PREPARATION OF MAGNETIC POLYHYDROXYBUTYRATE MICROPARTICLES AND APPLICATION FOR INVERTASE IMMOBILISATION

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Abstract

A new original technology of production of PHB hollow microspheres with addition of nanoparticular magnetite by carbon dioxide assisted nebulisation drying has been developed. Invertase enzyme (EC 3.2.1.26) was immobilised on the microparticles surface by a simple process exploiting hydrophobic interactions. The biocatalyst had very good activity and stability after repeated applications for sucrose hydrolysis to glucose and fructose. It can be used in effective biocatalytic production of inverted sugar syrup.

Keywords: Invertase, immobilisation, magnetic microparticles, polyhydroxybutyrate, spray nebulisation

1. INTRODUCTION

Invertase is a commercially important enzyme used for the hydrolysis of sucrose. The hydrolysis of sucrose yields an equimolar mixture of glucose and fructose, known as invert syrup, is widely used in food and beverage industries. This enzyme is also used for the manufacture of artificial honey, plasticizing agents used in cosmetics, pharmaceutical and paper industries as well as enzyme electrodes for the detection of sucrose [1]. Soluble invertases have been highly used for immobilisation. Invertase was probably the first enzyme to be used on a large industrial scale in an immobilised form. Tate & Lile Company used clarified yeast autolysate absorbed on bone char for production of the Golden Syrup during World War II, because sulfuric acid as the preferred reagent was unavailable at that time [2]. Some of the gel matrices currently used for soluble invertase immobilisation by entrapment is κ-carrageenen, alginate, gelatin, few synthetic polymers [1] and other materials, such as polyacrylamide-gelatine carrier system [3], Dowex® anionic exchange resin [4], microporous montmorillonite [5], or wood sawdust waste [6].

Recent interest in nanotechnology has provided a wealth of diverse nanostructured carriers that could potentially support enzyme immobilisation due to their potential applications in biotechnology, immunosensing, and biomedical areas. Immobilisation of enzymes is advantageous for commercial application due to convenience in handling, ease of separation of enzymes from the reaction mixture and reuse, low product cost, and a possible increase in thermal and pH stability [7]. Various nanostructures have been examined as hosts for enzyme immobilisation via approaches including enzyme adsorption, covalent attachment, enzyme encapsulation, and sophisticated combinations of methods [8]. Magnetic particles have been increasingly used as carriers for binding proteins, enzymes, and drugs. These smart materials exhibit different types of response to external magnetic field. In most cases they can be described as composite materials, where the magnetic properties are caused by the presence of iron oxides nano- or microparticles. Such materials are of great importance for new scientific and technological applications [9]. Enzyme immobilisation methods, such as adsorption, affinity binding, chelation, or metal binding or covalent binding, enable the preparation of efficient and stable enzyme bound to magnetic nanoparticles. Enzyme loaded magnetic nanoparticles have been reviewed by Seenuvasan, et al. [10]. Magnetic particles have already been used for invertase immobilisation, too. Akgöla, et al., immobilised invertase onto magnetic polyvinylalcohol microspheres [11].

Invertase immobilised on magnetic diatomaceous earth nanoparticles with high sucrolytic activity was described by Cabrera, et al. [12]. Uzun, et al., immobilised invertase covalently on PAMAM-dendrimer modified superparamagnetic iron oxide nanoparticles [13]. Polyhydroxyalkanoates (PHAs) belong among the promising materials for enzyme immobilisation. PHAs are a group of water insoluble biodegradace biopolymers. Among PHAs, polyhydroxybutyrate (PHB) is a short-chain-length polymer synthesised by bacteria and it is the most frequently studied among the PHA polymers [14]. PHB particles or beads have already been used for immobilisation of nattokinase [14] and lipases [15.16]. Beran, et al., used PHB nanofibers for immobilisation of endoinulinase [17]. In this study we have developed a cheap, simple, and effective method of the preparation of PHB magnetic microspheres by a new technology of spray nebulisation drying. Engineering efforts of researches of the Food research institute Prague and the Czech Technical University in spray drying technologies led to introduction of a demonstrator ATOMIZER. The ATOMIZER demonstrator combines the spray drying technology, when the liquid to be dried is atomized by a rotary atomizer, with carbon dioxide assisted nebulisation process in an original way. The atomization process takes place in two steps. In the first step, primary droplets are produced at the outlet of the rotary atomizer of special construction. In the second step, the primary droplets are divided in secondary droplets by the carbon dioxide expansion from the inside of primary droplets. The secondary droplets, usually in the form of microbubbles and nanobubles, are rapidly dried by warm air stream in a drying chamber at temperatures up to 60 ºC.

