Bioresource Technology 282 (2019) 464–474

Contents lists available at ScienceDirect

Bioresource Technology

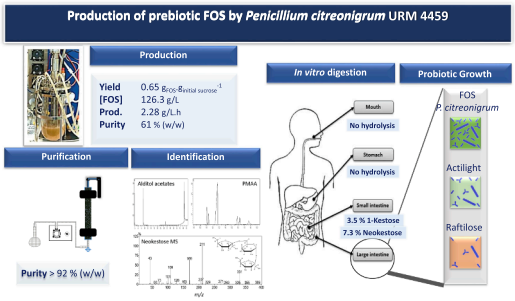
journal homepage: www.elsevier.com/locate/biortech

Process development for the production of prebiotic fructo-oligosaccharides by *penicillium citreonigrum*

Clarisse Nobre[a,*,1], Ana Karoline Caitano do Nascimento[b,1], Soraia Pires Silva[c], Elisabete Coelho[c], Manuel A. Coimbra[c], Maria Taciana Holanda Cavalcanti[b], José António Teixeira[a], Ana Lúcia Figueiredo Porto[b]

[a] Centre of Biological Engineering, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal
[b] Department of Morphology and Animal Physiology, Federal Rural University of Pernambuco-UFRPE, Av. Dom Manoel de Medeiros, s/n, 52171-900 Recife, PE, Brazil
[c] QOPNA & LAQV-REQUIMTE, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Fructo-oligosaccharides
Penicillium citreonigrum
Production yield optimization
In vitro prebiotic evaluation
Glycosidic linkage analysis

ABSTRACT

A new isolated *P. citreonigrum* URM 4459 was selected to produce fructooligosaccharides (FOS) in an efficient, economical and fast one-step fermentation. Optimal culture conditions were stablished by experimental design. Experiments run in bioreactor resulted in a high yield, content, productivity and purity of FOS (0.65 ± 0.06 $g_{FOS}/g_{initial\ Sucrose}$, 126.3 ± 0.1 g/L, 2.28 ± 0.08 g/L.h and 61 ± 0%). The FOS mixture was purified up to 92% (w/w) with an activated charcoal column. FOS produced were able to promote lactobacilli and bifidobacteria growth. Higher bacteria cell density was obtained for microbial-FOS mixtures than commercial Raftilose® P95. Some strains grew even faster in the FOS mixture produced than in all other carbon sources. FOS were resistant to the simulated gastrointestinal conditions. A high amount of a reducing trisaccharide was identified in the FOS produced mixture, possibly neokestose, which may explain the great prebiotic potential of the FOS.

1. Introduction

The daily ingestion of prebiotic sugars, such as fructo-oligosaccharides (FOS), has demonstrated a great impact in human health. Among the health benefits associated to prebiotics consumption are the positive effect on development of beneficial intestinal bacteria -

* Corresponding author.
E-mail addresses: clarissenobre@deb.uminho.pt (C. Nobre), soraiapiressilva@ua.pt (S.P. Silva), ecoelho@ua.pt (E. Coelho), mac@ua.pt (M.A. Coimbra), jateixeira@deb.uminho.pt (J.A. Teixeira).
[1] Clarisse Nobre and Ana Karoline Caitano do Nascimento contributed equally to this work.

https://doi.org/10.1016/j.biortech.2019.03.053
Received 31 January 2019; Received in revised form 8 March 2019; Accepted 9 March 2019
Available online 12 March 2019
0960-8524/

through the intestinal modulation of the microflora, reduction of colorectal cancer and other tumours, support of the immune system, risk reduction of obesity and metabolic syndrome, and protection against infection (Markowiak and Śliżewska, 2017).

Although FOS occur naturally in more than 36,000 plants, usually stored in tubers, bulbs, and tuberous roots of plants, they are present in low concentrations and are season-limited (Nobre et al., 2015; Singh et al., 2019). On a larger scale, FOS have been produced by enzymes with transfructosylation activity, able to convert sucrose into FOS. The industrial FOS process production is conducted in two-steps. The first step consists in the microbial production of β-fructofuranosidase and fructosyltransferase enzymes, with fructosyltransferase activity, and the second step consists in the production of FOS by the purified enzymes (Sangeetha et al., 2005). The main drawback of this process is the low FOS production yield ranging from 0.5 to 0.6 $g_{FOS}/g_{intitial\ Sucrose}$ (Ganaie et al., 2014). Alternatively, FOS can be produced in a one-step bioprocess, using the whole cells of microorganisms (Mano et al., 2018). The production of the enzyme and FOS occurs simultaneously resulting in several advantages, such as the possibility of increase FOS production yield and productivity, the use of lower sucrose concentrations, and avoid separation steps for enzyme purification (Castro et al., 2019, 2017; Nobre et al., 2018a,b).

The increasing demand of consumers for food that have an impact not only in the daily diet but also in the health has exponentially increased the market size of prebiotics in the last decade, with an expectancy to reach approximately $10.55 billion by 2025 (Research and Markets, 2017). To increase FOS production and produce FOS with differentiated properties, it is now necessary seeking among the microbial diversity for new strains, with great transfructosylation activity, as well as to explore new production strategies. Among the most common fungi used for FOS production are *Aspergillus* spp. and *Aureobasidium* spp. (Bali et al., 2015; Cruz et al., 1998; Ganaie et al., 2014; Shin et al., 2004). New isolated *Penicillium* strains have great potential as fructosyltransferase enzyme producers, with high enzyme concentration and low fermentation time (Nascimento et al., 2016).

To assure health claims related with FOS, the prebiotic potential of the FOS produced by the new isolated microorganisms must be evaluated and related with the oligosaccharide structural features. In practice, the physiological properties of new screened potential prebiotics may be reasonably well explored with *in vitro* tests so that their nondigestibility and fermentability can be established (Cummings et al., 2001). Oligosaccharides must be resistant to the human gastrointestinal harsh conditions, since the hydrolysis of FOS influences to a great extent the dose of substrate that effectively reaches the distal regions of the colon, as well as its potential to exert the expected prebiotic effect through metabolism by the colonic microbiota. In the colon, FOS must promote the bifidobacteria and lactobacilli growth. These bacteria produce carbohydrate degrading enzymes which ferment the oligosaccharides and produce short chain fatty acids, such as acetate, propionate and butyrate, which provide metabolic energy and help in the acidification of the gut, conferring a health benefit on the host (Younis et al., 2015).

Since it is not clear which prebiotics are the most suitable substrates for a selective growth of specific bacteria, it is important to conduct *in vitro* tests to evaluate the associations between bacteria species growth and the type of FOS under evaluation (Chung et al., 2016; Huebner et al., 2007), as FOS fermentability and hydrolysis are largely affected by their chemical structure (Nobre et al., 2018c).

In this context, the present work aimed to screen and identify new isolated strains of *Penicillium* fungi with potential for FOS production at high yields. The fermentation process was conducted in one-single step, using the whole cells of active microorganisms, for simultaneously production of enzymes and FOS. After selection of the *Penicillium* strain, the FOS production process was optimized in shake flask and scaled-up to bioreactor size. FOS mixtures obtained were purified with activated charcoal for prebiotic evaluation and structure characterization using different gas chromatographic techniques. The prebiotic potential of the purified FOS mixture was evaluated by fermentation with several *Bifidobacterium* and *Lactobacillus* strains and their resistance under *in vitro* gastrointestinal simulation conditions was also determined.

2. Material and methods

2.1. FOS production

2.1.1. Microorganisms and culture conditions

The potential of eight strains from the *Penicillium* genus, previously reported by Nascimento et al. (2016) as β-fructofuranosidase producers, were herein tested for their ability to produce FOS, namely *Penicillium aurantiogriseum* URM 5139, *Penicillium citrinum* URM 2725, *Penicillium commune* URM 4939, *Penicillium aurantiogriseum* URM 5126, *Penicillium implicatum* URM 5426, *Penicillium citreonigrum* URM 4459, *Penicillium glabrum* URM 4757, and *Penicillium islandicum* URM 5073). All strains were obtained from the Micoteca of the Federal University of Pernambuco (URM - UFPE) and maintained at 4 °C on Czapek Dox Agar medium (CZ) (Himedia, India).

2.1.2. Inoculum development and screening of fungi for FOS production

The inoculum was prepared from a 7-day-old fungi grown in CZ plates. A sterilized solution of 0.1 % (v/v) Tween 80 (Merck, German) was used for scrapping the spores from the plates. The number of spores in the suspension was determined by counting in a microscope, using an improved *Neubauer* chamber. The suspension was finally diluted to a concentration of 10^4 spores/mL and inoculated in a 250 mL shake flask containing 100 mL of medium composed by (% w/v): sucrose, 20.0; yeast extract, 2.75; $NaNO_3$, 0.2; $MgSO_4{\cdot}7H_2O$, 0.05; K_2HPO_4, 0.5; and KCl, 0.05 (Prata et al., 2010). Sucrose solutions were previously sterilized by filtration (0.2 μm) and the other components were autoclaved at 121 °C for 15 min. The fermentation medium pH's was adjusted to 5.5 before inoculation. Fermentations were carried out at 30 °C under an agitation of 150 rpm. Samples were harvested at 0, 12, 16, 20, 24 and 36 h of fermentation. Fermentation samples were firstly vacuum filtered with a Whatman.1 paper, for major cell pellets removal and further refiltrated through a 0.2 μm cellulose acetate membrane, for sugars purification and qualitative/quantitative analysis.

2.1.3. Effect of the operational conditions on FOS production in shake flask

The effects and interactions of variables affecting the FOS production (fermentation time, temperature, pH, and yeast extract concentration) were evaluated using a 2^4 central composite design (CCD), with three central points. The levels of the variables were defined according to other studies on FOS production optimization (Dominguez et al., 2012; Prata et al., 2010; Silva et al., 2011). The range and the levels of the independent variables studied were (−1, 0, 1): fermentation time (36, 48, 60 min), temperature (25, 30, 35 °C), pH (4, 5, 6) and yeast extract concentration (0, 1.375, 2.750 g/L).

Variables with statistical influence on FOS production were selected for further optimization experiments.

