List of publications and presentations
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PUBLICATIONS:


5. **Mohammad Jahir Khan** and Qayyum Husain. High yield immobilization and stabilization of *Bacillus amyloliquefaciens* α-amylase by SnO$_2$ nanoparticles. *Journal of Nanoscience and Nanotechnology*. (Communicated).

6. **Mohammad Jahir Khan** and Qayyum Husain. Potential applications of soluble and immobilized amylases: A review. (Communicated).

PRESENTATIONS:

1. **Mohammad Jahir Khan**, Shariq Qayyum, Fahad Alam and Qayyum Husain. “Effect of tin oxide nanoparticle binding on the structure and activity of α-amylase from *Bacillus amyloliquefaciens*”. Poster presented in Silver Jubilee Symposium under the auspices of UGC-DRS-II Programme organized by Department of Biochemistry, Faculty of Life Sciences, AMU, Aligarh, India, March 6, 2012.

2. **Mohammad Jahir Khan**, Qayyum Husain and Ameer Azam. “Immobilization of porcine pancreatic α-amylase on magnetic Fe$_2$O$_3$
nanoparticles: Applications to the hydrolysis of starch”. Poster presented in International Conference on Chemistry: Frontiers and Challenges; organized by Department of Chemistry, Faculty of Science, AMU, Aligarh, India, March 5-6, 2011.


5. Participated in SFFR Satellite India Conference organized by Department of Biochemistry, AIIMS, New Delhi, India, February 11-12, 2008.

Effect of tin oxide nanoparticle binding on the structure and activity of α-amylase from *Bacillus amyloliquefaciens*

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Effect of tin oxide nanoparticle binding on the structure and activity of α-amylase from Bacillus amyloliquefaciens

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Abstract
Proteins adsorbed on nanoparticles (NPs) are being used in biotechnology, biosensors and drug delivery. However, understanding the effect of NPs on the structure of proteins is still in a nascent state. In the present paper tin oxide (SnO₂) NPs were synthesized by the reaction of SnCl₄·5H₂O in methanol via the sol–gel method and characterized by x-ray diffraction (XRD), Fourier transform infrared spectroscopy (FT-IR) and transmission electron microscopy (TEM). The binding of these SnO₂–NPs with α-amylase was investigated by using UV–vis, fluorescence and circular dichroism (CD) spectroscopic techniques. A strong quenching of tryptophan fluorescence intensity in α-amylase was observed due to formation of a ground state complex with SnO₂–NPs. Far-UV CD spectra showed that the secondary structure of α-amylase was changed in the presence of NPs. The Michaelis–Menten constant (Kₘ), was found to be 26.96 and 28.45 mg ml⁻¹, while Vₘₐₓ was 4.173 and 3.116 mg ml⁻¹ min⁻¹ for free and NP-bound enzyme, respectively.

1. Introduction
α-Amylases (alternative names 1,4-α-D-glucan glucohydrolase, glycogenase, E.C.3.2.1.1) are endo-acting enzymes which belong to the glycoside hydrolase family 13 (GH13) [1]. These enzymes catalyze random hydrolysis of α-1,4-glycosidic bonds in amylose, amylpectin and related polysaccharides to low molecular weight products such as dextrins, maltose and glucose molecules [2, 3]. α-Amylases are very important commercial enzymes with a number of applications including their use in food, paper, detergent, pharmaceutical and textile industries [4]. Although α-amylases can be derived from various sources, their industrial demand has been satisfied by microbial and recombinant enzymes. The most abundantly used bacterial α-amylases for industrial purposes are derived from Bacillus species [5]. These enzymes have been characterized from a wide variety of organisms and almost all of them have a similar structure and catalytic mechanism [6, 7].

The interaction of enzymes with ligands offered stimulating opportunities for a wide variety of applications in the field of biotechnology and medicine [8–10]. Generally proteins undergo structural changes when interacting with ligands. Metal oxide NPs serves as novel candidates for the binding of protein due to their large surface-to-volume ratio, high biocompatibility, non-toxicity, chemical stability and ease of preparation [11, 12]. Adsorption of proteins on solid surfaces strongly depends on the nature of the protein and surface geometry and physicochemical characteristics of the solid surface. Binding of enzymes directly on the naked surface of bulk materials may result in their denaturation and
loss of activity. However, enzymes adsorbed onto NP surfaces retained their activity due to their biocompatible nature [13, 14]. Various specific and nonspecific interactions such as electrostatic, hydrogen bonding and hydrophobic interactions are involved in the adsorption of protein on NP surfaces which affect the structure and stability of proteins [15, 16].

The present study is focused on the interaction of α-amylase with tin oxide NPs. Enzyme adsorbed NPs were characterized by x-ray diffraction (XRD), Fourier transform infrared spectroscopy (FT-IR) and transmission electron microscopy (TEM). Structural changes in enzymes after binding with NPs were analyzed by UV–vis, fluorescence and circular dichroism (CD) spectroscopy. Kinetic parameters of free and NP-bound enzyme were also evaluated.

2. Materials and methods

2.1. Materials

α-Amylase (from Bacillus amylo liquefaciens, EC.3.2.1.1) and tin tetrachloride pentahydrate (SnCl4·5H2O) were purchased from Sigma–Aldrich Co. (USA). Starch, maltose, glucose and 3,5-dinitro-salicylic acid (DNS) were obtained from SRL Chemicals (Mumbai, India). All other chemicals and reagents were of analytical grade and used without further purification.

All the experiments were performed in 50 mM sodium phosphate buffer, pH 6.0 except where specified. The concentration of protein was determined spectrophotometrically using $E_{280\text{ nm}}^1\% = 2.493$ on a Shimadzu UV-1700 UV–vis Spectrophotometer and alternatively by a protein–dye binding method [17].

2.2. Synthesis of nanoparticles

Tin oxide NPs were synthesized by the sol–gel method according the procedure described Adnan et al with some modifications [18]. The sol solution was prepared by dissolving 3.0 g of SnCl4·5H2O in 100 ml methanol (CH3OH) under vigorous stirring. Aqueous ammonia solution (4.0 ml) was added drop wise to the above solution under similar experimental conditions. The resulting gel was filtered and washed with methanol to remove impurities and dried over 80°C for 5 h in order to remove water. Finally, the dried gel powder was calcined at 400°C for 2 h which resulted into formation of SnO2-NPs.

2.3. Enzyme–nanoparticle interaction studies

A stock solution of protein (5.0 mM) was prepared in 50 mM sodium phosphate buffer, pH 6.0 and diluted with the same buffer as necessary. The α-amylase concentration was kept constant at 24.4 μM, 7.0 μM and 5.0 μM, respectively, for UV–vis, CD and fluorescence measurements, while the concentration of sol–gel synthesized SnO2-NPs was varied from 0.2 to 1.0 mM. The reaction mixture was equilibrated for the optimal incubation time at 25°C for 1 h. Subsequently, UV–vis absorption, fluorescence and CD spectra of the solution were recorded to monitor the interaction between α-amylase and NPs.

2.4. Physical characterization of NPs

The interactions of enzyme with NPs were investigated by FT-IR analysis on an INTERSPEC 2020 (USA) in the range of 400–4000 cm$^{-1}$. The calibration was done with polystyrene film. The samples were injected with a Hamlet 100 μl syringe into the attenuated total reflectance (ATR) box. The syringe was first washed with acetone followed by distilled water. The morphology and size of the SnO2-NPs with and without conjugated α-amylase was observed with a JEOL JEM-2100F transmission electron microscope with an accelerating voltage of 200 kV. Samples for TEM analysis were prepared by drop-coating diluted NP solution on carbon-coated copper grids at normal atmospheric conditions. XRD analysis of NPs was done with a Rigaku Miniflex x-ray diffractometer, using a monochromated x-ray beam with nickel-filtered Cu Kα radiation. The diffraction angle was set at between 20° and 80°. The crystalline size was determined from corresponding x-ray spectral peaks by the Debye–Scherrer formula:

$$D = 0.9\lambda/B \cos \theta \quad (1)$$

where $\lambda$ is the x-ray wavelength (1.54060 Å), $B$ is the full width at half-maximum of SnO2(110) line and $\theta$ is the diffraction angle.

