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An efficient continuous flow process for the synthesis of a non-conventional mixture of fructooligosaccharides

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ABSTRACT

A sustainable and scalable process for the production of a new mixture of fructooligosaccharides (FOS) was developed using a continuous-flow approach based on an immobilized whole cells-packed bed reactor. The technological transfer from a classical batch system to an innovative flow environment allowed a significant improvement of the productivity. Moreover, the stability of this production system was ascertained by up to 7 days of continuous working. These results suggest the suitability of the proposed method for a large-scale production of the desired FOS mixture, in view of a foreseeable use as a novel prebiotic preparation.

1. Introduction

Biocatalysis and flow reactor technology are widely considered to be two of the key technologies intrinsically compatible with the principle of green chemistry (Bryan et al., 2013; Ley, 2012). However, even if the effectiveness of their combination has been recently demonstrated (Itabaiana, de Mariz e Miranda, & De Souza, 2013; Itabaiana et al., 2013; Tamborini et al., 2012, 2013), the potential of biocatalysis in flow chemistry reactors is far from being fully exploited.

In the present paper, we describe an application of this innovative approach in the food field, by proposing a method for preparing fructooligosaccharides (FOS).

FOS are alternative sweeteners with a number of interesting nutritional properties: they are calorie free, non-cariogenic and are considered as soluble dietary fibers (Barclay, Ginic-Markovic, Cooper, & Petrovsky, 2012). The energy value of FOS is 4.2–9.5 kJ/g. The sweetness of FOS depends on the composition of the mixture and the sweetness of the main components, *i.e.*, 1-kestose, 1-nystose, and 1-fructofuranosylnystose, relative to 10% sucrose solution is 31%, 22%, and 16%, respectively (Antošová & Polakovič, 2001). Furthermore, they induce important beneficial physiological effects, such as a prebiotic effect, an improved mineral absorption and decreased levels of serum cholesterol, triacylglycerols and phospholipids (Daubioul et al., 2002; Giacco et al., 2004). Currently, FOS are increasingly included in food products and infant formulas due to their prebiotic effect that stimulates the growth of non-pathogenic intestinal microflora (Sabater-Molina, Larqué, Torrella, & Zamora, 2009).

FOS are found in several kinds of plants and vegetables such as banana, onion, asparagus root and artichoke, however, the supply is rather limited owing to their limited content in natural sources. Therefore, they are industrially produced following two different approaches. The first is based on inulin degradation, whereas the second approach employs sucrose transformation catalyzed by fructosyltransferase (FTase) or β-fructofuranosidase (FFase) from microbial sources. Industrial scale production of FOS is commonly performed by either soluble enzymes in batch reactions (Hidaka, Eida, Adachi, & Saitoh, 1987) or by immobilized enzymes using continuous fixed-bed reactors (Park, Lim, Kim, Park, & Kim, 2005; Yun, Kang, & Song, 1995). Immobilized FTase from *Aureobasidium pullulans* and the immobilized whole cells have been used in

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packed bed reactors for the continuous production of FOS at a plant scale (Jung, Bang, Oh, & Park, 2011; Vaňková, Onderková, Antošová, & Polakovič, 2008).

Recently, we used the strain of *Cladosporium cladosporioides* MUT 5506 endowed with transfructosylating activity to produce a new mixture of FOS from a 600 g/l solution of sucrose in high yields (Zambelli et al., 2014, 2015). In this mixture, it was possible to identify and fully characterize the *non*-conventional disaccharide blastose (6-O-β-D-fructofuranosyl-α,β-D-glucopyranoside), whose prebiotic activity was unknown (Zambelli et al., 2014).

In the present work, we aimed to demonstrate the advantages of performing the previously described biotransformation in a flow-chemistry reactor, with the final aim of predisposing a suitable process for the sustainable and scalable production of the desired FOS mixture, whose potential use as a novel prebiotic preparation can be easily foreseen and is at present under investigation by us. To this end, a *C. cladosporioides* MUT 5506 strain was used as an immobilized mycelium in a packed bed reactor to improve the productivity, the efficiency and the scalability of the reported batch biotransformation. The innovative application of whole microbial cells into a flow chemistry reactor combines the advantages of an easy to produce biocatalyst with a process-intensification technology. Moreover, the use of a continuous-flow approach based on a packed bed reactor guarantees improved mass transfer and recyclability of the solid catalyst (Kirschning, Solodenko, & Mennecke, 2006). The produced FOS mixture and the isolated *non*-conventional blastose were submitted to a preliminary *in vitro* study to assess their ability to promote the growth – as sole carbon sources – of selected probiotic strains, thus giving a first indication of their suitability for a potential application as prebiotics.

