Journal of Colloid and Interface Science 482 (2016) 159–164

Contents lists available at ScienceDirect

Journal of Colloid and Interface Science

journal homepage: www.elsevier.com/locate/jcis

Regular Article

Immobilization of invertase on chitosan coated γ-Fe_2O_3 magnetic nanoparticles to facilitate magnetic separation

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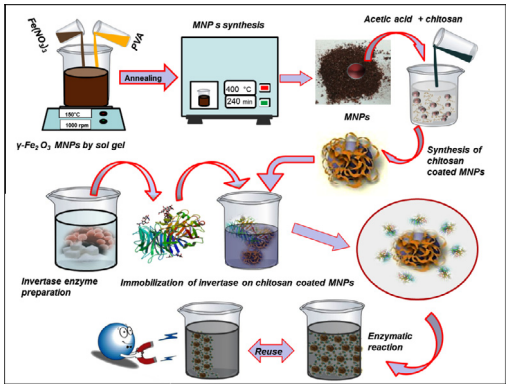
GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 9 June 2016
Revised 29 July 2016
Accepted 29 July 2016
Available online 30 July 2016

Keywords:
Sol–gel processes
Magnetic properties
Magnetic separation
Invertase

ABSTRACT

Industrially important invertase enzyme was immobilized on chitosan coated sol gel derived γ-Fe_2O_3 magnetic nanoparticles (MNPs) to enable it for repetitive use by magnetic separation. MNPs were characterized by X-ray diffraction (XRD), dynamic light scattering (DLS), field emission scanning electron microscope (FE-SEM), Fourier transform infrared (FTIR) spectrometer and magnetic measurements. FTIR studies confirmed successful immobilization of invertase on MNPs. The ability to convert sucrose into invert syrup was enhanced in immobilized invertase compared to that of free enzyme. Further it was found that invertase immobilized on MNPs (IIMNPs) were more stable at varying pH and temperature conditions. Magnetic separation technique was successfully employed for reuse of the IIMNPs for 20 times without significant loss of activity.

1. Introduction

The applications of magnetic nanoparticles in various bioprocesses have been the focus of an intensive research for more than a decade. Nanomagnetic materials have a special relevance to bioapplications due to their size compatibility with cells (10–100 pm), viruses (20–450 nm), proteins (5–50 nm) and genes (2 nm wide by 10–100 nm long). Intensive research into magnetic nanomaterials has accelerated the development of magnetically separable catalysts for reuse. Magnetic separation has been studied to facilitate the handling and recovery of proteins, enzymes and organocatalysts [1]. Biomagnetic materials have

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http://dx.doi.org/10.1016/j.jcis.2016.07.082
0021-9797/

different constraints than materials used for other applications. In addition to biocompatibility, materials must be capable of being functionalized with cells and other biological entities, must retain their magnetic properties for a reasonable period of time in aqueous media with varying pH and temperature and must form stable, non-aggregating dispersions [2,3].

Most of the research in this area involves iron-oxide particles, because they are highly biocompatible, can be made in a variety of ways and sizes, and can be made as superparamagnets or ferrimagnets. However these particles need to be stabilized to obtain magnetic colloidal ferrofluids that are stable against aggregation in both a biological medium and in a magnetic field. Magnetic nanoparticles must remain suspended in fluid (or be easily re-dispersed when needed) and should not form aggregates due to van der Waals or magnetic interactions. Stabilization of particles can be done by coating the particles with different surfactants. Such coating can also functionalize the particles so that different biomolecules can be immobilized or attached to particles. Monomeric functional groups, such as carboxylates, phosphates, and sulfates can be bind to the surface of magnetite [4,5]. Iron oxide nanoparticles can also be coated with inorganic materials like silica [6–10], gold [11–13], or gadolinium (III) [14–16]. In addition to stability of the nanoparticles in solution, this coating also helps in binding various biological ligands to the nanoparticle surface. Grafting the polymer on the magnetic particles is also a good strategy. Polymeric coatings such as dextran, polyethylene glycol (PEG), polyvinyl alcohol (PVA), and chitosan are commonly used [17,18]. Out of these chitosan is an alkaline, nontoxic, hydrophilic, biocompatible, and biodegradable polymer [19–21]. Due to this the preparations of magnetic nanoparticles encapsulated in chitosan are of great interest [22–25].