2.5. UV–vis, fluorescence and CD spectroscopy

Absorbance measurements were carried out with Shimadzu UV-1700 spectrometer operated at a resolution of 2.0 nm (Shimadzu, Kyoto, Japan). Enzyme concentration was kept constant at 24.4 μM in each reaction mixture while the NP concentration varied from 0.2 to 1.0 mM. Unless otherwise stated, the UV–vis spectra were recorded in a wavelength range of 250–350 nm. For sample measurements, the baseline was always set with a relevant blank. The final spectrum is an average of three independent measurements.

Fluorescence analyses were performed on a Shimadzu Spectrofluorophotometer (RF-5301) equipped with a DR-3 data recorder. The fluorescence spectra were measured at 25 ± 0.10°C with a 1.0 cm path length. Both excitation and emission slits were set at 5.0 nm. The intrinsic fluorescence of a protein sample was measured by exciting at 280 nm and emission spectra were recorded in the range of 300–500 nm.

The CD measurements were carried out on Jasco Spectropolarimeter (J-720). The instrument was calibrated with d-10-camphorsulfonic acid. All the CD measurements were carried out at 25°C using a thermostatically controlled cell holder attached to a Neslab RTE-110 water bath with an accuracy of 0.01°C. Far-UV CD spectra were measured at a protein concentration of 7.0 μM. The path length of the cell was 1.0 mm.

Mean residue ellipticity (MRE) = $\theta_{obs} / (10 \times n \times l \times c_p) \quad (2)$

where $\theta_{obs}$ is the CD in millidegrees, $N$ is the number of amino acid residues (483), $l$ is the path length of the cell and $c_p$ is the mole fraction. The % α-helical content was calculated from the MRE values at 222 nm using the following equation as described by Chen et al [19].

$$\% \alpha \text{-helix} = (\text{MRE}_{222 \text{ nm}} - 2340/30300) \times 100. \quad (3)$$
Figure 1. XRD pattern of SnO₂-NPs mediated in methanol. The x-ray diffraction pattern of SnO₂-NPs was recorded at room temperature using a Rigaku Miniflex x-ray diffractometer with Cu Kα radiation ($\lambda = 1.540 \, \text{Å}$) in $2\theta$ ranging from $20^\circ$ to $80^\circ$.

2.6. Assay of $\alpha$-amylase activity

$\alpha$-Amylase activity was determined according to the procedure of Bernfeld et al with some modifications [20]. Appropriately diluted enzyme was added to 500 $\mu$l of gelatinized starch (1%) solution. After incubation at 50°C for 10 min in a shaking water bath, the reaction was stopped by adding 1.0 ml of DNS reagent. The tubes were kept in boiling water for 5 min to develop color. After cooling, 1.0 ml distilled water was added and finally absorbance was recorded at 540 nm.

One unit (1.0 U) of $\alpha$-amylase activity is defined as the amount of enzyme that liberates 1.0 mM of maltose min$^{-1}$ under standard assay conditions. A standard curve of absorbance against amount of maltose was prepared to calculate the amount of maltose released during the assay.

2.7. Statistical analysis

Each value represents the mean for three independent experiments performed in duplicate, with average SD of <5%. Data were analyzed by one-way ANOVA and $p$-values < 0.05 were considered statistically significant.

3. Results and discussion

3.1. Biophysical characterization of free and enzyme bound SnO₂-NPs

Among various methods for chemical synthesis of nanomaterials, the sol–gel process offered several advantages like lower processing temperature, better homogeneity, controlled stoichiometry and flexibility of forming nanoparticles. Metal oxide NPs have attracted much attention owing to their remarkable physicochemical properties like high surface area, high electrical conductivity, good chemical stability and significant mechanical strength, therefore these NPs have been widely applied for the immobilization of wide range of biomolecules [21–23].

Figure 1 shows XRD patterns of SnO₂-NPs calcined at 400°C for 2 h using methanol as a solvent. Samples exhibited a wide diffraction peak at the same position, which can be indexed to the tetragonal structure of SnO₂-NPs (JCPDS no. 88-0287). The XRD diffractogram showed broad peaks at $2\theta$ of 27°, 34°, and 53° which correspond to the diffraction of 110, 101 and 211 planes of SnO₂ crystal, respectively. The size of the nanocrystals was calculated by using Scherrer’s equation (1). Based on the full width at half-maximum of the 110 diffraction plane, the average crystallite size of SnO₂-NPs was found to be 11.2 nm.

The binding of $\alpha$-amylase with SnO₂-NPs was confirmed by FT-IR analysis. Figure 2 shows the IR spectra of NPs, $\alpha$-amylase and enzyme-bound NPs. The intense and broad peak
Figure 3. TEM images of SnO\textsubscript{2}-NPs (A) and enzyme-bound SnO\textsubscript{2}-NPs (B) were recorded with a JEOL JEM-2100F transmission electron microscope with an accelerating voltage of 200 kV.

Figure 4. UV–vis absorption spectra of \(\alpha\)-amylase in the presence of SnO\textsubscript{2}-NPs. A fixed concentration of \(\alpha\)-amylase (24.4 \(\mu\)M) was mixed with varying concentrations of SnO\textsubscript{2}-NPs and their spectra were recorded in the UV–visible region.

TEM images of NPs before and after conjugation with enzyme further support the binding of \(\alpha\)-amylase with SnO\textsubscript{2}-NPs. Figure 3(A) shows an electron micrograph of free NPs at a magnification of 50000\(\times\), and it is revealed that nearly spherical SnO\textsubscript{2}-NPs with a mean diameter of 25 nm are distributed uniformly with some aggregation due to overlapping of some bigger particles with the smaller particles. The NPs remain spherical after adsorption of enzyme but the mean diameter increased from 25 to 32 nm (figure 3(B)). This suggests that the conjugation process did not change the morphology of the NPs while the size has increased. This might be due to the adsorption of a significant amount of enzyme on the NP surfaces.

3.2. UV–vis and fluorescence spectra of native and NP bound \(\alpha\)-amylase

UV–vis spectroscopy is an effective and simple tool to explore structural change in protein molecules [27, 28]. The UV–vis spectra obtained by the NP titration experiment demonstrate that the protein microenvironment at pH 6.0 was perturbed (figure 4). Upon addition of an increasing concentration of SnO\textsubscript{2}-NPs a consecutive regular increase in the absorbance maxima of protein at 280 nm is shown. This might be due to the partial or full adsorption of enzyme on the surface of the NPs. These results indicate that there is an interaction between SnO\textsubscript{2}-NPs and \(\alpha\)-amylase through ground state complex formation [29].

The conformational changes of \(\alpha\)-amylase in the presence of SnO\textsubscript{2}-NPs were evaluated by measurement of the intrinsic fluorescence intensity of tryptophan residues of protein (figure 5). NPs having diameter in the range 30–70 nm efficiently quench \(\alpha\)-amylase, as evidenced by the progressive decrease in the emission maximum intensity. This quenching effect indicated that SnO\textsubscript{2}-NPs strongly interact with the chromophore residues of protein (figure 6). The tendency of red-shift to occur in the protein upon binding of NPs suggests that the interaction was specific and involved only a
Figure 5. Fluorescence spectra of α-amylase were recorded in the range 300–500 nm in the presence of varying concentrations of SnO2-NPs (from a → e are 0.0, 0.2, 0.4, 0.6 and 1.0 mM, respectively). Inset: change in fluorescence intensity (FI) at 340 nm with NPs as monitored by intrinsic fluorescence by exciting protein at 280 nm.

