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Continuous production of chitooligosaccharides by an immobilized enzyme in a dual-reactor system

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ABSTRACT

A chitosanolytic activity found in a commercial α-amylase from *Bacillus amylolyquefaciens* (BAN) was covalently immobilized onto glyoxal agarose beads (25% recovery of activity) and assessed for the continuous production of chitooligosaccharides (COS). The immobilization did not change the reaction profile (with chitotriose and chitobiose as major products, using chitosans of different polymerization and deacetylation degrees), but significantly increased the enzyme thermostability. A two-step process was proposed, in which chitosan was first hydrolyzed in a batch reactor to a viscosity that could flow through a packed-bead reactor (PBR), thus avoiding clogging of the column. The relationship between hydrolysis degree of chitosan (1% w/v) and viscosity of the solution was assessed in a batch reactor. A 50% hydrolyzed chitosan did not cause any clogging of the PBR. Under these conditions, the productivity of the PBR at the lowest dilution rate was 37 $g_{COS}\,L^{-1}\,h^{-1}$, with a conversion yield of 73%. In contrast, at the highest dilution rate, the productivity was nearly 200 $g_{COS}\,L^{-1}\,h^{-1}$, but the conversion yield dropped to around 40%.

1. Introduction

Chitosan is a polysaccharide composed of D-glucosamine (GlcN) and *N*-acetylglucosamine (GlcNAc) moieties with β(1 → 4) linkages that is obtained by partial deacetylation of chitin,, the second most abundant polysaccharide in nature after cellulose [1]. Chitosan is widely used as a biopolymer in the pharmaceutical, cosmetic, textile, and food industries [2], either as a carrier for drugs due to its physicochemical characteristics, or as a preservative because of its antibacterial properties. The biological activities of chitosan depend on its molecular weight (related to the degree of polymerization, DP) and its charge (associated to the deacetylation degree, DD) [3]. However, some of the potential applications of chitosan are limited by its high viscosity and low solubility at neutral pH [4].

Chitooligosaccharides (COS), obtained by chemical or enzymatic hydrolysis of chitosan, are more soluble and exhibit enhanced biological activities compared with the polysaccharide [5,6]. COS show antimicrobial, antioxidant, antiangiogenic, antitumoral, anti-inflammatory and prebiotic properties, among others [7–11].

The bioactivity of COS also depends mostly on their DP, DD and pattern of acetylation (PA) which, in turn, depend on the source of chitosan and the hydrolysis strategy. Enzymatic hydrolysis offers significant advantages over chemical methods, especially with respect to the mild conditions employed and the possibility to control the DD, DP and PA of the resulting COS on the basis of the enzyme specificity [12–14]. Chitosanolysis can be catalyzed mainly by chitosanases, but also by other enzymes including *exo*-β-glucosaminidases, *exo*-β-*N*-acetylglucosaminidases, chitinases and, non-specifically, by proteases, pectinases, cellulases or lysozyme [15–19].

For the development of a continuous process for COS synthesis, the immobilization of the chitosanolytic enzymes is essential for the separation of the biocatalyst from the reaction mixture, facilitating downstream processes and potential enzyme reuse, which may lead to an overall economic-effectiveness [20]. For this type of enzyme, covalent binding is preferred over simple adsorption because the substrate chitosan possesses an extraordinary ability –

Abbreviations: COS, chitooligosaccharides; paCOS, partially acetylated chitooligosaccharides; DD, deacetylation degree; DP, degree of polymerization; ESI, electrospray; GlcN, D-glucosamine; GlcNAc, *N*-acetyl-glucosamine; $(GlcN)_2$, chitobiose; $(GlcN)_3$, chitotriose; $(GlcN)_4$, chitotetraose; $(GlcN)_5$, chitopentaose; HMWC, high molecular weight chitosan; HPAEC, high-performance anion-exchange chromatography; MS, mass spectrometry; PAD, pulsed amperometric detection; TOF, time-of-flight; BAN-Glx, BAN covalently immobilized on glyoxal agarose beads; PBR, packed-bed reactor; CSTR, continuous stirred tank reactor; d, dilution rate.

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partly due to the presence of amino groups – to bind biomolecules, which may cause the desorption of the enzyme from the carrier. In fact, most of the strategies reported to immobilize chitosanases involve the formation of covalent bonds between the enzyme and the carrier, using silica gel [21], agar gel [22,23], agarose [24], polyacrylonitrile [25], DEAE-cellulose [26], amylose-coated magnetic nanoparticles [27] and even chitin itself [28,29]. Other methodologies, in particular alginate entrapment, have been also explored [30,31]. However, the continuous production of COS with immobilized chitosanases has been scarcely studied [32–34].

Chitosan is a non-Newtonian viscous polymer, which adds a difficulty to the design of processes for the production of COS [35]. Some approaches have been proposed to overcome this limitation, but the problem of viscosity has not been entirely solved, leading to low productivities [36–39]. For example, Ming and coworkers immobilized a chitosanase on an agar gel-coated multidisc impeller, and obtained yields of between 20 and 45% for the sum of chitopentaose and chitohexaose, depending on the surface activity of the enzyme [23]. Jeon and Kim set up a dual reactor system with a packed-bed reactor (PBR) to decrease viscosity of chitosan followed by an ultrafiltration membrane reactor to produce COS of the desired DP [33].

