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Metallic/bimetallic magnetic nanoparticle functionalization for immobilization of α-amylase for enhanced reusability in bio-catalytic processes

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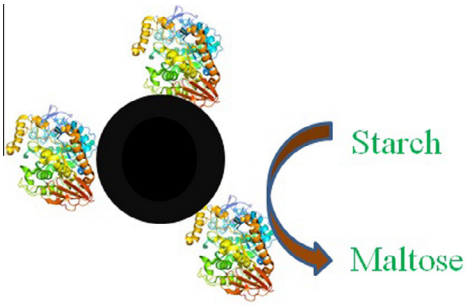
HIGHLIGHTS

- Bimetallic based magnetic nanoparticles have been developed to immobilize the enzyme.
- Enhanced reusability in bio-catalytic processes has been discussed.
- Activity and kinetic parameters have been calculated and compared.
- The properties of immobilized amylase were enhanced than the free enzyme.

GRAPHICAL ABSTRACT



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ABSTRACT

Novel magnetic nanoparticles coated with silica and gold were synthesized for immobilization of α-amylase enzyme and characterized with Fourier transform infrared spectroscopy, transmission electron microscopy. Effect of various limiting factors such as substrate concentration, temperature, and pH on the catalytic activity of enzyme was investigated. The optimum pH for free and immobilized enzyme was found unaffected (7.0), whereas optimum temperature for the enzyme activity was increased from 60 °C for free enzyme to 80 °C for immobilized counterpart. The gains in catalytic attributes concomitant to ease of recovery of the enzyme reflect the potential of the approach and the product to be useful for the enzymatic bioprocessing. The Michaelis–Menten constant (K_m) value of the immobilized α-amylase was higher than that of free α-amylase, whereas maximum velocity (V_{max}), and turn over number (K_{cat}), values were almost similar. Immobilized α-amylase maintained 60% of the enzyme activity even after recycling ten times.

1. Introduction

α-amylase (1,4-α-D-glucan-glucanhydrolase, EC. 3.2.1.1) catalyzes hydrolysis of internal α-1, 4-glycosidic linkages of starch resulting in formation of low molecular weight products such as maltotriose and maltose from amylose, or maltose, glucose and "limit dextrins" from amylopectin. Human salivary α-amylase contains aromatic residues, Trp58, Trp59, Tyr151 and Phe256 in its active site that play a crucial role in determining substrate binding, catalytic reaction and reaction rate. The free enzyme uses in bioprocessing has many shortcomings that include instability, poor

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recovery, and poor reusability thereby increasing bioprocessing cost (D'Souza, 1999). Therefore, for unremitting production of malto-oligosaccharides, immobilized α-amylase would have numerous advantages like ease of recovery of the biocatalyst from end reaction mixtures cost-benefits, convenience. In addition, by selecting appropriate support material, immobilization methods such as adsorption, entrapment, cross linking and covalent attachment can provide the improved retention of catalytic activity, stability and durability. Different types of support materials such as functionalized glass beads (Kahraman et al., 2007), mesoporous silica (Pandya et al., 2005), amberlite MB 150, chitosan beads (Tripathi et al., 2007), gelatin (Bayramoglu et al., 1992), alginate (Ertan et al., 2007), agarose and agar matrices (Prakash and Jaiswal, 2011), poly-acrylamide (González-Sáiz and Pizarro, 2001), modified poly-N-isopropylacrylamide (Sun et al., 1999), polyaniline (Khan et al., 2013), porous nitrocellulose (Tanyolaç et al., 1998), silver nanoparticles doped gum acacia–gelatin-silica nanohybrid, and tamarind gum-silica nanohybrid enhance its stability and have been used for the effective immobilization of α-amylase to improve its selectivity and reusability under harsh conditions by preventing adverse conformational changes that could be adverse to catalytic ability or efficiency.

Among such nanomaterials, magnetic nanoparticles also have attractive attributes such as biocompatibility, superparamagnetism, small size, large extinction and scattering coefficient, catalytic activity, efficient Brownian motion in solution and outstanding mechanical strength for enzyme immobilization (Gubin et al., 2005). In current years, they have been used for many important applications such as magnetic resonance (Yallapu et al., 2011), imaging contrast agent (Biederer et al., 2009), protein separation, data storage, drug delivery, enzyme immobilization (Ren et al., 2011), biomedicine (Pankhurst et al., 2003), filtration (Tang and Lo, 2013), and cation sensors (Rocha-Santos, 2014).