Figure 6. Stern–Volmer plots of NP quenching for α-amylase. The enzyme concentration was kept constant (5.0 μM) while the NP concentration increased from 0.0 to 1.0 mM. Values shown are the ratios of fluorescence in the absence of NPs (Fo) to the fluorescence at that concentration of quencher (F).

Figure 7. Far-UV CD spectra of α-amylase at different NP concentrations. Spectra were recorded in the wavelength region 200–250 nm. A constant enzyme concentration 7.0 μM was used in each set of experiments while the NP concentration was 0.0 to 1.0 mM. Inset: change in MRE value of α-amylase at 222 nm with increasing concentration of NPs. The NP concentration was 0.0 to 1.0 mM, while the protein concentration was 7.0 μM.

Table 1. Effect of SnO2-NP binding on the secondary structure of α-amylase. [Note: the MRE value in deg cm2 dmol−1 is calculated by equation (2). The % α-helix content was calculated by the Chen et al method (equation (3)) [19]. The % β-sheet content was calculated by online K2d software [38].]

<table>
<thead>
<tr>
<th>MRE222 nm (%)</th>
<th>α-helix (%)</th>
<th>β-sheet (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native α-amylase</td>
<td>−12 533</td>
<td>49</td>
</tr>
<tr>
<td>α-amylase +0.2 mM SnO2-NPs</td>
<td>−13 625</td>
<td>52</td>
</tr>
<tr>
<td>α-amylase +0.6 mM SnO2-NPs</td>
<td>−13 975</td>
<td>53</td>
</tr>
<tr>
<td>α-amylase +1.0 mM SnO2-NPs</td>
<td>−15 203</td>
<td>57</td>
</tr>
</tbody>
</table>

3.3. Effect of NP binding on the secondary structure of the enzyme

CD is a powerful analytical tool for studying the interaction of proteins with other molecules and determining the protein conformation in solution or adsorbed onto other molecules [34]. Figure 7 shows CD spectra of α-amylase recorded in the wavelength range of 200 to 250 nm; the results are expressed as mean residual ellipticity in millidegrees. The result showed that a relatively more compact structure of protein was obtained in presence of NPs, as shown by a more negative spectrum in far-UV CD (figure 7). The CD spectra displayed characteristic peaks (intensive positive peak at around 190 nm and two negative double humped peaks at 208 and 222 nm) of a high α-helical content in native enzyme [35]. In the presence of NPs, an increase in the α-helix was observed (table 1), which suggested the interaction between SnO2-NPs and α-amylase. The increased percentage of α-helical protein structure showed that NPs bound to the amino acid residues of the polypeptide chain of α-amylase, thus increasing the hydrogen bonding networks.

3.4. Kinetic studies

Catalytic properties of free and NP-bound enzymes were evaluated with soluble starch as a substrate. The Michaelis–
corresponds to 1 of a variable amount of starch. The intercept on the $y$-axis corresponds to $1/V_{max}$ and the intercept on the $x$-axis to $-1/K_m$. Data presented are an average of values ±SD of $n = 3$ experiments.

Figure 8. Lineweaver–Burke plot for free $\alpha$-amylase (open square) and NP-bound $\alpha$-amylase (filled square). All the samples were assayed in 50 mM phosphate buffer, pH 6.0 at 50°C in the presence of a variable amount of starch. The intercept on the $y$-axis corresponds to $1/V_{max}$ and the intercept on the $x$-axis to $-1/K_m$. The intercept on the y-axis corresponds to $1/V_{max}$ and the intercept on the x-axis to $-1/K_m$. Data presented are an average of values ±SD of $n = 3$ experiments.

Menten constant ($K_m$) and maximum activity ($V_{max}$) were determined at pH 6.0 and 50°C (figure 8). The values of $K_m$ in the present enzymatic assay were found to be 26.96 and 28.45 mg ml$^{-1}$ for the free and immobilized $\alpha$-amylase, respectively. The smaller value of $K_m$ in the case of free enzyme indicates that free enzyme has a higher affinity for the substrate. The value of $V_{max}$ obtained for the immobilized $\alpha$-amylase was 3.116 mg ml$^{-1}$ min$^{-1}$ which is one order of magnitude lower than that of free enzyme (4.173 mg ml$^{-1}$ min$^{-1}$). The increasing $K_m$ value of NP-bound enzyme is might be due to structural changes of the enzyme in the presence of NPs brought about by a higher rigidity and compactness in protein structure and diffusion limitations of the substrate [36, 37]. The secondary and tertiary structure of the enzyme is known to play an important role in the enzyme activity, as the rearrangement and conformational changes in these structures may result in the enhancement or suppression of enzyme activity.

4. Conclusions

Enzyme dysfunction is related to many diseases. It is desirable to be able to regulate enzyme conformation and function. NPs can be selected to specifically bind enzymes and control their functions after surface modifications, providing a promising strategy for therapy. In this study, SnO$_2$-NPs were synthesized using a simple sol–gel method and were characterized by XRD, FT-IR and TEM analysis. The interaction between $\alpha$-amylase and NPs was evaluated by UV–vis, fluorescence and CD spectroscopic studies. The analysis of CD spectra indicated that the $\alpha$-helical content increases considerably in the presence of NPs. In comparison with native enzyme, NP-bound enzyme revealed significant improvement in the stability of the $\alpha$-amylase which could henceforth contribute to its better use in various analytical, diagnostic and clinical applications. Furthermore, the reactors containing such SnO$_2$-NP adsorbed enzyme could be exploited for the hydrolysis of starch in batch systems as well as in continuous systems in the near future.

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Abstract  Enzymes play a pivotal role in catalyzing diverse reactions. However, their instability upon repetitive/prolonged use, as well as their inhibition by high substrates and product concentration, remains an area of concern. In this study, porcine pancreatic α-amylase was immobilized on magnetic Fe$_2$O$_3$ nanoparticles (Fe$_2$O$_3$-NPs) in order to hydrolyze starch. The magnetic nanoparticle bound enzymes retained 94% of their initial enzyme activity. X-ray diffraction and atomic force microscopy analyses showed that the prepared matrix had advantageous microenvironment and a large surface area for binding significant amounts of protein. Functional groups present in enzyme and support were monitored by Fourier transform infrared spectroscopy. Immobilized enzyme exhibited lowered pH optimum (pH 6.0) to a greater degree than its soluble counterpart (pH 7.0). Optimum temperature for the immobilized enzyme shifted towards higher temperatures. The immobilized enzyme was significantly more resistant to inactivation caused by various metal ions and chemical denaturants. Immobilized α-amylase hydrolyzed 92% starch in a batch process, after 8 h at 40°C; while the free enzyme could hydrolyze only 73% starch under similar experimental conditions. A reusability experiment demonstrated that the immobilized enzyme retained 83% of its original activity even after its 8$^{th}$ repeated use.

Keywords: α-Amylase, immobilization, nanoparticles, stabilization, starch hydrolysis

1. Introduction

The enzymes α-amylases (E.C. 3.2.1.1) catalyze the hydrolysis of α-1,4 glycosidic bonds present in starch, glycogen, and other related carbohydrates [1]. They are present in plants, animals, and microorganisms and have extensive applications in medicine, textiles, fermentation, and the food industry [2]. The exploitation of soluble enzymes in industries is limited due to product inhibition, instability, non-reusability, and difficult recovery from a reaction system [3]. In order to overcome these limitations, enzyme immobilization has been considered one of the best alternatives to using enzymes on a large scale, in industrial and environmental applications [4]. Immobilization also protects enzymes from denaturation and helps to retain them in biochemical reactors in order to further catalyze the subsequent feed and to offer more economical use of biocatalysts in industry, waste water treatment, and the development of bioprocess monitoring devices, such as biosensors [5,6].

