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High throughput synthesis of ethyl pyruvate by employing superparamagnetic iron nanoparticles-bound esterase

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ABSTRACT

Surface-modified with tetraethoxysilane (TEOS) Superparamagnetic Nanoparticles (SNPs) were successfully synthesized to enhance their hydrophobicity and bio-affinity for an esterase. The obtained SNPs possessed large surface area, high conductivity and improved antimicrobial activity. An esterase from *Bacillus pumilus* was efficiently immobilized on silane functionalized SNPs and its successful binding was confirmed by both FTIR and FE-SEM. The low Michaelis Menten constant (K_m) (~12% lesser than free enzyme) of the SNPs-immobilized/bounded esterase (SNPs-Est) suggested its preeminent compatibility for the substrate (*p*-nitrophenyl acetate) than the free esterase. SNPs-Est retained half of its original activity even after 11.3 h of incubation and remained vigorous over 14 cycles of repetitive use at 45 °C. SNPs-Est was also checked out for successful synthesis of ethyl pyruvate (96% conversion of pyruvic acid into ethyl pyruvate), a flavor ester using High Performance Liquid Chromatography (HPLC) and ^{1}H-Nuclear Magnetic Resonance (^{1}H-NMR) studies.

1. Introduction

Enzyme, as is distinguished, is superior over chemical catalyst because of its bio-catalytic nature, superior specificity and placid reaction conditions. Esterase is a kind of omnipresent enzyme that hydrolyze esters into acid and an alcohol with simultaneous release of water in a biochemical reaction. An esterase can also catalyze the ester synthesis and trans-esterification in water-free or water-restricted medium [1–5]. Esterases are imperative in a diversity of commercial applications primarily in foods, pharmaceuticals, detergents, textiles, paper, leather and cosmetics [5–7]. Esterase has been extracted from different sources including plants, animals and micro-organisms [4,5,8–10]. Microbial enzymes are attractive because the asking price to produce and sustain them is low and they are also convenient to manipulate [5–8,11,12]. However, all these distinguished features and industrialized applications of enzymes are primarily restricted by their everlasting long-term storage stability, recovery and reusability. Enzyme immobilization on an appropriate matrix ameliorate is the solitary approach to conquer these restrictions.

Immobilization of enzyme(s) onto a variety of solid matrices remains attractive since immobilization cut down the expenditure of a process and often improves robustness of biocatalyst [2,9,12–14]. Immobilized enzymes also possessed excellent functioning stability at various ionic strengths, pH values and temperatures which makes them extra stable than the native enzymes [9,15–17]. Immobilized enzymes thus facilitate their use at high temperature, harsh pH and extend their effortless separation, economical reuse and good suitability for practical applications. Many researchers have effectively explored diverse techniques for immobilization taking into account processibility and feasibility, such as immobilization of the biocatalyst by adsorption, entrapment, covalent binding and membrane confinement [16,17]. Amongst the carrier materials now accessible, magnetic nanoparticles (NPs) have established substantial attention because they being heterogeneous could be effortlessly separated from the reaction system by a magnet and hence reusable [17,18].

Modern advances in nano-technology develop a variety of nano materials which have contributed an efficient support system(s) for efficient immobilization of enzymes. The enormous benefits of NPs as immobilization support are like increased in immobilization efficiency, enhanced durable storage stability, thermal stability and recycling capability of the enzyme [19–21]. Several studies have estimated the utilization of SNPs directly as supports for enzyme immobilization [15,16]. Acetylated or amino-functionalized SNPs with super paramagnetic peculiarities have been described [19]. Covalent binding of an esterase onto suitable matrix may be advantageous as it minimizes the loss of esterase from the support. Researchers have reported the use of macromolecules immobilized to modified magnetic NPs [17,20,21]. Diverse physiochemical features of SNPs such as surface area to volume

