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Development of a thermo-stable and recyclable magnetic nanobiocatalyst for bioprocessing of fruit processing residues and D-allulose synthesis

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ABSTRACT

The aim of the study was to covalently immobilize Smt3-D-psicose 3-epimerase onto functionalized iron oxide magnetic nanoparticles. After immobilization, K_m of the immobilized enzyme increased, however, V_{max} was nearly the same as that of its free form, indicating that immobilization has no detrimental effects on its catalytic output. The covalent immobilization caused a reduction in the deactivation rate constant (k_d) values leading to 4–5 fold enhancement in its half-life at 50–65 °C, indicating significant thermal stability of the iron-enzyme nanobioconjugate. The immobilized enzyme showed excellent storage stability by losing only 20% activity even after 60 days of storage at 4 °C. The immobilized enzyme retained up to 90% of its initial activity even after 10 consecutive cycles of catalyzing D-fructose epimerization reactions. Thus, after immobilization the enzyme exhibited remarkable improvements in thermal tolerance, storage stability and recycling efficiency, useful for development of industrially exploitable process for D-allulose production.

1. Introduction

D-allulose (or D-psicose), a carbon-3 epimer of D-fructose, has been widely recognized as an ultra-low calorie sweetener of rare abundance in nature (Matsuo et al., 2002). Besides being a nearly zero calorie bulk sugar, it has been demonstrated to exert several additional health benefits such as anti-oxidative (Suna et al., 2007), anti-dyslipidemic (Ochiai et al., 2014), hypoglycemic (Chung et al., 2012), anti-obesity (Nagata et al., 2015), anti-diabetic (Hossain et al., 2015) and neuro-protective (Takata et al., 2005) effects. Furthermore, D-allulose improves the texture of food materials and provides a pleasant flavour through the Maillard reaction with food proteins (Sun et al., 2004; O'Charoen et al., 2015). With the aforementioned physiological functions and its GRAS (Generally Regarded as Safe) status (FDA, GRN No. 400), D-allulose is a potential sucrose substitute and a functional biomolecule useful in food industries.

As chemical synthesis of D-allulose is difficult, production of this rare sugar through catalytic inter-conversion of D-fructose to D-allulose using bio-catalysts, such as Ketose 3-epimerases, is the most attractive approach. Ketose 3-epimerases have been identified from various microbial sources such as *Pseudomonas cichorii* (Itoh et al., 1995), *Rhodobacter sphaeroides* (Zhang et al., 2009), *Agrobacterium tumefaciens* (Kim et al., 2006), *Clostridium cellulolyticum* (Mu et al., 2011), *Ruminococcus* sp. (Zhu et al., 2012), *Clostridium* sp. (Mu et al., 2013), *Clostridium scindens* (Zhang et al., 2013a), *Desmospora* sp. (Zhang et al., 2013b), *Clostridium bolteae* (Jia et al., 2014), *Dorea* sp. (Zhang et al., 2015), and *Treponema primita* (Zhang et al., 2016a). Additionally, modified enzyme systems have been developed with improved stability and catalytic efficiency (Choi et al., 2011; Yeom et al., 2011; Bosshart et al., 2015; Patel et al., 2016; Bosshart et al., 2016; Zhang et al., 2016b).

However, the industrial application of free Ketose 3-epimerase enzymes is limited by short half-life, insubstantial operational stability, lack of post-reaction enzyme recovery, and difficulties in reusability for multiple and/or continuous operational cycles with minimal loss of activity (Lim et al., 2009). Enzyme immobilization has been an effective approach for enhancing the activity, stability, and substrate specificity of a biocatalyst. Immobilization of Ketose 3-epimerases have been demonstrated on chitopearl beads (Itoh et al., 1995; Takeshita et al., 2000), sodium alginate (Oh et al., 2011), amberlite resins (Lim et al., 2009), sephadex resins (Lim et al., 2009), and duolite resins (Lim et al., 2009; Choi et al., 2011) to establish continuous production of D-allulose in packed-bed reactor or bioreactor. Moreover, D-psicose 3-epimerase was immobilized on yeast spores (Li et al., 2015) and oil bodies (Tseng et al., 2014) to achieve recovery and recycling of the enzyme. However, to the best of our knowledge, immobilization of Ketose 3-epimerase

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enzyme on magnetic nanoparticles for production of D-allulose has not been reported yet.

