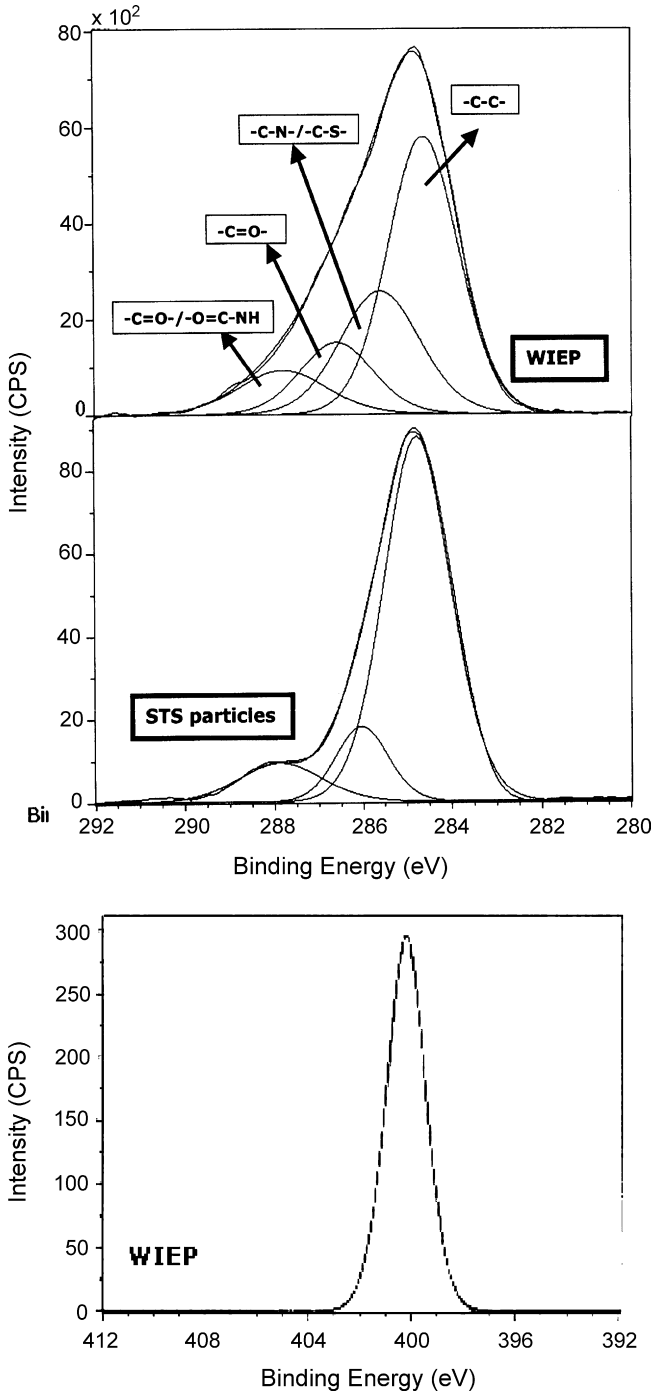


Fig. 3. High-resolution narrow scan for C 1s (resolved into component peaks).

C–, –C–O– and –C=O–, respectively, which clearly indicate that the silica surface is modified with octadecanoic acid. C 1s signal from WIEP samples shows similar binding energy peaks at 284.6, 285.6, 286.2, and 287.4 eV, which can be attributed to –C–C–, –C–S–/–C–N–, –C–O– and –C=O–/–O=C–NH bonds, respectively.

The presence of the binding energy peak at ≈400 eV in the high resolution narrow scan for N 1s in WIEP sample further provides chemical details of the transformation of the surface due to the modification of OAS sample with diastase enzyme. This is visualized by the presence of NH_2 groups on surface. Characteristic binding energy peak due to S 2p was not observed which could be due to the orientation of the enzyme molecule such that they do not appear on the surface of WIEP.

