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 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.

Keywords:

Fructooligosaccharides, Flow chemistry, *Cladosporium cladosporioides*, Dried Alginate Entrapped Enzymes (DALGEE), Prebiotics.

1. Introduction

Biocatalysis and flow reactor technology are widely considered some 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 (Tamborini, Romano, Pinto, Bertolani, Conti & Molinari, 2012; Tamborini et al., 2013; Itabaiana, de Mariz e Miranda & De Souza, 2013a; Itabaiana, et al., 2013b), 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 nutritional interesting properties: they are calorie free, non-cariogenic and are considered as soluble dietary fibres (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 roots and artichokes, 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 one 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) and by immobilized enzymes using continuous fixed-bed reactors (Yun, Kang & Song, 1995; Park, Lim, Kim, Park & Kim, 2005). Immobilized FTase from *Aureobasidium pullulans* and the immobilized whole cells have been used in a 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; Zambelli et al., 2015). In this mixture, we were able 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 at demonstrating 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 purpose, a *C. cladosporioides* MUT 5506 strain was used as an immobilized mycelium into 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, as well as 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

A 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 to 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 independent controlled air-circulating heating zones, with a 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 to 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 post the pumps. Finally, at the top, a large drip tray is located for reagents 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, *Lactobacillus 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 hours. 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 to the stationary phase). The medium used to test the growth with different sugars was API 50 CHL medium without glucose, prepared at different 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®) (Taveriniti 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 µMax 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 ºC and 90 rpm. 50 μL of the solution, conveniently diluted to fit into the calibration curve, were added to each well. Then, 50 μL of 10 g/L DNS were added. The plate was incubated for 20 min at 80 ºC to develop colour 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 catalysing the formation of 1 μmol reducing sugar per minute under the above described conditions.

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 ratio 1:1 (w/w) 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).

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158 *2.6. Biotransformation with DALGE-mycelium in batch*

159 40 g/L of DALGE-mycelium was added to a 600 g/L sucrose solution in sodium acetate
160 buffer 20 mM pH 6.0 in a final volume of 2 mL, incubated at 50 °C in an orbital shaker at 90
161 rpm. The biotransformations were followed for 144 h and analyzed by HPLC.

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163 *2.7. Analytical HILIC-HPLC*

164 The analytical data were collected with a HPLC system composed by a Hitachi LaChrom
165 L-7100 pump available from Merck (DE) equipped with a Sedex 75 evaporative light
166 scattering detector (ELSD) available from Alfatech (FR). The chromatographic separation of
167 products was performed with a Luna NH_2 100 Å column (250 × 4.6 mm, particle size 5 µm,
168 Phenomenex, Aschaffenburg, Germany) equipped with the corresponding guard column (4 ×
169 3.0 mm), and kept at 30 °C with a Merck T-6300 column thermostat. The mobile phase was
170 acetonitrile/water (80:20 v/v) at a flow rate of 1.0 mL/min. The temperature of detection was
171 set to 52 °C. EZ Chrome Elite software by Agilent was used for data management.

172

173 *2.8. Semi-Preparative HILIC-HPLC*

174 The semi-preparative purifications were performed with a commercially HPLC system
175 composed by a Hitachi LaChrom L-7100 pump available from Merck equipped with a Sedex
176 75 evaporative light scattering detector (ELSD) available from Alfatech. The
177 chromatographic separation of products was performed with a Luna NH_2 100 Å column (250
178 × 10 mm, particle size 5 µm, Phenomenex, Aschaffenburg, Germany) at room temperature.

179 The following gradient of was used:

180 - 0'-30' acetonitrile/water (80:20 v/v)

181 - 30'-35' switch to acetonitrile/water (50:50 v/v)

- 35’-40’ acetonitrile/water (50:50 v/v)

- 40’-45’ switch to acetonitrile/water (80:20 v/v)

- 45’-60’ acetonitrile/water (80:20 v/v).

The flow rate was set to 4.7 mL/min. The temperature of detection was set to 52 °C. EZ Chrome Elite software by Agilent was used for data management.