2. MATERIALS AND METHODS

2.1. Material

Commercial polyhydroxybutyrate (PHB) *Biomer* microparticles, Lot T21, were purchased from Biomer (Krailling, Germany). Invertase enzyme, >10000 U/g, was purchased from Carolina, USA. Chloroform, absolute ethanol, 4- hydroxybenzoic acid hydrazide (PAHBAH), sucrose, magnetite nanoparticles< 50 nm, sodium azide and other chemicals were purchased from Sigma-Aldrich (Prague, Czech Republic).

2.2. Production of magnetic polyhydroxybutyrate microparticles by spray nebulisation

The 2.5 % (w/w) solution of PHB in chloroform was prepared by dissolution of the polymer under reflux at 70 ºC for 24 h. The magnetite nanoparticles were added to the solution under intensive mixing before drying. The suspensions prepared were dried by the Carbon Dioxide Spray Nebulisation Drying (CASND) process using the Atomizer demonstrator (see **Figure 1**) under following conditions: Liquid flow rate, 10 ml.min^{-1}; Carbon dioxide flow rate, 10 ml.min^{-1}; Pressure, 5 MPa; Inlet air temperature, 60 ºC; Outlet air temperature, 48 ºC; Air flow rate: 400 m^3.h^{-1}.

2.3. Immobilisation of invertase onto polyhydroxybutyrate microparticles

Initially, 1 g of PHB magnetic microparticles were soaked into 50 ml of anhydrous ethanol for 30 min at room temperature, following a previously published procedure [15,17]. After the swelling step, the excess of ethanol was removed and the fibres were washed thoroughly with demineralised water. The washed microparticles were incubated in 100 ml of 2 % (w/w) solution of invertase in 5 mM phosphate buffer, pH 7.0, with 0.05 % (w/w) sodium azide, under continuous agitation in an orbital shaker (200 rpm) at 30°C for 12 hours. Finally, the microparticles were washed thoroughly with demineralised water, dried at room temperature, and weighed. A neodymium magnet was used to separate the microparticles from the solution.

2.4. Scanning electron microscopy

The morphology of PHB microspheres before and after invertase immobilisation was evaluated by scanning electron microscopy (SEM). The samples were evaluated in a Phenom G2 scanning electron microscope (Phenom-World BV, Eindhoven, Netherlands).

2.5. Determination of invertase activity

Activities of the free invertase and the invertase immobilised on the magnetic microparticles were determined by sucrose hydrolysis to glucose and fructose in 5 % (w/w) sucrose solution in 100 mM acetate buffer, pH 4.5, with 0.05 % (w/w) sodium acide, under continuous agitation in an orbital shaker (200 rpm) at 55 °C. One ml samples were collected in given time intervals. The enzymatic reaction in the samples was stopped by boiling the samples for 10 min in a water bath. Standardized Enzymatic Assay of Invertase of Sigma-Aldrich (http://www.sigmaaldrich.com/technical-documents/protocols/biology/enzymatic-assay-of-invertase.html) was used to determine invertase activity, based on the PAHBAH reducing sugar assay. Besides the PAHBAH assay, a HPLC method with a refractometric detector was also used for sucrose, glucose and fructose determination under the following conditions: Hema-Bio 1000 SB (30 × 3 mm, 10 µm) guard columns; Ostion LGKS 0800 Ca form (250 × 8 mm) column; column temperature 80°C; mobile phase demineralised water, flow rate of 0.5 ml/min. The HPLC system consisted of Waters 515 pump, In-line degaser AF Waters, Autosampler Waters 717 plus and RI detector Waters 2414.

