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Optimized endodextranase-epoxy CIM® disk reactor for the continuous production of molecular weight-controlled prebiotic isomalto-oligosaccharides

Seltanna Chalane[a,b], Cédric Delattre[a,b], Philippe Michaud[a,b], André Lebert[a,b], Christine Gardarin[a,b], Damini Kothari[c], Catherine Creuly[a,b], Arun Goyal[c], Aleš Štrancar[d], Guillaume Pierre[a,b,*]

[a] Université Clermont Auvergne, Institut Pascal, BP 10448, F-63000 Clermont-Ferrand, France
[b] CNRS, UMR 6602, IP, F-63178 Aubière, France
[c] Department of Biotechnology, Indian Institute of Technology Guwahati, Guwahati 781039, Assam, India
[d] BIA Separations d.o.o, Teslova 30, SI-1001 Ljubljana, Slovenia

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ABSTRACT

An epoxy-activated monolithic Convective Interaction Media (CIM®) disk was used for the immobilization of endodextranase D8144 from *Penicillium* sp. (EC 3.2.1.11) in order to produce on-line isomalto-oligosaccharides (IMOs) from Dextran T40. Enzymatic parameters, molecular weight of IMOs and performance of the IMmobilized Enzymes Reactor (IMER) were investigated. The immobilization yield of enzymes was about 45.3% (w/w), and the real specific activity close to 3.26 U mg^{-1}. The K_m values did not significantly change between free (12.8 g L^{-1}) and immobilized enzymes (14.2 g L^{-1}), due to the absence of diffusional limitation. The IMER system presented more than 80% of its residual activity after 5000 column volumes, highlighting the high stability of the immobilized endodextranases. Response surface methodology was used to enhance the performance of the IMER. Depending on dextran concentrations and flow rates, specific patterns of IMOs distributions were observed during the enzymatic hydrolysis. Finally, prebiotic activity was also investigated on IMOs produced by medium conditions (flow rate 0.3 mL min^{-1} and dextran concentrations 4% w/w) against *Lactobacillus rhamnosus* GG (ATCC 53103). Their scores were at least as good as two commercialized fructo-oligosaccharides (FOS), Fibrulose® F97 and Orafti® P95.

1. Introduction

The known biological functions of polysaccharides had long been limited to energy and structural roles until the advent of glycomics revealed numerous more complex biological properties of polysaccharides, and especially of oligosaccharides [1]. The possibility of changing their biological behavior by modulating their structures made them particularly attractive, but the lack of high-performance industrial processes for producing active oligosaccharides still limits their exploitation. Specific pathways have already been described for the production of oligosaccharides, *e.g.* the synthesis of oligosaccharides from monosaccharides and the depolymerization of polysaccharides. Chemical or enzymatic synthesis has some advantages, but it is complex to implement and requires expensive substrates. Hence the depolymerization of polysaccharides by physical, chemical and/or enzymatic treatments is better-suited to an industrial context, as numerous enzymes, processes and polysaccharides are commercially available [2,3]. Enzymatic cleavage, using glycoside hydrolases (EC 3.2.1.-) and polysaccharide lyases (EC 4.2.2.-) is currently described as a viable way of toll-manufacturing oligosaccharides [4]. More recently the use of immobilized enzymes has offered a stand-in for biocatalysis and production of oligosaccharides [5,6]. Overall, classical limitations described for free enzyme reactors can be overcome by using IMmobilized Enzymes Reactors (IMERs) (enzyme efficiency, recycling of biocatalysts, enzyme stability, working in a continuous flow system and scale-up of reduction costs [7–10]). The performance of IMERs depends on parameters such as (i) the immobilization procedure, (ii) the thermodynamic properties of the support, (iii) the biocatalyst, (iv) the structure and concentration of the substrate, and (v) flow rate [11]. As described by Bartolini et al. [12], the choice of the support is of prime importance since it determines surface area, thermal and chemical stabilities, mass transfer characteristics and cost. Recently,

* Corresponding author at: Université Clermont Auvergne, Institut Pascal, BP 10448, F-63000 Clermont-Ferrand, France.
E-mail address: guillaume.pierre@uca.fr (G. Pierre).