2.9. Calculation of the T/H index

The transfructosylation/hydrolysis ratio (T/H index) was calculated using the following equation (Eq. 1):

$$T/H = ([glucose] - [fructose])/[fructose].$$

Eq. 1. Transfructosylation/hydrolysis ratio (T/H)

2.10. Productivity

The productivities for batch and flow biotransformations were calculated at the same degree of conversion using the following equations (Eq. 2 and Eq. 3):

$$r_{batch} = m_p / (t \times m_E)$$

Eq. 2. r_{batch}: batch productivity; m_p: amount of the product of interest (mg); t: reaction time (min); m_E: mass of the catalyst used (g).

$$R_{flow} = [P] \times f / m_E$$

Eq. 3. r_{flow}: flow productivity; [P]: concentration of the product of interest (mg/mL); f: flow rate (mL/min); m_E: mass of the catalyst packed in the reactor (g).

2.11. Continuous flow biotransformation using alginate mycelia in a packed bed column

Alginate beads and celite (1:1 w/w) were packed into an Omnifit® glass Column (15 mm id × 150 mm length). Each gram of alginate beads, mixed with the same volume of celite, can

fill about 6 mL of the inner volume of the column. The swelling of the dried beads was achieved with an acetate buffer solution (20 mM, pH 6) before connecting the column. A flow stream of 600 g/L sucrose solution in 20 mM sodium acetate buffer (pH 6) was pumped through the packed bed column, at different flow rates, in order to obtain residence times from 5 hours to 30 hours. The appropriate temperature was set by the R4 block heater. An aliquot (100 µL) of the exiting flow stream was diluted with water (1:3), the sample was filtered on a 0.45 µm nitrocellulose filter and analysed by HLIC-HPLC.

2.12. Glucose oxidation with Gluzyme MONO 10000 BG

The mixture obtained under optimized conditions after 20 hours of residence time has been exposed to the biocatalytic activity of a glucose oxidase preparation from Novozymes (Gluzyme MONO 10.000 BG) in batch (Sheu, Lio, Chen, Lin & Duan, 2001). To a 600 g/L mixture (20 mL) in acetate buffer 20 mM (pH 6) Gluzyme powder (800 mg, 400 U/mL) and $CaCO_3$ (800 mg) were added. The resulted suspension was stirred at 35 °C insufflating air. The produced gluconic acid precipitated as calcium salt in the reaction flask. At different times, aliquots (100 µL) were withdrawn, diluted with water (1:3) and incubated for 10 min at 90 ºC to inactivate the enzyme. Each sample was filtered on a 0.45 µm nitrocellulose filter and analysed. After 24 h the whole suspension was centrifuged at 14000 rpm for 30 min, filtered on a cellulose filter paper and used for preparative HPLC purification.

3. Results and discussion

In our previous work, we reported a method for the batch production of a new mixture of FOS, starting from a 600 g/L solution of sucrose using lyophilized mycelium of *C. cladosporioides* MUT 5506 in 20 mM acetate buffer (Zambelli, et al., 2014). The FOS mixture was characterized by the presence of a high variety of FOS, *i.e.*, kestose (1-, 6- and

neo-), nystose (1-, 6-, and neo-), 1-fructofuranosylnystose and, interestingly, an unusual disaccharide named blastose. Given our great interest in investigating the potential prebiotic properties of this new type of FOS mixture, we decided to exploit the flow chemistry facilities to implement its production, with the aim of setting up a suitable process for the sustainable and scalable production of the desired FOS mixture on a large scale. Thus, we decided to perform the biotransformation into a meso-flow reactor, using immobilized mycelia of *C. cladosporioides*. Dried alginate entrapped (DALGE)-mycelium was identified as the suitable kind of immobilization for the application in a continuous flow reactor, due to the good stability over the time and during subsequent cycles of biotransformations. Moreover, alginates are economic and easy to prepare (Fernandez-Arrojo, et al., 2013).