2.6. Determination of specific activity of the immobilised invertase

For calculation of the specific activity of the immobilised invertase, protein concentration on the magnetic microparticles after the enzyme immobilisation was calculated from the difference between protein concentrations in the invertase solutions before and after immobilisation onto the PHB microparticles. Absorbance measured at 280 nm using Helios α (Thermo Scientific, USA) spectrophotometer was used to calculate protein concentration by comparison with standard curve. Invertase activities were expressed relative to the protein concentrations. All the assays were performed in triplicate for each sample.

2.7. Determination of the biocatalyst reusability

To evaluate the reusability of the biocatalyst, the magnetic microparticles with immobilised invertase were removed from the sucrose solution, washed thoroughly with demineralised water and dried at laboratory temperature. The biocatalyst was stored at laboratory temperature and reintroduced into a fresh reaction medium.

3. RESULTS

3.1. Characterisation of the biocatalyst by SEM

Figure 2 shows SEM image of the PHB hollow porous microspheres with diameters in the range 0.5 - 20 µm. The magnetite nanoparticles are noticeable as small white dots at the surface of the microspheres. No morphology change was observed after the enzyme immobilisation.

3.2. Determination of specific activity of the immobilised invertase

Time course of sucrose hydrolysis determined by the HPLC method is shown in **Figure 3**. **Figure 4** shows separation of the biocatalyst with neodymium magnet. The full line in the **Figure 5** shows a linear time course of reducing sugars production from sucrose by the freshly prepared biocatalyst. The activities of the free and immobilised invertase enzymes were calculated from the line slopes. The activity of the original free invertase was 18520 ± 382 U/g. Protein concentration in the biocatalyst was 4.15 ± 0.12 % (w/w). The specific activity of the immobilised invertase was 21569 ± 412 U/g. Immobilisation of the enzyme onto the PHB magnetic microspheres increased the specific activity by 16 %.

