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Production of fructo-oligosaccharides by *Aspergillus ibericus* and their chemical characterization

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ABSTRACT

A great demand for prebiotics is driving the search for new sources of fructo-oligosaccharides (FOS) producers and for FOS with differentiated functionalities. In the present work, FOS production by a new isolated strain of *Aspergillus ibericus* was evaluated. The temperature of fermentation and initial pH were optimized in shaken flask to yield a maximal FOS production, through a central composite experimental design. FOS were produced in a one-step bioprocess using the whole cells of the microorganism. The model ($R^2 = 0.918$) predicted a yield of 0.56, experimentally 0.53 ± 0.03 $g_{FOS}.g_{initial\ sucrose}^{-1}$ was obtained (37.0 °C and a pH of 6.2). A yield of 0.64 ± 0.02 $g_{FOS}.g_{initial\ sucrose}^{-1}$ was obtained in the bioreactor, at 38 h, with a content of 118 ± 4 $g.L^{-1}$ in FOS and a purity of 56 ± 3%. The chemical structure of the FOS produced by *A. ibericus* was determined by HPLC and NMR. FOS were identified as 1-kestose, nystose, and 1^F-fructofuranosylnystose. In conclusion, *A. ibericus* was found to be a good alternative FOS producer.

1. Introduction

Several aspects of human health are influenced by the microbial communities colonizing the different regions of the human gut. Via fermentation of non-digestible carbohydrates, e.g. oligosaccharides, the gut microbiota contributes with energy and nutrient supply to the host (Flint, Scott, Louis, & Duncan, 2012). The daily intake of specific oligosaccharides, namely fructo-oligosaccharides (FOS), has proved to be effective in the manipulation of the composition and functionality of the colonic microbiota (Scheid, Moreno, Maróstica Junior, & Pastore, 2013; Rastall et al., 2005). FOS consumption by the probiotic bacteria results in (a) an increase of the expression or change in the composition of short-chain fatty acids; (b) an increased fecal weight; (c) a mild decrease in luminal colon pH; (d) a decrease in nitrogenous end-products; (e) an increased expression of the binding proteins or active carriers associated with mineral absorption, and immune system regulation (Younis, Ahmad, & Jahan, 2015). These changes are reflected in a great number of health benefits for the humans such as reducing or preventing gastroenteritis, inflammatory bowel disease, colon cancer, allergies, obesity, cardiovascular disease, osteoporosis, among others (Sabater-Molina, Larqué, Torrella, & Zamora, 2009; Slavin, 2013; Wang, 2009). Therefore, FOS are one of the most commonly commercialized prebiotics (Nobre, Cerqueira, Rodrigues, Vicente, & Teixeira, 2015).

FOS are produced from the transfructosylation of sucrose by enzymes contained in a number of microorganisms. Fungi are the most studied microorganisms for FOS production, particularly *Aureobasidium pullulans* (a yeast-like fungus), *Aspergillus* sp. and *Penicillium* sp. Detailed information on microorganisms with transfructosylating activity that are able to produce FOS can be found in recent reviews (Bali, Panesar, Bera, & Panesar, 2015; Dominguez, Rodrigues, Lima, & Teixeira, 2013; Ganaie, Lateef, & Gupta, 2014).

The main drawback in the production of FOS using microbial enzymes is the low yields achieved between 0.55 and 0.60 $g_{FOS}.g_{initial\ sucrose}^{-1}$ (Nishizawa, Nakajima, & Nabetani, 2001; Sangeetha, Ramesh, & Prapulla, 2005). Fructosyltransferase (FTase) enzymes are inhibited by glucose, which is the main product released in the fermentative broth during the FOS synthesis. Moreover, the FOS formed are simultaneously hydrolyzed back to the single monomer forms by the action of the same enzymes. Therefore, many attempts have been done

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to increase the production yields of FOS in fermentative broths such as the use of immobilized enzymes and treatment with pressurized fluids (Castro, Nobre, Duprez, De Weireld, & Hantson, 2017; Silva et al., 2013), microbial treatments or mixed enzymes (Nobre et al., 2016; Nobre, Teixeira, & Rodrigues, 2015). One-step fermentation, where the whole cells of *A. pullulans* instead of the isolated enzymes are used, has also shown to increase yields up to 0.63 $g_{FOS}.g_{initial\ sucrose}^{-1}$ (Dominguez et al., 2012; Nobre et al., 2016).