In the recent past, nanosized materials have been widely employed for enzyme immobilization. Due to the large surface area of nanosized materials, they provide superior loading capacity and low mass transfer resistance [7,8]. The magnetic nanoparticles have now been used in conjunction with biological materials, such as proteins, enzymes, and nucleic acids, as they result in complete and easy recovery of these materials from reaction systems, thereby reducing the operational costs of the processes [9-
In the present work, an attempt has been made to immobilize α-amylase on Fe₂O₃-NPs. The immobilized enzyme was characterized by X-ray diffraction (XRD), Fourier transform-infrared (FT-IR) spectroscopy, and atomic force microscopy (AFM). Thermal behavior of both soluble and immobilized α-amylase was studied by thermo-gravimetric (TGA) and differential thermal analysis (DTA). Effects of various metal ions and denaturing agents on the activity of soluble and immobilized α-amylase were monitored. Hydrolysis of starch by soluble and immobilized enzymes was performed in batch processes. The reusability of the immobilized enzyme was also examined.

2. Materials and Methods

2.1. Materials
Materials used included α-Amylase (Porcine pancreas), starch, maltose, glucose and 3,5 dinitro-salicylic acid (DNS); these were purchased from SRL Chemicals (Mumbai, India). Ferric nitrate, mono hydrated citric acid, and metal ions were obtained from Sigma-Aldrich Co. (USA). All other chemicals and reagents were of analytical grade and were used without further purification.

2.2. Synthesis of Fe₂O₃-NP by sol-gel method
Ferric oxide nanoparticles (Fe₂O₃-NPs) were synthesized according to the procedure described by Raming et al, with several modifications [12]. Ferric nitrate (200 mL, 0.1 M) was used as a precursor solution and was gelated with 800 mL of mono hydrated citric acid (0.05 ~ 0.2 M); as ligand and triple distilled water were used as solvents. Ferric nitrate was added, dropwise, to the citric acid solution; it was vigorously shaken. The solution was heated, at 70°C, with continuous stirring until the gel was formed and the water was completely evaporated. The dried gel was then annealed at 100°C for 16 h in order to solidify the gel, which was grounded for 30 min. The formed Fe₂O₃-NP was used for further studies.

2.3. XRD analysis of Fe₂O₃-NP and Fe₂O₃-NP adsorbed α-amylase
The crystal structure of Fe₂O₃-NP and Fe₂O₃-NP, which adsorbed the α-amylase, was obtained with an X-ray diffractometer (Rigaku Miniflex X-ray diffractometer), using a monochromatized X-ray beam with nickel-filtered Cu Kα radiation. A continuous scan mode was used to collect 2θ data from 5° to 80°. The particle size (D) of the samples was determined by Scherer’s formula as:

\[ D = \frac{0.9 \lambda}{B \cos \theta} \]

where \( \lambda \) is the X-ray wavelength (1.54060 Å), B is the full width at half-maximum of Fe₂O₃ (400) line, and \( \theta \) is the diffraction angle.

2.4. Immobilization of α-amylase
Fe₂O₃-NP (40 mg) was added to α-amylase (2,083 U), and this mixture was stirred overnight in 0.1 M sodium phosphate buffer, pH 7.0 at 4°C. The enzyme bound on nanosupport was collected by centrifugation at 5,000 × g for 10 min at 4°C. The unbound enzyme was removed by washing it three times, with a 0.1 M sodium phosphate buffer, pH 7.0. The immobilized enzyme was stored in an assay buffer at 4°C for further use.

2.5. AFM analysis
The tapping mode AFM experiment of free nanoparticles and enzyme bound nanoparticles was performed using commercial etched silicon tips as an AFM probe, with a characteristic resonance frequency of ca. 300 Hz (RTESP, Veeco). The samples were placed, dropwise, on a mica wafer, air dried at room temperature for 12 h, and the images were recorded with a Veeco Innova nanoscope II AFM. Microscopic scans were carried out on several surface positions in order to check the surface uniformity.

2.6. FT-IR analysis of enzyme, Fe₂O₃-NP and Fe₂O₃-NP adsorbed α-amylase
The FT-IR spectra of the enzyme, Fe₂O₃-NP and Fe₂O₃-NP, adsorbed α-amylase; these were recorded through the potassium bromide pellet method on INTERSPEC 2020 (USA) in the range of 400 ~ 4,000/cm. The calibration was performed by polystyrene film. Samples were injected with a Hamlet 100 µL syringe in an ATR box. The syringe was washed with acetone; this was followed by distilled water. FT-IR analysis was performed in order to examine the functional groups present in the enzyme and support.

2.7. Thermogravimetric and differential thermal analysis
TGA was performed using a Mettler-3000 thermal analyzer with a 2.0 mg sample, with heating rate of 10°C/min in N₂ atmosphere. DTA analysis was also carried out in a similar heating range, using TA-DSC, Q 200 (USA).

2.8. Effect of pH and temperature
Enzyme activity of soluble and immobilized α-amylase (51.0 U) was assayed in the buffers of different pHs (4.0 ~ 8.0). The buffers used were sodium acetate (pH 4.0, 5.0), sodium phosphate (pH 6.0, 7.0), and Tris-HCl (pH 8.0). The molarity of each buffer was 0.1 M. The activity at pH 7.0 and pH 6.0 were taken as control (100%) for the calculation of the remaining percentage of activity for the soluble and immobilized enzymes, respectively.
The activity of soluble and immobilized α-amylase (51.0 U) was measured at various temperatures (20 ~ 80°C). Enzyme activity at 40 and 50°C was taken as control (100%) for the calculation of the remaining percentage of activity for the soluble and immobilized enzymes, respectively.

2.9. Effect of metal ions on activity of soluble and immobilized α-amylase
Soluble and immobilized α-amylase (51.0 U) was independently incubated, with a 5.0 mM solution of metal ions (chlorides of Na⁺, K⁺, Mg²⁺, Cu²⁺, Co²⁺, Ba²⁺, Zn²⁺, Ca²⁺, and Fe³⁺); their activity was determined in a 0.1 M sodium phosphate buffer, pH 7.0 at 40°C. The activity of enzymes in the absence of metal ions was taken as control (100%) for the calculation of the remaining percentage of the activity for the soluble and immobilized enzymes.

2.10. Effect of urea on activity of soluble and immobilized α-amylase
Soluble and immobilized α-amylase (51.0 U) was incubated with a 4.0 M urea for different time intervals. The enzyme activity without urea was taken as control (100%) for the calculation of the remaining percentage activity for the soluble and immobilized enzymes.

2.11. Reusability of immobilized α-amylase
Immobilized α-amylase (51.0 U) was taken in triplicates in order to assay its activity. After each assay, the immobilized enzyme was taken out from assay tubes and stored in a 0.1 M sodium phosphate buffer, pH 7.0 at 4°C. This procedure was repeated for eight consecutive days. The activity determined on first day was considered as the control (100%) for the calculation of the remaining percentage activity after each use.

2.12. Hydrolysis of starch in batch process
Starch solution (1.0%, w/v) was incubated independently, with soluble and immobilized α-amylase (4,850 U) at 40°C with constant stirring for 8 h. The aliquots (500 µL) were taken at indicated time intervals, hydrolysis of starch and the formation of maltose was assayed by the DNS method [13].

The activity of α-amylase was assayed by the DNS method [13], using a 1.0% soluble starch as a substrate. The enzyme in a 0.1 M sodium phosphate buffer, pH 7.0 (250 µL) and starch (250 µL), was incubated for 30 min at 40°C. The reaction was stopped by adding 1.5 mL DNS reagent and heating the solution in boiling water for 5 min. DNS is a coloured reagent, and the reducing groups released from starch by α-amylase action were measured by the reduction of this reagent. After cooling, 1.5 mL of distilled water was added and, finally, absorbance was recorded spectrophotometrically, at 540 nm.

