JPP JOURNAL OF PROTEINS AND PROTEOMICS
4(2), December 2013, pp. 115-121

Research Article

BIOSYNTHESIS, CHARACTERIZATION AND APPLICATION OF TIO_2 NANOPARTICLES IN BIOCATALYSIS AND PROTEIN FOLDING

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Abstract: The nano-TiO_2 was synthesized using *Lactobacillus* sp. and characterized by XRD and TEM. The X-ray diffraction showed that TiO_2 nanoparticles were crystalline in nature. TEM images revealed that these particles are irregular in shape with an average particle size of 50–100 nm. The biosynthesized nanoparticles were used for the immobilization and refolding of thermally inactivated alpha amylase enzyme. The enzyme after adsorption on TiO_2 nanoparticles retained 71% of enzyme activity. The immobilized enzyme was found to be thermally more stable as compared to the free enzyme. When the enzyme was heated to 60°C for 60 min the free enzyme loses all of its activity whereas the adsorbed enzyme retained 82% of its activity.The adsorbed/immobilized protein could be reused five times without any loss in enzyme activity. The operational stability data also shows that after immobilization the stability of alpha amylase increases. To study the nanoparticles-protein interaction, alpha amylase enzyme was inactivated by heating at 60°C for 1 hour. The thermally inactivated alpha amylase when incubated with the biosynthesized TiO_2 nanoparticles regains nearly 65% activity after 2.0 hour. Thus TiO_2 nanoparticles assist in refolding of the enzyme.

Keywords: Biosynthesis; XRD and TEM of TiO_2 nanoparticles; alpha amylase; immobilization.

Introduction

Nanotechnology is the development of reliable experimental protocols for the synthesis of nanomaterials over a range of chemical compositions, sizes and high monodispersity. The syntheses of titanium nanoparticles and nanostructures have been in the focus of interest due to their attractive material properties and applications in various fields like optical devices, sensors, and photocatalysis, organic pollutants and antibacterial coatings (Mills *et al.*, 1993; Wei *et al.*, 1994; Busca *et al.*, 2008: Guo *et al.*, 2010; Li and Hi, 2013). TiO_2 nanoparticles are considered to be among the best photocatalytic materials due to their long-term thermodynamic stability, strong oxidizing power, and relative non-toxicity (Krishna *et al.*, 2006; Xu *et al.*, 2010). Presently, there are chemical, physical and biological (including the use of microorganisms) routes available for the synthesis of metal oxide nanoparticles (Chen and Mao, 2007; Bansal *et al.*, 2005). This is now well known that many organisms can produce inorganic materials either intra or extracellularly (Senapati *et al.*, 2004). Biological synthesis of metal oxide nanoparticles is hallmarked by ambient experimental conditions of temperature, pH, and pressure (Bansal *et al.*, 2005; Jha and Prasad, 2010). There is a need to develop environmentally safe protocols for the synthesis of TiO_2 nanoparticles. So, far only few reports are available on the biosynthesis of TiO_2 nanoparticles (Bansal *et al.*, 2005; Luckarift *et al.*, 2006; Jha *et al.*, 2009; Jha and Prasad, 2010; Johnson *et al.*, 2012). Thus in the present study synthesis of TiO_2 nanoparticles using *lactobacillus* species is described. The biosynthesized nanoparticles were

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Received: November 12, 2013
Accepted: December 5, 2013
Published: December 14, 2013

used further as an immobilization matrix for alpha amylase enzyme having a macromolecular substrate.

Nanoparticles provide an ideal remedy to the usually contradictory issues encountered in the optimization of immobilized enzymes: minimum diffusional limitation, maximum surface area per unit mass and high effective enzyme load (Aubin and Hammad, 2008; Yang *et al.*, 2008). There is an increasing need for techniques for placing large amount of biological activity on a small surface area, such as microfluidics, bionanofabrication, biosensor designs, molecular gates and drug delivery (Sardar *et al.*, 2008). In biotechnology the desirability of reducing the bioreactor size has led to the immobilization of proteins/enzymes on nanoparticles (Aubin and Hammad, 2008). They have significant adsorption capacities due to their relatively large surface area; therefore they are also able to bind other molecules such as chemical compounds, drugs, probes and proteins attached to the surface by covalent bonds or by adsorption.

