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Synthesis of amino-silane modified superparamagnetic Fe_3O_4 nanoparticles and its application in immobilization of lipase from *Pseudomonas fluorescens* Lp1

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ABSTRACT

Superparamagnetic nanoparticles (Fe_3O_4–magnetite) were prepared by chemical co-precipitation method and their surface was functionalized with 3-aminopropyltriethoxysilane via silanization reaction to obtain amino functionalized magnetic nanoparticles. The purified lipase from *Pseudomonas fluorescens* Lp1 was immobilized onto functionalized magnetite using glutaraldehyde as the coupling agent. The characterization of the nanoparticles was done by scanning electron microscopy, transmission electron microscopy, powder X-ray diffraction, vibrating sample magnetometry and Fourier transformed infrared spectroscopy. The size of the magnetite was measured about 10–30 nm. The results of characterization study revealed the successful immobilization of lipase on to functionalized magnetite. The saturation magnetization of magnetic nanoparticles was found to be 28.34 emu/g whereas the immobilized magnetic nanoparticle was 17.074 emu/g. The immobilized lipase had greater activity at 50 °C and thermal stability upto 70 °C. It exhibited excellent reusability for 4 cycles and storage stability upto 15 days by retaining 75% of its initial activity.

1. Introduction

Lipases (triacylglycerol ester hydrolases, EC 3.1.1.3) hydrolyze triglycerides to fatty acids and glycerols are widely distributed in nature. Although lipases have been found in many species of animals and plants the lipase from microorganisms are most interesting since they posses potential applications in various industries such as food, dairy, pharmaceutical, detergents, textile, biodiesel, and cosmetic industries, and in synthesis of fine chemicals, agrochemicals, and new polymeric materials [1,2]. Lipases accept a wide range of substrates other than triglycerides such as aliphatic, alicyclic, bicyclic, and aromatic esters and even esters based on organometallic sandwich compounds which helps them in various synthetic reactions. Lipases are quite stable in non-aqueous organic solvents hence depending on the solvent system used, can be applied to hydrolysis reactions or ester synthesis [3] and they are very effective biocatalysts for the synthesis of optically pure compounds [4–7].

The industrial application of the biocatalysts is economically unattractive due to their high cost and inconvenience in their separation, recycling and reusing. The use of immobilized lipase onto insoluble or solid support is a possible solution to this problem since the enzyme in an immobilized form provides convenient handling of the enzyme, facile separation from the product, thereby minimizing or eliminating protein contamination of the product. Immobilization also facilitates the efficient recovery and reuse of costly enzymes, and enables their use in continuous, fixed-bed operation. A further benefit is the enhanced stability under storage and operational conditions [8,9].

The research on the development of immobilization method using new carrier is one of the main thirst in enzyme engineering. The continuous industrial applications of lipases are limited since they are often inactivated due to the wide variety of environmental conditions and difficult to separate from the reaction system for reuse [10]. The new immobilization methods have been designed and new supports have also been synthesized to overcome these problems [11]. In recent years, nanostructured materials have been used as supports for enzyme immobilization since the high surface area: volume ratios of nanoparticles can effectively improve the enzyme loading and the catalytic efficiency of the immobilized enzyme [12]. Among the various supports, magnetic nanoparticles have received considerable attention due to their higher specific surface area for the binding of larger amount of enzymes [13], lower mass transfer resistance and less fouling [14,15], ease in the separation of immobilized lipase from the reaction mixture by the application of a magnetic field [16] and reduction in capital and operation costs [17].

Magnetic nanoparticles (MNPs) are a class of engineered particulate materials of less than 100 nm that can be manipulated under the influence of an external magnetic field [18]. They exhibit

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a phenomenon of superparamagnetism, not keeping magnetized after the action of magnetic field giving the advantage of reducing the risk of particle aggregation [19]. Superparamagnetic nanoparticles have a high potential as a carrier for oligonucleotides and macromolecules including enzymes in different life science applications [20]. The functionalized coating on the nanoparticles enables selective recognition and capture of specific molecules [21]. Magnetite is one of the nontoxic paramagnetic nanomaterial accepted for biotechnological and biomedical applications because of their smaller size of the core, the larger the surface area for functionalization [22], superparamagnetic behavior and low toxicity [23]. The functionalized magnetic nanoparticles have been used by many researchers to immobilize lipase for various applications due to its facile synthesis, chemical stability, nontoxic and noncarcinogenic nature [24–29]. Hence, Fe_3O_4 superparamagnetic nanoparticles were chosen to immobilize the lipase in this study.

In the present study, magnetite was prepared by chemical co-precipitation method and subsequently coated with 3-aminopropyltriethoxysilane to derive amino-functionalized magnetic nanoparticles (AFMNPs). The purified lipase from *Pseudomonas fluorescens* Lp1 was immobilized onto AFMNPs. The characterization of magnetite was done by scanning electron microscopy (SEM), transmission electron microscopy (TEM), powder X-ray diffraction (PXRD), vibrating sample magnetometry (VSM) and Fourier transformed infrared spectroscopy (FT-IR). The properties of the immobilized lipase such as thermal stability, reusability and storage stability were also investigated.