One unit (1.0 U) of α-amylase activity is defined as the amount of enzyme that liberates 1.0 µM of maltose per min, under standard assay conditions. A standard curve of absorbance against the amount of maltose was prepared to calculate the amount of maltose released during assay.

2.14. Estimation of protein
Protein concentration was determined by a dye binding method [14]. Bovine serum albumin was used as a standard protein.

2.15. Statistical analysis
Each value represents the mean for three independent experiments performed in duplicates, with average SDs of < 5%. Data were analyzed by one-way ANOVA, and p-values of < 0.05 were considered statistically significant.

3. Results and Discussion

3.1. Immobilization efficiency of α-amylase
Several methods have previously been employed for the immobilization of α-amylase. Among these methods, physical adsorption onto insoluble materials has been considered one of the preferred choices, due to its simplicity and regeneration of support. Moreover, this method of enzyme immobilization does not require pre-activation steps for support [15].

Table 1 demonstrates the immobilization efficiency of α-amylase. Fe₃O₄-NP adsorbed α-amylase preserved 94% of its initial enzyme activity which was higher compared to previous reported immobilization studies [16,17].

3.2. XRD analysis
The diffraction patterns of Fe$_2$O$_3$-NP and Fe$_2$O$_3$-NP adsorbed $\alpha$-amylase are represented in Fig. 1. The results showed that nanoparticles have hexagonal crystal symmetry with an average crystalline size of 26.90 nm (Fig. 1A), which was decreased to 24.25 nm (Fig. 1B) after enzyme adsorption. The intensity peaks of the NPs were masked after immobilization as the enzyme covered significant parts of the NP surfaces [18]. The binding of magnetic nanoparticles to bioactive substances involved a number of interactions, including interactions between organic ligands and those between amino acid side chains of protein and metal centers. This might be accredited better enzyme-substrate interactions on immobilization, with the availability of larger surface areas on nanoparticle and possible structural modulation or better exposure of the active site [19]. Moreover, such coupling of biomolecular entities with nanoparticles provides enhanced thermal stability of the enzyme, allowing its use in various analytical, biotechnological, and industrial applications [20].

3.3. AFM and FT-IR analysis

Morphological information presents a physical picture of how $\alpha$-amylase molecules are assembled on nanoparticle surfaces (Fig. 2). The roughness shown in these images can also be analyzed and related to the pattern of immobilization. We used the peak-to-valley distance in these images as an indicator of surface roughness. As shown (Fig. 2A), the nanoparticle surface is relatively smooth and has small peak-to-valley distance. Once an enzyme molecule is adsorbed (Fig. 2B), there was a significant increment in peak-to-valley distance. Thus, we can conclude that nanoparticle surfaces were fully covered with enzymes after immobilization, which corresponds to high enzyme loading [21,22].

Fig. 3 shows FT-IR spectra of Fe$_2$O$_3$-NP, $\alpha$-amylase and Fe$_2$O$_3$-NP adsorbed $\alpha$-amylase. FT-IR spectroscopy revealed that the formation of hydrogen bonds between amino groups and water may result in tremendous water solubility, which makes them ideal for enzyme immobilization [23]. A sharp peak was observed at 538/cm (Figs. 3A and 3C), which is characteristic of Fe-O vibrations. The amide I peak appeared between 1,600 and 1,700/cm due to C=O stretching vibrations of the protein backbone. This peak is sensitive to short range, imposed by distinct hydrogen bonding arrangements in the secondary structure of the proteins [24]. The peak at 1,108/cm (Fig. 3B) is most likely reflecting an imidazole ring of the histidine side chain. The interactions of NPs with C-N groups of enzymes were also evident with the shifting of the amide A band from 3,341/cm toward a higher frequency, 3,349/cm (Figs. 3B and 3C) [25].

3.4. TGA and DTA analysis

TGA and DTA results of free and Fe$_2$O$_3$-NP bound $\alpha$-amylase are shown in Fig. 4. The TGA spectra of enzyme showed a 5% loss in weight at 200°C, followed by a sharp
Immobilization of Porcine Pancreatic α-amylase on Magnetic Fe₂O₃ Nanoparticles: Applications to the Hydrolysis of Starch

peak at 400°C; this indicates a weight loss of over 65% (Fig. 4A). However, Fe₂O₃-NP adsorbed α-amylase exhibited very slight (5%) weight loss (Fig. 4C) under a similar temperature range. The significant weight loss in the free enzyme might be due to the removal of free and bound water and solvent.

The DTA curve showed that thermal decomposition of enzymes begins at 180°C (Fig. 4B) while Fe₂O₃-NP adsorbed α-amylase retained a significantly higher stability of up to 300°C (Fig. 4D). This showed that the immobilized enzyme preparation can be employed at a higher temperature for various applications.

3.5. Effect of pH and temperature

The pH optimum for soluble enzymes was found at pH 7.0, which shifted to pH 6.0 upon immobilization (Fig. 5). A shift in pH optimum upon immobilization depends on enzyme microenvironment as well as the structure and charge of the matrix [26]. The change in pH optimum for immobilized α-amylase has also been reported by several earlier investigators [27,28].

The temperature optimum for α-amylase shifted from 40 to 50°C after immobilization (Fig. 6). However, as the temperature increased, activity of free enzymes decreased more rapidly, compared with the immobilized counterpart. The inability of a free enzyme to enhance its thermal stability is one of the major limitations for its application in continuous reactors. An increase in temperature optimum of immobilized enzymes revealed that the enzyme might be more rigid to structural changes induced by heat [29,30].
3.6. Effect of metal ions

Our studies showed that the activity of immobilized enzymes was less affected in the presence of various metal ions, compared with free enzymes (Table 2). However, we observed that Cu$^{2+}$ caused maximum inhibition in the activity of both free and immobilized enzymes [31]. The relative activity of the immobilized $\alpha$-amylase was higher than that of the soluble enzyme in the presence of NaCl, KCl, CaCl$_2$, and CoCl$_2$ [32].

<table>
<thead>
<tr>
<th>Metal ions (5.0 mM)</th>
<th>Remaining activity (%)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Soluble enzyme</td>
</tr>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>NaCl</td>
<td>106.9 ± 2.90</td>
</tr>
<tr>
<td>KCl</td>
<td>103.6 ± 1.11</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>96.3 ± 0.97</td>
</tr>
<tr>
<td>CuCl$_2$</td>
<td>21.7 ± 2.00</td>
</tr>
<tr>
<td>CoCl$_2$</td>
<td>81.4 ± 1.94</td>
</tr>
<tr>
<td>BaCl$_2$</td>
<td>112.2 ± 2.54</td>
</tr>
<tr>
<td>ZnCl$_2$</td>
<td>53.0 ± 1.27</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>97.5 ± 1.11</td>
</tr>
<tr>
<td>FeCl$_3$</td>
<td>63.1 ± 2.46</td>
</tr>
</tbody>
</table>

$^a$The percent activity of an enzyme compared with the activity without the presence of metal ions.

3.7. Effect of urea

The immobilized $\alpha$-amylase was more stable than the soluble enzyme upon exposure to 4.0 M urea for a longer duration (Fig. 7). The result showed an 81% loss in enzyme activity for soluble enzymes, while the immobilized enzyme retained more than 40% with identical exposure. Earlier studies have indicated that proteins become unfolded due to direct interaction of urea molecules with the peptide backbone, via hydrogen bonding or hydrophobic interactions [33]. Molecular crowding/confinement theory has predicted that Fe$_3$O$_7$-NPs provide a beneficial confined space that resists conformational changes to the immobilized enzyme, even at higher concentration of urea [34].
Table 3. Hydrolysis of starch by soluble and immobilized α-amylase in batch processes at 40°C

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Remaining activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Soluble</td>
</tr>
<tr>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>30</td>
<td>14 ± 0.69</td>
</tr>
<tr>
<td>60</td>
<td>20 ± 2.11</td>
</tr>
<tr>
<td>90</td>
<td>25 ± 1.34</td>
</tr>
<tr>
<td>120</td>
<td>36 ± 2.08</td>
</tr>
<tr>
<td>180</td>
<td>47 ± 1.22</td>
</tr>
<tr>
<td>240</td>
<td>61 ± 1.34</td>
</tr>
<tr>
<td>300</td>
<td>67 ± 0.98</td>
</tr>
<tr>
<td>360</td>
<td>73 ± 1.75</td>
</tr>
<tr>
<td>420</td>
<td>73 ± 1.11</td>
</tr>
<tr>
<td>480</td>
<td>73 ± 2.43</td>
</tr>
</tbody>
</table>

ND: Not determined.