The great current demand for prebiotics, in particular FOS, requires a continuous search for new microorganisms capable of producing FTase with a good transfructosylation activity that can be further used to produce FOS. *Aspergillus ibericus* MUM 03.49 was isolated from Portuguese wine grapes (Serra et al., 2006). The strain exhibits potential for FOS formation since a pronounced positive colour reaction was obtained while screening the transfructosylation activity of the microorganism in plate tests (Dominguez, Santos, Teixeira, & Lima, 2006). Therefore, the aim of this work was to evaluate the production of FOS by the new isolated *A. ibericus* MUM 03.49 strain, by means of a one-step fermentation. Fermentation conditions, namely temperature and pH were optimized in shaken flasks using a central composite design and the process was further scaled-up to a 2 L bioreactor. Since this is the first report on FOS obtained by this strain, the sugar mixture obtained was also analyzed by HPLC and chemically characterized by NMR to identify the chemical structures and compare it with other commercially available mixtures, namely regarding the linkage between the sugar monomers that may have an important impact on the prebiotic functionality of the oligosaccharide (Li et al., 2015).

2. Materials and methods

2.1. Microorganisms and culture conditions

The fungus *Aspergillus ibericus* MUM 03.49 from *Micoteca da Universidade do Minho* (MUM) culture collection (Braga, Portugal) was used. The strain was revived on Czapeck Dox (Oxoid, UK) at 25 °C from a frozen glycerol stock and maintained on agar Petri plates with the same medium at 4 °C. Every month, the strain was sub-cultured. A concentrated spore suspension was prepared from a 7-day-old culture plate by scrapping the spores with a 0.1% (w/v) solution of Tween 80 (Panreac, AppliChem, Spain). Afterwards, the spore concentration of the suspension was adjusted to 9×10^6 spores.mL^{-1} using an improved *Neubauer* chamber.

2.2. Experimental design and data analysis

Temperature and pH conditions were optimized by experimental design. Parameters were selected according to preliminary studies where a broader range of temperature (20.9–49.1 °C) was investigated under pH 6.0 (Gomes, 2009). The influence of agitation was not evaluated at this stage since the main goal of the work was to scale-up the process to a bioreactor size, in which the agitation mode is significantly different from the one obtained in an orbital shaker. The influence of temperature and pH (both independent variables) on the FOS production yield (dependent variable) was assessed through a 2^2 full-factorial central composite design (CCD), with 3 central points. For the statistical analysis, the independent variables were coded according to Eq. (1), where each independent variable is represented by xi (coded value), Xi (real value), X0 (real value at the central point), and ΔXi (step change value):

$$x_i = (X_i - X_0) / \Delta X_i \tag{1}$$

The range and the levels of the independent variables studied are given in Table 1.

Experimental results were fitted with a second-order polynomial equation by multiple regression analysis. The quadratic mode for predicting the optimal point was expressed according to Eq. (2), where Y represents the response variable (FOS production yield), β0 is the interception coefficient, β_i are the regression coefficients, and X_1 and X_2 represent the independent variables (temperature and pH, respectively):

$$Y = \beta 0 + \beta_1 X_1 + \beta_2 X_2 + \beta_1 X_1^2 + \beta_1 \beta_2 X_1 X_2 + \beta_2 X_2^2 \tag{2}$$

Table 1
Experimental range and levels of the independent process variables according to the 2^2 full-factorial central composite design.

Independent variables	Symbol	Range and levels		
		−1	0	1
Temperature (°C)	X_1	25	30	35
pH	X_2	5.5	6	6.5

The Statistica 10.0 software (Statsoft, USA) was used for the experimental design 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 the *F*-test analysis of variance (ANOVA). The coefficients of the response surface were evaluated using the student *t*-test.