First, the reaction was performed in batch, following the procedure reported above. The higher conversion (total FOS amount ~ 51% w/w) was achieved after a prolonged reaction time (96 hours) and blastose appeared only after 48 hours with a maximum (4.9% w/w) at 168 hours. We then moved to flow. To this aim, a glass column (10 mm id × 100 mm length) was packed with the DALGE-mycelium beads and a 600 g/L sucrose solution in 20 mM acetate buffer (pH 6.0) was flowed through it (Scheme 1). To avoid any unwanted and uncontrolled increase of the pressure due to a clogging, celite was mixed to the alginates before the packaging (alginate beads: celite = 1:1 v/v). In this way, no over-pressure was observed over the reaction time.

Scheme 1 to be inserted here.

We initially optimized the reaction parameters using the transfructosylation/hydrolysis ratio (T/H index) as an index of the transglycosylation rate. First, the temperature effect on the T/H index was evaluated (Figure 1). The residence time was kept constant at 5 h and the temperature was varied between 25 °C and 70 °C. The highest T/H index was obtained at 35 °C, with a value of 4.0 ± 0.2. Then, the effect of the pressure on the biotransformation was

evaluated, keeping the temperature constant at 35 °C. Pressurization of the system was easily achieved applying in-line different backpressure regulators (40, 75, 100 and 250 psi). No significant differences in the T/H index were observed (Figure 1) and, for this reason, we decided not to use any back pressure regulator to avoid any negative influence of the prolonged pressurization on the stability of the enzyme and/or alginates over the time.

Figure 1 to be inserted here.

After having set the optimal operating conditions (*i.e.* T = 35 °C, atmospheric pressure), we analyzed the kinetic profile of the packed bed continuous-flow biotransformation and compared it to that obtained in batch with *C. cladosporioides* alginates in buffer. Considering the biotransformation catalyzed by DALGE-mycelium in acetate buffer (pH 6), the main products were 1-kestose, 1-nystose, 1-fructofuranosylnystose and blastose, with a maximum concentration of 139 g/L (23% w/w), 96 g/L (16% w/w), 15 g/L (2.5% w/w), and 30 g/L (5% w/w), respectively. Neo-kestose, neo-nystose and 6-kestose were found in a later stage of the biotransformation (after 48 h of reaction time), with a concentration below 12 g/L (< 2% w/w).

In flow, working at atmospheric pressure and at a temperature of 35 °C, we considered the residence time and we evaluated the conversion in terms of total amount of FOS formed (Figure 2). The maximum amount of FOS was reached at 20 h of residence time and remained constant up to 30 h. At 20 h of residence time, the concentration of FOS was 313 g/L (52.2 % w/w), as represented in figure 2, with a concentration of 1-kestose, 1-nystose, 1-fructofuranosylnystose and blastose of 139 g/L (23.2 % w/w), 86 g/L (14.3 % w/w), 39 g/L (6.5% w/w) and 28 g/L (4.7% w/w) respectively, and a concentration of neo-kestose, 6-kestose and neo-nystose of 9 g/L (1.5% w/w), 6 g/L (1% w/w) and 6 g/L (1% w/w), respectively. It is worth noting that, after only 10 h of residence time, a 47.4 % (w/w) of conversion was already reached and that the total amount of FOS only slightly increased in

the following 10 hours. A similar conversion (48% w/w) was obtained in batch after 72 hours of reaction. A significant reduction of the reaction time is a typical benefit achieved on passing from batch to flow conditions, both for heterogeneous chemo- or bio-catalyzed reactions (Kirschning, Solodenko & Mennecke 2006; Puglisi, Benaglia & Chiroli, 2013).

Focusing on blastose formation, as shown in Figure 2, blastose could be identified in the reaction flow stream after only 5 hours (residence time), and it reached a concentration of 28 g/L (4.7% w/w) after 20 hours, whereas a similar concentration (30 g/L) could be obtained in batch only after 168 hours. Moreover, running the reaction in flow for additional ten hours (30 hours of total residence time), the amount of blastose increased up to 31 g/L (5.2% w/w) of the total solution.

Figure 2 to be inserted here.