3.8. Hydrolysis of starch in batch processes by soluble and immobilized α-amylase

Table 3 describes the hydrolysis of starch by the soluble and immobilized α-amylase for various time intervals at 40°C. The soluble and immobilized enzymes hydrolyzed 61 and 68% starch after 4 h of incubation, respectively. Additionally, the maximum hydrolysis of starch, 74% by the free enzyme, was attained after 8 h, while the immobilized enzyme hydrolyzed a higher concentration of starch (92%) under similar incubation time. This was due to the fact that the soluble enzyme was more accessible to the substrate for first few hours but, after a prolonged period of time, the rate of hydrolysis decreased more quickly. This might be due to the enzyme unfolding or the inhibition of enzyme activity by its own product [35,36]. Satish et al. reported similar results, where starch was hydrolyzed by α-amylase immobilized on super porous cellulose beads [37].

3.9. Reusability

Enzymes are quite expensive products, so reusability and operational stability are important criteria for enzyme application at the industrial level. Immobilized α-amylase showed 83% residual activity even after the 8th consecutive use (Fig. 8). The significant retention in immobilized enzyme activity on repeated uses might be due to the multipoint non-covalent attachment of the enzyme to nanosupport.

4. Conclusion

In this study, α-amylase was immobilized onto Fe₂O₃-NPs by a simple adsorption mechanism with excellent catalytic efficiency. Immobilized α-amylase was found to be stable against various types of physical and chemical denaturants. The increased temperature optimum of immobilized enzyme results in several benefits; higher temperature decreased the viscosity of both substrate and product, increased the reaction rate with less operational time, and minimized the risk of microbial contamination. Reusability experiments further supported that the adsorbed enzyme did not detach from the nanosupport with repeated use and, therefore, such preparations could be considered for the continuous hydrolysis of starch for longer durations, even at higher temperatures. Thus, such immobilized enzymes could prove useful for the efficient conversion of starch in continuous reactors at the industrial level.

Acknowledgement

Aligarh Muslim University, Aligarh is gratefully acknowledged for providing UGC, scholarship to one of us (M. J. Khan).

Nomenclature

AFM : Atomic force microscopy
DNS : 3,5 Dinitro-salicylic acid
DTA : Differential thermal analysis
FT-IR : Fourier transform-infrared spectroscopy
NPs : Nanoparticles
TGA : Thermo-gravimetric analysis
XRD : X-ray diffraction
Application of Immobilized *Ipomoea batata* β Amylase in the Saccharification of Starch

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**Abstract**  
Present study demonstrates the immobilization of acetone fractionated *Ipomoea batata* (sweet potato) β amylase on an inorganic support, Celite-545 by simple adsorption mechanism. The adsorbed enzyme exhibited an activity yield of 244 U g⁻¹ of the matrix with effectiveness factor η 0.83. Interaction between Celite-545 and enzyme was confirmed by fourier transform infrared spectroscopy and atomic force microscopy. The binding efficiency of enzyme to the support was analyzed by eluting it with 1.0 M NaCl. Both soluble and immobilized β amylase exhibited same pH optima while temperature optimum of immobilized enzyme was shifted from 50°C to 60°C. The immobilized β amylase preparation was superior to the free enzyme in hydrolyzing starch in a batch process: it hydrolyzed starch to 88% and 96% at 40°C and 50°C, respectively whereas soluble enzyme hydrolyzed only 83% and 80% of starch under similar experimental conditions. Immobilized β amylase retained 84% of its original activity after 30 days storage at 4°C, while the soluble enzyme showed only 41% of the initial activity under identical conditions. Immobilized β amylase retained 79% activity even after its 7th repeated use.

**Keywords**: β amylase; Celite-545; immobilization; sweet potato; batch process

**Abbreviations**: AFM, atomic force microscopy; DNS, 3,5 dinitro-salicylic acid; FT-IR, fourier transform infrared spectroscopy

**INTRODUCTION**

β Amylase (E.C. 3.2.1.2) is an exoamylase which is widely distributed in higher plants and microorganisms. Among plant sources sweet potato is thought to be a promising source of β amylase with fair thermostability [1]. It catalyzes the hydrolysis of α-1, 4 glycosidic linkages at non reducing end of starch and related carbohydrates [2]. The biotechnological application of β amylase includes the production of maltose and high maltose syrups. Maltose is widely applied in food and pharmaceutical industries, since its properties are represented by mild sweetness, good thermal stability and low viscosity in solution [3,4].

An effective application of free enzymes is hindered due to several drawbacks like thermal instability, rapid loss of catalytic activity during operation and storage period and sensitivity to numerous denaturing agents [5,6]. Such drawbacks can be circumvented by using enzymes in their immobilized form [7-9].

Several methods including entrapment, adsorption, encapsulation in membranes, chemical cross linking by using bifunctional or multifunctional reagents and bioaffinity based procedures have been employed previously for enzyme immobilization [10,11]. Among these methods, adsorption of enzymes on particulate carriers is one of the simplest and cost effective immobilization techniques [12].

Celite-545 has recently been used as an immobilization matrix owing its inexpensive nature and other desirable physical properties like large surface area with high enzyme loading and enormous porosity which increase enzyme accessibility to the substrate. Moreover, it shows higher thermal and chemical stabilities with greater resistance to microbial degradation [13,14].

In this study, *I. batata* β amylase has been immobilized on an inexpensive support, Celite-545. The immobilized enzyme preparations were characterized by fourier transform infrared spectroscopy (FT-IR) and atomic force microscopy (AFM) in order to monitor the functional groups and surface topography, respectively. The stability of soluble and immobilized β amylase has been investigated against several physical and chemical denaturants. Hydrolysis of starch in batch processes at varying temperatures by soluble and immobilized enzyme was also evaluated.

**MATERIALS AND METHODS**

**Materials**

Celite-545 (20-45 µ mesh) was obtained from Serva Labs (Heidelberg, Germany). Starch, maltose, glucose and DNS were purchased from SRL Chemicals (Mumbai, India). Acetone was obtained from Merck (Darmstadt, Germany). *I. batata* was purchased from local market. Other chemicals and reagents employed were of analytical grade and used without any further purification.
Extraction and partial purification of β amylase from sweet potato

Mature, healthy Ipomoea batata roots (200 g) were washed thoroughly with distilled H_2O, cut into small pieces and homogenized in 100 ml of 0.1 M sodium phosphate buffer, pH 6.0. The suspension was filtered through 4 layers of cheesecloth. Filtrate thus obtained was centrifuged at 10 000 x g for 30 min at 4°C. The crude extract was initially fractioned by 50% (v/v) chilled acetone saturation. This solution was continuously stirred overnight at 4°C for complete precipitation of proteins. After centrifugation at 10 000 x g for 30 min, precipitated pellets were collected and resuspended in two-pellet volume of cold buffer. The solution was dialyzed against 0.1 M phosphate buffer of pH 6.0 for overnight. Undissolved particles were removed by centrifugation and the clear solution was stored in assay buffer at 4°C for further use.