TiO_2 being non porous solid particles, small in size may generate less diffusion limitations than the porous analogous do in the solution and can be the best candidate for immobilization of enzymes having macromolecular substrates. Recently, we reported the immobilization of peroxidase on amino silane modified chemically synthesized TiO_2 nanoparticles (Ahmad *et al.*, 2013). The immobilized enzyme showed 83% activity as compared to the equivalent free peroxidase enzyme. The immobilized enzyme has a higher V_{max}/ K_m (166.7) as compared to the free enzyme (100). It also showed enhanced thermal stability at 60 ^oC compared to its soluble counterpart. Here we report that biosynthesized TiO_2 nanoparticles can also be used for immobilization as well as in protein folding.

Material and Methods

Alpha amylase enzyme and 3,5-Dinitrosalicylic acids were purchased from Hi-media Ltd., Mumbai, India. TiO_2nanopowder was purchased from Sigma. All other chemicals and solvents used were of analytical grade and used without further purification.

Preparation of culture medium of *Lactobacillus* sp. from yoghurt: The culture medium was prepared by following the method of Jha and Prasad with slight modification (Jha and Prasad, 2010). Briefly, the homogenized full cream yoghurt was filtered through pre-sterilized muslin cloth under laminar flow. The filtrate was diluted five times with sterile de-ionized water and pH was adjusted to 4. 10% of glucose solution prepared in de-ionized and autoclaved water was added to the filtrate solution and the culture was allowed to grow overnight at 25 ^oC on an orbital shaker. 25 mL of the above culture solution was taken and 75 mL of sterile 10% glucose solution was added. This culture solution was again allowed to grow for another 24 h and was treated as the culture medium for biosynthesis of TiO_2.

Biosynthesis of TiO_2 nanoparticles: Twenty milliliters of 0.25 M Titanium Hydroxide solution was added to the culture solution and it was heated on steam bath up to 60°C. After 20 min the white deposition starts to appear at the bottom of the flask, indicating the initiation of transformation. The culture solution was cooled and kept at 25°C. After 24 hours the culture solution has distinct white clusters of TiO_2 nanoparticles deposited at the bottom of the flask. The TiO_2 nanoparticles were purified by the simple process of centrifugation (5000g, 5 min at 4° C). The loosely bound proteins and other macromolecules were removed by washing the TiO_2 nanoparticles with distilled water.

XRD analysis: The formation of metal oxide TiO_2 nanoparticles was confirmed by X-ray diffraction (XRD) technique using an X-ray diffractometer over a wide range of Bragg angles ($20° \leq 2\theta \leq 80°$).

TEM measurements: Transmission electron microscope (TEM) measurements were performed on a JEOL, F2100 instrument operated at an accelerating voltage at 200kV. The sample was suspended in distilled water, sonicated and the drops of the suspension deposited onto carbon coated copper grids. An EDX (Model EVO-40, ZEISS) spectrum was also recorded for elemental analysis of above prepared sample.

Activity measurement of alpha amylase: **A**ctivity of alpha amylase was estimated using starch as the substrate (Decker, 1977). The activity of the immobilized enzyme on TiO_2 nanoparticles

was determined in the similar way the immobilized enzyme was continuously shaken for the entire duration of the assay. One enzyme unit is defined as the amount of the enzyme that catalyzes the conversion of 1 µmole of substrate per minute.

Immobilization of alpha amylase on TiO_2 nanoparticles: Alpha amylase (184 U dissolved in 20mM sodium acetate buffer of pH 4.0, containing 6mM NaCl) was incubated with 20 mg of biosynthesized TiO_2 nanoparticles (washed and equilibrated with the sodium acetate buffer of pH 4.0) at 25 ºC with constant shaking. After 2 hour the mixture was centrifuged at 3000 g for 5 min at 4 ºC. The TiO_2 nanoparticles were washed with the sodium acetate buffer to remove the loosely bound enzyme and resuspended in 2 ml of sodium acetate buffer. The enzyme activity was calculated in the supernatant, washings and in the immobilized enzyme. To calculate the immobilization efficiency the enzyme load was varied (45 U – 368 U) and the immobilization was carried as above. A control was run for both the enzymes in which the adsorbed enzyme was washed with 1M NaCl and 50% Ethylene glycol.