The hydrolysis of chitosan is not the only case where the viscosity of the substrate represents a hurdle for engineering a process. In the context of biorefineries [40], the high viscosity of the feed solution (starch, cellulose, hemicellulose, etc.) limits the implementation of fixed-bed bioreactors for the continuous hydrolysis of the biopolymers [41]. However, continuous processes would enable control to a much greater extent and, in particular, to settle the degree of hydrolysis by adjustment of the flowrate, and hence residence time. Recently, Rakmai and Cheirsilp proposed a dual reactor system for the production of β-cyclodextrin, composed of a continuous stirred tank reactor (CSTR) for reduction of viscosity of liquefied starch, followed by a PBR with a productivity of 3.98 $g\,L^{-1}\,h^{-1}$ [42]. On this basis we argued that this strategy could also serve as a model for the production of COS.

In this work, we report the characterization of a chitosanolytic activity found in a commercial α-amylase from *Bacillus amylolyquefaciens* (BAN). The enzyme was covalently immobilized onto glyoxal agarose beads and assessed both in batch and fixed-bed reactors for continuous production of COS.

2. Materials and methods

2.1. Enzyme and reagents

BAN 480L (α-amylase from *Bacillus amylolyquefaciens*) was kindly donated by Novozymes (Bagsvaerd, Denmark). High Density Glyoxal 4BCL (spherical, 50–150 μm diameter, 40–60 $\mu mol_{glyoxyl}\,m_{gel}^{-1}$, coupling capacity 15–20 $mg_{BSA}\,mL_{gel}$), was purchased from Agarose Bead Technologies (Miami, FL, USA). Chitosan QS1 (98.5 kDa, 81% DD) was kindly provided by InFiQus (Madrid, Spain). Chitosan CHIT100 (100–300 kDa, DD ≥ 90%) and CHIT600 (600–800 kDa, DD ≥ 90%) were acquired from Acros Organics (Thermo Fischer Scientific Inc., Waltham, MA, USA). D-Glucosamine (GlcN) and *N*-acetyl-glucosamine (GlcNAc) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fully deacetylated COS with DP from 2 to 5, and fully acetylated COS with DP from 2 to 4 were purchased from Carbosynth Ltd. (Berkshire, UK). All other reagents were of the highest purity grade.

2.2. Activity assays

Chitosanolytic activity was determined by detection of reducing sugars with a modified 3,5-dinitrosalicylic acid (DNS) method. For the soluble enzyme, and prior to the assay, low-molecular-weight contaminants in the commercial sample were removed with a DP-10 desalting column (GE Healthcare, Uppsala, Sweden). Activity assays were performed in 1.5 mL centrifuge tubes by adding 200 μL of enzyme to 800 μL of 1% (w/v) chitosan CHIT100 dissolved in 50 mM sodium acetate buffer (pH 5.0). Tubes were incubated at 50 °C and 900 rpm in a Thermo Shaker TS-100 (Boeco, Hamburg, Germany) and reactions were stopped by addition of 0.25 M NaOH in a 1/1 (v/v) ratio. The addition of NaOH also caused the precipitation of the remaining polysaccharide, which was removed by centrifugation at 5000 × *g* for 10 min.

For the immobilized enzyme, the activity assay was carried out following a method developed in our laboratory that involves the use of filtered micro-centrifuge tubes [43]. 50–60 mg of immobilized biocatalyst was placed on the filter and 500 μL of 1% (w/v) chitosan CHIT100 dissolved in 50 mM sodium acetate buffer (pH 5.0) was added. The reaction was incubated in the same conditions as the soluble enzyme and it was stopped by centrifugation (5 min, 5000 × *g*) that separated the substrate from the enzyme. To inactivate the possible leaked enzyme, NaOH was added to the supernatant, in the same conditions as above.

In both cases, the quantification of reducing sugars in the supernatant was carried out by the DNS method in a 96-well microplate. A calibration curve of D-glucosamine was done. One unit of activity (U) corresponded to the release of one μmol of reducing sugars per minute.

2.3. Optimal temperature of soluble enzyme

For the determination of optimal temperature of the enzyme, a mixture of 20 μL of BAN and 80 μL of 1% (w/v) CHIT100 in 50 mM acetate buffer (pH 5.0) was incubated at different temperatures (35–80 °C) in a thermocycler (BioRad), using PCR microplates. Reactions were stopped by addition of 100 μL of 0.25 M NaOH to each well and the plates were centrifuged for 20 min at 3000 × *g*. The supernatant was transferred to a flat-bottom 96-well plate and the concentration of reducing sugars was measured by the DNS assay. All the experiments were performed in triplicate and the error was expressed as the standard deviation of the three measurements.

2.4. Enzyme immobilization

The immobilization of BAN on glyoxal agarose was carried out following the protocol of the supplier with slight modifications. Prior to immobilization, the glyoxal agarose beads were washed with distilled water to remove any preservatives. Simultaneously, contaminants present in the enzymatic preparations were removed with a DP-10 desalting column, and the enzyme was eluted with 100 mM $NaHCO_3$ (pH 10.0), in order to perform immobilization at such pH. The enzyme solution was added to 1 g of glyoxal agarose and incubated for 1 h at room temperature in a roller shaker (JP Selecta S.A., Spain). This immobilization time was optimized taking into account the compromise between the formation of covalent bonds and the stability of the enzyme at pH 10. After this incubation, 10 mg of $NaBH_4$ were added for the reductive amination of the Schiff base bonds and the mixture was incubated at room temperature for 30 min to yield the immobilized biocatalyst BAN-Glx. The immobilization mixture was filtrated with acetate/nitrate cellulose filters (0.45 μm, Merck Millipore, Billerica, MA) to separate the immobilized biocatalyst from the remaining solution. The immobilized biocatalyst was washed thoroughly with 50 mM sodium acetate buffer (pH 5.0) in order to remove any loosely adsorbed enzyme.