2.1.4. Optimization of FOS production in shake flask

To determine the optimal experimental conditions for FOS production, the variables with statistical significance on the FOS production were optimized by Response Surface Methodology (2^2). A 3 levels (−1, 0 and +1) combination was tested, with two central points. The regression coefficients for linear, quadratic and interactions for each variable were determined and adjusted to a polynomial second order equation (Eq. (1)).

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_1 X_1^2 + \beta_1\beta_2 X_1 X_2 + \beta_2 X_2^2 \quad (1)$$

where Y is the dependent variable, FOS production concentration; β_0 is the interception coefficient; β_1 and β_2 are coefficients estimated by the

model; X_1 and X_2 are the independent variables, fermentation time and temperature, respectively.

2.1.5. FOS production in bioreactor

Bioreactor fermentations were conducted with the selected *Penicillium* strain. The inoculum was prepared with the following procedure: a spore suspension with 10^4 spores/mL concentration was added to 100 mL of fermentation medium, containing (% (w/v)): sucrose, 10.0; $NaNO_3$, 0.2; $MgSO_4{\cdot}7H_2O$, 0.05; K_2HPO_4, 0.5; and KCl, 0.05, in 250 mL shake flask. After 3 days growing at 30 °C and 150 rpm, the inoculum was transferred to a 3.75 L stirred bioreactor (Autoclavable Benchtop Fermenter Type R'ALF, Bioengineering AG, Wald, Switzerland) containing 2 L of culture medium with the same composition of the inoculum, except for sucrose, which concentration was adjusted for 20% (w/v). Fermentations were performed under an agitation of 150 rpm. Temperature and fermentation time conditions previously optimized for the FOS production in shake flask were used. Samples were collected at 0, 14, 19, 24, 38, 43, 48, 63, 72 and 86 h. Fermentative broth was harvested at the optimal point for further use in the purification of FOS. Before the chromatographic process, the broth was purified by vacuum filtration, with a Whatmann.1 paper, for major cell pellets removal, and further re-filtrated through a 0.2 µm cellulose acetate membrane. Fermentation samples were firstly vacuum filtered with a Whatman.1 paper for major cell pellets removal, and further re-filtrated through a 0.2 µm cellulose acetate membrane, for further sugars purification and qualitative/quantitative analysis.

2.2. FOS purification

FOS mixtures harvested at the maximum FOS production time were purified using the methodology described by Nobre et al. (2012) with minor modifications. Briefly, an amount of 180 g of the extra pure activated charcoal supplied by Merck (Darmstadt, Germany) in a granular form, with 1.5 mm of mean, was used in the purification experiments. A glass column (33 × 4.5 cm length × internal diameter) was filled with activated charcoal and further loaded with 455 mL of fermentative broth, at a flow rate of 25 mL/min and 25 °C. The retained sugars were recovered by elution with a gradient of ethanol (up to 100% v/v). Fractions collected in the desorption phase were evaporated at 60 °C in a rotary evaporator (B. Braun Biotech International, Germany) to remove ethanol and concentrate the sugars. Finally, FOS samples were lyophilized and used for chemical analyses and *in vitro* tests.

2.3. Chemical characterization

2.3.1. FOS identification and quantification

FOS mixture produced by *P. citreonigrum* URM 4459 were analysed according to Nobre et al. (2018c) and Ribeiro et al. (2018). A mixture of commercial FOS Actilight® (Beghin-Meiji Industries, France) was used as standard. The samples FOS and Actilight® were derivatized in duplicate by adding 200 µL of a sodium borohydride solution (15% (m/v) in NH_3 3 M) and incubated at 30 °C for 60 min. The acetylation of the alditols was performed with acetic anhydride and 1-methylimidazole as catalyst for 30 min at 30 °C. The alditol acetates were extracted with dichloromethane and evaporated to dryness. The alditol acetates were dissolved in anhydrous acetone and analysed by gas chromatography-quadrupole mass spectrometry (GC-qMS) equipped with a DB-1 (J & W Scientific, Folsom, CA, USA) capillary column (30 cm length, 0.25 mm of internal diameter and 0.10 µm of film thickness). The samples were identified according the mass spectra pattern and standards injection by GC-MS (Nobre et al., 2018c). Monosaccharides quantification is presented in equivalents of glucose, and di-trisaccharides are depicted as equivalents of sucrose, i.e. they were calculated with the response factor of glucose and sucrose, respectively. For the quantification of tetrasaccharides, the alditol acetates were dissolved in anhydrous acetone (50 µL) and analysed by GC–FID equipped with a 400-5HT, dimethylpolysiloxan capillary column (Quadrex, New Haven, CT, USA) with 25 m length, i.d. of 0.22 mm and film thickness of 0.05 µm. The oven temperature program used was: initial temperature of 100 °C; rise in temperature at a rate of 10 °C/min until 200 °C, standing for 2 min; rise in a rate of 1.0 °C/min until 250 °C and maintaining this temperature 2 min; final linear increase of 15 °C/min until 400 °C, standing for 1 min. The injector and detector temperatures were 300 and 400 °C, respectively. The flow rate of the carrier gas (H_2) was set at 1.7 mL/min (Ribeiro et al., 2018).

2.3.2. Glycosidic linkage analysis

The glycosidic linkages established between the monomers of the *P. citreonigrum* URM 4459 FOS and Actilight® were identified by methylation analysis, using the same procedure as Nobre et al. (2018c). Briefly, the free hydroxyl groups of FOS were methylated, followed by the hydrolysis of the glycosidic linkages. The resultant monosaccharides were then reduced and the hydroxyl groups formed, previously involved in the glycosidic linkages or ring formation, were acetylated. The partially methylated alditol acetates were analysed by GC-qMS.

2.4. In vitro evaluation of the prebiotic effects

2.4.1. Gastrointestinal digestion of FOS

The gastrointestinal digestion of the FOS mixture produced was evaluated according to a methodology described by Minekus et al., (2014). An Actilight® FOS mixture, obtained from a microbial source, (Beghin-Meiji Industries, France) was used as positive control. Briefly, 5 mL of each carbohydrate sample (FOS and Actilight®), at a concentration of 94% of FOS in total sugars, was exposed to conditions simulating the environment of the mouth, stomach, and small intestine.

To simulate the oral phase a simulated salivary fluid (SSF) was added to a $CaCl_2.(H_2O)_2$ solution (to obtain 1.5 mM in the fluid). The SSF was prepared as follows: KCl 15.1 mM, KH_2PO_4 3.7 mM, $NaHCO_3$ 13.6 mM, $MgCl_2.(H_2O)_6$ 0.15 mM, $(NH_4)_2.CO_3$ 0.06 mM, and HCl 1.1 mM. The reaction mixture with the sample was incubated in a shaking bath at 37 ± 1 °C for 2 min.

The gastric phase was mimicked by mixing a simulated gastric fluid (SGF) with porcine pepsin solution (2000 $U.mL^{-1}$ in the final sample), $CaCl_2.(H_2O)_2$ (to obtain 0.15 mM in the fluid) and HCl to adjust the pH to 3.0. The SGF was prepared as follow: KCl 6.9 mM, KH_2PO_4 0.9 mM, $NaHCO_3$ 25 mM, NaCl 47.2 mM, $MgCl_2.(H_2O)_6$ 0.1 mM, $(NH_4)_2.CO_3$ 0.5 mM, and HCl 15.6 mM. Samples were incubated in a shaking bath at 37 ± 1 °C for 2 h.

The intestinal phase was mimicked by mixing a simulated intestinal fluid (SIF) with a pancreatin suspension in SIF (based on trypsin activity of 100 U/mL in the final sample), bile solution in SIF (to obtain 10 mM in the fluid), $CaCl_2.(H_2O)_2$ (to obtain 0.6 mM in the fluid) and NaOH to adjust the pH to 7.0. The SIF was prepared as follow: KCl 6.8 mM, KH_2PO_4 0.8 mM, $NaHCO_3$ 85 mM, NaCl 38.4 mM, $MgCl_2.(H_2O)_6$ 0.33 mM, and HCl 8.4 mM. Samples were incubated in a shaking bath at 37 ± 1 °C for 2 h.

Samples were collected after each phase (oral, gastric and intestinal). The reaction of gastric phase was stopped by raising the sample pH to 7.0 with $NaHCO_3$ (1 M). At the end of digestion, the reaction was stopped by adding the enzyme inhibitor pefabloc (1 mM) (10 µL per 1 mL of sample). All samples were tested at least in triplicate.

The hydrolysis degree was calculated according to the equation (2):

$$\%Hydrolysis = \frac{FOS\ mass}{Total\ sugars\ mass} \times 100 \quad (2)$$

Porcine pepsin, pancreatin (8 × USP), bile extract porcine, HCl, $NaHCO_3$, $(NH_4)_2.CO_3$ and pefabloc SC (4-(2-aminoetyl) benenesulfonyl fluoride) were purchased from Sigma-Aldrich (St. Louis, MO). NaOH, KCl, $CaCl_2.(H_2O)_2$, KH_2PO_4 and NaCl were obtained from Panreac (Spain) and $MgCl_2.(H_2O)_6$ was purchased from Merck.

2.4.2. Fermentability of FOS by probiotic bacteria

Lactobacillus acidophilus CECT *903*, *Lactobacillus paracasei* CECT 277, *Bifidobacterium bifidum* CECT 870, and *Bifidobacterium adolescentis* CECT 5781 were obtained from Spanish Type Culture Collection (CECT) of the University of Valencia. *Lactobacillus rhamnosus* ATCC 7469, *Lactobacillus delbrueckii delbrueckii* ATCC 9649, *Lactobacillus bucheneri* ATCC 4005, and *Lactobacillus delbrueckii lactis* ATCC 12375 were obtained from The Global Bioresource Center (ATCC). *Lactobacillus plantarum* NCIMB 8827 and *Lactobacillus paracasei paracasei* CCUG 27320 were obtained from National Collections of Industrial, Marine and Food Bacteria Ferguson Building (NCIMB) and Culture Collection University of Göteborg (CCUG), respectively. All cultures were maintained at −80 °C in Man-Rogosa-Sharpe (MRS; Biokar Diagnostics, France), both supplemented with glycerol (30% v/v), and sub-cultured in MRS broth by overnight incubation at 37 °C for the prebiotic activity assay. *Bifidobacterium* strains required 0.5 g.L of L-cysteine-HCl (Merck, Germany) to grow in the MRS medium. *Bifidobacterium* were cultured in anaerobic jars generating an anoxic atmosphere by means of AnaeroGen sachets.