Adsorption of β amylase on Celite-545

Celite-545 (1.0 g) was suspended in 50 ml of 0.1 M phosphate buffer and stirred for 1 h at room temperature. The fine particles present in suspension were removed by decantation and similar procedure was repeated thrice [15]. The binding of β amylase on support was carried out by incubating 1232 U of enzyme g⁻¹ of Celite-545 and this mixture was stirred overnight in 0.1 M sodium phosphate buffer, pH 6.0 at 4°C. The enzyme bound on Celite-545 was collected by centrifugation at 3 000 x g for 15 min at 4°C. Unbound enzyme was removed by washing thrice with buffer and immobilized enzyme was stored in assay buffer at 4°C for further use.

FT-IR spectra of Celite-545 and Celite-545 adsorbed β amylase

FT-IR spectra of Celite-545 and Celite-545 adsorbed β amylase were recorded by the potassium bromide pellet method on INTERSPEC 2020 (USA) in the range of 400-4000 cm⁻¹. The calibration was done by polystyrene film. The samples were injected by Hammet 100 µl syringe in ATR box. The syringe was first washed with acetone followed by distilled water. FT-IR analysis was done to examine the functional groups present in enzyme and support.

AFM analysis

Tapping mode AFM experiments of Celite-545 and Celite-545 adsorbed β amylase were performed using commercial etched silicon tips as AFM probes with typical resonance frequency of ca. 300 Hz (RTESP, Vecco). The samples were placed drop wise on a mica wafer, air dried at room temperature for 12 h and the images were recorded with a Vecco Innova nanoscope II AFM. AFM scans were carried out on several surface positions to check the surface uniformity.

Effect of NaCl on immobilized enzyme

The Celite-545 adsorbed β amylase (1.0 U) was incubated with 1.0 M NaCl in 0.1 M sodium phosphate buffer, pH 6.0 at 50 °C for varying times. Activity of untreated enzyme was considered as control (100%) for the calculation of remaining percent activity.

Effect of pH

Soluble and immobilized β amylase (1.0 U) was assayed in the buffers of different pH (pH 2.0-8.0). The buffers used were glycine-HCl (pH 2.0), sodium acetate (pH 3.0, 4.0), sodium phosphate (pH 5.0-7.0) and tris-HCl (pH 8.0). The molarity of each buffer was 0.1 M. Maximum activity obtained at pH 6.0 was taken as control (100%) for the calculation of remaining percent activity.

Effect of temperature

Effect of temperature on soluble and immobilized β amylase (1.0 U) was studied by measuring activity of enzyme preparations at various temperatures (20-80°C) in 0.1 M sodium phosphate buffer, pH 6.0.

In another set of experiment, soluble and immobilized β amylase (1.0 U) was independently incubated at 60°C in 0.1 M sodium phosphate buffer, pH 6.0, for varying times. Aliquots of each preparation were taken at indicated time intervals, chilled quickly in ice for 5 min and activity was measured. The activity obtained without incubation at 60°C was taken as control (100%) for the calculation of remaining percent activity.

Storage stability

Soluble and the immobilized β amylase were stored at 4°C in 0.1 M sodium phosphate buffer, pH 6.0 for over 30 days. The aliquots from each preparation (1.0 U) were taken in triplicates at the gap of 5 days and were then analyzed for the remaining enzyme activity. The activity determined on the first day was taken as control (100%) for the calculation of remaining percent activity.

Reusability of immobilized β amylase

Immobile enzyme was taken in triplicates for assaying the activity of enzyme. After each pass the immobilized enzyme preparation was taken out, washed, and stored overnight in 0.1 M sodium phosphate buffer, pH 6.0, at 4°C. The activity was assayed for seven successive days. The activity determined for the first day was considered as control (100%) for the calculation of remaining percent activity after each use.

Starch hydrolysis in batch process

Starch solution (1% w/v) was independently incubated with soluble and immobilized β amylase (500 U) at 50°C and 60°C respectively under stirring condition for 6 h. Aliquots were taken out at different time intervals and assayed for the formation of maltose by DNS method [16].

Measurement of β amylase activity

Activity of β amylase was assayed by DNS method with slight modifications [16]. 250 µl of enzyme in buffer was added to 250 µl substrate (1% w/v) and the resulting mixture was incubated for 30 min at 50°C. Reaction was stopped by adding 1.5 ml of DNS solution and then heated in a boiling water bath for 5 min. After cooling, reaction mixture was diluted with distilled water. Absorbance was measured spectrophotometrically at 540 nm with maltose as standard.
One unit (1.0 U) of β amylase activity is defined as the amount of enzyme that liberating 1 mg of maltose min⁻¹ under the standard assays conditions. A standard curve of absorbance against amount of maltose was constructed to calculate the amount of maltose released during assay.

**Estimation of protein**

Protein concentration was estimated according to the procedure described by Lowry et al [17]. BSA was used as a standard protein.

**Statistical analysis**

Each value represents the mean for three independent experiments performed in duplicates, with average SDs, < 5%. Data were analyzed by one-way ANOVA. P-values < 0.05 were considered statistically significant.

**RESULTS AND DISCUSSION**

**Immobilization efficiency of β amylase on Celite-545**

The present study involves direct immobilization of partially purified β amylase from *I. batata* on an inexpensive support, Celite-545. Thus, the cost of enzyme purification is minimized. Celite-545 is an inorganic mechanically stable, non-toxic and non-biodegradable diatomaceous earth which has been used widely to immobilize various enzymes and proteins [15,18]. The binding of β amylase on support was significantly affected by change in pH. Enzyme was maximally adsorbed at pH 6.0 and retained 244 U β amylase activity g⁻¹ of Celite-545 with 83% preserved activities (Table 1). In the literature, for immobilized β amylase, various values for binding capacities and preserved activities are given. For example, when immobilization of sweet potato β amylase was achieved on chitosan beads and sphenon based support, preserved activities were reported as 59% and 76%, respectively [19,20]. Furthermore, 49% immobilized efficiency was observed on immobilizing barley β amylase on polyacrylamide polymer [21].

**Table 1. β Amylase Immobilized on Celite-545**

<table>
<thead>
<tr>
<th>Enzyme Loaded (X) (U)</th>
<th>Enzyme activity in washes (Y) (U)</th>
<th>Activity bound g⁻¹ of celite 545 (U)</th>
<th>Activity yield % (B/A×100)</th>
<th>Theoretical (X-Y=A)</th>
<th>Real (B)</th>
<th>Effectiveness factor (B/A)- (q)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1232</td>
<td>939</td>
<td>292</td>
<td>244</td>
<td>1232</td>
<td>939</td>
<td>244</td>
</tr>
</tbody>
</table>

Each value represents the mean for three independent experiments performed in duplicates, with average standard deviations, < 5%.

**FT-IR analysis**

The FT-IR spectra of Celite-545 and Celite-545 adsorbed β amylase are used to investigate the interaction between enzyme and support (Fig. 1). The premier intensity peak at 1079.63 cm⁻¹ is due to asymmetric Si-O-Si stretching vibration of Celite-545. The band at 791.57 cm⁻¹ is attributed to the symmetric stretching of the ring structure of (SiO)₄ tetrahedra [22]. The vibration of H₂O caused by the hydrogen bonds of protein with silanol groups is presented at 1639.48 cm⁻¹ [23]. Furthermore, peak at 3362.70 cm⁻¹ due to C-H stretching indicated strong interactions of enzyme with Celite-545 [24]. Intensity peak of Si-O-Si stretching vibration at 1079.63 cm⁻¹ was decreased to 1074.20 cm⁻¹ when enzyme was adsorbed to support surface.

**AFM analysis**

Visualization of surface topography of support and enzyme adsorbed on it with AFM revealed a significant amount of enzyme molecules immobilized on the support (Fig. 2). The functional groups existing on Celite-545 surface were also verified by FT-IR spectroscopy. We used the peak-to-valley distance in these images as an indicator of the surface roughness. The support surface, before β amylase immobilization, was smooth (Fig. 2a), compared with the enzyme immobilized surfaces. It is evident that as the immobilization progressed the roughness of support surface increases, which could be seen from the increase of peak-to-valley value (Fig. 2b). The roughness of the sample surface is an important feature and should play an important role in affecting the enzyme activity. This observation is consistent with those in previous reports [25].