3.1.4. Enzyme binding and analysis

Quantification of enzyme loading was investigated by estimating the enzyme in terms of protein content present in WIEP after loading of the enzyme onto OAS particles. It was noticed that, enzyme loading was influenced by the pH of the medium and maximum enzyme binding was noticed at pH 3 in the entire pH range (pH 3.0–8.0) investigated. However, when the enzyme activity was measured, maximum activity was noticed at pH 4 (Fig. 4). To clarify further free enzyme activity was measured at different pH conditions and observed that maximum activity at pH 4.0 indicating there is no relation with enzyme binding pattern onto WIEP and bound enzyme activity with respect to medium pH. Hence, further experiments were performed at pH 4.0.

Influence of temperature on enzyme loading was evaluated by incubating the OAS and enzyme at different temperature ranging from 30 to 80 °C with an increment of 10 °C at pH 4.0. The enzyme loading was found to be the maximum at 40 °C and incubation at either side of this temperature reduced the enzyme loading concentration with the same of amount of OAS particles. WIEP evaluation for enzyme activity was performed at 40 °C irrespective of loading temperature. It indicated that the maximum biocatalytic activity was noticed with 40 °C loaded OAS particles. As the loading temperature increased, though binding was present on OAS particles but the activity decreased (Fig. 5). This may be explained based on the fact that biocatalyst looses its catalytic activity due to conformational change caused by temperature.

Enzyme loading process on OAS particles was investigated as a function of time using enzyme and OAS particles in the ratio of 1:1 to understand the adsorption levels and the results are indicated in Fig. 6. It is clear from the data that the enzyme immobilization process was instantaneous and 95% of enzyme loading on OAS particles was occurred within 5 min and fur-

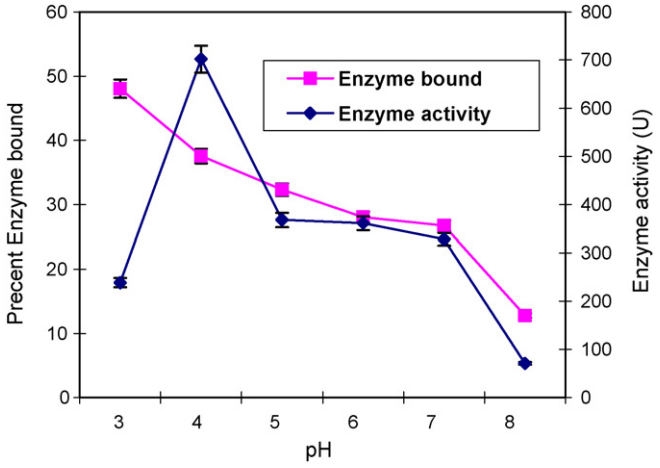


Fig. 4. Effect of pH on enzyme loading and its catalytic activity.

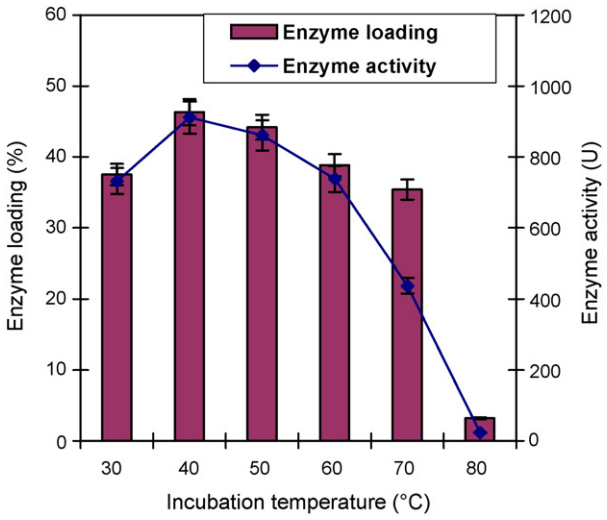


Fig. 5. Influence of incubation temperature on enzyme loading and activity.

ther incubation could not improve much of the enzyme loading. These results are in accordance with the lysozyme loading on to mesoporous silica particles where 95% of the enzyme loading was observed within 10 min [23]. The data further reveal that the enzyme loading on to OAS particles was higher compared to literature reports. In the present investigation, the immobilization efficiency with increasing enzyme amounts in solution indicated that the maximum enzyme loading noticed was 46% with 1:1 (octodecanoic acid anchored silica:enzyme (w/w)), while <45% and <30% loading was noticed on to mesoporous silica [23] and SBA 15 nanoscale MPS particles [28], respectively.