Enzyme immobilization on magnetic nanoparticles has several advantages over beads such as high surface area, large surface-to-volume ratio, high stability against oxidation, high loading capacity, low toxicity, high biocompatibility and easier recovery of enzyme by employing an external magnetic field that facilitates repeated and continuous use of the catalyst (Vaghari et al., 2016). Thus, the product mixture can be easily recovered, without contamination, from the enzymatic reaction for efficient utilization, while at the same time the retrieved enzyme can be put to next duty cycles in industrial processes. However, small size, large specific surface area and high chemical reactivity of magnetic nanoparticles make them sensitive to oxidation and agglomeration. Coating of thin oxide layers over nanoparticles may reduce these problems, however, it sometimes causes alteration in the properties of nanoparticles (Atacan et al., 2016). Nevertheless, simplicity and cost-effectiveness of a bioprocess are the keys to success of magnetic nanoparticle immobilized biocatalysts in industrial applications (Vaghari et al., 2016).

We previously demonstrated that N-terminal fusion of yeast Smt3 conferred favoured enzymatic properties, such as elevated optimal temperature, improved stability and better catalytic efficiency, to D-psicose 3-epimerase enzyme (Patel et al., 2016). In the present study, Smt3-D-psicose 3-epimerase was immobilized onto Fe_3O_4 magnetic nanoparticles to enhance its thermal-stability, and recovery and reusability of the enzyme for multiple duty cycles, leading to wide-ranging of its bioprocessing applications. The nanobiocatalyst was characterized by transmission electron microscopy (TEM), zeta potential analysis, X-ray diffraction (XRD) spectra profiling, and Fourier transform infrared spectroscopy (FTIR). The catalytic and stability parameters were evaluated for immobilized Smt3-D-psicose 3-epimerase. The enzyme nanocarriers were operated for the production of D-allulose using D-fructose and fruit processing residues as feedstocks.

2. Materials and methods

2.1. Materials

Iron(III) chloride hexahydrate, Iron (II) chloride tetrahydrate, Tetramethylammonium hydroxide solution, 3-phosphonopropionic acid, N-hydroxysuccinimide (NHS), 1 ethyl-3-(dimethylaminopropyl) carbodimide (EDC), sodium hydroxide, D-fructose, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer were sourced from Sigma Aldrich Chemicals Co. (St. Louis, USA). Manganese (II) chloride tetrahydrate was acquired from Himedia laboratory Pvt Ltd. (Mumbai, India). The entire reagents were of analytical grade. All the chemical solutions and buffers were prepared in deionized distilled water with high resistivity (18.2 MΩ cm at 25 °C).

2.2. Enzyme preparation

D-psicose 3-epimerase gene from *Agrobacterium tumefaciens* was cloned in Champion™ pET SUMO (Invitrogen, USA) vector for heterologous expression of Smt3-D-psicose 3-epimerase conjugate protein in *Escherichia coli* BL21 (DE3) (Novagen, Germany), as described previously (Patel et al., 2016). The recombinant *E. coli* cells were grown in luria bertani (LB) medium supplemented with antibiotic selection (Kanamycin 20 μg mL^{-1}), at 37 °C and 220 rpm. Protein expression was induced by the addition of 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at ~0.6 OD_{600}, and the culture was incubated at 16 °C at 150 rpm for 20 h.

