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Immobilization and some properties of commercial enzyme preparation for production of lactulose-based oligosaccharides

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ABSTRACT

The commercial enzymatic preparation, Pectinex Ultra SP-L, was successfully immobilized on activated chitosan particles for production of lactulose-based galacto-oligosaccharide (LuOS). The main degrees of polymerization (DP) of LuOS produced in this bioreactor were DP3 and DP4. The immobilization yield in term of expressed activity and recovery activity were 71% and 36.05%, respectively. The optimum temperature (60° C) was not affected by immobilization process, while the optimum pH was shifted from pH 5.5 to pH 4.5. In addition, a thermal stability of the immobilized enzyme was better than that of free enzyme. The half-life time of hydrolytic activity increased from 2.5 days (free enzyme) to 14 days (immobilized enzyme). Hydrolytic K_M values were 651.3 mM and 187.9 mM lactulose for free enzyme and immobilized enzyme, respectively. Furthermore, the immobilized enzyme showed high reusability. The yield of LuOS formation was more than 60% after 33 cycles of reuses. In the case of *trans*-galactosylation, a model with 8 reaction rates was build using five-step reaction pathways with different initial lactulose concentrations. This catalytic model was validated well for a long-term period (27 days). These results strongly support to develop a continuous bioconversion to synthesize functional food ingredients, prebiotic LuOS.

1. Introduction

Galacto-oligosaccharides (GOS) are non-digestible oligosaccharides and known as prebiotics (Rycroft et al., 2001; Tuohy et al., 2002) that selectively stimulate the growth and activity of probiotic bacteria (*bifidobacteria* and *lactobacilli*). The potential prebiotic properties of these oligosaccharides depend on their chemical structure, such as number and type of monomers; type, position and conformation of the glycosidic linkages (Sanz et al., 2005, 2006; Martinez-Villaluenga et al., 2008; Hernández-Hernández et al., 2011; Styevkó et al., 2013). The prebiotic actions of lactulose (Lu) on the colonic motility pattern as well as its ability to promote the selective growth of beneficial intestinal bacteria located in human gut has been reported (Schumann, 2002; Tuohy et al., 2002; De Preter et al., 2006). Unfortunately, at high doses, the Lu has laxative effects and the fact that fermentation occurs mainly in the proximal colon, which results in uncomfortable gas production (Salminen and Salminen, 1997; Tuohy et al., 2002), thus this limits the use of Lu. Moreover, the prebiotic index of lactulose-based oligosaccharides (LuOS) may higher than of the Lu due to the higher degree of polymerization. Fermentation of these oligosaccharides is going slowly

Abbreviations: GOS, galacto-oligosaccharides; Lu, lactulose; LuOS, lactulose-based oligosaccharides; IE, immobilized enzymes; FE, free enzyme; CTS, chitosan; ILC, initial lactulose concentration.

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and their metabolism takes place more distally in the colon (Tuohy et al., 2005). In addition, LuOS showed a better anti-inflammatory profile than Lu in a model of experimental colitis (Algieri et al., 2014), thus production and application of LuOS are currently attracting the attention of the scientific community (Cardelle-Cobas et al., 2008, 2009; Martinez-Villaluenga et al., 2008).

Technically, LuOS can be produced by *trans*-galactosylation reactions catalyzed by β-galactosidases from different fungal sources, including *Aspergillus aculeatus* (Cardelle-Cobas et al., 2008; Guerrero et al., 2013), *Kluyveromyces lactis* (Martinez-Villaluenga et al., 2008; Guerrero et al., 2013) and *Aspergillus oryzae* (Guerrero et al., 2013). In the synthesis process of LuOS, Lu plays both the roles of donor and acceptor of the galactosyl residue. The galactose unit (from a donor) can link to C-6 of the galactose moiety or C-1 of the fructose moiety (Martinez-Villaluenga et al., 2008). The degree of polymerization of LuOS may depend on the source of enzyme. Hernández-Hernández et al. (2011) reported that β-galactosidases from *A. aculeatus*, *K. lactis*, and *A. oryzae* were able to synthesize LuOS with DP up to DP5, DP4, and DP6, respectively.