Kinetic properties evaluation for free and immobilized enzyme on OAS particles depicted that the K_m values for WIEP and free enzyme were noticed to be 6.41 and 4.02 mM while V_{max} values were noticed to be 6.6 and 55.5 μmol/(min g) substrate, respectively. Activation energies for free and bound

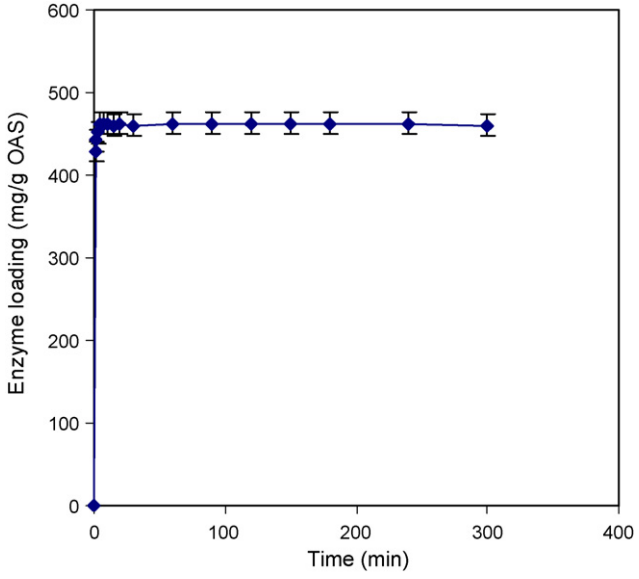


Fig. 6. Adsorption of enzyme on OAS particles as a function of time.

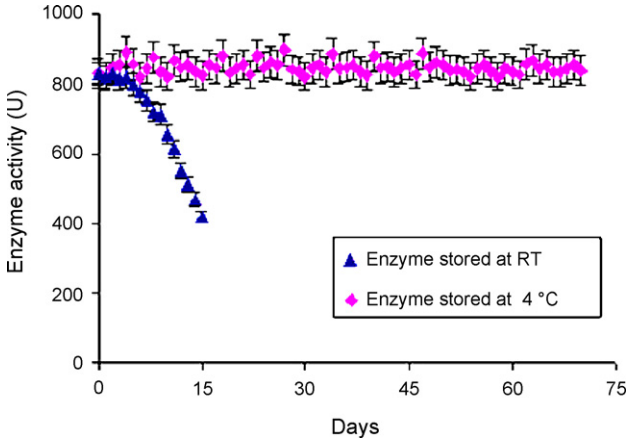


Fig. 7. Biocatalytic evaluation of WIEP.

enzyme were found to be 4.85E−3 and 3.82E−3 kcal/mol indicating efficient catalytic activity of enzyme present in WIEP.

4. WIEP storage stability studies

Storage stability of the prepared WIEP was analyzed by incubating these particles for prolonged periods at ambient and low temperatures and analyzing the enzyme activity at every 24 h of interval using the reaction mixture containing 1.0 mL of 1% soluble starch in 0.01 M phosphate buffer (pH 4.0). The results indicated that the enzyme stored at 4 °C is highly stable in bound form for more than 70 days without any change in enzyme catalytic activity (Fig. 7). Whereas, storing at ambient temperature resulted in gradual reduction (50%) of enzyme activity within 15 days (Fig. 7).

5. Conclusion

In conclusion, we have studied the enzyme immobilization using simple system composed of hydrophobic alkyl chain anchored on porous silica particles. These water insoluble enzyme particles and octadecanoic acid anchored silica particles were analyzed using different analytical instruments such as FT-IR, XPS and X-ray diffraction for investigating the conformational changes before and after binding the enzyme. These enzyme bound particles were evaluated for their biocatalytic function.

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