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Packed bed enzyme microreactor: Application in sucrose hydrolysis as proof-of-concept

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ABSTRACT

A continuous flow enzyme microreactor was designed, assembled and run that allowed for operation with immobilized enzymes in particulate form. As a proof-of-concept, invertase was covalently bound to silane-coated silica carriers and used for the hydrolysis of sucrose into invert sugar syrup. Once glutaraldehyde solution and enzyme load were optimized, and the kinetic behavior of the immobilized biocatalyst was established, the particles were loaded in the sandwich type microreactor, resulting in a packed bed form. The microreactor was fed with substrate solution within 1.0–8.0% (w/v), at flow rates ranging from 17.5 to 259.0 μL/min. Apparent kinetics were evaluated using the Lilly–Hornby model. Accordingly $K_{m(app)}$ values decreased with increasing flow rates, whereas, for higher flow rates, the $K_{m(app)}$ tends to stabilize at values close to that observed for the enzyme in the free form. Full conversion was observed up to 8.0% (w/v) of sucrose. Moreover, the immobilized invertase formulation packed in the microreactor displayed high operational stability, as it retained roughly 100% of its initial activity during 30 days.

1. Introduction

Over the last decade, a consistent trend towards the use of microscale processing techniques in biocatalysis has emerged and has been contributing to speed up the development of enzyme based systems [1–3]. The high level of parallelization that is achieved in microfluidic devices allows the high throughput required at the different phases of bioprocess development. Operation in microfluidic environment is characterized by low reagent consumption and energy requirements, concomitantly contributing to reduce the cost of process development and the environmental impact. Moreover, the minute diffusion lengths result in enhanced heat and mass transfer, which coupled to the typical continuous mode of operation under laminar flow, allow for a better control over process conditions and enhanced safety. Finally, production scale may be achieved by numbering-up rather than scaling-up. Altogether, the outcome is a faster transfer of the development stage into production [2–4].

The present work is within the context of the use of microscale platforms on the development of a biocatalytic continuous process anchored in invertase immobilization. Invertase (β-fructofuranosidase, EC 3.2.1.26) is mainly used to catalyze the hydrolysis of sucrose in the production of an equimolar mixture of glucose and fructose (invert sugars) that is 20% sweeter and less prone to crystallization than sucrose [5,6]. Invert sugars are widely used in bakery and pastry, where shelf life is improved; in the manufacture of artificial honey; and as plasticizing agent in cosmetics [7–9]. Continuous flow enzyme reactions require enzyme immobilization, and among the diverse methodologies used for invertase immobilization [10], some have also been used in the design of continuous hydrolytic systems, yet overlooking the potential of microreactors. Thus, Albertini et al. immobilized invertase on glass-ceramic support and proceeded with experiments in a packed bed reactor with alternate-flow [11]. Tomotani and co-workers evaluated the performance of a membrane reactor with invertase adsorbed on anionic polystyrene beads [7]. Invertase, chemically modified with chitosan, was immobilized on pectin coated chitin support by Gómez et al. and packed into a column reactor [12]. Cadena et al. covalently immobilized invertase on polyurethane rigid adhesive foam and subsequently covered the internal surface of a metallic column. The resulting structure was used on the

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continuous hydrolysis of sucrose [13]. Kumar et al. developed a simple flow-through reactor by chemical activation of a polyvinyl chloride tube where invertase was immobilized afterwards [14]. Invertase was encapsulated in magnetic polyvinyl alcohol microspheres with glutaraldehyde as crosslinking agent and used in a packed bed reactor by Akgöl et al. [15]. Nylon-6 microbeads were used for the covalent immobilization of invertase by Amaya-Delgado et al. and the resulting formulation was tested in a tubular fixed bed reactor [16].

The extensive accessible data together with the industrial relevance of invert sugars and the wide availability of invertase, make this reaction both an interesting model and a relevant target system. In the present work, the development of a continuous biocatalytic process combining enzyme immobilization and the use of miniature devices is presented. Invertase from *Saccharomyces cerevisiae* was covalently immobilized on CPC (controlled porosity carrier) silica carrier silane coated particles through the use of glutaraldehyde as crosslinking agent. Due to CPC silica carriers mechanical instability, preliminary optimization of the immobilization protocol was performed in a static basket reactor configuration, where the effect of pH and concentration of both glutaraldehyde and invertase solutions on biocatalyst activity were evaluated. The same reactor configuration was used to assess the impact of immobilization on the optimal operational conditions of the enzyme and to test biocatalyst reuse in successive batch runs.

The immobilized enzyme was used in a packed-bed enzymatic microreactor for the production of invert sugars, where mass transfer and kinetic parameters were evaluated. Finally, the operational stability under continuous flow was also studied.