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new polymeric macroporous monolithic supports have been suggested for IMER design [11,13–15]. Based on the literature, these matrices possess large surface areas, good mass transfer characteristics, high chromatographic efficiency and also low back-pressure [15,16]. Convective Interaction Media® (CIM) disks are made of macroporous poly (glycidyl methacrylate-co-ethylene dimethacrylate). Vlakh and Tennikova have published two extensive reviews concerning the potential of CIM® disks for designing new IMERs. Stability of enzymes, activity, yields, cost and potential industrial applications were addressed by the authors [17,18]. These supports have been used with immobilized dextranases, pectin lyases and glucuronan lyases for the production of respectively isomalto-oligosaccharides (IMOs) [11], oligogalacturonans [5] and oligoglucuronans [19]. None of these studies sought to optimize IMER designs for toll-manufacturing of oligosaccharides. Tavernier et al. [19] highlighted the possibility of controlling the degree of polymerization (DP) of oligosaccharides through the substrate flow rate, as confirmed by Bertrand et al. [11] for IMO production. Low Molecular Weight (LMW) IMOs possess simple structures enabling easy chemical modifications, such as sulfation, and also nutraceutical [20,21], antitumor [22], clinical [23] or prebiotic activities [24–26]. In this paper, an endodextranase (EC 3.2.1.11) was covalently immobilized on a macroporous epoxy-activated monolithic support to produce IMOs from dextran, an extracellular homopolysaccharide composed of α-D-glucopyranose (d-Glc*p*) with mainly α-(1,6) linkages and α-(1,2), α-(1,3) and α-(1,4) branching in the backbone. Our main goal was to demonstrate how the optimization of this IMER would allow the production of sought-after sizes of IMOs. Data analyses and surface response methodology were used to optimize experiments to obtain specified conditions for the production of different LMW IMOs. Specific models were also established for the production of DP 7–10 (1152, 1314, 1476 and 1638 Da) IMOs by varying two critical parameters: flow rate and substrate concentration. Finally, the prebiotic potential of the IMOs produced was assessed using *Lactobacillus rhamnosus* GG (ATCC 53103) as a healthy bacterial model found in the gut.

2. Materials and methods

2.1. Reagents and enzymes

1,6-α-D-Glucan 6-glucanohydrolase D8144 (EC 3.2.1.11) from *Penicillium* sp. was purchased from Sigma-Aldrich (Lyon, France). Epoxy-activated CIM® disks (213.7175) and housing support were obtained from BIA separations (Slovenia). A low-branched (1.3%) Dextran T40 (Dextranum 40 for injection, 40 kDa) was obtained from Pharmacosmos (Denmark) [27]. Eluents used for High Pressure Anion Exchange Chromatography – Pulsed Amperometric Detection (HPAEC-PAD) analysis were purchased from Fisher Scientific (Illkirch, France). Other chemicals (analytical grade) were from Sigma-Aldrich.

2.2. Colorimetric analysis

The 2,2-bicinchoninate method was used to assay reducing sugars, with isomaltose as standard [28]. Protein content was determined using the Lowry protein assay with bovine serum albumin as standard [29]. All the measurements were performed in triplicate on a Shimadzu UV-1700 spectrophotometer (Duisburg, Germany).