Data were compared using one-way ANOVA followed by a Tukey's multiple comparison test with 95% confidence level. Positive effects were considered significant for *p-values* lower than 0.05.

2.3. FOS production in shaken flasks

The experimental design runs were performed in 250 mL glass flasks containing 50 mL of the following fermentation medium: 200 g.L^{-1} sucrose, 5.0 g.L^{-1} $NaNO_3$, 4.0 g.L^{-1} KH_2PO_4, 0.5 g.L^{-1} KCl, 0.35 g.L^{-1} K_2SO_4, 0.5 g.L^{-1} $MgSO_4.7H_2O$ and 0.01 g.L^{-1} $FeSO_4.7H_2O$ (Nobre et al., 2016). Sucrose and $FeSO_4.7H_2O$ solutions were sterilized by filtration (0.2 μm) and the other salt solutions were autoclaved at 121 °C for 15 min. The fermentation medium was inoculated with 1 mL of the spore suspension solution (9×10^6 spores.mL^{-1}) and agitated at 150 rpm, in an orbital shaker (Certomat®R, B.Braun Biotech International GmbH, Germany), for 44 h.

Several samples were taken at different time points for further determination of sugar concentration. Different combinations of temperature and initial pH were tested according to the experimental design (Table 1). All chemicals used were of analytical grade.

2.4. FOS production in bioreactor

The inoculum was prepared in a 250 mL flask, containing 100 mL of fermentation medium (same composition as described in section 2.3 except for sucrose (100 g.L^{-1})). The fermentation medium was seeded with 1 mL of the spore suspension solution (9×10^6 spores.mL^{-1}) and grown for 3 days at 35 °C and 150 rpm. Fermentations were carried out in a 2 L bioreactor (Autoclavable Benchtop Fermenter Type R'ALF, Bioengineering AG, Wald, Switzerland) with a working volume of 1 L of medium with the same composition as described in section 2.3. Assays were conducted for 62 h (time point at which it can be assured that the maximal production of FOS has been reached), at 200 rpm under the optimized temperature and pH conditions obtained by the experimental design. Samples were collected during the fermentation for sugar concentration determination. Fermentations were carried out in triplicate.

2.5. Sugar analysis

The sugars concentration of the samples was determined by high-performance liquid chromatography (HPLC). A modular liquid chromatograph (Shimadzu) equipped with a Prevail Carbohydrate ES column (5 μm, 25 × 0.46 cm length x diameter) from Alltech, was used at 25 °C. Samples were eluted with a mixture of acetonitrile (HPLC Grade, Carlo Erba, France) and 0.04% ammonium hydroxide (HPLC Grade, Sigma-Aldrich, Germany) in water (70:30, v/v), at a flow rate of 1.0 $mL.min^{-1}$ (Nobre et al., 2009). Samples were detected with a Sedex 55 evaporative light scattering detector (ELSD) (Sedere, Alfortville, France) working with a drift tube temperature set at 50 °C and nitrogen gas as nebulizing gas, at a pressure of 3.5 bar. The chromatographic signal was recorded and further integrated using the software LabSolutions (Shimadzu).

FOS standards were acquired from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Sucrose and fructose standards were obtained from Merck Co. (Darmstadt, Germany) and glucose from VWR International (Belgium).

2.6. FOS mixtures

The chemical structure of FOS obtained from three different sources was analyzed by Nuclear Magnetic Resonance (NMR). The mixtures herein studied were: (IB) the FOS mixture produced in this work by *A. ibericus*; (AP) a FOS mixture produced by *A. pullulans* CCY 27-1-94 in our previous work (Nobre et al., 2016), identified as 1-kestose, 1-nystose and 1^F-fructofuranosylnystose (Vandáková, Platková, Antošová, Báleš, & Polakovič, 2004); and the commercially available Actilight mixture (Beghin Meiji, France) that is produced from sucrose through fructosyl-transferase of the *Aspergillus niger*. The chemical structure of each FOS was compared with the standard FOS from Wako, identified as 1-kestose (GF_2), 1-nystose (GF_3) and 1^F-fructofuranosylnystose (GF_4) (Neuss, Germany).