To investigate the oligosaccharides utilization and growth of the bacteria, MRS medium without glucose (MRS-), was prepared containing: 10 g/L of peptone, 10 g/L of meat extract, 5 g/L of yeast extract, 2 g/L of K_2HPO_4, 1.08 g/L of Tween 80, 5 g/L of sodium acetate, 2 g/L of ammonium citrate tribasic, 0.2 g/L of $MgSO_4$, 0.05 g/L of $MnSO_4$. This medium was sterilized at 121 °C for 15 min. Glucose was used as positive control (MRS+). Two commercial FOS mixtures, Raftilose® P95 (Beneo-Orafti Group, Belgium) and Actilight® (Beghin-Meiji Industries, France), were tested and compared with FOS mixtures produced in our laboratory by *P. citreonigrum* URM 4459. These oligosaccharides were added to the MRS broth at final concentration of 2% (w/v) in a 1:9 ratio (v/v) by filter-sterilized at 0.2 µm. As negative control, a volume of 10% of filter-sterilized deionized water was added to the MRS broth.

Bacterial strains, at 2% (v/v), were cultured overnight in a 96-well microplate, containing 300 µL of the MRS broth with the different carbon sources. Strains were grown in an automated microplate reader (Varioskan Flash, Thermo Electron Corporation, Finland), in duplicate, at 37 °C for 36 h. The optical density at 600 nm (OD_{600}) was recorded each 30 min intervals.

2.5. Saccharides quantification by HPLC

Sugars were quantified by high-performance liquid chromatography (HPLC) using a LC-10 equipment (Jasco, Japan) equipped with a Prevail Carbohydrate ES column (5 µm particle size, 250 × 4.6 mm length × diameter) from Alltech. Sugars were detected with an evaporative light scattering detector – Sedex 55 (Sedere, Alfortville, France). Samples were eluted at a flow rate of 1.0 mL/min and 25 °C with a mixture of acetonitrile (HPLC Grade, Carlo Erba, France) and water (70:30 v/v), containing 0.04% ammonium hydroxide (HPLC Grade, Sigma-Aldrich, Germany) in water (Nobre et al., 2009). The chromatographic signal was recorded and integrated using the software LabSolutions (Shimadzu).

2.6. Commercial FOS composition

The composition of the Actilight® FOS mixture used was (g/L): Fructose (1.99); Glucose (1.69); Sucrose (8.25); 1-Kestose (81.47); Nystose (88.97); and Fructofuranosylnystose (28.12).

The composition of the Raftilose® FOS mixture used was (g/L): Fructose (5.25); Glucose (0.38); 1-Kestose (2.08); Nystose (6.5); and Fructofuranosylnystose (16.07). This mixture was also composed by non-identified sugars as follow: 5.20 g/L disaccharide; 24.69 g/L trisaccharide; 21.38 g/L tetrasaccharide; 7.28 g/L pentasaccharide; 28.70 g/L of two hexasaccharides; and 4.64 g/L of two heptasaccharides.

2.7. Statistical analysis

The *Statistica* 8.0 software (Statsoft, USA) was used for the experimental designs and regression analysis of the experimental data. The effects of linear, quadratic and interactive terms of the independent variables on the chosen dependent variables were evaluated by the model. The quality of the fitted polynomial model was statistically checked by the magnitude of the coefficient of determination R^2 and its statistical significance was checked by analysis of variance (ANOVA). The coefficients of the response surface were evaluated using the student *t*-test. Results were considered significantly different for *p*-values < 0.05. All statistical tests were conducted at a 5% significance level. Data are presented as mean ± Standard Deviation.

3. Results and discussion

3.1. FOS production

3.1.1. Screening of fungi strains for FOS production

Results on the production of FOS obtained for the eight screened *Penicillium* fungi are shown in Fig. 1. The highest FOS yields (> 0.5 $g_{FOS}/g_{initial\ Sucrose}$) were obtained for the following strains: *P. aurantiogriseum* URM 5139 and URM 5126, *P. citreonigrum* URM 4459, and *P. glabrum* URM 4757.

Among the strains tested, *P. citreonigrum* URM 4459 obtained, beyond the highest FOS yield (0.55 ± 0.02 $g_{FOS}/g_{initial\ Sucrose}$), also the highest FOS concentration (100 ± 3 g/L), and productivity (3.9 ± 0.2 g/L.h). The highest content of FOS in total sugars (57 ± 1%, w/w) was also obtained for this strain. Thus, the *P. citreonigrum* URM 4459 was selected for further study the influence of the fermentation operational conditions into the FOS production (Section 3.1.2), process optimization (Section 3.1.3), and scale-up to bioreactor size (Section 3.1.4). The mixture of FOS produced by the *P. citreonigrum* URM 4459 was purified (Section 3.2) for structure analysis (Section 3.3) and prebiotic potential evaluation – *in vitro* digestibility in a simulated human harsh digestion conditions (Section 3.4.1) and fermentability by probiotic bacteria (Section 3.4.2).

3.1.2. Effect of the operational conditions on FOS production in shake flask

The influence of the fermentation time, temperature, pH and amount of yeast extract in the FOS concentration, purity, yield and productivity obtained in fermentations conducted with the *P. citreonigrum* URM 4459 are shown in Table 1. Twenty independent assays were performed.

Within the range of study, pH and yeast extract did not affect FOS production by the *P. citreonigrum* URM 4459 significantly ($p > 0.05$). Indeed, Nobre et al. (2016) also reported that the presence of yeast extract did not significantly affect the production of FOS with an *Aureobasidium pullulans* strain. On the other hand, temperature and fermentation time have a statistically significant effect on FOS production ($p < 0.05$). Pareto chart showed that the temperature is the parameter that most influences the FOS production yield, followed by the fermentation time, with an estimated effect of −23.52 and 12.98, respectively. The negative effect of the temperature means that lower temperatures led to higher FOS production yields. Optimal growth/production temperature parameters are dependent on the type of microorganism used. Hence, using the same temperature levels, different effects may be found on the FOS production, in accordance with the results reported by Nobre et al. (2018a) for an *Aspergillus ibericus* strain, where the increasing of temperature had a positive effect on the FOS produced.

Based on the results obtained, the influence of temperature and fermentation time was further optimized by experimental design. Since the effect of using yeast extract in the fermentative broth was not statistically significant, the yeast extract was not added to the culture medium used in the further fermentations to make the process more

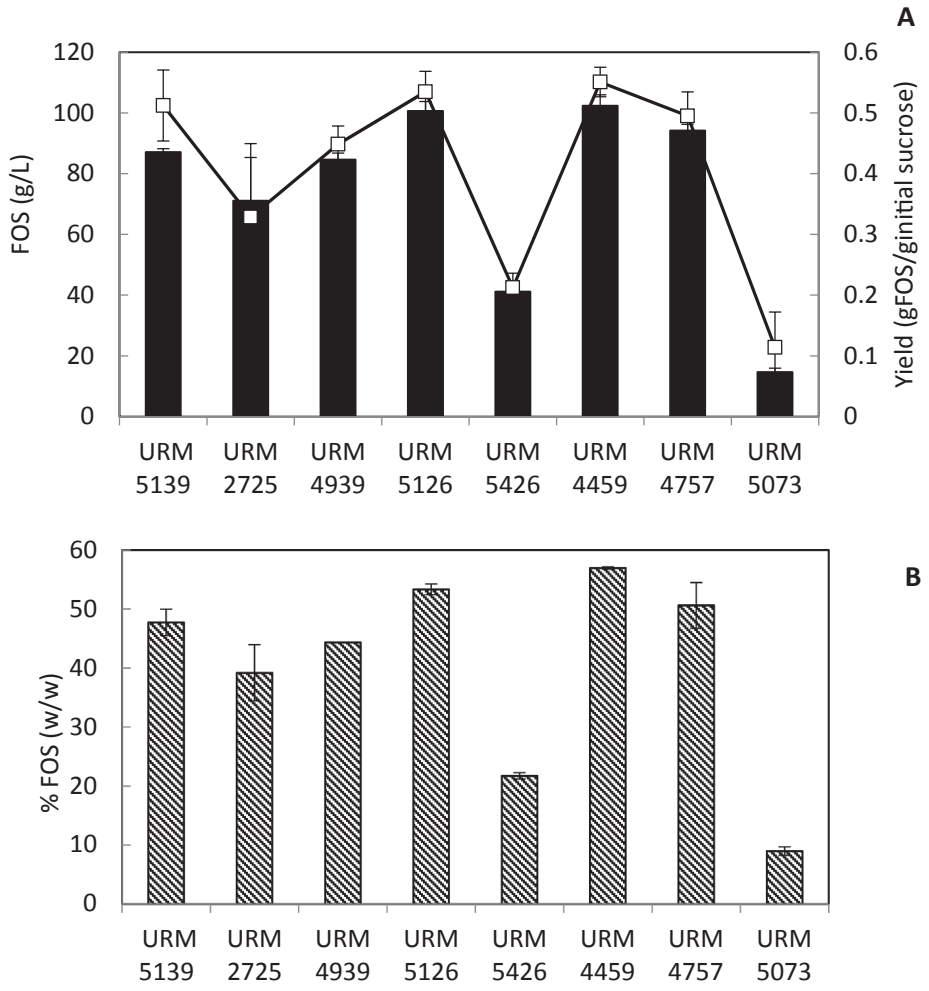


Fig. 1. Fructo-oligosaccharides (FOS) production at 36 h fermentation by the *Penicillium* strains: URM 5139 – *P. aurantiogriseum,* URM 2725 – *P. citrinum,* URM 4939 – *P. citrinum,* URM 5126 - *P. aurantiogriseum,* URM 5426 – *P. implicatum,* URM 4459 – *P. citreonigrum,* URM 4757 - *P. glabrum,* URM 5073 – *P. islandicum*. Fig. A: concentration of FOS produced (black bars) and yield (92). Fig. B: % (w/w) of FOS produced in total sugars. Results correspond to the average ± standard deviation of triplicated independent experiments.