2. Materials and methods

2.1. Materials

Sucrose, glucose, fructose and *p*-anisaldehyde were purchased from Sigma–Aldrich. Standards of 1-nystose and 1-kestose were purchased from Fluka (DE). A standard of 1-F-fructofuranosylnystose was purchased from Megazyme. Actilight® was kindly donated by Beghin Meiji. Gluzyme MONO10000 preparation was kindly donated by Novozymes. Yeast extract was purchased from Difco (Difco, MD, USA) and barley malt flour from Diagermal (IT).

2.2. Flow chemistry equipment

An R2+/R4 combination flow reactor commercially available from Vapourtec was used. The main R2+ system is driven by two integrated HPLC pumps; the flow rates can be regulated and set at any value between 0.01 and 10 ml/min working with a system pressure of up to 30 bar without any risk. The R4 heater guarantees a precise temperature control over the range room temperature to 150 °C in four independently controlled, air-circulated heating zones, with rapid temperature ramping and cooling (80 °C/min). The four reactor zones can each accept either a packed column or a flow tube arrangement, providing reaction volumes of 0.1–10 ml (or 40 ml in a linked sequential operation). A back-pressure regulator is applied in-line, if necessary. The system is also outfitted with a pair of injection loops that are positioned after the pumps. Finally, at the top, a large drip tray is located for reagent bottles and collection vessels, giving the whole system a very small compact foot print which fits comfortably into any fume cupboard.

2.3. Strains and growth conditions

C. cladosporioides, previously isolated and deposited at *Mycotheca Universitatis Taurinenesis* (MUT) as strain MUT 5506, was maintained as previously described (Zambelli et al., 2014).

Five probiotic *Lactobacillus* strains (*Lactobacillus paracasei* DG, *Lactobacillus rhamnosus* GG, *L. paracasei* SHIROTA, *Lactobacillus johnsonii* LC1, *Lactobacillus reuteri* ATCC55730) used in this study were cultivated in MRS broth (Difco) and incubated at 37 °C for 24 h. The bacterial cell concentration of an overnight culture was determined microscopically with a Neubauer improved counting chamber (Marienfeld GmbH, Lauda-Königshofen, Germany). For the assessment of *in vitro* blastose fermentation, strains were inoculated in triplicate at a final concentration of $4 * 10^5$ bacteria/ml (from cultures grown overnight on the stationary phase). The medium used to test the growth with different sugars was API 50 CHL medium without glucose, prepared at various dilutions. The medium was added with a 0.5% concentration of different sugars (di- and oligosaccharides): glucose (as positive control), inulin, blastose, FOS-mixture, FOS-mixture without blastose and a commercially available mixture of FOS (Actilight®) (Taverniti et al., 2012). *In vitro* experiments were carried out in 384 well plates, filled by means of an automated pipetting system epMotion 5070 (Eppendorf, Germany). The microbial growth was monitored with a spectrophotometer (MicroWave RS2, Biotek, USA) programmed for 145–290 readings (OD 600 nm) every 10 min for 24–48 h at 37 °C. At the end of the incubation, the μ_{max} and the final OD at 600 nm were calculated using the software Gen5 (Biotek, USA) and reported as the mean of three independent measurements ± standard deviation (Arioli et al., 2014).

2.4. Fructofuranosidase activity assays

The enzymatic activity towards sucrose was determined by measuring the initial rate of reducing sugar formation using the dinitrosalicylic acid (DNS) assay adapted to 96-well microplates (Rodrıguez, Perez, Ruiz, & Rodrıguez, 1995). Dried alginate entrapped (DALGE) mycelium (10 g/l) was incubated with 1 ml of a sucrose solution (100 g/l) in acetate buffer (20 mM pH 6.0) for 20 min at 50 °C and 90 rpm. A 50 µl aliquot of the solution, conveniently diluted to fit into the calibration curve, was added to each well. Then, 50 µl of 10 g/l DNS were added. The plate was incubated for 20 min at 80 °C to develop color with a seal plate tape (GeneMate). After cooling, 150 µl of water were added to each well, and the absorbance measured at 540 nm using a microplate reader (model Versamax, Molecular Devices). One unit (U) of activity was defined as that catalyzing the formation of 1 µmol of reducing sugar per minute under the conditions described above.

2.5. Dried alginate entrapped (DALGE) mycelium

The gel beads were prepared by ionotropic gelation following a slightly modified protocol previously developed by us (Fernandez-Arrojo et al., 2013). A 4% (w/v) sodium alginate solution was prepared in distilled water and stirred until a homogeneous clear solution was formed. The solution was left to settle for 2 h in order to eliminate all the air bubbles. The alginate solution was then gently mixed in a 1:1 (w/w) ratio with 40 g/l of lyophilized mycelia in 20 mM sodium acetate buffer (pH = 6.0) after 5 cycles of sonication at 15 kHz for 1 min (Soniprep 150, MSE). The resulting mycelia–alginate mixture was then used as previously described (Fernandez-Arrojo et al., 2013).