The FOS mixtures AP and IB were purified in an activated charcoal column, as described by Nobre, Teixeira, & Rodrigues, 2012. Fractions desorbed with 20% of ethanol were further used for the characterization tests. The AP mixture exhibited the following composition in FOS (% w/w): 33% of GF_2, 53% of GF_3 and 7% of GF_4. The IB mixture was composed by 39% of GF_2, 50% of GF_3 and 4% of GF_4.

Actilight is a food ingredient commercialized by Beghin Meiji (France), which is produced from sucrose through fructosyl-transferase from *Aspergillus niger*. The commercial mixture Actilight 950S (Beghin Meiji, France) comprises (% in dry matter): 4.7% fructose + glucose + sucrose, 37.1% GF_2, 47.7% GF_3, and 15.2% GF_4 (information obtained from the supplier).

2.7. NMR analysis

The NMR experiments were performed in an Agilent 600-MHz spectrometer equipped with a 5 mm (H-F/^{15}N-^{31}P) inverse detection One Probe™ with actively shielded Z-gradient. The ^{1}H NMR spectra were performed using the PRESAT pulse sequence for non-deuterated water suppression (δ 4.77). The data were acquired with the RF pulse (p1) calibrated and 8 scans, 65.536 of time domain points for a spectral window of 10 ppm, acquisition time of 5.0 s and a relaxation delay of 10.0 s. The spectra were calibrated externally to the TMSP-d_4 resonance (δ 0.0) and temperature controlled to 298 K. The ^{13}C NMR spectra were acquired 10k scans, 32.768 of time domain points for a spectra window of 250 ppm, acquisition time of 0.87 s and a relaxation delay of 1.0 s.

Two-dimensional (2D) NMR experiments were acquired using the standard spectrometer library pulse sequences. ^{1}H-^{1}H COSY experiments were obtained with spectral width of 18,028.1 Hz in both dimensions; 1442 × 200 data matrix; 16 scans per t1 increment and relaxation delay of 1.0 s. One-bond ^{1}H-^{13}C HSQC experiments were acquired with an evolution delay of 1.7 ms for an average $1J$(C,H) of 145 Hz; 1442 × 200 data matrix; 32 scans per t1 increment; spectral widths of 9615.4 Hz in f2 and 30,165.9 Hz in f1 and relaxation delay of 1.0 s. Long-range ^{1}H-^{13}C HMBC experiments were recorded with an evolution delay of 50.0 ms for ^{LR}J(C,H) of 10 Hz; 1442 × 200 data matrix; 64 scans per t1 increment; spectral widths of 9615.4 Hz in f2 and 30,165.9 Hz in f1 and relaxation delay of 1.0 s.

3. Result and discussion

3.1. Effect of the operating conditions on FOS production

A maximum temperature of 35 °C was established since higher values influence negatively the growth of the microorganism as found in a previous work (Gomes, 2009). The pH variation was around 6.0 as good FOS production by whole cells microorganisms were reported at this pH (Dominguez et al., 2012; Gomes, 2009; Nobre et al., 2016).

After identifying the variables affecting the FOS production yield, the experimental values were fitted to a second-order equation, obtained by multiple regression analysis.

The *F* test and ANOVA analysis were used as significance criteria for the fitted model. The model was considered statistically significant at 95% confidence level since the calculated *F* value (11.22) was higher than the listed one ($F_{5,5}$ = 5.05). The quality of the quadratic fit was analyzed based on the coefficient of determination R^2. The model explained 91.8% of the dependent variable's variability (R^2 = 0.918) with a good adjusted determination coefficient ($R^2_{adjsuted}$ = 0.836). Given the R^2 value, the prediction performance of the proposed model in the experimental region was found to be accurate.