Table 1
Experimental conditions defined by a 2^4 central composite design and respective responses obtained experimentally for fermentations run with the *Penicillium citreonigrum* URM 4459 fungi.

Independent variables (real and coded values)					Dependent variables			
Run	Fermentation time (h)	Temperature (°C)	pH	Yeast extract (% (w/v))	FOS (g/L)	% FOS (w/w)	Yield ($g_{FOS}/g_{initial\ Sucrose}$)	Q_p (g/L.h)
1	36 (−1)	25 (−1)	4 (−1)	0 (−1)	5.54	3.43	0.03	0.15
2	60 (+1)	25 (−1)	4 (−1)	0 (−1)	86.01	46.31	0.46	1.43
3	36 (−1)	35 (+1)	4 (−1)	0 (−1)	0.00	0.00	0.00	0.00
4	60 (+1)	35 (+1)	4 (−1)	0 (−1)	2.87	1.61	0.02	0.05
5	36 (−1)	25 (−1)	6 (+1)	0 (−1)	39.67	22.27	0.21	1.10
6	60 (+1)	25 (−1)	6 (+1)	0 (−1)	92.36	54.09	0.49	1.54
7	36 (−1)	35 (+1)	6 (+1)	0 (−1)	2.11	1.36	0.01	0.06
8	60 (+1)	35 (+1)	6 (+1)	0 (−1)	65.25	35.70	0.35	1.09
9	36 (−1)	25 (−1)	4 (−1)	2.750 (+1)	50.72	26.95	0.27	1.41
10	60 (+1)	25 (−1)	4 (−1)	2.750 (+1)	110.03	58.62	0.59	1.83
11	36 (−1)	35 (+1)	4 (−1)	2.750 (+1)	14.48	9.07	0.08	0.40
12	60 (+1)	35 (+1)	4 (−1)	2.750 (+1)	7.78	5.00	0.04	0.13
13	36 (−1)	25 (−1)	6 (+1)	2.750 (+1)	73.59	38.23	0.39	2.04
14	60 (+1)	25 (−1)	6 (+1)	2.750 (+1)	54.32	45.14	0.36	1.12
15	36 (−1)	35 (+1)	6 (+1)	2.750 (+1)	2.01	1.19	0.01	0.06
16	60 (+1)	35 (+1)	6 (+1)	2.750 (+1)	0.00	0.00	0.00	0.00
17	48 (0)	30 (0)	5 (0)	1.375 (0)	90.8	55.89	0.53	2.08
18	48 (0)	30 (0)	5 (0)	1.375 (0)	97.88	55.73	0.52	2.04
19	48 (0)	30 (0)	5 (0)	1.375 (0)	96.59	55.25	0.52	2.01
20	48 (0)	30 (0)	5 (0)	1.375 (0)	88.69	56.71	0.54	2.12

FOS: fructo-oligosaccharides; Qp: productivity.

Table 2
Experimental conditions defined by the 2^2 central composite design and respective responses obtained experimentally for fermentations run with the *Penicillium citreonigrum* URM 4459, at pH 6.0.

Independent variables (real and coded values)			Dependent variables				
Run	Fermentation time (h)	Temperature (°C)	FOS (g/L) (Experimental)	FOS (g/L) (Predicted)	% FOS(w/w)	Yield ($g_{FOS}/g_{initial\ Sucrose}$)	Q_p (g/L.h)
1	43 (−1)	25 (−1)	74.14	76.08	41.82	0.40	1.72
2	67 (+1)	25 (−1)	93.84	94.52	54.70	0.50	1.40
3	43 (−1)	35 (+1)	7.99	8.15	4.27	0.04	0.19
4	67 (+1)	35 (+1)	10.68	9.59	5.73	0.6	0.16
5	55 (0)	25 (−1)	97.96	95.34	57.17	0.52	1.78
6	55 (0)	35 (+1)	17.98	18.91	9.42	0.10	0.33
7	43 (−1)	30 (0)	87.19	85.08	50.00	0.47	2.03
8	67 (+1)	30 (0)	94.62	95.02	55.22	0.51	1.41
9	55 (0)	30 (0)	100.28	100.09	55.66	0.54	1.82
10	55 (0)	30 (0)	98.20	100.09	57.05	0.53	1.79

FOS: fructo-oligosaccharides; Qp: productivity.

cost effective. The pH was set at 6.0, following the positive effect of the previous design.

3.1.3. Optimization of FOS production in shake flasks

For the optimization of the FOS production process, the effect of temperature and fermentation time on FOS concentration, purity, yield and productivity was evaluated. Following the trend of the effects found in the first design, the fermentation time was set at 43, 55 and 67 h, and temperature at 25, 30 and 35 °C. Ten independent assays were performed at initial pH 6.0.

Experimental results obtained by the 2^2 central composite design, for the FOS production by the *P. citreonigrum* URM 4459, using the different combinations of temperature and fermentation time, are shown in Table 2. The highest FOS production and yield found, 100.28 g/L and 0.54 $g_{FOS}/g_{initial\ Sucrose}$, respectively, were obtained for the central point of the design, corresponding to 55 h of fermentation and 30 °C (Table 2).

High temperature levels have generally an adverse effect on the metabolic activity of microorganisms (Arumugam et al., 2014). However, similar effects might be found to lower temperatures. Thus, the working temperature is an important parameter of the fermentation process, especially when working with the whole-cells of fungi, since it influences not only the product formation, but also the spore germination and the microorganism growth itself (Nascimento et al., 2016). Results found in the present work, concerning the evaluation of temperature on FOS production, are in agreement with the findings of other authors while using an *Aspergillus japonicus* strain (Mussatto et al., 2013), where temperatures between 26 and 30 °C were found to raise FOS production, while temperatures above 30 °C had an opposite effect. Accordingly, for an *A. pullulans* strain, an optimum temperature of 32 °C and 48 h of fermentation time were reported as the optimal conditions (Dominguez et al., 2012).

ANOVA analysis was used to evaluate the fitting of the model obtained by a multiple regression analysis of the experimental data. The model was found to be statistically significant ($p = 0.027$) and did not show lack-of-fit ($p = 0.397$). It also explained 99.8% of the dependent variable's variability ($R^2 = 0.998$) with a good adjusted determination coefficient ($R^2_{adjusted} = 0.997$) that indicates a good agreement between the experimental and predicted values. The R^2 value ensured the satisfactory adjustment of the polynomial model to the experimental data. The $R^2_{adjusted}$ corrects the R^2 value for sample size and number of terms in the model. If there are many terms in the model and if the sample size is not very large, the $R^2_{adjusted}$ must be lower than R^2. Hence, the model equation (Eq. (3)) adequately approximates the response surfaces and can be suitably used to predict the concentration of FOS with any other values of the tested parameters within the experimental domain.

The response surface obtained by the model within the studied experimentally region is showed in Fig. 2. By increasing the fermentation time and reducing the temperature values, a favorable FOS production may be achieved. The variation of temperature (linear and quadratic effect) was significant on the FOS production response, while the fermentation time did not have any significant effect ($p > 0.05$). The temperature effect was negative (linear: −63.72, quadratic: −44.68), indicating that an increase of temperature above the studied limits would lead to a decrease on FOS production.

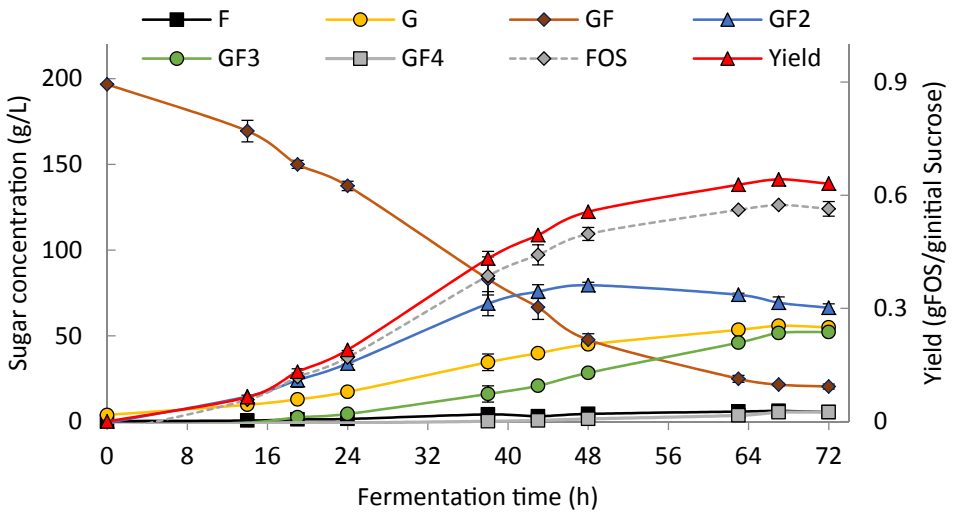


Fig. 2. Profile of sugars concentrations and FOS yield obtained during the fermentation carried out in bioreactor, with *Penicillium citreonigrum* URM 4459 (F: Fructose; G: Glucose; GF: Sucrose; GF_2: 1-Kestose; GF_3: Nystose; GF_4: fructofuranosylnystose; FOS: Fructo-oligosaccharides). Results correspond to the average ± standard deviation of triplicated independent experiments.

The simplified model obtained by a multiple regression analysis of the experimental data, using the least squares method ($p < 0.05$), is represented in the following equation:

$$Y = 100.09 - 38.22X_2 - 42.97X_2^2 \quad (3)$$

The optimal conditions found, using the proposed model (Eq. (3)), for FOS production by *P. citreonigrum* URM 4459, were 28 °C and 61 h of fermentation time. Under these conditions, the model predicted a FOS production of 109.8 g/L and a yield of 0.60 $g_{FOS}/g_{initial\ Sucrose}$.

To validate the model, three independent fermentations were conducted at the optimized conditions. An amount of 102.4 ± 0.1 g/L of FOS were produced in a fermentation that yielded 0.55 ± 0.00 $g_{FOS}/g_{initial\ Sucrose}$ and a productivity of 1.69 ± 0.01 g/L.h. These results allowed to observe experimental values approaching the predicted ones.