To compare the batch and flow efficiencies for the FOS production, we determined the productivity values for both total FOS and blastose, at different reaction times, in these two different environments. In batch conditions (72 hours, total FOS ~ 48.0% w/w), we obtained a complex mixture of different FOS, with different degree of polymerization and structures, and a productivity of 1.66 mg/g*min. In flow, a similar FOS concentration (47.4%) was reached with a residence time of 10 hours. In these conditions, the productivity value was equal to 2.84 mg/g*min, about 1.7 times the productivity obtained in batch. Considering blastose, the maximum value (30 g/L) in batch was reached after 168 hours. This corresponds to a productivity 0.07 mg/g*min. In flow, a similar concentration of blastose was obtained after 20 hours of residence time: the productivity is 0.14 mg/g*min, two times the productivity obtained in batch.

After the optimization of reaction parameters, we exploited the packed bed flow system for the continuous production of the new mixture of FOS. First, we evaluated the stability of the packed bed alginate beads over the time; to this aim, the conversion at 5 h of residence

time was evaluated during 7 days of continuous work. The reaction outcome was checked the first time, after 5 hours of residence time and, then, every 24 hours, for one week. A similar concentration of residual sucrose was observed in the monitored reaction time (Figure 3). The same packed column was then washed with the acetate buffer 20 mM and conserved at 4 °C for two months. The column was used again under the same reaction conditions and a similar conversion was obtained. This outcome highlights the possibility of using this set up for a continuous production over the time, without the need of replacing or recycling the biocatalyst.

Figure 3 to be inserted here.

Subsequently, we selected a residence time of 20 h, because the highest amount of total FOS is formed, including a 4.6% (w/w) of blastose, and we followed the production over 7 days of continuous work, as represented in figure 4. It is important to note that, using the flow system, once fixed the residence time, a constant mixture of FOS can be produced. Indeed, the total FOS amount remains in a range of 51.7-53.0% (w/w) during the monitored reaction time.

Figure 4 to be inserted here.

Finally, we set up a suitable method for the purification of the FOS mixture from the reaction medium and for the isolation of the *non*-conventional sugar blastose, in order to submit them to biological investigation. Glucose is the main undesirable by-product in the synthesis of FOS catalyzed by *trans*-fructofuranosydases (Duan, Chen & Sheu, 1994). In the exiting flow stream, glucose represents the 26% (w/w) of the mixture and its presence complicates the purification of the FOS mixture, and, in particular, the isolation of blastose. Therefore, we exploited a method reported in the literature for glucose removal based on its enzymatic oxidation to gluconic acid catalyzed by a commercially available glucose oxidase (Gluzyme® mono 10000 BG, from Novozymes); the formed gluconate can be precipitated as

calcium salt and easily removed (Sheu, Lio, Chen, Lin & Duan, 2001; Biyela, Du Toit, Divol, Malherhe & Van Renshurg, 2009). A final semi-preparative HPLC step was necessary to obtain either the pure FOS mixture or the blastose (97% purity).

In the last decade the concept of "prebiotic", substrate that selectively stimulates the growth and activity of health-promoting *Lactobacilli* and *Bifidobacteria*, has assumed much interest in terms of improving human host health (Gimeno-Pérez, Linde, Fernández-Arrojo, Plou & Fernández-Lobato, 2014). In order to investigate the ability of probiotic strains to use blastose as the sole carbon source and thus its suitability for a potential application in symbiotic-prebiotic mixtures, an *in vitro* fermentation study was carried out on 5 well characterized probiotic *Lactobacillus* strains, easier to cultivate than Bifidobacteria: *L. paracasei* DG (Ferrario et al, 2014), *L. rhamnosus* GG (Segers and Leeber, 2014); *L. paracasei* SHIROTA (Aoki et al., 2014); *L. johnsonii* LC1 (Isobe et al., 2012); *L. reuteri* ATCC55730 (Di Nardo et al, 2014; Valeur, Engel, Carbajal, Connolly, & Ladefoged, 2004). In detail, *Lactobacillus* strains were grown in presence of glucose (considered as positive control) and two well known prebiotic substrates, *i.e.* inulin and FOS Actilight®. Then, the growth kinetic parameter (μ_{max} value) and the final OD after 48 h of incubation were compared to that obtained in presence of our new FOS-mixture, our FOS-mixture without blastose and only blastose. The results are summarized in Table 1.