2. Materials and methods

2.1. Materials

Invertase (Maxinvert L 10000, batch number 611181801), from *S. cerevisiae*, was from DSM Food Specialties; CPC (controlled porosity carrier) – silica carrier silane coated reported as 30–45 mesh with a pore size of 375 Å was purchased from Fluka Analytical; Glutaraldehyde 24% (w/w) aqueous solution was acquired from Acros Organics. Glacial acetic acid (purity ≥ 99.7%) and dipotassium hydrogen phosphate (purity ≥ 99.0%) were purchased from Panreac; Sodium acetate (purity ≥ 99%) and sodium dihydrogen phosphate dehydrate (purity ≥ 98.0%) were obtained from Merck. A solution of sodium cyanoborohydride (5.0 M) was purchased from Sigma–Aldrich. Analytical grade Sucrose and Fructose were obtained from Fisher Chemicals and Sigma–Aldrich, respectively. Pierce BCA Protein Assay Kit was purchased from Thermo Scientific. Bradford reagent was acquired from Sigma–Aldrich.

2.2. Biotransformation with free enzyme

Experiments with free enzyme were carried out in magnetically stirred (600 rpm) 25 mL vessels. 10 μL of invertase were added to 10 mL of a 5.0% (w/v) sucrose solution prepared in acetate buffer 100 mM pH 4.5 at a temperature of 50 °C. Samples (10 μL) were collected periodically, quenched in dinitrosalicylic acid (DNS) reagent and assayed for quantification of reducing sugars. Enzyme activity was determined through the initial reaction rates. All trials were performed in triplicates.

2.3. Free enzyme kinetic parameters

Batch runs were performed in the presence of sucrose solutions with concentrations ranging from 0.5 to 10% (w/v), and assessing the corresponding initial reaction rates. Solutions were prepared in acetate buffer 0.1 M pH 4.5 and trials were performed at 50 °C. 10 μL samples were collected every minute until a maximum of 10% substrate conversion was reached. Enzyme activity was determined through the initial reaction rates. Kinetic parameters, V_{max} and K_m, were obtained through Hyper32® software.

2.4. Invertase covalent immobilization

Unless indicated otherwise, 1 g of CPC silica carriers (dry weight) were immersed in a 10 mL solution of 0.3% (w/w) glutaraldehyde prepared in phosphate buffer (100 mM, pH 8) followed by degasification under vacuum for 1 h and overnight incubation at room temperature in an orbital shaker at 20 rpm. Subsequently, the CPC silica carriers were thoroughly washed with acetate buffer (100 mM, pH 5) to remove excess of glutaraldehyde and immersed in 10 mL acetate buffer (100 mM, pH 5) solution containing 1.5 g/L invertase and 50 mM of sodium cyanoborohydride. The mixture was incubated for 20 h at room temperature in an orbital shaker at 20 rpm. Finally, the CPC carriers were again thoroughly washed with acetate buffer (100 mM, pH 5) to remove any loosely bound enzyme and stored in the same buffer at 4 °C until further use.

Immobilized invertase quantification was performed by mass balance, where the mass of invertase in solution was assayed by BCA method [26] prior and after incubation with CPC silica carriers.

2.5. Biotransformation with immobilized enzyme

Given the mechanical instability of the CPC silica carriers when exposed both to magnetic and orbital stirring, a static basket reactor configuration was chosen to carry immobilization protocol optimization and characterization. Within this approach, a triangular polyethylene terephthalate (PET) basket (2 cm × 2 cm × 2.82 cm) was used to hold 100 mg (wet weight) of CPC silica carriers with immobilized invertase. Experiments were carried out in the same conditions used on the biotransformations with free enzyme.

2.6. Immobilization protocol optimization

The conditions used on the immobilization protocol were optimized. In particular, pH and concentration of the glutaraldehyde and enzyme solutions were varied and the obtained immobilized invertase assayed for sucrose hydrolysis in 15 min batch runs.

On these trials and along the present work, values for the relative activity were calculated as follows:

$$\text{relativeactivity} = \frac{\text{activity}}{\text{maximumactivity}} \times 100$$

2.7. Effect of pH and temperature on the activity of free and immobilized invertase

The effects of pH and temperature on enzyme activity were evaluated in batch runs by incubating either form of the enzyme in sucrose solutions, 5.0% (w/v) in acetate buffer 100 mM, in a pH range of 3–6, and in a temperature range of 40–70 °C. The determination of enzyme activity was performed as described previously.

2.8. Operational stability in static basket reactor configuration

The operational stability of the immobilized invertase in the static basket reactor configuration was evaluated by performing 10 consecutive 15 min batch runs. In-between runs, the immobilized invertase was recovered from the reaction system and washed with acetate buffer (100 mM, pH 4.5). Samples were collected and analyzed for reducing sugars and protein. Initial reaction rates were calculated for each run and results are presented as relative activities towards the first batch reaction.