Similar results were obtained in shake flask in a work conducted also in one-step fermentation, using the whole-cells of a *Penicillium expansum* strain, and starting with the same amount of sucrose (0.58 $g_{FOS}/g_{initial\ Sucrose}$) (Mussatto et al., 2009; Prata et al., 2010). On the other hand, works conducted in two-step process, i.e. using pure enzymes, seems to reach lower FOS yields. For example, a crude enzyme of *P. citrinum* yielded 0.47 $g_{FOS}/g_{initial\ Sucrose}$ while using 37.5% sucrose as substrate (Tashiro et al., 2017), and a lyophilized mycelium of *Penicillium sizovae* only obtained 0.31 $g_{FOS}/g_{initial\ Sucrose}$, starting with 60% sucrose (Zambelli et al., 2014). Results found in the present work reinforce that fermentations run in one-step tends to lead to higher production yield values, highlighting the positive impact on the global process economy, together with the reduced downstream processing.

Following experiments run in the bioreactor were performed at 28 °C.

3.1.4. FOS production in bioreactor

Sucrose consumption, FOS produced, and monosaccharides released in a fermentation conducted in bioreactor, under the optimized conditions found in the shake flask experiments, with the *P. citreonigrum* URM 4459, are shown in Fig. 2. A concentration of 126.3 ± 0.1 g/L of FOS with a yield of 0.65 ± 0.06 $g_{FOS}/g_{initial\ Sucrose}$ was obtained at 67 h fermentation. The maximum productivity of 2.28 ± 0.08 g/L.h was obtained at 48 h fermentation time.

FOS yield and productivity obtained in fermentations run in bioreactor represent an important improvement as compared to results from shake flask experiments (0.55 $g_{FOS}/g_{initial\ Sucrose}$ and 1.69 ± 0.01 g/L.h, respectively). The increase of the fermentation productivity has an important impact not only in the efficiency of the bioprocess, but also in its global cost, since a small period of time is necessary to produce the same amount of product. The high productivity obtained in bioreactor might be explained by the considerably different mixture patterns obtained in the bioreactor, due to the shape of the impellers and the bioreactor configuration itself, which means different levels of aeration. Also, the pH in the bioreactor is controlled during the fermentation. Pellets may also be damaged by the bioreactor impellers, contributing to a high biomass level, which produces more enzymes and, ultimately, more FOS (Nobre et al., 2018a).

The yield obtained in this work (0.65 $g_{FOS}/g_{initial\ Sucrose}$) was considerably higher than the maximum yield usually found for FOS production with purified (Nishizawa et al., 2001) and crude (Sangeetha et al., 2005) microorganism enzymes, in a two-step fermentation (0.55–0.60 $g_{FOS}/g_{initial\ Sucrose}$). For example, in a work conducted in a batch basket reactor using an adsorbed and partially purified β-fructofuranosidase from *Rhodotorula* sp., a FOS yield of 0.60 $g_{FOS}/g_{initial\ Sucrose}$ was obtained after 96 h fermentation (Detofol et al., 2015). The highest yield found in the present study represents an improvement that may have high impact at industrial level, since the lower yield of the two-step fermentation is the main drawback of the process.

In processes using one-step fermentation, where the whole-cell microorganisms are used instead of purified or crude enzymes, higher yield values have been reported such as: 0.58 $g_{FOS}/g_{initial\ Sucrose}$ for *P. expansum* (Prata et al., 2010), 0.61 $g_{FOS}/g_{initial\ Sucrose}$ for *A. japonicus* (Mussatto et al., 2009), 0.63 $g_{FOS}/g_{initial\ Sucrose}$ for *A. pullulans* (Dominguez et al., 2012; Nobre et al., 2016), and 0.64 $g_{FOS}/g_{initial\ Sucrose}$ for *A. ibericus* (Nobre et al., 2018a). The high yields of FOS production obtained are of interest from the purification point of view, since the prebiotic sugars are present in higher concentration in the final product, facilitating the separation process.

The degree of purity obtained in this work (61 ± 0% of FOS in total sugars) was considerably high as compared to previous works conducted in bioreactor with *A. pullulans* (54%) and *A. ibericus* (56%), under one-step FOS production, which might be an advantage, facilitating the further purification steps envisaging the use of the FOS mixtures in e.g., diabetics and dietetic products.

3.2. FOS purification

To purify the FOS produced in bioreactor it was used an activated charcoal fixed bed column. Fig. 3 illustrates the amount and purity of the FOS recovered within each eluted ethanol fraction.

A global amount of 36 g of FOS were recovered in the fractions between 10 and 100%, representing 62% (w/w) of the initial sugars fed into the activated charcoal column. In the fraction of 20% ethanol, 25 g of sugars were recovered with a purity of 92% (w/w), representing an increase of 31% (w/w) in FOS purity as compared to the untreated mixture. The aim of the purification in the present study was the recovering of a significant amount of FOS with high purity, for further use in prebiotic activity analysis.

In this study, fructose and glucose were the first sugars eluting from the column, followed by sucrose (Kuhn et al., 2014; Nobre et al., 2012). The affinity of small sugars by the activated charcoal is smaller than the higher molecular weight sugars, hence, monosaccharides and disaccharides are easily desorbed using water or lower concentrations of ethanol. On the other hand, to desorb oligosaccharides, higher ethanol concentrations are needed. Polymerized sugars hold more CH bonds in the structure, which are responsible for the hydrophobic character of the sugar, resulting in less affinity to water. Accordingly, the non-polar surfaces of FOS are capable of interacting with non-polar adsorbents, ensuring higher adsorption of FOS in activated charcoal (Boon et al., 2000; Nobre et al., 2012). In the present work, FOS with lower degree of polymerization (GF_2 and GF_3) were preferentially desorbed with 20% ethanol, while GF_4 was mainly desorbed with 50% ethanol.

The FOS mixture obtained with 20% ethanol (fraction that yielded higher amount of FOS) presented as *P. citreonigrum* URM 4459 FOS mixture, was used for chemical characterization (Section 3.3) and *in vitro* evaluation of the prebiotic effects (Section 3.4).

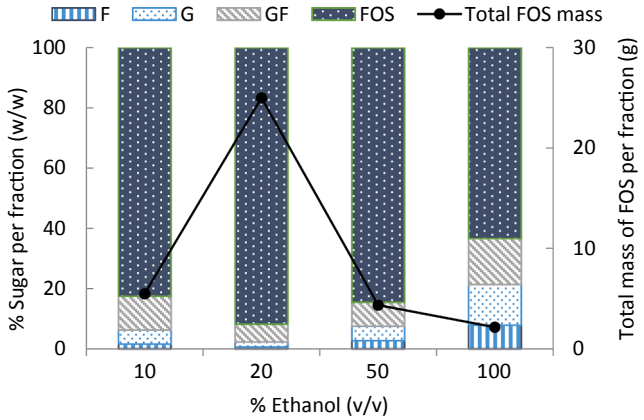


Fig. 3. Mass of total fructo-oligossacharides (FOS) recovered per fraction desorbed (—●—) and respective percentage of sugar (FOS; GF: sucrose; G: glucose; F: fructose) in each collected fraction.

Table 3
Carbohydrate content (mg/g) found for *Penicillium citreonigrum* URM 4459 FOS and Actilight®.

Assignment		Actilight®		FOS (*P. citreonigrum* URM 4459)	
		$\bar{X}$	RSD (%)	$\bar{X}$	RSD (%)
DP 1	Fructose	12.1	25	1.1	1
	Glucose	2.9	21	7.7	6
DP 2	Anhydrosugars	tr[c]		tr	
	Sucrose	46.8	2	108.8	13
	Inulobiose	1.9	11	1.4	15
	Blastose	0.4	14	1.7	11
DP 3	Anhydrosugars	tr			
	1-kestose	283.0	13	202.0	6
	NR[a]	15.5	12	80.1	4
	R[b]	3.0	8		
	R	1.8	5		
DP4	Nystose	480.4	7	469.0	17
	Other tetrasaccharides	55.8	1	128.0	19

[a] NR – non-reducing;[b]R – reducing; [c]traces (< 0.2%).

Table 4
Glycosidic linkage composition (mol%) present in the fructo-oligosaccharides (FOS) produced from *Penicillium citreonigrum* URM 4459 and Actilight®.

Glycosidic linkages		Fraction (mol%)	
		Actilight®	FOS (*P. citreonigrum* URM 4459)
t-Fru		13.6	28.7
1-Fru		24.4	21.2
1,6-Fru		0.2	–
	Total	38.2	49.9
t-Glc		51.6	29.2
4-Glc		0.7	0.2
6-Glc		8.9	20.1
2,3-Glc		–	tr[a]
3,4-Glc		–	tr
3,6-Glc		–	0.3
4,6-Glc		0.1	0.3
	Total	61.3	50.1
t-Gal		0.1	tr
	Total	0.1	tr
t-Man		0.3	–
2,6-Man		0.1	–
	Total	0.4	–

[a] Traces (< 0.05%).

3.3. Chemical characterization

3.3.1. Identification, quantification and glycosidic linkage analysis of FOS

The composition of the *P. citreonigrum* URM 4459 FOS mixture, determined by HPLC analysis was (g/L): Fructose (0.06); Glucose (1.15); Sucrose (6.67); Non-identified trisaccharide (14.93); 1-Kestose (35.77); Nystose (61.57); and Fructofuranosylnystose (10.77).

Table 3 shows the concentration of the mono- and oligosaccharides produced. The major FOS produced by *P. citreonigrum* were 1-kestose (202.0 mg/g) and nystose (469.0 mg/g), comparable to what was observed for Actilight® commercial FOS. Their amount was similar to the *A. ibericus* FOS produced mixture (Nobre et al., 2018c), whereas the inulin derived FOS (Raftilose® P95) contained only 31.7 mg/g of this GF_2 (Nobre et al., 2018c). Besides the detection of sucrose, used as substrate, the reducing disaccharides inulobiose and blastose were identified in *P. citreonigrum* produced FOS, as well as in Actilight® commercial FOS. Inulobiose was already observed as a result of *A. ibericus* fermentation, and was also detected in the commercial Raftilose® P95, with a minimum content of 3%, while blastose was only observed for fungi derived FOS, with an abundance of 1% (Nobre et al., 2018c).