**Effect of 1.0 M NaCl on immobilized β amylase**

The activity of immobilized enzyme was evaluated after incubating it with 1.0 M NaCl for 4 h at 50°C (Fig. 3). The adsorbed enzyme exhibited retention of significant enzyme activity even in presence of 1.0 M NaCl. Result showed that binding of β amylase with Celite-545 was quite strong and such type of immobilized enzyme preparations could be easily exploited for industrial applications. Ashraf and Husain, showed similar results with radish peroxidase when immobilized on DEAE cellulose [26].
Effect of pH

A shift in enzyme activity upon immobilization towards acidic or basic directions is natural since the microenvironment of the free and immobilized enzyme is quite different. Charge and structure of support material, along with nature of the activators impart a significant effect on enzyme activity. Therefore, comparing the activity of soluble and immobilized enzyme as a function of pH forms an important part of the study [27].

Fig. 4 demonstrates the pH-activity profiles for soluble and immobilized β amylase. Both soluble and immobilized enzyme preparations showed the same pH-optima, pH 6.0. However, immobilized β amylase retained significantly higher enzyme activity at alkaline sides as compared to soluble under similar experimental conditions. Several earlier investigators have previously reported the broadening in pH optima for the immobilized amylases [18,28].

Effect of temperature

The inability to enhance the thermal stability of a native enzyme is one of the most important limitations for their application in continuous reactors. Studies showed an increase in temperature-optimum from 50°C to 60°C for immobilized enzyme (Fig. 5). At 70°C the activity retained by immobilized enzyme was significantly higher (83%) as compared to soluble enzyme (28%). Thermal inactivation studies showed 88% of the initial activity retained by immobilized enzyme after 2 h exposure at 60°C whereas the free enzyme exhibited 62% activity under identical thermal exposure (Fig 5). Further incubation of soluble enzyme at 60°C for 4 h resulted in a loss of 64% activity whereas immobilized enzyme retained significantly higher activity, 66%.

Noda et al in their studies observed higher temperature optima for immobilized β amylase in comparison to its soluble counterpart [18]. Similarly an increase in temperature-optima was noticed when soybean β amylase was immobilized on chitosan beads. The shift in temperature optima of immobilized enzyme might be due to conformational changes at higher temperatures that might expose active sites more appropriate for substrate interaction thereby increasing its apparent enzymatic activity [29,30].

Reusability of immobilized β amylase

Enzymes are quite expensive products. Immobilization as a technique ensures the recycling and reusability of the enzyme. The most important advantage of immobilization is its repeated uses. Reusability pattern of immobilized β amylase showed about 79% of initial activity retention even after its 7th repeated use (Fig. 7). The trivial activity loss upon reuse could be due to frequent encountering of substrate into the same active site which might distort it and this distortion could dwindle the catalytic efficiency either partially or fully [31].
Starch hydrolysis in batch processes

Starch is a very important raw material for the production of sweeteners, adhesives, thickening and binding agents. Table 3 illustrates the hydrolysis of starch in batch process by soluble and immobilized β amylase at 40°C and 50°C, respectively. It was noticed that the hydrolysis of starch by soluble enzyme was 82% after 5 h at 40°C whereas immobilized enzyme exhibited 88% of the hydrolytic activity under identical conditions. Hydrolysis of starch with free and immobilized β amylase at 50°C after 3 h incubation was 74% and 85% respectively. It was observed that only 80% starch was hydrolyzed by the free enzyme after 4 h at 50°C. However, the hydrolysis of starch by soluble enzyme beyond this limit did not exhibit any significant increase whereas the maximum starch hydrolysis achieved by immobilized enzyme reached to 96% in 6 h (Table 3). The results showed that the rate of hydrolysis was more in case of soluble enzyme for the first few hours as compared to immobilized enzyme. It was due to the fact that soluble enzyme was more accessible to starch for first few hours but after prolonged incubation, the rate of hydrolysis decreased. It might be due to enzyme unfolding or inhibition of enzyme activity by its own product [32,33]. Satish et al have reported similar results where starch was hydrolyzed by immobilized α-amylase on super porous CELBEADS [34].

Table 2. Storage stability of soluble and immobilized β amylase at 4°C

<table>
<thead>
<tr>
<th>Number of days</th>
<th>Remaining activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SβA</td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>05</td>
<td>91 ± 1.41</td>
</tr>
<tr>
<td>10</td>
<td>77 ± 1.25</td>
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<tr>
<td>15</td>
<td>60 ± 1.13</td>
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<tr>
<td>20</td>
<td>52 ± 2.31</td>
</tr>
<tr>
<td>25</td>
<td>47 ± 1.88</td>
</tr>
<tr>
<td>30</td>
<td>43 ± 1.09</td>
</tr>
</tbody>
</table>

SβA; Soluble β amylase
IβA; Immobilized β amylase

Soluble and the immobilized β amylase were stored at 4 °C in 0.1 M sodium acetate buffer, pH 6.0 for over 30 days. The aliquots from each preparation were taken in triplicates at the gap of 5 d and were then analyzed for the remaining enzyme activity. Each value represents the mean for three independent experiments performed in duplicates, with average standard deviation, < 5%.
Table 3. Hydrolysis of starch by soluble and immobilized β amylase in batch process at different temperatures

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>RA%_{a = 40°C}</th>
<th>RA%_{a = 50°C}</th>
</tr>
</thead>
<tbody>
<tr>
<td>SβA</td>
<td>IβA</td>
<td>SβA</td>
</tr>
<tr>
<td>0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>30</td>
<td>59 ± 1.21</td>
<td>46 ± 1.71</td>
</tr>
<tr>
<td>60</td>
<td>64 ± 1.53</td>
<td>53 ± 2.44</td>
</tr>
<tr>
<td>90</td>
<td>67 ± 1.33</td>
<td>59 ± 1.22</td>
</tr>
<tr>
<td>120</td>
<td>67 ± 1.88</td>
<td>64 ± 0.79</td>
</tr>
<tr>
<td>150</td>
<td>72 ± 2.34</td>
<td>70 ± 1.57</td>
</tr>
<tr>
<td>180</td>
<td>81 ± 1.09</td>
<td>82 ± 1.63</td>
</tr>
<tr>
<td>240</td>
<td>82 ± 0.97</td>
<td>82 ± 0.20</td>
</tr>
<tr>
<td>300</td>
<td>82 ± 1.86</td>
<td>88 ± 1.11</td>
</tr>
<tr>
<td>360</td>
<td>82 ± 1.14</td>
<td>88 ± 2.34</td>
</tr>
</tbody>
</table>

RA; Remaining activity
ND; Not determined

Starch hydrolysis was performed as described in text. Each value represents the mean for three independent experiments performed in duplicates, with average standard deviations, < 5%.

CONCLUSION

Amylases are among the most important enzymes used for industrial purposes, and now in the light of biotechnology they are considered useful for biopharmaceutical applications. Here, an attempt has been made to obtain a simple, inexpensive and stable immobilized β amylase. The proteins were directly immobilized by adsorption from the crude homogenate, thus avoiding the high cost of enzyme purification. The immobilized β amylase exhibited better thermostability than the free enzyme which resulted in several benefits including low viscosity of substrates and products, minimized bacterial contaminations, increased reaction rates and decrease of operation time. Furthermore, immobilized enzyme significantly hydrolyzed starch in batch processes at high temperatures. Thus the reactors containing such types of inexpensive immobilized enzyme preparations could be exploited for the continuous hydrolysis of starch at large scale.

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REFERENCES


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