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ratios, aquaphilicity, hydrophobicity, particle size & shape, topography and chemical reactivity coupled with inherent chemical, electrical and magnetic properties of the NPs make them appropriate support for immobilization of many scientifically considerable enzymes [20,21]. Ester synthesis might be achieved by using an esterase and residual stability of esterase may be immensely enhanced by its attachment to suitable NPs. Ethyl pyruvate a derivative of the metabolite pyruvic acid, can be synthesized from pyruvic acid and ethanol *via* esterification reaction in a water-free or water-restricted solvent system. Ethyl pyruvate, a flavor ester causes a number of the structural and functional modifications associated with mesenteric ischemia and reperfusion in rats [22]. Ethyl pyruvate also inhibits the glyoxalases enzymes which promote the growth of glycolysis-driven tumor cells [23,24]. Consequences indicated that ethyl pyruvate might serve as another lead compound or a promising contender for the remedy of trypanosomiasis [25].

In the present study, a purified esterase from *Bacillus pumilus* strain formerly isolated from ear wax [5] was successfully immobilized on silane-functionalized. The immobilization process was optimized and the properties of free and immobilized esterase were systematically investigated. Finally, the improved SNPs-Est was interrogated for successful synthesis of ethyl pyruvate.

2. Experimental

p-Nitrophenyl acetate (*p*-NPA), *p*-nitrophenyl benzoate (*p*-NPBz), *p*-nitrophenyl laurate (*p*-NPL), *p*-nitrophenyl palmitate (*p*-NPP) and *p*-nitrophenol (*p*-NP) were purchased from Alfa Lancaster Synthesis, White Lund, Morecamber, England; $FeCl_2$ and $FeCl_3$were purchased from S.D. Fine-Chem. Ltd., Hyderabad, India. Tetraethoxy silane (TEOS) was procured from Sigma-Aldrich Chemical Co., St Louis, USA. Pyruvic acid, ethyl pyruvate, molecular sieves and methanol were purchased from HIMEDIA Laboratory Ltd., Mumbai, India. DMSO, toluene, *n*-hexane, *n*-heptane, ethanol, *iso*-propanol, butan 2-ol, decane 1-ol, phenol, acetonitrile and Tris buffer were purchased from Merck Darmstadt, Germany. All the chemicals were of analytic grade and were used.

2.1. Facile synthesis of SNPs and covalent immobilization of purified esterase onto SNPs

Fe_3O_4 magnetic NPs were synthesized by co-precipitation method [26]. Aqueous solution of ferric chloride hexahydrate ($FeCl_3.6H_2O$) (0.6 M) and ferrous chloride tetrahydrate ($FeCl_2.4H_2O$) (0.3 M) were assorted. Ammonia (aqueous) was added into the mixture drop-wise under shaking accompanied by steady bubbling of N_2 in the reaction mixture till the pH was in the range 9–11. Subsequently the solution was stirred at 40 °C for 30 min, 150 rpm followed by heating at 80 °C for another 30–40 min with constant shaking. The consequential SNPs were filtered and separated by magnetic decantation and were washed with distilled water to bring the pH to neutral. Different methods were applied to dry magnetic NPs *i.e.* vacuum-drying and freeze-drying. A portion of thick NPs suspension was spread on aluminum foil and kept in an incubator at 37 °C, overnight.

Magnetic NPs suspension was given ultrasonication treatment (35% amplitude for 5 min at room temperature) and 1 mL of deoxygenated water and 2 mL of TEOS were added to these suspension. Suspension was then kept on a shaker at 150 rpm for 12 h at 37 °C. Magnetic NPs so obtained were collected through magnet and were given thorough ethanol washing to remove traces of un-immobilized silane. So obtained SNPs were dried at room temperature under vacuum for 1 h and were stored under N_2. An esterase from *B. pumilus* was purified as previously reported [5] and covalently immobilized on SNPs using glutaraldehyde (2%) as a cross-linker [27–29].

2.2. Enzyme activity

The activity and protein concentrations of free esterase and SNPs-Est were measured by previously reported methods [30,31,9,10].