The recombinant cells were harvested by centrifugation at 6000 rpm for 5 min at 4 °C. After washing of the pellet with 0.85% NaCl solution, the cells were suspended in 50 mM HEPES buffer and 300 mM Nacl (pH 8). The cells were lysed by the addition of 0.5 mg mL^{-1} lysozyme followed by sonication at 30 amplitude (3 s 'on' and 7 s 'off') for 3–5 min (Q sonica, USA). After centrifugation (12,000 rpm for 20 min at 4 °C) the supernatant was clarified by passing through 0.45 μm syringe filter. The clarified flow through was loaded on nickel-nitrilotriacetic acid (Ni-NTA) agarose affinity chromatography matrix (Qiagen, Germany), pre-equilibrated with 300 mM Nacl, 50 mM HEPES buffer and 10 mM Imidazole (pH 8). Then, the column was washed with 3 bed volume of 300 mM Nacl, 50 mM HEPES buffer and 70 mM imidazole (pH 8). The bound protein were eluted by 300 mM NaCl, 50 mM HEPES buffer and 300 mM imidazole (pH 8). The eluted protein was further purified by Q-Sepharose ion exchange column, and elution was done using 300 mM Nacl and 50 mM HEPES buffer (pH 8). The protein concentration was determined by the Bradford method using bovine serum albumin (BSA) as a standard.

2.3. Synthesis of functionalized magnetic nanoparticles and enzyme immobilization

The magnetic nanoparticles were prepared by the co-precipitation method (Maity and Agrawal, 2007; Singh et al., 2016). The iron chloride solution was prepared in 0.4 M HCl by mixing $FeCl_2{\cdot}4H_2O$ (0.4 M) and $FeCl_3{\cdot}6H_2O$ (0.8 M) in 1:2 ratio. The mixture was heated at 120 °C for 15 min under vigorous stirring. The clear solution of iron chlorides was added drop by drop into 250 ml of 1.5 M NaOH solution with vigorous stirring at 80 °C, along with nitrogen purging to maintain the inert environment. A black precipitate appeared and the reaction mix was further incubated at 80 °C for 30 min under vigorous stirring. After this, the reaction mixture was allowed to cool to room temperature. The precipitate containing Fe_3O_4 magnetic nanoparticles was retrieved from the solution using a magnetic field, followed by sequential washing with water and 0.1 M tetramethylammonium hydroxide pentahydrate (TMAOH), and finally suspended in 0.1 M TMAOH solution and stored in dark at room temperature.

The Fe_3O_4 magnetic nanoparticles were mixed with 3-phosphonopropionic acid (3-PPA) solution in 1:1 M ratio and pH of the solution was adjusted to 8, followed by ultra-sonication at amplitude 30 (10 s 'on' and 10 s 'off') for 30 min. The solution was placed on a permanent magnet for several hours (24–40 h) to settle down the magnetic nanoparticles grafted with 3-PPA. The PPA grafted magnetic nanoparticles were washed with de-oxygenated water and then suspend in 0.1 M MES buffer (pH 6). The carboxyl group of 3-PPA was activated by addition of EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) (~2 mM) and N-hydroxysuccinimide (NHS) (~5 mM) in 0.1 M MES buffer containing 1 mg mL^{-1} $Fe_3O_{4\text{-}3PPA}$ magnetic nanoparticles. The mixture was incubated at room temperature for 15 min. The activated $Fe_3O_{4\text{-}3PPA}$ magnetic nanoparticles were harvested by applying a magnetic field.

Smt3-D-psicose 3-epimerase enzyme and magnetic nanoparticles were mixed in 1:1.5 wt ratio in 50 mM HEPES buffer (pH 8), and incubated at room temperature for 2 h with tapping after every 15 min. The nanoparticles with immobilized enzyme were retrieved by applying an external magnetic field, and washing of nanoparticles was done by 50 mM HEPES buffer (pH 8). Finally, the magnetic nanoparticles with immobilized enzyme molecules were stored in 50 mM HEPES buffer (pH 8) at 4 °C.

The percentage of enzyme immobilized onto the nanoparticles was determined by calculating the amount of enzyme in unbound and washing fractions, and comparing them with initial protein concentration as follows:

$$\text{Immobilized enzyme } (\%) = (\text{SPEi} - \text{SPEs}/\text{SPEi}) \times 100$$

SPEi = Initial protein concentration.
SPEs = Protein concentration in unbound fraction + washings.