2.3. Determination of free endodextranase activity

Endodextranase D8144 (1.6 U) was added to 20 mL of 1–8% (w/v) Dextran T40 solution prepared beforehand in potassium phosphate buffer (100 mM, pH 5.5) at 37 °C with stirring (400 rpm); 500 µL aliquots were regularly taken and boiled for 30 min at 100 °C to inactivate the enzymes. The samples were finally stored at −20 °C before colorimetric analysis. One unit (U) of endodextranase activity was defined as the amount of enzyme necessary to release reducing sugars equivalent to 1 µmol of isomaltose per min from Dextran T40 (37 °C; pH 5.5). Initial velocity (V_i), maximal velocity (V_m) and Michaelis constant (K_m) were estimated for free endodextranase D8144. All the experiments were done in triplicate using a Radley Carousel 12 plus instrument (Radley Discovery Technologies, Shire Hill, UK).

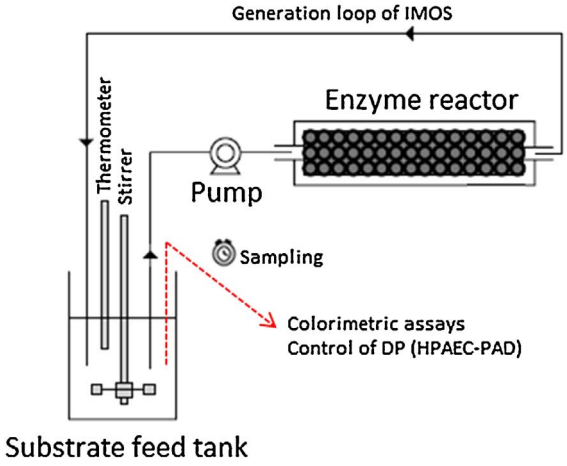

Fig. 1. IMER system used for the generation of IMOs, running with a Dextran T40 in phosphate buffer solution (100 mM; pH 5.5); a control of pH, temperature, flow rate and concentration; a peristaltic pump and the immobilized endodextranases D8144 on an epoxy-activated CIM® disk.

2.4. Production of IMOs by the immobilized endodextranases reactor

2.4.1. Design of the IMER

The IMER (Fig. 1) was composed of a Gilson Minipuls 3 peristaltic pump (Middleton, WI, USA) providing flow rates up to 5.5 mL min^{-1}, an epoxy-activated CIM® disk (diam. 12 mm, thickness 3 mm, vol. 0.34 mL) placed on its housing support, and a 10 mL mixed feed tank. The IMER was placed in a temperature-controlled oven and sealed to prevent evaporation.

2.4.2. Immobilization of endodextranases D8144

The CIM® disk was equilibrated with a potassium phosphate buffer solution (100 mM, pH 8) for 30 min at 21 °C under a flow rate of 0.24 mL min^{-1} and with stirring (120 rpm); 100 U of endodextranase D8144 (266 µg of proteins) was dissolved in 2.8 mL of potassium phosphate buffer solution (100 mM, pH 8); the solution was then eluted at 0.24 mL min^{-1} through the epoxy-activated CIM® disk in a closed loop for 24 h at 21 °C. This dynamic immobilization step was followed by a static step in which the disk was immersed in the same endodextranase D8144 solution for 14 h under gentle stirring (150 rpm). In order to eliminate non-covalently bound enzymes, the system was flushed three times with 5 mL of potassium phosphate buffer solution (0.1 M, pH 8). Residual epoxy sites were inactivated by flushing the CIM® disk for 8 h with 5 mL of 30 mM ethanolamine in a 100 mM potassium phosphate buffer (pH 8) at 0.24 mL min^{-1} and 21 °C. The immobilization procedure was ended by washing the CIM® disk for 150 min with a potassium phosphate buffer solution (100 mM, pH 5.5) at 0.24 mL min^{-1} and 21 °C. Three epoxy CIM® disks were used to confirm the robustness of the immobilization procedure. The systems were kept at 4 °C in potassium phosphate buffer solution (100 mM, pH 5.5) until used.

2.4.3. IMOs production assays

The IMER was incubated at 35 °C and flushed for 30 min with potassium phosphate buffer (100 mM, pH 5.5) at a flow rate of 0.3 mL min^{-1}; 8 mL of each Dextran T40 solution (2, 4 and 8% w/v)