Table 1 to be inserted here.

All the *Lactobacillus* probiotics were able to growth in the presence of blastose as carbon source, except for *L. reuteri* strain, able to growth only in presence of glucose. Among the probiotics tested, *L. paracasei* DG and *L. johnsonii* LC1 showed the most efficient growths in presence of the *non*-conventional disaccharide, with an increase of the final OD of 50% and 109% and higher μ_{maxs} (>62% and >77%) in respect to glucose. These data indicate a growth stimulation effect of blastose. No increases of biomass production or μ_{max} values were

observed for *L. rhamnosus* GG and *L. casei* Shirota, probably due to a less efficient disaccharide transport system in these species. More in general, the microorganisms, in the presence of blastose as sole carbon source, are able to reach higher OD and μ_{max} than in the presence of other prebiotic substrates such as inulin and the FOS mixture Actilight®. Surprisingly, our new FOS mixture seems to be better metabolized than FOS Actilight® by *L. rhamnosus* and *L. casei* cells, reaching a major final OD value and an increased μ_{max}. This effect is probably due to a different 1F-FOS amount compared to Actilight®, as well as the presence of about 5% of blastose (Zambelli et al., 2014). These positive results provide a first preliminary evidence on the potential *in vitro* prebiotic effect of the newly isolated blastose and of our new FOS mixture. Nevertheless, further *in vitro* and *in vivo* studies are needed in order to assess the effect of the new compound on other probiotic microorganisms and its possible use in prebiotic formulations.

4. Conclusions

A continuous production of a new mixture of fructooligosaccharides was performed exploiting the innovative combination of immobilized whole cells with a continuous flow chemistry reactor, leading to a significant improvement of the total FOS productivity (1.7 times) and blastose productivity (2 times), compared to classical batch methods. Moreover, we ascertained the stability of our production system over the time and, indeed, we performed a 7 days continuous production cycle, being able to produce a mixture of FOS characterized by a constant composition. Hence, the use of a continuous flow reactor allowed to overcome some of the typical limitations of batch biotranformations, such as long reaction times, product inhibition, and scalability.

These results suggest the suitability of the proposed method for a sustainable and scalable production of the desired FOS mixture, whose potential use as a novel prebiotic preparation

382 can be easily foreseen and is at present under investigation by us. The results presented here,
383 even if only preliminary, indicate that blastose has prebiotic properties similar or higher than
384 the reference prebiotics, *i.e.*, inulin and a commercialized FOS mixture (Actilight®),
385 depending on the probiotic species analyzed. This result represents the first indication that
386 blastose has a positive influence on the growth of probiotic microorganisms and this can
387 justify further *in vivo* assays conducted on animal models or human volunteers to ensure and
388 better assess its prebiotic properties.
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394
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Figure and scheme captions

Scheme 1. Schematic representation of the continuous system configuration used for pressure and temperature optimization. BPR: back pressure regulator.

Figure 1. Graph of the T/H index in function of temperature (A) and pressure (B). A) Conditions: [sucrose]: 600 g/L in acetate buffer 20 mM (pH 6); alginate beads: 1 g; celite: 1 g; residence time: 5 h; atmospheric pressure. B) Conditions: [sucrose]: 600 g/L in acetate buffer 20 mM (pH 6); alginate beads: 1 g; celite: 1 g; residence time: 5 h; T = 35 °C. Results are the average of three trials with standard deviations were lower than 5%.

Figure 2. Total (A) and single (B) FOS amount at different residence times using a packed bed continuous flow reactor. Conditions: [sucrose]: 600 g/L in acetate buffer 20 mM (pH 6); alginate beads: 2 g; celite: 2 g; T = 35 °C; atmospheric pressure. Results are the average of three trials with standard deviations were lower than 5%.

Figure 3. Sucrose residual concentration during 7 days of continuous work. Conditions: [sucrose]: 600 g/L in acetate buffer 20 mM (pH 6); alginate beads: 2 g; celite: 2 g; T = 35 °C; atmospheric pressure. Results are the average of three trials with standard deviations were lower than 5%.