Thermal stability of free enzyme and adsorbed enzyme on TiO_2 nanoparticles: The stability of alpha amylase in free and in immobilized states was studied at 60 ºC at different time interval. Free and immobilized enzymes (dissolved in sodium acetate buffer of pH 4.0) were incubated separately at 60 ºC. Appropriate aliquots of sample were taken out at different time interval, the samples were cooled and activities were determined using starch as substrates. The activity of enzyme at 25 °C was taken as 100%.

Reusability of the adsorbed/immobilized enzyme: Immobilized enzyme (1.0 ml, containing 66 U) was incubated with 0.5 ml of the substrate under shaking condition at 25ºC. After 10 minute the supernatant was removed by centrifugation at 3000 g for 5 minute at 4 ºC and the enzyme activity was estimated in the supernatant. The immobilized enzyme was washed three times with 1.5 ml of assay buffer. For second cycle the immobilized enzymewas incubated with 0.5 ml of fresh substrate and the reaction was carried out as before.

Thermal inactivation/denaturation of enzymes: Alpha amylase (184 U dissolved in sodium acetate buffer, pH 4.0 containing 6mM NaCl) was kept at 60 ºC for 1 hour.

Reactivation of thermally denatured enzymes on TiO_2 nanoparticles: The thermally inactivated/denatured alpha amylase was incubated with 20 mg of biosynthesized TiO_2 nanoparticles at 25 °C with constant shaking. Appropriate aliquot was withdrawn after regular intervals of time and the enzyme activity was determined using starch as substrates. In a control the thermally inactivated enzyme without TiO_2 nanoparticles was incubated at 25° C and after regular intervals of time the enzyme activity was determined.

Results and Discussion

In the present study non-pathogenic, environmentally safe and easily available *Lactobacillus* sp. was used for the biosynthesis of TiO_2 nanoparticles. Extra cellular proteins and other biomolecules present in the culture of *Lactobacillus* mediated the hydrolysis of the anionic complexes and results in the synthesis of titania nanoparticles (Bansal *et al.*, 2005; Jha and Prasad, 2010). *Lactobacilli*, like most of the bacteria, have a negative electro-kinetic potential; which readily attracts the cations and this step probably acts as a crux of the procedure of biosynthesis. Earlier, such a possibility of biosorption and bioreduction had been reported in case of silver iodide by the *Lactobacillus* sp. A09* (Jha and Prasad, 2010). A mildly acidic pH and lowered oxidation potential (which is formed due to the presence of glucose) activates the membrane bound oxidoreductase and makes the requisite ambience for an oxide nanoparticle synthesis (Jha and Prasad, 2010).

XRD studies (Figure 1) showed that TiO_2 nanoparticles synthesized by bacteria were in anatase phase and are crystalline in nature. The average particle size estimated by XRD is in order of 37 nm. The phases were found to be in good agreement with the available literature reports (PCPDF No. #84-1285). Figure 2 show the TEM image of TiO_2 nanoparticles. The size ranges 50-130 nm in diameters and is irregular in shape. The nanoparticles synthesized by biological methods