Moreover, another non-reducing trisaccharide was identified with higher quantity for FOS produced by *P. citreonigrum* (80 mg/g) and in lower amount for Actilight® (15.5 mg/g). The presence of a non-identified trisaccharide was also quantified by HPLC with an abundance of 121 mg/g for *P. citreonigrum* FOS. Additionally, this non-reducing trisaccharide was also reported in the *A. ibericus* fermentation resultant mixture (18.8 mg/g), but was absent in Raftilose® P95 (Nobre et al., 2018c). The tentatively identification of this compound was supported on the glycosidic linkage results (Table 4), and three possible assignments were considered, neokestose [Fru(β2 → 6)Glc(α1 ↔ β2)Fru], [Fru(β2 → 6)Glc(α1 ↔ α1)Glc], and theanderose [Glc(α1 → 6)Glc (α1 ↔ β2)Fru]. As neokestose is already reported as a product of sucrose transformation by fungi species (Yasuda et al., 1986), it is highly probable to be the assignment of this trisaccharide, showing that *P. citreonigrum* should contain an invertase with fructosyltransferase activity (Goosen et al., 2007). This assignment is also supported by the absence of two reducing trisaccharides in *P. citreonigrum* FOS, while present in small amounts (< 1%) in Actilight®.

Concerning tetrasaccharides, their identification and quantification was carried out by GC-FID, which only allowed to identify compounds whose retention time was known by comparison with standards. Thus, only nystose was identified with certainty, and the remaining tetrasaccharides were quantified and grouped, as the methodology used does not allow further characterization. FOS mixture produced by fungi had more than twice the amount of these unidentified tetrasaccharides than the commercial mixture (Table 3).

The glycosidic linkage analysis (Table 4) is consistent with the oligosaccharides identified, with the residues found in higher proportions (*t*-Fru, *t*-Glc, and (2 → 1)-Fru) being suggestive of FOS with the inulin structure, such as nystose (GF_3) and 1-kestose (GF_2), which appeared as the major constituents (Table 3). In addition, the proportion of *t*-Glc and (2 → 1)-Fru being almost 1:1 in FOS produced by fungi and 2:1 in Actilight®, support that both mixtures have a FOS-inulin type with a low degree of polymerization (DP), having predominantly DP3 and DP4.

Also, (1 → 6)-Glc, which is present in neokestose (GF_2), was found in FOS produced by *P. citreonigrum* URM 4459 in higher quantity when compared with Actilight®. The non-reducing trisaccharide was also found in higher proportion in *P. citreonigrum* FOS, which further supports the previously assignment proposed, namely neokestose. In fact, the production of neokestose by *P. citrinum* (Hayashi et al., 2000) and *P. oxalicum* GXU20 (Xu et al., 2015) was also reported by other authors.

Aside from the identified di-, tri-, and tetrasaccharides, other components were quantified but in very small amounts. They have a retention time inferior to that of sucrose, and their mass spectra (MS) have the same fragmentation ions of sucrose (namely ions at *m/z* 169, 109, 211, and 331), differing on their intensity (data not shown). Additionally, the ion at *m/z* 229 is also present in these MS peaks, and is indicative of anhydrosugars (Šimkovic et al., 2003), as it corresponds to the mass of a triacetylated anhydrohexose. Therefore, it can be suggested that these structures are anhydrosugars. It is also possible that some of these compounds have in their composition galactose and mannose, as residues of these monosaccharides were found by glycosidic linkage analysis, but in small or trace amounts (Table 4). A compound with the same fragmentation pattern was identified for Actilight®, with a retention time inferior to 1-kestose. Thus, it can be an anhydrotrisaccharide where the hexose of the reducing end is dehydrated. In fact, it is reported that 1,5-anhydro-D-fructose is produced with α-1,4-glucan lyase (EC 4.2.2.13) on glycogen, starch and maltooligosaccharides (Fiskesund et al., 2010). It is possible that these anhydrosugars have origin on the thermal treatment required to sterilize the medium.

The sugar composition and glycosidic linkage type can impact on the prebiotic functionality of the carbohydrate, considering that there is a strong link between the oligosaccharide chemical structure and its

potential bioactivities (García-Cayuela et al., 2014).

3.4. In vitro evaluation of the prebiotic effects

3.4.1. Gastrointestinal digestion of FOS

Although oligosaccharides are known to be resistant to hydrolysis by human digestive enzymes, once they are specific for α-glycosidic linkages (Roberfroid, 2000), the low pH conditions associated to the digestive system may affect their hydrolysis resistance.

After purification, the FOS mixture produced by the *P. citreonigrum* URM 4459 was exposed to the harsh conditions of the digestive system namely oral, gastric, and small intestinal phases. The hydrolytic resistance of the produced mixture was also compared with a commercial FOS mixture, obtained also from a microbial source – the Actilight® mixture. Results achieved are shown in Table 5. Only results with a degree of hydrolysis higher than 2.0% were considered statistically significant ($p < 0.05$).

According to the prebiotic criteria, the sugar resistance to the salivary fluid is a pre-requisite for a prebiotic candidate so that it can reach the caeco-colon for further use by the probiotic bacteria (Wang et al., 2015). None of the oligosaccharides studied were hydrolyzed either in the oral, either in the gastric phases.

Once the FOS mixture had been through the simulated gastric phase of digestion it was transferred to a simulation of the digestion that occurs in the small intestine (Minekus et al., 2014). In opposite to the previous phases, 1-kestose (GF_2) showed lower resistance to the hydrolysis during intestinal digestion. 1-Kestose from both Actilight® mixture and from the mixture produced by the *P. citreonigrum* URM 4459 was hydrolyzed up to an amount of 4.9% and 3.5%, respectively. The other trisaccharide present only in the mixture produced by the *P. citreonigrum* URM 4459, probably neokestose, was also reduced in 7.3% from its initial amount.

Both nystose (GF_3) and fructo-furanosylnystose (GF_4) showed more resistance to the hydrolysis and were not significantly digested in any of the gastrointestinal phases, which can be attributed to their higher degree of polymerization.

According to the bibliography, in a similar study, using a FOS mixture produced by *A. ibericus* and other from *A. pullulans*, 1-kestose is resistant to the small intestine conditions, although nystose was reported to be slightly hydrolysed during this phase (5.5% and 4.3% for *A. ibericus* and *A. pullulans*, respectively) (Nobre et al., 2018c).

In spite of the slight decrease of 1-kestose and the other trisaccharide (possibly neokestose) contained in the produced FOS mixture, during the small intestine phase, the hydrolysis rates obtained were relatively low, thus, more than 90% of FOS remained undigested. These results suggest that the FOS mixture from *P. citreonigrum* URM 4459 is stable at low pH, and is able to reach the colon with unchanged structure, for further stimulate the growth of probiotic bacteria.

3.4.2. Fermentability of FOS by probiotic bacteria

FOS are excellent substrates for the colonic probiotic bacteria, since they are neither absorbed in the upper gastrointestinal tract nor hydrolyzed by human enzymes, due to their chemical structure configuration, reaching the colon almost intact (Gibson and Roberfroid, 1995). The probiotic bacteria produce specific β-fructofuranosidase enzymes that are responsible for the hydrolysis of the FOS fructan chains in the terminal position (β2 → 1) (Karimi et al., 2015). FOS are therefore selectively fermented by the probiotic strains, promoting their growth and/or activity, and thus improving the host health.

The growth stimulation of ten *Lactobacillus* and *Bifidobacterium* strains by the FOS produced by *P. citreonigrum* URM 4459 was herein evaluated to determine their capacity to be used as prebiotic compounds. Bacteria growth profile was obtained for a negative control (no added sugar), positive control (glucose), two commercial sugars – Raftilose® (derived from inulin) and Actilight® (microbial-FOS), and for the *P. citreonigrum* URM 4459 FOS mixture (Table 6). After 36 h fermentation, all *Lactobacillus* and *Bifidobacterium* strains were able to grow independently on the tested carbon source, except in the negative control. Overall, *Bifidobacterium* strains grew more than *Lactobacillus* ones. As expected, glucose was the preferential carbon source, obtaining the highest cellular growth: 2.17 ± 0.00 for *Lactobacillus* (*L. rhamnosus* ATCC 7469) and 2.71 ± 0.02 for the *Bifidobacterium* (*B. bifidum* CECT 870). An exception was the *L. acidophilus* CECT 903 that grew more in the FOS mixture from *P. citreonigrum* URM 4459 and Actilight® than in glucose, and both *L. paracasei* strains, that grew similarly in all carbon sources studied.

Higher cell density was found for bacteria growing in FOS mixture from *P. citreonigrum* URM 4459 and Actilight® rather than in Raftilose® P95. Similar cell density was obtained for FOS mixture from *P. citreonigrum* URM 4459 and Actilight®. The similar results obtained might be explained due to the similar source of both mixtures, since Actilight® is also a microbial-derived FOS. The two mixtures present similar monomeric composition, polymerization degree and type of glycosidic linkages. The main difference is the presence of the trisaccharide, possibly assigned as neokestose, in higher quantity in the *P. citreonigrum* URM 4459 FOS sample. On the other hand, Raftilose® P95 is obtained by partial enzymatic hydrolysis of inulin, yielding inulin-type saccharides (Nobre et al., 2018c). *L. delbrueckii lactis* ATCC 12,375 was not able to ferment Raftilose® P95, reaching only 0.47 ± 0.00 maximum optical density. Lower cell density was also observed for *Lactobacillus gasseri* CECT 5714 and *Lactobacillus fermentum* CECT 5716 grown in Raftilose® P95 as compared to Actilight® 950P (Bañuelos et al., 2008). Likewise, Nobre et al. (2018c) observed a higher grow with the FOS synthesized by microorganisms rather than in Raftilose® P95, for all studied strains.