2.3. Characterization of SNPs-Est

The dimensions and morphology of free and SNPs-Est were observed by Field Emission- Scanning Electron Microscope (FE-SEM; Nova Nano SEM-450, USA) and AFM (Atomic force microscopy; NTEGRA NT-MDT Scanning probe microscope, Russia). The SNPs were observed for phase purity *via* X-ray diffraction (XRD; Philips PAN Analytical, Netherland). An ultraviolet-visible spectrum (UV–vis Spectrophotometer; Lambda Bio 20, Perkin Elmer, USA) was recorded in the range of 200–800 nm. Fourier-transform infrared spectroscopy spectra of naked NPs, SNPs and SNPs-Est (FTIR; Shimadzu FTIR-8400S, Japan) were recorded *via* potassium bromide (KBr) pellet method (Wavelength (λ) 3000–300 cm^{-1}). Vibrating Sample Magnetometer (VSM; Microsense EV9) was also done to confirm magnetic actions of SNPs as well as SNPs-immobilized esterase. The SNPs were studies for electric conductivity by a coductivity metre (Coductivity metre; Eutech Instruments, Singapore).The particle size examination and zeta potential measurements of synthesized NPs were recorded *via* Dynamic Light Scattering analyzer (DLS; Microtac).

2.3.1. Hydrolytic properties of free esterase and SNPs-Est

Substrate specificity of the free and SNPs-Est was determined *via* using substrates namely *p*-NPA, *p*-NPBz, *p*-NPL and *p*-NPP (5 mM in *iso*-propanol). SNPs-Est activity was also assayed at selected reaction temperature (25, 37, 45, 55 and 65 °C) with *p*-NPA (5 mM). The reactions were performed independently using 40 µL of free and ~20 mg of SNPs-Est in Tris buffer (0.05 M; pH 8.0) for 20 min.

Ideally, a biocatalyst must remain functional in different organic solvents in synthetic reactions efficiently [10]. The esterases might be extra destabilized in polar water-miscible solvents than in water-immiscible solvents [32,33]. Hence, to screen the stable esterases with high activity in different types of toxic organic solvents efforts are being made. The free and SNPs-Est were pre-exposed to organic solvents (2%, v/v) *i.e.* DMSO, toluene, *n*-hexane, *n*-heptane, ethanol, *iso*-propanol, butan 2-ol, decane 1-ol, phenol and acetonitrile at 45 °C for 10 min [34]. The hydrolysis of *p*-NPA was performed using 40 µL of free and ~20 mg of SNPs-Est in Tris buffer (0.05 M; pH 8.0) at 37 °C and 45 °C, respectively.

2.3.2. Kinetic studies of free and SNPs-Est

Considerable kinetic parameter like Michaelis Menten constant (*K*m), Maximum Velocity (*V*max), Turn over number (*K*cat) and specificity constant (*K*si) were calculated by calibrating the reaction velocities of free and SNPs-Est with different concentrations of the *p*-NPA (1–30 mM) through Lineweaver-Burk plot. Thermal stability studies were also performed separately in test tubes for 2 *h* intervals at 45 °C. Reusability of the SNPs-Est was examined by recycling SNPs-Est up to 21 cycles of hydrolysis of *p*-NPA.

2.4. Synthesis of ethyl pyruvate using magnetic SNPs-Est

The SNPs-Est (100 mg) was added to 50 mM pyruvic acid in 5 mL ethanol in the occurrence of molecular sieves (1 mg/mL) and the reaction was subjected to shaking for 12 h at 45 °C and 100 rpm. The synthesized ester was obtained by drying to recover ethyl pyruvate. The SNPs were recovered from reaction mixture *via* a magnet. HPLC analysis of recovered ethyl pyruvate (dissolved in methanol) was accomplished *via* 515 HPLC pump (Waters) equipped with a reverse phase Lichrosorb C18-5 µm (4 mm × 125 mm) column (Merck) and 2998 Photodiode Array Detector (Waters) at 265 nm. Methanol: water (60:40) solvent system was used as mobile phase (flow rate of 1 mL/