The effect of temperature and pH on the production yield is provided in Table 3. Results showed that both temperature and pH present a statistically significant effect on the FOS production yield. The temperature is the parameter that most influences the yield, with an estimated effect of 7.046. The positive effect of the temperature means that an increase of the temperature level will lead to higher FOS production yields. On the other hand, the effects of the interaction between the studied variables were not significant at a 95% confidence level. Therefore, a simplified model is proposed by the elimination of the statistically insignificant terms. The coefficients determined for the model are given in Eq. (3), where X_1 and X_2 represent the coded levels for the initial temperature and pH, respectively:

$$\text{FOS yield production} = -582.53 + 2.67X_1 + 190.33X_2 - 16.06X_2^2 \quad (3)$$

The values predicted by the model are presented in Table 2 along with the values observed experimentally. A good agreement between the predicted values and the experimental ones was found. Therefore, the central composite design and regression analysis were effective in identifying the optimal pH and temperature conditions to maximize the

Table 2
Experimental runs using coded levels of Temperature (°C) (X_1) and pH (X_2) according to the 2^2 full factorial central composite design and yields of FOS production obtained under those conditions.

Runs	Independent variables		Yield (% (g_{FOS}·$g_{initial\ sucrose}^{-1}$))		
	X_1	X_2	Experimental	Predicted	Residues
1	−1	−1	42.80	44.16	1.37
2	−1	0	48.62	45.98	−2.63
3	−1	1	46.41	47.68	1.27
4	0	−1	47.14	46.25	−0.89
5	0	0	53.56	53.40	−0.16
6	0	0	53.56	54.67	1.10
7	0	0	53.56	54.21	0.65
8	0	1	51.95	51.25	−0.70
9	1	−1	48.64	48.17	−0.47
10	1	0	55.66	56.71	1.04
11	1	1	54.65	54.08	−0.57

Table 3
Statistical analysis of the full Central Composite Design with two factors used for the optimization of FOS production.

Variables and interactions	Estimated effects	Standard errors	*t*-value	*p*
X_1	7.046	1.415	4.980	0.004[a]
X_1^2	−2.840	2.177	−1.304	0.249
X_2	4.811	1.415	3.400	0.019[a]
X_2^2	−8.030	2.177	−3.688	0.014[a]
X_1X_2	1.200	1.733	0.692	0.520

X_1 = coded values of temperature; X_2 = coded values of pH.
[a] Significant influence at 95% confidence level.

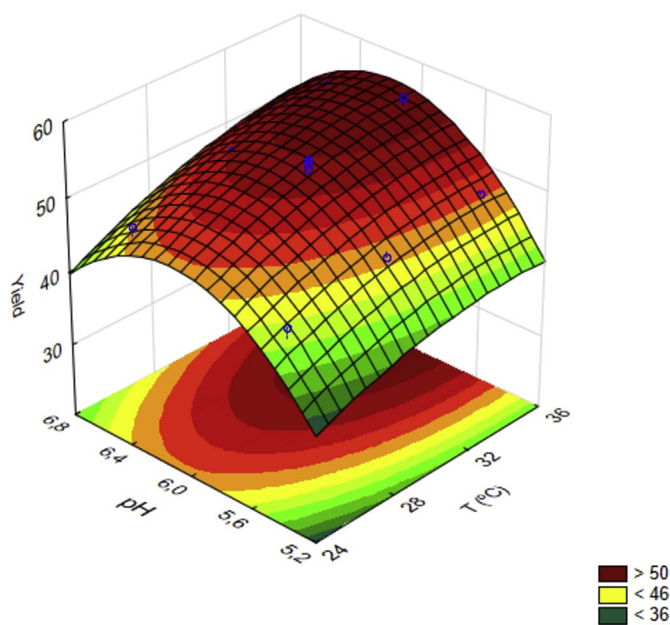

Fig. 1. Response surface of the full Central Composite Design for the optimization of the FOS production yield ($g_{FOS}.g_{initial\ sucrose}^{-1}$) as a function of the pH and the temperature (°C) conditions.

production of FOS.

The effect of the independent variables (pH and Temperature) on FOS production yield can be better visualized by examining the surface plot shown in Fig. 1.

The response surface (Fig. 1) clearly shows that the FOS production yield is favoured by the linear increase of temperature, while for pH, an optimum point was found after which higher pH values did not improve the FOS production yields.