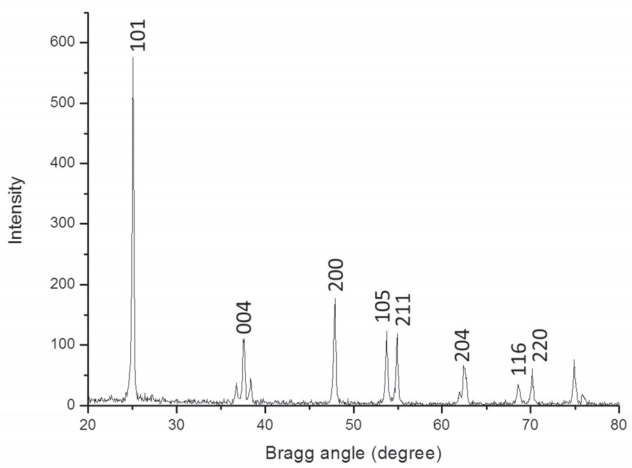

Figure 1: **X-ray diffraction pattern of TiO_2 nanoparticles.**

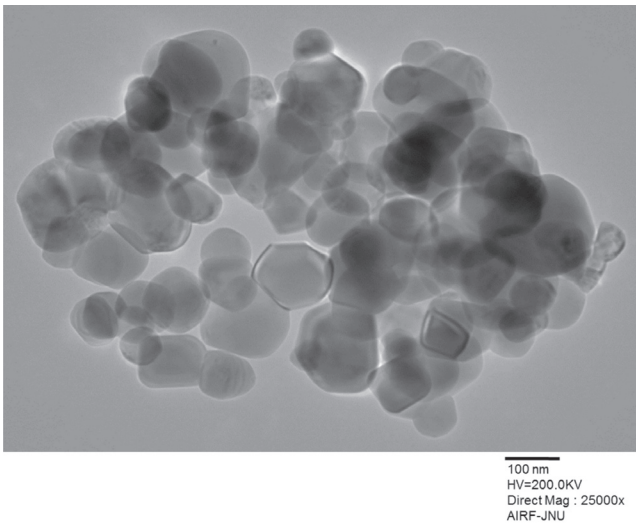

Figure 2: **Transmission Electron Microscopy (TEM) image of TiO_2 nanoparticles.**

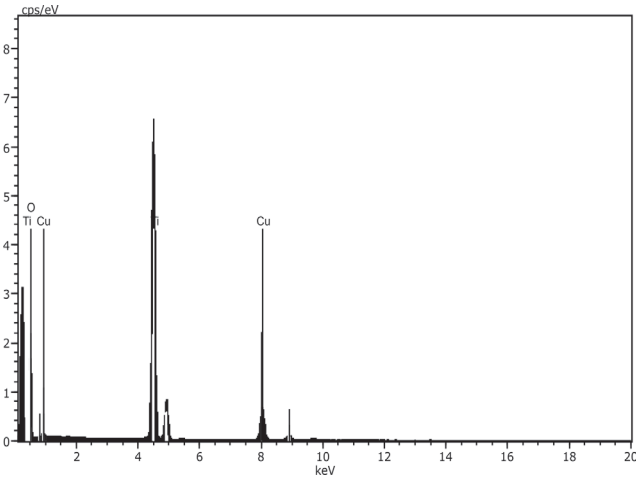

Figure 3**: Energy-dispersive X-ray spectroscopy (EDX) spectra of TiO_2 nanoparticles.**

have different size and shapes; this has been observed by others also (Mishra and Sardar, 2012; Kumar *et al.*, 2007; Bansal *et al.*, 2005). The elemental composition of the nanoparticles was determined by EDX. The spectrum shows the nanoparticles are composed of titanium and oxygen (Figure 3).

The biosynthesized TiO_2 nanoparticles were used for the immobilization and reactivation of alpha amylase. Alpha amylase is industrially important enzyme (Gupta *et al.*, 2003) but, in general, soluble enzymes do not fulfill the requirements for industry: they are unstable; they are soluble; they undergo inhibitions; they may be poorly selective on non-natural substrates, etc. Therefore, most enzymes have to be greatly improved before industrial implementation (Katchalski, 1993). Immobilization of enzymes makes these biocatalysts reusable and stable and thus turns the enzyme-based process into a more economically viable approach (Gupta and Mattiasson, 1992; Guisan, 2006). Recently, nanoparticles are used as immobilization matrices to overcome the diffusional constraints specially enzymes having the macromolecular substrates (Gupta *et al.*, 2011). In the present study the alpha amylase enzyme was immobilized on TiO_2 nanoparticles by a simple process of adsorption. The enzyme adsorbed strongly to the TiO_2 nanoparticles as no enzyme activity was determined when the adsorbed enzyme was washed with 1M NaCl and 50% Ethylene glycol. Table 1 shows the Immobilization efficiency of the adsorbed alpha amylase enzyme on TiO_2 nanoparticles. The immobilization efficiency (B/A) is defined as the ratio of the measurable enzyme activity (calculated by subtracting the unbound activity in the supernatant and wash) in the immobilized enzyme (B) to the total bound activity (A). The immobilization efficiency increases as the load of the enzyme unit on nanoparticles was increased. The maximum immobilization efficiency (0.71) was achieved at a load of 184 units. The variation of immobilization efficiency with increasing enzyme load observed here is similar to what has been reported earlier (Roy *et al.*, 2003; Ahmad *et al.*, 2013). Table 2 shows that immobilized enzyme could be reused five times without any loss of enzyme activity. The immobilized enzyme