The rate at which a microorganism grow on a particular carbon source influences its ability to compete with other bacteria in the colon. In general, the *Lactobacillus* and *Bifidobacterium* strains grew faster or similarly in the FOS mixture from *P. citreonigrum* URM 4459 rather than Raftilose® P95, except for *L. rhamnosus* (Table 6). Four of the studied bacteria strains (*L. delbrueckii delbruecki* ATCC 9649, *L. delbrueckii lactis* ATCC 12375, *L. plantarum* NCIMB 8827 and *B. adolescentis* CECT 5781) grew faster in the FOS mixture from *P. citreonigrum* URM 4459 than in Actilight®, while all the other strains presented similar grow rates on both carbon sources. *B. adolescentis* CECT 5781 and *L. plantarum* NCIMB 8827 grew faster in the FOS mixture from *P. citreonigrum* URM 4459 rather than in all other carbon sources tested, including glucose (the positive control).

Table 5
Hydrolysis effect of the gastrointestinal simulation on the microbial-fructo-oligosaccharides. The values are expressed as rate of hydrolysis at different phases of the digestion.

Digestion phase	Actlight®			FOS (*Penicillium citreonigrum* URM 4459)			
	GF_2	GF_3	GF_4	GF_2	Tri	GF_3	GF_4
Mouth	< 2.0%	< 2.0%	< 2.0%	< 2.0%	< 2.0%	< 2.0%	< 2.0%
Stomach	< 2.0%	< 2 .0%	< 2.0%	< 2.0%	< 2.0%	< 2.0%	< 2.0%
Small Intestine	4.9%	< 2.0%	< 2.0%	3.5%	7.3%	< 2.0%	< 2.0%

FOS: Fructo-oligosaccharides; GF_2: 1-Kestose; Tri: Non-identified trisaccharide; GF_3: Nystose; GF_4: Fructofuranosylnystose.

Table 6
Maximum specific growth rates (μmax (h^{-1})) and maximum optical density (ODmax 600 nm) for *Lactobacillus* and *Bifidobacterium* strains grown in various carbon sources.

Strains		Glucose	FOS (*P. citreonigrum*)	Actilight®	Raftilose® P95
L. acidophilus CECT 903	μmax	0.43 ± 0.01	0.42 ± 0.00	0.41 ± 0.04	0.30 ± 0.01
	ODmax	1.58 ± 0.01	1.64 ± 0.01	1.68 ± 0.02	0.87 ± 0.00
L. plantarum NCIMB 8827	μmax	0.62 ± 0.01	0.69 ± 0.01	0.63 ± 0.01	0.47 ± 0.00
	ODmax	2.05 ± 0.00	1.75 ± 0.03	1.86 ± 0.02	0.91 ± 0.01
L. rhamnosus ATCC 7469	μmax	0.56 ± 0.05	0.35 ± 0.00	0.34 ± 0.00	0.58 ± 0.01
	ODmax	2.17 ± 0.01	1.84 ± 0.02	1.94 ± 0.02	1.03 ± 0.01
L. delbrueckii delbrueckii ATCC 9649	μmax	0.75 ± 0.01	0.47 ± 0.03	0.36 ± 0.01	0.48 ± 0.03
	ODmax	1.86 ± 0.03	1.65 ± 0.08	1.77 ± 0.05	0.82 ± 0.20
L. delbrueckii lactis ATCC 12375	μmax	0.77 ± 0.00	0.65 ± 0.06	0.55 ± 0.01	0.40 ± 0.00
	ODmax	2.11 ± 0.00	1.85 ± 0.05	1.83 ± 0.00	0.47 ± 0.00
L. bucheneri ATCC 4005	μmax	0.89 ± 0.00	0.89 ± 0.02	0.92 ± 0.10	0.79 ± 0.01
	ODmax	1.96 ± 0.01	1.52 ± 0.01	1.57 ± 0.02	1.28 ± 0.12
L. paracasei CECT 277	μmax	0.51 ± 0.00	0.49 ± 0.01	0.52 ± 0.00	0.48 ± 0.00
	ODmax	2.07 ± 0.01	2.07 ± 0.01	2.06 ± 0.02	2.04 ± 0.00
L. paracasei paracasei CCUG 27320	μmax	0.53 ± 0.00	0.54 ± 0.00	0.57 ± 0.02	0.53 ± 0.00
	ODmax	2.10 ± 0.00	2.10 ± 0.00	2.08 ± 0.00	2.07 ± 0.00
B. bifidum CECT 870	μmax	0.67 ± 0.12	0.31 ± 0.03	0.33 ± 0.05	0.25 ± 0.09
	ODmax	2.71 ± 0.02	2.27 ± 0.00	2.27 ± 0.02	2.01 ± 0.02
B. adolescentis CECT 5781	μmax	0.44 ± 0.09	0.50 ± 0.02	0.45 ± 0.03	0.44 ± 0.00
	ODmax	2.61 ± 0.01	2.29 ± 0.00	2.31 ± 0.00	2.03 ± 0.00

Results correspond to the average ± standard deviation of triplicated independent experiments.

Neokestose has been shown to improve the population of bifidobacteria and lactobacilli as compared to other commercial FOS as well as to inhibit *Clostridia* and the development of cancer cells (Miranda-Molina et al., 2017). Therefore, the high amount of the neokestose quantified in the FOS produced by *P. citreonigrum* could justify the higher prebiotic activity of this mixture. In a similar study, a FOS mixture of *A. ibericus,* containing neokestose sugar, obtained also higher prebiotic activity as compared to other FOS mixtures.

FOS mixture produced from *P. citreonigrum* URM 4459 revealed high potential as prebiotic-like sugars and are good candidates for inclusion in symbiotic food.

4. Conclusions

Results gathered in this study highlight the possibility of using a new isolated fungus – *P. citreonigrum* URM 4459 – as a prebiotic producer at a large scale. High production yields and purity of FOS were obtained in a one-step fermentation.

It was possible to assign a high amount of neokestose in the FOS produced mixtures, which may justify the greater growth of probiotic bacteria with the produced FOS, as compared to other commercial FOS mixtures. Produced FOS showed also resistance to the hydrolysis under gastrointestinal simulated conditions.

Acknowledgments

The authors acknowledge the financial support from the CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brasília, Brazil) the approved project in network Renorbio – 140366/2014-8, Facepe (Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco, Recife, Brasil, AMD-0273-2.00/14) and Capes (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brasília, Brazil) for the 88881.119817/2016-01 funding. This study was also supported by the Portuguese Foundation for Science and Technology (FCT) under the scope of the strategic funding of UID/BIO/04469/2019 unit and BioTecNorte operation (NORTE-01-0145-FEDER-000004) funded by the European Regional Development Fund under the scope of Norte2020 – Programa Operacional Regional do Norte also, Project ColOsH 02/SAICT/2017 (POCI-01-0145-FEDER-030071). The authors acknowledge also University of Aveiro and FCT/MCT for the financial support for the QOPNA research Unit (FCT UID/QUI/00062/2019) through national founds and, where applicable, co-financed by the FEDER, within the PT2020 Partnership Agreement. Elisabete Coelho and Soraia P. Silva acknowledge FCT for the postdoctoral grant SFRH/BPD/70589/2010 and PhD grant SFRH/BD/136471/2018, respectively. This work was also funded by national funds (OE), through FCT, in the scope of the framework contract foreseen in the numbers 4, 5 and 6 of the article 23, of the Decree-Law 57/2016, of August 29, changed by Law 57/2017, of July 19.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2019.03.053.

References

Arumugam, G.K., Selvaraj, V., Gopal, D., Ramalingam, K., 2014. Solid-state fermentation of agricultural residues for the production of antibiotics. In: Biotransformation of Waste Biomass into High Value Biochemicals. Springer, New York, New York, NY, pp. 139–162.

Bali, V., Panesar, P.S., Bera, M.B., Panesar, R., 2015. Fructo-oligosaccharides: production, purification and potential applications. Crit. Rev. Food Sci. Nutr. 55, 1475–1490.

Bañuelos, O., Fernández, L., Corral, J.M., Valdivieso-Ugarte, M., Adrio, J.L., Velasco, J., 2008. Metabolism of prebiotic products containing β(2–1) fructan mixtures by two Lactobacillus strains. Anaerobe 14, 184–189.

Boon, M.A., van't Riet, K., Janssen, A.E.M., 2000. Enzymatic synthesis of oligosaccharides: product removal during a kinetically controlled reaction. Biotechnol. Bioeng. 70, 411–420.

Castro, C.C., Nobre, C., Duprez, M.-E., De Weireld, G., Hantson, A.-L., 2017. Screening and selection of potential carriers to immobilize *Aureobasidium pullulans* cells for fructo-oligosaccharides production. Biochem. Eng. J. 118, 82–90.

Castro, C.C., Nobre, C., De Weireld, G., Hantson, A.-L., 2019. Microbial co-culturing strategies for fructo-oligosaccharide production. N. Biotechnol. 51, 1–7.

Chung, W.S.F., Walker, A.W., Louis, P., Parkhill, J., Vermeiren, J., Bosscher, D., Duncan, S.H., Flint, H.J., 2016. Modulation of the human gut microbiota by dietary fibres occurs at the species level. BMC Biol. 14, 3.

Cruz, R., Cruz, V.D., Belini, M.Z., Belote, J.G., Vieira, C.R., 1998. Production of fructo oligosaccharides by the mycelia of *Aspergillus japonicus* immobilized in calcium alginate. Bioresour. Technol. 65, 139–143.

Cummings, J.H., Macfarlane, G.T., Englyst, H.N., 2001. Prebiotic digestion and fermentation. Am. J. Clin. Nutr. 73, 415–420.

da Silva, J.B., Fai, A.E.C., dos Santos, R., Basso, L.C., Pastore, G.M., 2011. Parameters evaluation of fructooligosaccharides production by sucrose biotransformation using an osmophilic *Aureobasium pullulans* strain. Procedia Food Sci. 1, 1547–1552.

Detofol, M.R., Aguiar-Oliveira, E., Bustamante-Vargas, C.E., Soares, A.B. de J., Soares, M.B.A., Maugeri, F., 2015. Modeling and simulation of fructooligosaccharides synthesis in a batch basket reactor. J. Biotechnol. 210, 44–51.