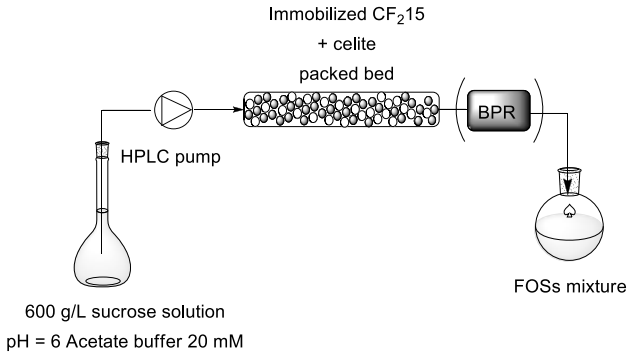
Figure 4. Continuous production of FOS under optimized conditions. Conditions: [sucrose]: 600 g/L in acetate buffer 20 mM (pH 6); alginate beads: 2 g; celite: 2 g; T = 35 °C; atmospheric pressure. Results are the average of three trials with standard deviations were lower than 5%.

536

537 **Table 1**. Growth parameters (OD 600 nm and μ_{max}) of probiotics during the *in vitro*

538 prebiotic assay.

539



Scheme 1

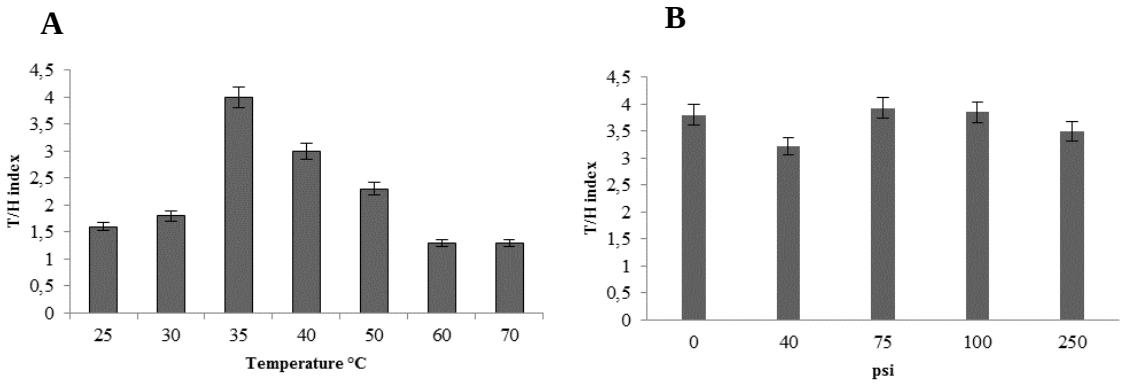
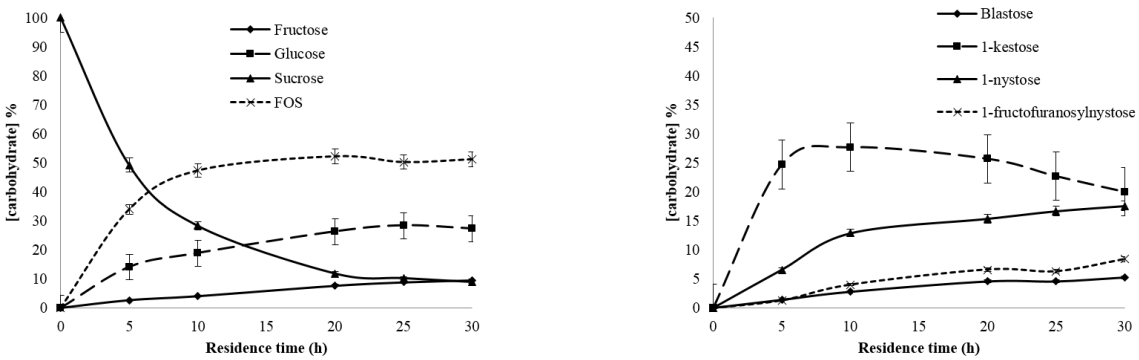


Figure 1.