Table 1
Adsorption and optimization of activity of alpha amylase on TiO_2 nanoparticles

Unit Expressed Actual (B)	*Total Units Bound Theoretical (A)*	*Immobilization efficiency η = B/A*
20.4	46	0.44
54.4	92	0.59
132	184	0.71
170	276	0.61
202	368	0.54

All experiments were done in duplicate and the results within each pair differed by <3%.

Table 2
Reusability of the adsorbed/immobilized enzyme. The enzyme activity was determined using starch as a substrate as given in materials and method section

No. of cycles	*Residual activity (%)*
1	100
2	100
3	100
4	100
5	100

preparations were found to be thermally more stable as compared to their soluble enzymes. Figure 4 shows that at temperature of 60 °C the free enzyme loses almost all of its activity whereas adsorbed/immobilized enzyme retained 82% of its activity after 60 minutes. The immobilized preparation can be successively used for the continuous hydrolysis of starch at 60 °C (Figure 5). The amount of reducing sugars formed by physically immobilized enzyme after 60 min at 60 °C is 1.5 times more than the reducing sugars formed by free enzyme.

Further, the use of enzymes in industry is limited because of its inactivation at high temperature and in presence of denaturants. Several approaches for refolding of enzymes/ proteins have been described in the literature; most of these involve use of additives for assisting correct refolding. A variety of additives such as PEG (Cleland *et al.*, 1992), smart polymers (Roy and Gupta, 2003; Mondal *et al.*, 2007) and small molecular weight additives (Tsumoto *et al.*, 2003) have been reported to assist protein refolding. Nanoparticles have been used to assist protein folding (De and Rotello, 2008; Shah and Gupta, 2008; Raghava *et al.*, 2009) as well as protein immobilization (Roy *et al.*, 2003; Khan *et al.*, 2012; Ansari and Husain, 2012; Ahmad *et al.*, 2013). Therefore TiO_2 nanoparticles were also used to study the refolding of thermally inactivated alpha amylase. Alpha amylase enzyme was completely inactivated by incubating at 60 °C for 1 hour. When the thermally inactivated enzyme was incubated with biosynthesized TiO_2 nanoparticles, the enzyme get adsorbed on the TiO_2 nanoparticles and regain its activity. Figure 6 shows that as the time of incubation of inactivated enzymes with TiO_2 nanoparticles

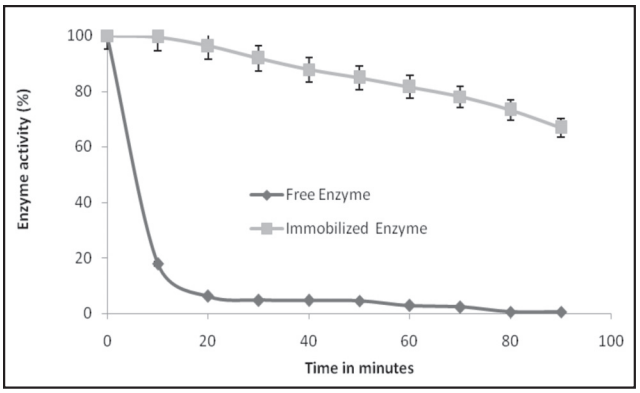

***Figure 4*: Thermal stability of free enzyme and adsorbed/ immobilized preparations. Free enzyme and immobilized preparations were incubated at 60°C and the thermal stability experiment was performed as described in materials and methods.Each point represents the outcome of a pair of readings, with many points showing errors smaller than the symbol.**