2. Materials and methods

2.1. Materials

The chemicals such as P-nitrophenyl palmitate (4-NPP), 3-aminopropyltriethoxysilane (APTES) and Sephadex G-100 purchased from Sigma Aldrich, USA and bovine serum albumin (Hi Media, India) were used. All other chemicals and reagents used were of analytical grade and used as such without further purification. Aqueous solutions were prepared in deionized water.

2.2. Methods

2.2.1. Microorganism and lipase preparation

P. fluorescens Lp1 (GenBank accession number JN123466), a gram negative bacterium isolated from oil contaminated soil was used in this study. The lipase production was carried out by shake flask fermentation using olive oil as substrate and carbon source [30]. The flasks were incubated overnight in a shaker incubator at 40 °C with 150 rpm. Culture supernatant was collected by centrifugation at 10,000 × *g* for 10 min at 4 °C. The purification of lipase carried out by ammonium sulfate fractionation, dialysis followed by applying onto a column packed with Sephadex G-100. The fractions showing highest lipase activity were pooled; the resultant concentrated lipase solution (284.7 U/ml of enzyme activity) was used directly for immobilization. Extracellular lipase activity was measured by photometric method by measuring the micromoles of 4-nitrophenol released from 4-NPP [31]. One unit of lipase activity was defined as the amount of enzyme releasing 1 μmol of 4-nitrophenol per minute. Protein analysis of the different supernatants was determined spectrophotometrically [32].

2.2.2. Synthesis and surface modification of magnetite

Naked Fe_3O_4 nanoparticles were prepared by chemical co-precipitation of $Fe^{3+}$and Fe^{2+} ions [33]. 500 ml of 0.2 M $FeCl_2$ was mixed with 500 ml of 0.3 M $FeCl_3$ in a flask containing 5.0 g of stearic acid and stirred vigorously. 200 ml of 0.4 M aqueous solution of NaOH was added drop wise into the flask till the pH reached 12.0 and black precipitate appeared. The final Fe_3O_4 precipitate was filtered, washed thrice with ethyl acetate and dried. Surface modification of magnetite was carried by using [3-(2-aminoethylamino) propyl] trimethoxysilane (APTS) and tetraethyl orthosilicate (TEOS) [34]. Two grams of magnetite was suspended in 100 ml distilled water. To this a mixture of 5.0 ml APTS, 15 ml of methanol and 5.0 ml of sodium fluoride (1%, w/w) aqueous solution was stirred vigorously for 10 min, then 20 ml of TEOS was added drop wise slowly into the flask and stirred vigorously at room temperature for 24 h. The black precipitate of AFMNPs obtained was collected by magnetic decantation, washed thrice with ethanol and water and dried.

2.2.3. Immobilization of lipase

The immobilization of lipase was performed [35] using glutaraldehyde as the coupling agent for making the covalent attachment of the lipase to the AFMNPs. AFMNPs were dispersed in phosphate buffer (100 mM, pH 7.0) and equal volume of glutaraldehyde (0.5%, w/v) was added shaken at 30 °C up to 12 h. The aldehyde-functionalized magnetic nanoparticles were then separated by magnetic decantation and washed thrice with phosphate buffer (100 mM, pH 7.0) to remove the unattached glutaraldehyde. The prepared aldehyde-functionalized magnetic nanoparticles were combined with purified lipase solution and then kept at 30 °C with 10,000 rpm agitation. Then immobilized AFMNPs were removed by magnetic decantation and washed thrice with phosphate buffer to remove the unbound lipase. The immobilized and free lipase activity was calculated by spectrophotometric method as described above.

The experiments were performed in triplicates and the mean values of the results were calculated. The efficiency of the immobilization technique η was estimated [36], the lipolytic activity of the lipase solution before (E_0) and after immobilization (E_f), and the volumes of the solution before (V_0) and after (V_f), using the relation:

$$\eta = \frac{E_0V_0 - E_fV_f}{E_0V_0} \times 100$$

The activities are given in U/ml, and the volumes are in ml. The available activity of the was calculated as

$$\text{relative activity } (\%) = \frac{\text{immobilized lipase activity}}{\text{free lipase activity}} \times 100$$

2.2.4. Characterization of magnetic nanoparticles

The dispersion ability of magnetic nanoparticles in various solvents such as water, ethyl acetate, ethanol, methanol, isopropanol and hexane was tested by adding in the respective solvents. The superparamagnetic behavior of magnetite was evaluated by applying external magnetic field.

The morphology of the nanoparticles was revealed by SEM, FEI Quanta 200F, Netherlands. The morphology and size of the magnetic nanoparticles was determined by TEM Tecnai-10, Philips at 120 keV. X-ray diffraction patterns of magnetic nanoparticles obtained with PXRD model Bruker AXS D8 Advance with Cu (Kαλ = 1.5406 Å) radiation, scanning range from 10° to 90°. FT-IR spectra of magnetic nanoparticles, AFMNPs and lipase bound AFMNPs were obtained using Nicolet FT-IR Avatar 370 with the wave number ranged from 4000 cm^{-1} to 400 cm^{-1} using KBr beam splitter. The magnetization curves of samples were measured with