552

553 **A** **B**



554

555 **Figure 2.**

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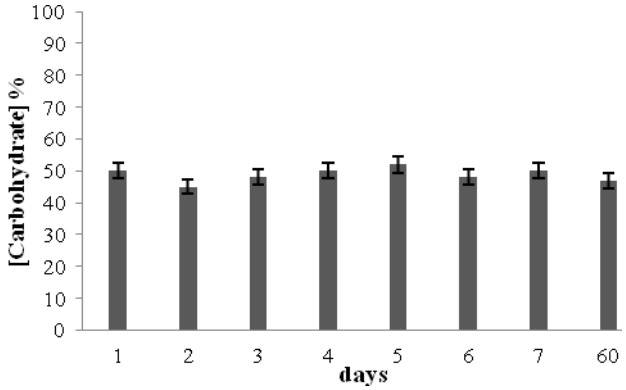

560

561 **Figure 3.**

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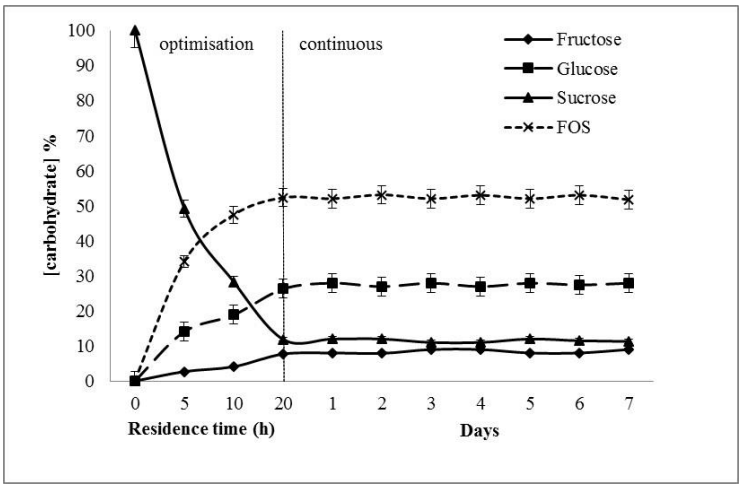

563

564 **Figure 4.**

565

566 **Table 1.** Growth parameters (OD_{600nm} and μ_{max}) of probiotics during the *in vitro* prebiotic
567 assay.

	L. paracasei DG		*L. rhamnosus* GG		*L. casei* SHIROTA		*L. johnsonii* LC1		*L. reuteri* ATCC55730	
Carbohydrate	OD_{600nm}	μ_{max} mOD/min	OD_{600nm}	μ_{max} mOD/min	OD_{600nm}	μ_{max} mOD/min	OD_{600nm}	μ_{max} mOD/min	OD_{600nm}	μ_{max} mOD/min
Glucose	1.2±0.07	0.29±0.07	1.9±0.03	0.71±0.002	1.6±0.08	0.43±0.09	1.1±0.01	0.49±0.05	1.5±0.01	0.1±0.01
Inulin	0.9±0.03	0.26±0.03	0.9±0.01	0.32±0.03	1.1±0.03	0.28±0.00	0.8±0.01	0.21±0.02	n.d	n.d
FOS Actilight®	1.5±0.02	0.34±0.03	0.74±0.05	0.13±0.03	n.d.	n.d.	1.8±0.05	0.86±0.02	n.d	n.d
FOS mixture	1.4±0.05	0.39±0.03	1.2±0.07	0.40±0.03	1.6±0.03	0.36±0.01	1.9±0.05	0.82±0.02	n.d	n.d
FOS mixture (-blastose)	1.4±0.04	0.38±0.09	1.1±0.07	0.35±0.02	1.1±0.04	0.35±0.03	2.0±0.01	0.21±0.02	n.d	n.d
Blastose	1.8±0.05	0.47±0.06	1.8±0.07	0.57±0.02	1.6±0.04	0.53±0.02	2.3±0.05	0.87±0.02	n.d	n.d

568 n.d: not detected growth after 48h of incubation at 37 °C

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