In the present study, invertase enzyme was used as a biological entity. Invertase acts as a catalysis in hydrolysis of sucrose to mixture of glucose and fructose (invert sugar) [26,27]. This is a very important process since invert sugar does not crystallize easily like sucrose and is preferred over sucrose in food and pharmaceutical industry. Use of free invertase for the hydrolysis of sucrose to invert sugar is costly due to impossibility of recovering and separating them from reaction medium after enzymatic process. Therefore the enzyme immobilization technology is beneficial which offers technical and economic advantages such as, easy separation from the reaction medium for storage, reuse and enhanced stability [28]. Uzun et al. have immobilized invertase on polyamidoamine (PAMAM) dendrimer modified iron oxide nanoparticles and found improved stability of the enzyme upon immobilization [29]. They have also reported invertase immobilization on metal-chelated polyvinylimidazole (PVI) grafted magnetic nanoparticles [30]. Herein, we report a study on immobilization of invertase on chitosan coated γ-Fe_2O_3 magnetic nanoparticles and detailed physicochemical evaluation.

2. Materials and methods

2.1. Chemicals

Invertase (b-fructofuranosidase) type V (EC No. 3.2.1.26) was purchased from Sigma and used as received. Ferric nitrate ($Fe(NO_3)_3{\cdot}9H_2O$) and polyvinyl alcohol (PVA) were purchased from sd fine-chem India. Chitosan from shrimp shells (>75% deacetylated) was purchased from HiMedia India. All other chemicals were of analytical grade and were used without further purification.

2.2. Synthesis of chitosan coated MNPs

γ-Fe_2O_3 magnetic nanoparticles were synthesized by modified sol-gel method [31]. Aqueous solutions of PVA (10 g/100 ml) and saturated ferric nitrate solution were prepared in Milli-Q water. Ferric nitrate solution (10 ml) was mixed with PVA (180 ml) and kept at room temperature for 2 h with constant stirring. The total water was allowed to evaporate at 150 °C under stirring condition to obtain gel. Pure γ-Fe_2O_3 magnetic nanoparticles (MNPs) were then obtained by annealing the gel at 400 °C for 4 h.

To coat MNPs with chitosan, first clear homogeneous chitosan solution was prepared by stirring mixture of 400 mg of chitosan and 20 mL of 0.1 M acetic acid for 5 h. Then 1 g of MNPs was added to chitosan solution and kept at 25 °C for 7 h with gentle shaking. After this chitosan coated MNPs were washed with Milli-Q water by magnetic decantation to remove the free chitosan.

2.3. Immobilization of invertase on chitosan coated MNPs

The chitosan coated MNPs (10 mg/ml) were immersed in invertase solution (1 mg/ml) and gently shaken at room temperature for 1 h. Chitosan coated MNPs are sonicated before and after addition of the enzyme for 15 min for the better dispersion of the MNPs. The invertase immobilized on MNPs (IIMNPs) was obtained by washing this mixture for several times with Milli-Q water by magnetic decantation to remove unbound invertase.

2.4. Enzyme activity and magnetic separation for reuse

The enzymatic activity of free and immobilized invertase was determined by adding 100 μl of enzyme solution to 400 μl of 1% sucrose in 50 mM acetate buffer, pH 5.0 for 10 min. Reaction was stopped with boiling the reaction mixture in water bath and released glucose was measured with the enzymatic glucose determination kit (Merck, India) using glucose as a standard. One unit of invertase activity is defined as the amount of enzyme required to release 1 μmol glucose from sucrose per minute under the described conditions. Invertase concentration was estimated from A280 nm (1 mg/ml) = 2.25 [32].

To determine the thermal stability of both the free enzyme and IIMNPs, assays were carried out at six different temperatures (30, 40, 50, 60, 70 and 80 °C) at pH 5.0. The pH stability was evaluated by measuring activity of free enzyme and IIMNPs at six different pH values (3, 4, 5, 6, 7 and 8) at 30 °C. For pH range of 3.0–6.0 acetate buffer of 50 mM was used and for pH range of 7.0–8.0 phosphate buffer of 50 mM was used.

For reuse of IIMNPs, separation was facilitated by the use of an external magnet, where the reaction mixture was removed while the IIMNPs were held in place with a magnetic field. The collected reaction mixture was used for measurement of enzymatic activity. These magnetically separated IIMNPs, were washed by Milli-Q water before reusing them for carrying out next evaluation.

2.5. Kinetic parameters K_m and V_{max}

The kinetic parameters of the both the free enzyme and IIMNPs were determined by Lineweaver-Burk plot using various concentration of sucrose (0–300 mM) in acetate buffer (50 mM, pH 5.0) at 35 °C. K_m and V_{max} values were calculated from the data obtained after 15 min.

2.6. Characterization

X-ray diffraction (XRD) measurements were done on Bruker AXS D2 phaser diffractometer using Cu K_α radiation (λ = 1.5406 Å). Dynamic light scattering (DLS) measurement was done by using a Malvern Instruments Zetasizer Nano ZS-90 instrument to obtain the size distribution of the particles. Field emission scanning electron microscope (FE-SEM) by a Mira-3, Tescan, was employed for the surface morphology and particle size investigations.