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Immobilization of *Bacillus circulans* β-galactosidase and its application in the synthesis of galacto-oligosaccharides under repeated-batch operation

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ABSTRACT

A highly active and stable derivate of immobilized *Bacillus circulans* β-galactosidase was prepared for the synthesis of galacto-oligosaccharides (GOS) under repeated-batch operation. *B. circulans* β-galactosidase was immobilized on monofunctional glyoxyl agarose and three heterofunctional supports: amino-, carboxy-, and chelate-glyoxyl agarose. Glyoxyl agarose was the support with highest immobilization yield and stability being selected for the optimization of immobilization conditions and application in GOS synthesis. A central composite rotatable design was conducted to optimize contacted protein and immobilization time, using maximum catalytic potential as the objective function. Optimal conditions of immobilization were 28.9 mg/g and 36.4 h of contact, resulting in a biocatalyst with 595 IU/g and a half-life 89-fold higher than soluble enzyme. Immobilization process did not alter the synthetic capacity of β-galactosidase, obtaining the same GOS yield and product profile than the free enzyme. GOS yield and productivity remained unchanged along 10 repeated batches, with values of 39% (w/w) and 5.7 g GOS/g of biocatalyst·batch. Total product obtained after 10 batches of reaction was 56.5 g GOS/g of biocatalyst (1956 g GOS/g protein). Cumulative productivity in terms of mass of contacted protein was higher for the immobilized enzyme than for its soluble counterpart from the second batch of synthesis onwards.

1. Introduction

Galacto-oligosaccharides (GOS) are non-digestible oligosaccharides with a recognized prebiotic capacity, promoting specific changes in the composition and/or the activity in the gastrointestinal microbiota that confer benefits upon host well-being and health [1,2]. These physiological effects together with their physicochemical characteristics have enabled them to be incorporated in a variety of design foods, especially in acidic beverages and fermented milk formulations since they exhibit higher thermal stability in acidic environments than fructo-oligosaccharides, another recognized and widely utilized prebiotic [3].

GOS are composed by a mixture of di-, tri-, and even higher oligosaccharides characterized by the presence of a terminal glucose, the remaining monosaccharide units being galactose, and also disaccharides comprising two units of galactose [4]. GOS may be enzymatically produced from lactose by β-galactosidases (β-D-galactoside galactohydrolases, EC 3.2.1.23), through a kinetically controlled reaction, caused by the competition between the reactions of hydrolysis and synthesis from the galactosyl-enzyme complex formed. The predominance of synthesis over hydrolysis depends mainly on the origin of the β-galactosidase [5,6] and the initial lactose concentration [7–9]. It has been reported that water thermodynamic activity also affects GOS synthesis [10,11]; however, no effect was observed when its value varied between 1 and 0.77 [9].

The synthetic capacity of β-galactosidase from *B. circulans* has been widely evaluated for the production of GOS [9,12–15] and other transgalactosylated products [16–19]. It has been reported that commercial β-galactosidase from *B. circulans* is a mixture of three monomeric isoenzymes with molecular weights of 212 kDa (I), 145 kDa (II), and 86 kDa (III). The optimal pH and temperature vary according to the isoenzyme evaluated. The optimal pH for isoenzyme I is 6 while for the other two isoforms is 4. The optimal temperatures of isoforms I, II and III are 44, 74, and 60 °C, respectively [20].

Even though the synthesis of GOS with the soluble β-galactosidase from *B. circulans* has been carried out at commercial scale, as in any enzymatic process, the immobilization of the biocatalyst may offer significant improvements from an economical and technical point of view. Among immobilization methods, covalent enzyme attachment to solid supports normally leads to most

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stable biocatalysts. Several supports have been tested for covalent immobilization of *B. circulans* β-galactosidase including silica [15,21,22], chitosan particles [14], polymeric membranes [23,24], and methacrylate supports [25–27] and many of those catalysts have been tested for the synthesis of GOS [14,15,22–24].

Glyoxyl-agarose is a widely used support for offering a high degree of stabilization to many enzymes [28]. Immobilization of enzymes in glyoxyl-agarose is produced through the protein surface area with the highest concentration of lysine residues; nevertheless, it has been reported that the presence of a second reactive group in the support allows the variation of the enzyme orientation during immobilization, resulting in some cases in a higher stabilization as a consequence of involving enzyme areas more sensitive to denaturing agents [29].

The objective of this work was to produce a highly loaded and stable biocatalyst of *B. circulans* β-galactosidase by immobilizing it in an agarose support, and its further application in GOS synthesis under repeated-batch operation mode. A previous step of selection of the best agarose activation method was considered before optimizing protein load and immobilization time. Effect of immobilization over GOS profile was analysed.