In the present work, synthesis of bare iron oxide magnetic nanoparticles magnetite (Fe_3O_4), hematite (Fe_2O_3), bimetallic gold (Fe_2O_3@Au) and silica (Fe_3O_4@Si) coated magnetic nanoparticles have been reported. The synthesized nanoparticles were functionalized with 3-phosphono propionic acid (3-PPA) to covalently immobilize α-amylase enzyme by using carbodiimide (EDC). The main problem in nanoparticles application is uncontrolled oxidation or irreversible aggregation processes. This demands proper surface modification by coating magnetite with surfactants, polymers or noble metals. Gold and silica coating on magnetic nanoparticles have been utilized due to their unique chemical and optical properties along with chemical inertness that prevents the particles from uncontrolled oxidation and irreversible aggregation processes. The immobilized enzyme was characterized by Fourier transform infrared spectroscopy, transmission electron microscopy, scanning electron microscopy, Zeta size analyzer, and X-ray diffraction. The immobilization was successfully carried out which also lead to the enhancement of enzyme activity to an extent within a specific pH (2–11) and temperature (30–100 °C) range. The reusability of the immobilized enzyme was also improved.

2. Experimental

2.1. Materials

Iron (III) chloride hexahydrate ($FeCl_3{\cdot}6H_2O$), Iron (II) chloride tetrahydrate ($FeCl_2{\cdot}4H_2O$), 3-phosphonopropionic acid (3-PPA), hydroxylamine hydrochloride ($NH_4OH{\cdot}HCl$), sodium citrate triabasicdihydrate, tetraethyl orthosilicate (TEOS), gold (III) chloride hydrate ($HAuCl_4$), tetramethylammonium hydroxide solution (TMAOH), α-amylase from *Aspergillus oryzae,* starch, maltose, Bradford reagent, 3,5-dinitrosalicylic acid (DNS), sodium potassium tartrate and NaOH pellets were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). All solvents and reagents like HCl, HNO_3, and ethanol were purchased from Sisco Research Laboratories (Mumbai, India). All the reagents were of analytical grade and were used without any further purification. All buffers and solutions were made in deionized double-distilled water having 18.2 MΩ resistivity.

2.2. Spectral analyses

UV/Visible spectra of different synthesized magnetic nanoparticles were obtained using double beam UV/Vis spectrophotometer (Shimadzu 2700) and the optical properties of bimetallic core/shell nanoparticles with the metallic iron oxide nanoparticles were compared. Enzyme functionalization of nanoparticles was determined using Fourier transformed infra-red spectroscopy at room temperature in the spectral range from 550 to 4000 cm^{-1} by using FTIR-ATR spectrophotometer (Agilent Cary 660 series with DTGS detector). Background spectra of a clean ATR surface were acquired prior to each sample measurement using the same acquisition parameters. Crystalline structure was obtained by X-ray diffraction (XRD) with a four circle diffractometer. Transmission electron microscopy (TEM, JEOL2100, and accelerating voltage 200 kV), high resolution transmission electron microscopy (HR-TEM, LIBRA 120, accelerating voltage 300 kV, Carl Zeiss (Germany) and scanning microscopy (FE-SEM, JEOL, JSM-5410LV) of the different synthesized bimetallic core/shell and metallic nanoparticles were obtained. The zeta potential measurements were made with Malvern Zeta Sizer (Nano-ZS) at 30 °C.

2.3. Synthesis of magnetic nanoparticles (Fe_3O_4)

Briefly the magnetic nanoparticles (Fe_3O_4) were prepared essentially by the method of (Lyon et al., 2004). The magnetic nanoparticles were obtained by reduction of $FeCl_2{\cdot}7H_2O$ and $FeCl_3{\cdot}6H_2O$ chlorides in aqueous ammonia solution under vigorous stirring. 5.4 g of $FeCl_3{\cdot}6H_2O$ followed by 2.0 g of $FeCl_2{\cdot}4H_2O$ was dissolved in 25 ml of 0.4 M HCl solution. The solution was added drop wise to 250 ml of a 1.5 M NaOH solution with vigorous stirring. A black precipitate was formed immediately. The precipitate was retrieved via magnetic decantation and washed twice with 100 ml of water, then twice with 100 ml of 0.1 M tetramethylammonium hydroxide pentahydrate and the final precipitate was again dissolved in 0.1 M TMAOH solution and stored in dark condition for further use.

2.4. Synthesis of gold coated magnetic nanoparticles (Fe_2O_3@Au)

For gold coating, firstly Fe_3O_4 nanoparticles were washed with water, dissolved in 250 ml of 0.1 M HNO_3 and the precipitate was retrieved by the magnet decantation (Lyon et al., 2004). The precipitate was dispersed in 250 ml of 0.01 M HNO_3 and heated with stirring to 90–100 °C. During heating, the solution color changed from dark brown–black to brown–red. After 30 min, the heating was stopped and the solution was allowed to cool to room temperature. To encapsulate with gold shell around Fe_2O_3, 7 mg of Fe_2O_3 nanoparticles were suspended in 0.1 M TMAOH and were added to 0.1 M citric acid, 0.2 M $NH_2OH{\cdot}HCl$ and 1% $HAuCl_4$ and stirred until the solution became purple in color.

2.5. Synthesis of silica coated magnetic nanoparticles Fe_3O_4@Si

The prepared Fe_3O_4 (containing 5 mg magnetite) was added (Quy et al., 2013) into a flask containing 250 μL of the 10 (w/v)% solution of aqueous tetraethylorthosilicate (TEOS) and 350 μL of