Dominguez, A., Nobre, C., Rodrigues, L.R., Peres, A.M., Torres, D., Rocha, I., Lima, N., Teixeira, J., 2012. New improved method for fructooligosaccharides production by

Aureobasidium pullulans. Carbohydr. Polym. 89, 1174–1179.
Fiskesund, R., Abeyama, K., Yoshinaga, K., Abe, J., Yuan, Y., Yu, S., 2010. 1,5-Anhydro-D-fructose and its derivatives: biosynthesis, preparation and potential medical applications. Planta Med. 76, 1635–1641.
Ganaie, M.A., Lateef, A., Gupta, U.S., 2014. Enzymatic trends of fructooligosaccharides production by microorganisms. Appl. Biochem. Biotechnol. 172, 2143–2159.
García-Cayuela, T., Díez-Municio, M., Herrero, M., Martínez-Cuesta, M.C., Peláez, C., Requena, T., Moreno, F.J., 2014. Selective fermentation of potential prebiotic lactose-derived oligosaccharides by probiotic bacteria. Int. Dairy J. 38, 11–15.
Gibson, G.R., Roberfroid, M.B., 1995. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. J. Nutr. 125, 1401–1412.
Goosen, C., Yuan, X., van Munster, J.M., Ram, A.F.J., van der Maarel, M.J.E.C., Dijkhuizen, L., 2007. Molecular and biochemical characterization of a novel intracellular invertase from *Aspergillus niger* with transfructosylating activity. Eukaryot. Cell 674–681.
Hayashi, S., Yoshiyama, T., Fujii, N., Shinohara, S., 2000. Production of a novel syrup containing neofructo-oligosaccharides by the cells of *Penicillium citrinum*. Biotechnol. Lett. 22, 1465–1469.
Huebner, J., Wehling, R.L., Hutkins, R.W., 2007. Functional activity of commercial prebiotics. Int. Dairy J. 17, 770–775.
Karimi, R., Azizi, M.H., Ghasemlou, M., Vaziri, M., 2015. Application of inulin in cheese as prebiotic, fat replacer and texturizer: a review. Carbohydr. Polym. 119, 85–100.
Kuhn, R.C., Mazutti, M.A., Albertini, L.B., Filho, F.M., 2014. Evaluation of fructooligosaccharides separation using a fixed-bed column packed with activated charcoal. N. Biotechnol. 31, 237–241.
Mano, M.C.R., Neri-Numa, I.A., da Silva, J.B., Paulino, B.N., Pessoa, M.G., Pastore, G.M., 2018. Oligosaccharide biotechnology: an approach of prebiotic revolution on the industry. Appl. Microbiol. Biotechnol. 102, 17–37.
Markowiak, P., Śliżewska, K., 2017. Effects of probiotics, prebiotics, and synbiotics on human health. Nutrients 9, 1021.
Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., Carrière, F., Boutrou, R., Corredig, M., Dupont, D., Dufour, C., Egger, L., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., Lesmes, U., Macierzanka, A., Mackie, A., Marze, S., McClements, D.J., Ménard, O., Recio, I., Santos, C.N., Singh, R.P., Vegarud, G.E., Wickham, M.S.J., Weitschies, W., Brodkorb, A., 2014. A standardised static in vitro digestion method suitable for food – an international consensus. Food Funct. 5, 1113–1124.
Miranda-Molina, A., Castillo, E., Lopez Munguia, A., 2017. A novel two-step enzymatic synthesis of blastose, a β-d-fructofuranosyl-(2↔6)-d-glucopyranose sucrose analogue. Food Chem. 227, 202–210.
Mussatto, S.I., Aguilar, C.N., Rodrigues, L.R., Teixeira, J.A., 2009. Fructooligosaccharides and β-fructofuranosidase production by *Aspergillus japonicus* immobilized on lignocellulosic materials. J. Mol. Catal. B Enzym. 59, 76–81.
Mussatto, S.I., Ballesteros, L.F., Martins, S., Maltos, D.A.F., Aguilar, C.N., Teixeira, J.A., 2013. Maximization of fructooligosaccharides and β-fructofuranosidase production by *Aspergillus japonicus* under solid-state fermentation conditions. Food Bioprocess Technol. 6, 2128–2134.
Nascimento, A.K.C., Nobre, C., Cavalcanti, M.T.H., Teixeira, J.A., Porto, A.L.F., 2016. Screening of fungi from the genus *Penicillium* for production of β- fructofuranosidase and enzymatic synthesis of fructooligosaccharides. J. Mol. Catal. B Enzym. 134, 70–78.
Nishizawa, K., Nakajima, M., Nabetani, H., 2001. Kinetic study on transfructosylation by β-fructofuranosidase from *Aspergillus niger* ATCC 20611 and availability of a membrane reactor for fructooligosaccharide production. Food Sci. Technol. Res. 7, 39–44.
Nobre, C., Santos, M.J., Dominguez, A., Torres, D., Rocha, O., Peres, A.M., Rocha, I., Ferreira, E.C., Teixeira, J.A., Rodrigues, L.R., 2009. Comparison of adsorption equilibrium of fructose, glucose and sucrose on potassium gel-type and macroporous sodium ion-exchange resins. Anal. Chim. Acta 654, 71–76.
Nobre, C., Teixeira, J.A., Rodrigues, L.R., 2012. Fructo-oligosaccharides purification from a fermentative broth using an activated charcoal column. N. Biotechnol. 29, 395–401.
Nobre, C., Cerqueira, M.Â., Rodrigues, L.R., Vicente, A.A., Teixeira, J.A., 2015. Production and extraction of polysaccharides and oligosaccharides and their use as New Food Additives. Chapter 19 In: Industrial Biorefineries & White Biotechnology. Elsevier B.V., pp. 653–679.
Nobre, C., Castro, C.C., Hantson, A.-L., Teixeira, J.A., De Weireld, G., Rodrigues, L.R., 2016. Strategies for the production of high-content fructo-oligosaccharides through the removal of small saccharides by co-culture or successive fermentation with yeast. Carbohydr. Polym. 136, 274–281.
Nobre, C., Alves Filho, E.G., Fernandes, F.A.N., Brito, E.S., Rodrigues, S., Teixeira, J.A., Rodrigues, L.R., 2018a. Production of fructo-oligosaccharides by *Aspergillus ibericus* and their chemical characterization. LWT 89, 58–64.
Nobre, C., Gonçalves, D.A., Teixeira, J.A., Rodrigues, L.R., 2018b. One-step co-culture fermentation strategy to produce high-content fructo-oligosaccharides. Carbohydr. Polym. 201, 31–38.
Nobre, C., Sousa, S.C., Silva, S.P., Pinheiro, A.C., Coelho, E., Vicente, A.A., Gomes, A.M.P., Coimbra, M.A., Teixeira, J.A., Rodrigues, L.R., 2018c. *In vitro* digestibility and fermentability of fructo-oligosaccharides produced by *Aspergillus ibericus*. J. Funct. Foods 46, 278–287.
Prata, M.B., Mussatto, S.I., Rodrigues, L.R., Teixeira, J.A., 2010. Fructooligosaccharide production by *Penicillium expansum*. Biotechnol. Lett. 32, 837–840.
Research and Markets, 2017. Global prebiotic ingredients market analysis & trends - Industry forecast to 2025. http://www.researchandmarkets.com/research/bcrhhf/global_ prebiotic. Accessed 16 Jan 2019.
Ribeiro, T., Cardoso, V., Ferreira, L.M.A., Lordelo, M.M.S., Coelho, E., Moreira, A.S.P., Domingues, M.R.M., Coimbra, M.A., Bedford, M.R., Fontes, C.M.G.A., 2018. Xylo-oligosaccharides display a prebiotic activity when used to supplement wheat or corn-based diets for broilers. Poult. Sci. 97, 4330–4341.
Roberfroid, M.B., 2000. Chicory fructooligosaccharides and the gastrointestinal tract. Nutrition 16, 677–679.
Sangeetha, P.T., Ramesh, M.N., Prapulla, S.G., 2005. Maximization of fructooligosaccharide production by two stage continuous process and its scale up. J. Food Eng. 68, 57–64.
Shin, H.T., Baig, S.Y., Lee, S.W., Suh, D.S., Kwon, S.T., Lim, Y.B., Lee, J.H., 2004. Production of fructo-oligosaccharides from molasses by *Aureobasidium pullulans* cells. Bioresour. Technol. 93, 59–62.
Šimkovic, I., Šurina, I., Vričan, M., 2003. Primary reactions of sucrose thermal degradation. J. Anal. Appl. Pyrolysis 70, 493–504.
Singh, R.S., Singh, T., Larroche, C., 2019. Biotechnological applications of inulin-rich feedstocks. Bioresour. Technol. 273, 641–653.
Tashiro, Y., Ueno, H., Takaba, M., Hayashi, S., 2017. Production of functional inulin-type fructooligosaccharides by an Enzyme from *Penicillium citrinum*. Curr. Microbiol. 74, 1114–1117.
Wang, X., Huang, M., Yang, F., Sun, H., Zhou, X., Guo, Y., Wang, X., Zhang, M., 2015. Rapeseed polysaccharides as prebiotics on growth and acidifying activity of probiotics in vitro. Carbohydr. Polym. 125, 232–240.
Xu, Q., Zheng, X., Huang, M., Wu, M., Yan, Y., Pan, J., Yang, Q., Duan, C.-J., Liu, J.-L., Feng, J.-X., 2015. Purification and biochemical characterization of a novel β-fructofuranosidase from *Penicillium oxalicum* with transfructosylating activity producing neokestose. Process Biochem. 50, 1237–1246.
Yasuda, H., Shitoh, T., Yamano, T., Itoh, Y., Shimura, S., 1986. Identification of neokestose produced from sucrose by an enzyme of *Penicillium oxalicum*. Agric. Biol. Chem. 50, 777–778.
Younis, K., Ahmad, S., Jahan, K., 2015. Health benefits and application of prebiotics in foods. J. Food Process. Technol. 06, 1–7.
Zambelli, P., Fernandez-Arrojo, L., Romano, D., Santos-Moriano, P., Gimeno-Perez, M., Poveda, A., Gandolfi, R., Fernández-Lobato, M., Molinari, F., Plou, F.J., 2014. Production of fructooligosaccharides by mycelium-bound transfructosylation activity present in *Cladosporium cladosporioides* and *Penicilium sizovae*. Process Biochem. 49, 2174–2180.