Immobilized enzymes (IE) have many advantages over soluble enzymes (free enzyme, FE) in biotechnological applications. Immobilization facilitates the efficient recovery and reuse of costly enzymes (Sheldon, 2007). Additionally, advantages include enhanced stability, great operational control, the flexibility of reactor design, and easy separation from the catalyst and unreacted substrate contamination. Covalent immobilization of enzyme onto the solid carriers is an attractive method as their benefits. The chitosan (CTS) is the deacetylated form of chitin (CT), the second most abundant polymer in nature after cellulose. This solid carrier exhibits many interesting properties such as a low cost, renewable, biodegradable natural product, which has very good biocompatibility, low toxicity, biocompatible and chemical stability (Dwevedi and Kayastha, 2009; Mendes et al., 2011). Furthermore, immobilization can be carried out by entrapment into chitosan beads (Freeman and Dror, 1994) or by covalent binding to transparent chitosan films (Çetinus and Öztop, 2000) or by chitosan particles with glutaraldehyde as a cross-linking agent. In addition, enzyme immobilization can be performed simply after chitosan cross-linking and activation by glutaraldehyde (Ravi Kumar, 1999). Thus, the CTS is considered to be an excellent carrier for covalent immobilization of enzyme (Ravi Kumar, 2000; Yazdani-Pedram et al., 2000; Krajewska, 2004).

In this work, covalent immobilization of β-galactosidase (in Pectinex Ultra SP-L preparation) onto activated chitosan particles, as well as characterization of immobilized enzyme preparation for the synthesis of prebiotic LuOS were focused.

2. Materials and methods

2.1. Materials

Chitin (No. C7170) was purchased from Sigma–Aldrich Inc. (Hungary). Pectinex Ultra SP-L commercial enzyme preparation with β-galactosidase from *A. aculeatus* was from Novozymes A/S (Denmark). Lactulose was from Panreact Applichem (Germany). All other chemicals and reagents were of analytical grades and purchased from either Sigma–Aldrich, Reanal (Hungary) or VWR (Hungary).

2.2. Methods

2.2.1. Immobilization of enzyme

Immobilization of Pectinex Ultra SP-L was done according to the method published by Nguyen et al. (2011).

2.2.2. Calculation of immobilization yield and recovery activity

Immobilization yield was determined in term of expressed activity (IY_{act}) according to Eq. (1), where A_{ini} and A_{fin} were enzyme activities (U/ml) in the supernatant (soluble enzyme) before and after immobilization, respectively (Silva et al., 2012).

$$IY_{act} = \frac{A_{ini} - A_{fin}}{A_{ini}} \times 100 \quad (1)$$

Recovery activity (A_{rec}) was calculated using Eq. (2), where A_{imm} is the activity of the IE (U/g support). The theoretical activity (A_{the}, U/g support) of IE on the activated CTS support could be calculated using the amount of enzyme offered per gram of support (A_{off}) and the IY_{act} (Silva et al., 2012).

$$A_{rec} = \frac{A_{imm}}{A_{the}} \times 100 = \frac{A_{imm}}{IY_{act} \cdot A_{off}} \times 100 \quad (2)$$

2.2.3. The effect of pH and temperature on enzyme activities

The optimal pH and temperature refer to the maximum enzymatic activity (total of LuOS product) at the defined reaction conditions. In the case of pH, 0.1 M McIlvaine buffer with the pH in the range of pH 4.5–6.5 on enzyme activity was studied respect to Lu substrate using. The concentration of FE and amount of immobilized enzyme as well as temperature (at 60 °C) were kept constant (Cardelle-Cobas et al., 2008).

The effect of temperature on enzyme activity was determined at the optimal pH condition. The reaction mixtures were placed at various temperatures (from 50 to 75 °C with steps of 5 °C).

2.2.4. Stability of enzyme preparation

Stability of both free and immobilized enzyme preparations was determined with standard procedure. Briefly, about 100 U of these enzyme preparations were transferred into the test tubes and then 5 ml 0.1 M McIlvaine buffer (pH 5.5 for FE and pH 5.0 for IE, respectively) were added. The test tubes were placed in the water incubator pre-set optimal temperature (60 °C). Samples were periodically taken every day and their hydrolytic activities were assayed using *p*-nitrophenyl-β-D-galactopyranoside (*p*-NPGal) as a substrate.

2.2.5. Kinetic modeling and estimation of model parameters

Multi-step reaction based on the Michaelis–Menten model was proposed for understanding of the kinetics of the bioconversion mechanism (Palai and Bhattacharya, 2013; Palai et al., 2012, 2014, 2015; Rodriguez-Fernandez et al., 2011). Consequently, several nonlinear ordinary differential equations that define the rate of change of reactants on time course including the constant rates of reactions (one of the kinetic parameters). The kinetic parameters were estimated by application of multiresponse nonlinear regression for the whole sets of experiments (Bates and Watts, 1988). These techniques were adapted and applied using the LSODA solver of COPASI version 4.16 (Hoops et al., 2006).

2.2.6. Determination of reusability of immobilized preparation

The immobilized enzyme was repeatedly used for several cycles in batch mode. All cycles were done in the same conditions (≈0.5 g support, 1.5 ml of 20% of lactulose substrate, temperature 60 °C, and 12 h of reaction for each batch). At the end of each cycle, the yield of LuOS was determined. Then the immobilized enzyme was washed for several times with the