Figure 1 Demonstrator Atomizer

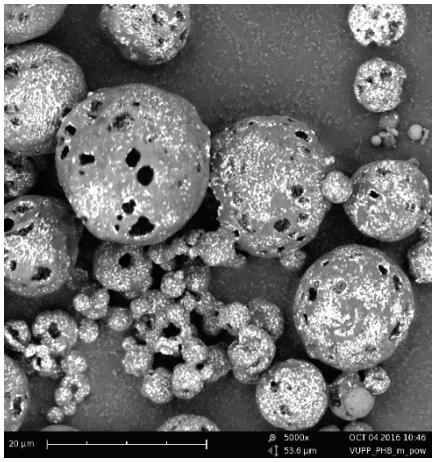

Figure 2 SEM image of of the PHB microspheres with magnetite nanoparticles

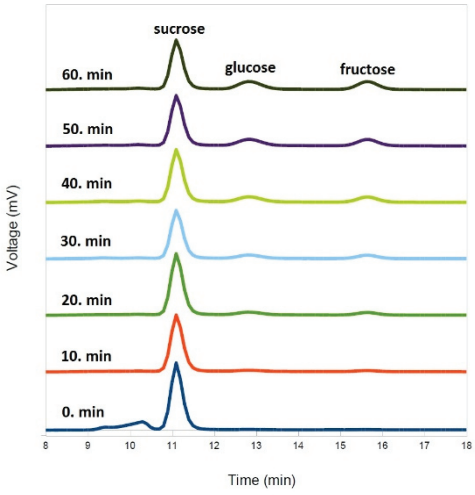

Figure 3 Time course of sucrose hydrolysis

Figure 4 Separation of the biocatalyst with neodymium magnet

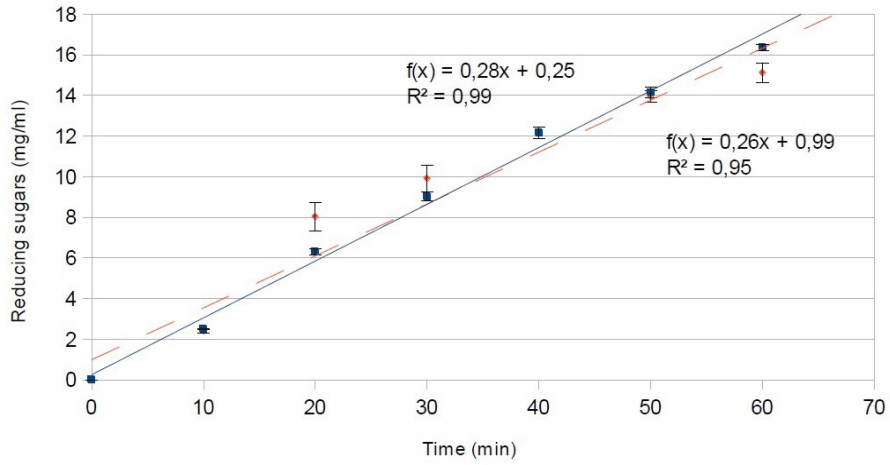

Figure 5 Time course of reducing sugars production. Full line: First experiment Dashed line: Repeated usage of the biocatalyst after 112 days

3.3. Determination of the biocatalyst reusability

The dashed line in the **Figure 5** shows a linear time course of reducing sugars production from sucrose by the reused biocatalyst after 112 days of storing at laboratory temperature. The specific activity of the immobilised invertase in the reused biocatalyst was 19,318 ± 296 U/g. The relative decrease of the specific activity was 10.4 % in comparison with the freshly prepared biocatalyst.

4. DISCUSSION

Immobilisation of the enzyme onto the PHB magnetic microspheres increased the specific activity by 16 % in comparison with the free enzyme. Increase in the specific activity after immobilisation is not unusual and can be explained by conformation change and stabilization of the enzyme. However, the immobilisation of invertase in alginate capsules resulted in decrease of relative activity by 13 % [18], immobilisation into polyacrylamide-gelatin carrier by 21 or 28 % [3], entrapment in polyvinyl alcohol hydrogel capsules by 14 % [19]. The retained activity of invertase immobilized on the magnetic polyvinylalcohol microspheres crosslinked by glutaraldehyde was 74% [11]. Cross-linking of invertase on aminoalkylsilylated magnetite with glutaraldehyde resulted in low yield due to the inactivation of the enzyme. However in the presence of 1% sucrose, the total activity recovered was 79% of the initial activity and the preparation contained 4.4 mg/g of active invertase [20]. A residual specific activity of invertase immobilised onto magnetic diatomaceous earth nanoparticles equalled to 92.5 % [12]. Crosslinking agents usually inactivate enzymes by denaturation, at least partially. Moreover, toxic properties of most of the crosslinking agents can limit utilization of the biocatalysts for food production. Activities of the immobilised were usually quite stable during storage in the range 1 - 8 moths and repeated usage [3, 5, 12, 18, 19, 21, and 22]. Immobilised samples were used 20 [3] or 25 [5] times without a considerable loss of activity.

5. CONCLUSION

Simple and cheap technology of production of the biocatalyst in the form of PHB hollow porous microspheres containing magnetite nanoparticles has been verified at pilot-plant scale. The PHB magnetic microspheres can be used for the immobilisation of a wide range of different enzymes in an easy way, without necessity of chemical crosslinking. The biocatalyst described in this paper has very good activity and stability after repeated applications. It can be used for the production of inverted sugar syrups. The magnetic particles can be easily manipulated in response to external magnetic field. Repeated separation and washing can be achieved using a simple, inexpensive magnet.

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