2. Materials and methods

2.1. Materials

A commercial preparation of β-galactosidase from *B. circulans* (Biolactasa-NTL CONC X2) from BIOCON (Barcelona, Spain) was used having 31.3 ± 0.5 mg of protein/mL and 1353 ± 11 IU/mL (definition of IU is in Section 2.2). *o*-Nitrophenyl-β-D-galactopyranoside (*o*-NPG) was purchased from Carbosynth Limited (Bershire, UK). Sodium metaperiodate, iminodiacetic acid, cupric sulfate, triethylamine, and glycerol were purchased from Merck (Darmstadt, Germany). Epichlorhydrine, glycidol, sodium borohydride, *o*-nitrophenol (*o*-NP), were from Sigma–Aldrich (St. Louis, MO, USA). Lactose monohydrate was from Quimatic S.A. (Santiago, Chile). Sepharose CL-6B bead (6% agarose, cross-linked with epichlorohydrin) was purchased from GE Healthcare (Uppsala, Sweden). All other reagents were of the highest available purity and used as purchased.

2.2. Analysis

The enzymatic activity was assayed using *o*-NPG as substrate and measuring *o*-NP release spectrophotometrically at 420 nm using a temperature-controlled cell with constant magnetic stirring. To initiate the reaction, 50 μL of β-galactosidase solution or suspension was added to 2 mL of substrate solution. One international unit of β-galactosidase activity (IU) was defined as the amount of enzyme producing 1 μmol of *o*-NP per minute from a 45 mM *o*-NPG solution in 0.1 M citrate-phosphate buffer pH 6 at 25 °C. The extinction molar coefficient of *o*-NP under assay conditions was 560 M^{-1} cm^{-1}.

Protein concentrations were determined by Bradford methodology [30].

Lactose and products of GOS synthesis (galactose, glucose and GOS) were determined in a Jasco RI 2031 HPLC delivery system, provided with refractive index detector, isocratic pump (Jasco PU2080) and autosampler (Jasco AS 2055), using BP-100 Ag columns (300 mm 7.8 mm) for carbohydrate analysis (Benson Polymerics, Reno, NV). Samples were eluted with Milli-Q water at a flow rate of 0.5 mL/min. Column and detector temperatures were 80 and 40 °C, respectively. The composition of the samples was determined by assuming that the area of each peak is proportional to the weight percentage of the respective sugar on the total sugars mass [31]. Standards of galactose, glucose, 4β-galactobiose (disaccharide), lactose and 3α-4β-3α-galactotetraose (tetrasaccharide) were used to determine their retention times and check the linear range of the measurements. Chromatograms were integrated using the software ChromPass.

2.3. Enzyme immobilization

Immobilization was monitored by measuring the enzyme activity in the suspension and in the supernatant. Immobilization yields were determined in terms of expressed activity (IY_A) and bound protein (IY_P):

$$IY_A = \frac{A_I}{A_C} \cdot 100 \quad (1)$$

$$IY_P = \frac{P_I}{P_C} \cdot 100 \quad (2)$$

where A_I is the activity expressed by the immobilized biocatalyst (measured by suspending the immobilized and washed biocatalyst in the activity buffer), A_C is the enzyme activity contacted per gram of support, P_I is the immobilized protein per gram of support (difference between contacted protein and unbound protein in the supernatant) and P_C is the contacted protein per gram of support.

2.3.1. Glyoxyl agarose

The support was prepared activating the agarose matrix with glycidol and subsequently oxidizing it with peryodate, as previously reported [32]. Immobilization of β-galactosidase was carried out contacting 10 mL of enzyme solution with 1 g of support at 25 °C. The enzyme solution (0.13–4.6 mg of protein/mL) was prepared in 0.1 M bicarbonate buffer pH 10, with 20% of glycerol to avoid enzyme inactivation at immobilization conditions. A final step of reduction for 30 min using 1 mg/mL of sodium borohydride was required to obtain stable amide bonds between enzyme and support.

2.3.2. Heterofunctional supports

Heterofunctional supports were prepared from agarose activated with epoxy groups, as previously described [29]. Immobilization of β-galactosidase was carried out in two steps. The first step was the adsorption of the enzyme to the support, which was done by contacting 10 mL of enzyme solution per 1 g of support at 25 °C. In the case of amino- and carboxy-glyoxyl agarose, the enzyme solution was prepared in 0.05 M phosphate buffer pH 7 to favour the ionic adsorption of the enzyme to the support. The second step was the multi-point covalent attachment of the already adsorbed protein by means of the ε-amino groups in their lysine residues and the aldehyde groups of the support, which was done by filtration of the suspension and incubation in 0.05 M bicarbonate buffer pH 10. For the immobilization of β-galactosidase in chelate-glyoxyl agarose, the enzyme solution was prepared in 0.1 M phosphate buffer pH 7 to promote the enzyme adsorption only by coordination of the histidine residues of the protein and the metal chelates.

2.4. Selection of the agarose activation method and optimization of immobilization conditions

In order to consider both the activity of the immobilized biocatalysts and their thermal stability, the lumped parameter maximum catalytic potential (MCP) was defined (Eq. (3)).

$$MCP = \int_0^{t_f} A_I \cdot dt \quad (3)$$

MCP was assessed evaluating the thermal stability of the immobilized biocatalysts under non-reactive conditions in 0.1 M