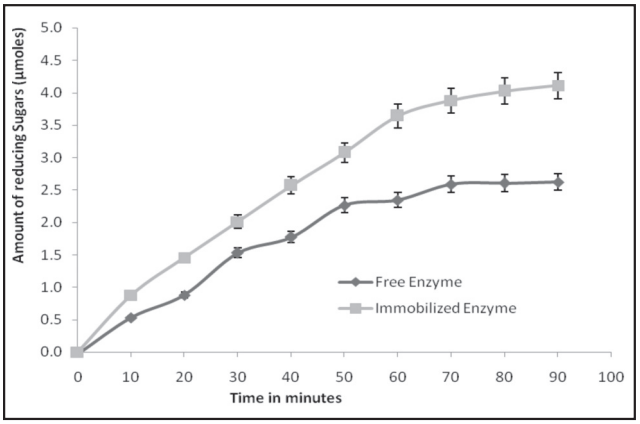

***Figure 5*: Operational stability of free enzyme and in adsorbed/immobilized preparations. Free enzyme and immobilized preparations were incubated with 0.5ml of 1% starch at 60°C. Aliquots were withdrawn at various intervals of incubation and the amount of reducing sugar was estimated by dinitrosalicyclic acid method. Each point represents the outcome of a pair of readings, with many points showing errors smaller than the symbol.**

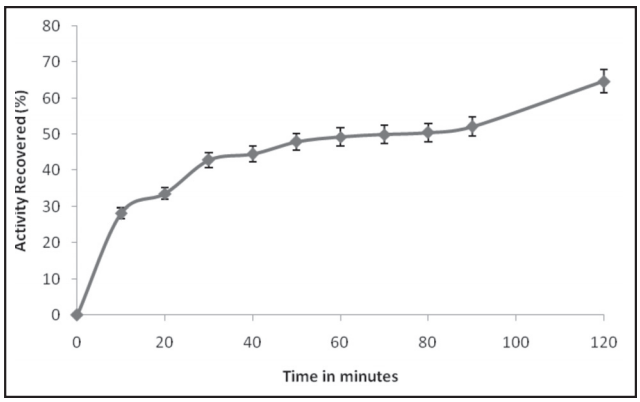

Figure 6: **Reactivation of thermally denatured alpha amylase enzyme on TiO_2 nanoparticles. Thermally denatured enzyme was incubated withTiO_2 nanoparticles with continuous shaking, appropriate aliquots were withdrawn at various interval of time and their activities were determined using starch as the substrate. Each point represents the outcome of a pair of readings, with many points showing errors smaller than the symbol.**

increases its activity increases. Alpha amylase regained 65% of the activity after 2.0 hours. When the thermally inactivated enzyme was incubated at 25°C without TiO_2 nanoparticles no enzyme activity was observed even after 2.0 hours. This shows that the TiO_2 nanoparticles assist in protein folding also. When a protein is in its native state, its hydrophobic core is buried, and the protein surface is generally rich in hydrophilic residues. Upon unfolding, exposure of hydrophobic surfaces readily leads to protein aggregation. Chaperones, like GroEL and GroES, selectively bind to unfolded proteins through hydrophobic or electrostatic interactions, stabilize them from aggregation and help them fold to the native conformation. Selective binding to the hydrophobic and hydrophilic regions of proteins provide the route to protein folding (De and Rotello, 2008). Highly charged nanoparticles based hosts could serve as refolding agents by interacting with charged residues on denatured proteins, facilitating refolding and preventing aggregation (De and Rotello, 2008).

Conclusions

The biosynthesized TiO_2 nanoparticles were effectively used for immobilization and protein folding. The biosynthesis is ecofriendly and can be easily scaled up using non-toxic *Lactobacillus* sp. The immobilized enzyme shows better thermal and operational stability as compared to the free enzyme. The TiO_2 nanoparticles assist in refolding of thermally inactivated enzyme. Further work on the refolding using TiO_2 nanoparticles can give some insight on the mechanism of refolding.

Acknowledgements

The financial support provided by ICMR, Government of India to Razi Ahmad in the form of SRF and UGC-CSIR to Nafeesa Khatoon in the form of JRF is acknowledged.

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