An estimate of the optimum point revealed that a temperature of 37 °C and a working pH of 6.2 may lead to a maximum FOS production yield. Under these conditions the model predicted a FOS production yield of 0.56 $g_{FOS}.g_{initial\ sucrose}^{-1}$.

In order to validate the fitted model, four assays were performed under the estimated optimum operating conditions. A FOS production yield of 0.53 ± 0.03 $g_{FOS}.g_{initial\ sucrose}^{-1}$ was obtained, with a content of 101.1 ± 8.2 g.L^{-1} of FOS and a purity of 50.8 ± 0.9%. The FOS production yield values found experimentally are in good agreement with the predicted ones, again confirming the significance of the model.

3.2. FOS production in bioreactor

To scale-up the bioprocess, assays were performed in a bioreactor under the optimized operating conditions previously discussed. Fig. 2 shows the concentration profile of the sucrose consumed and the respective FOS formed. The maximum amount of FOS formed (Total FOS)

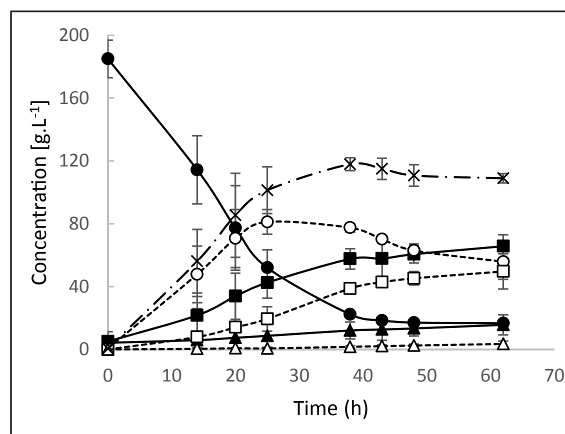

Fig. 2. Time course of the production of fructo-oligosaccharides (FOS) by *Aspergillus ibericus* in a bioreactor at 37 °C, pH 6.2 and 200 rpm. Fructose (▲), glucose (■), sucrose (•), GF_2 (◯), GF_3 (□), GF_4 (Δ) and total FOS (×). Results correspond to the average of 3 independent assays ± confidence interval (95% confidence level).

was obtained after 38 h of fermentation yielding 0.64 ± 0.02 $g_{FOS}.g_{initial\ sucrose}^{-1}$, with a content of 118 ± 4 g.L^{-1} in FOS and a purity of 56 ± 3%. At this time point, the FOS mixture composition was 37 ± 3% of GF_2, 18 ± 2% of GF_3 and 0.8 ± 0.3% of GF_4. The productivity of the process was found as 3.1 ± 0.1 02 g_{FOS}.L^{-1}.h^{-1}.

The time required to achieve the maximum concentration of GF_2 was 25 h (81 ± 8 g.L^{-1}). After this period, GF_2 concentration started to decrease due to the formation of the GF_3, by transfructosylation of fructose with GF_2, and due to the activity of the hydrolysis enzymes converting GF_2 back to fructose and sucrose. At 62 h of fermentation both GF_3 and GF_4 concentrations were still increasing, and the FOS total concentration did not decrease significantly from its maximum (109 ± 3 g.L^{-1} at 62 h). An average of 113 ± 4 g.L^{-1} of FOS was achieved from 38 to 62 h. Thus, through the selection of different harvesting fermentation times, it is possible to obtain a variety of mixtures containing different ratios of oligosaccharides although with an equivalent total amount of FOS (66 - 51% of GF_2 and 33 - 46% of GF_3 in total FOS, between 38 and 62 h).

The FOS production yield increased in the assays performed in bioreactor as compared to the shaken flasks. This increase is probably related with different mixture patterns obtained due to the reactor configuration, as well as with the pH control during fermentation. The agitation is substantially different, promoting different mixture conditions in the liquid and levels of aeration. To minimize the effect of the different volume headspaces and liquid surface areas in contact with the air of both set of experiments (Erlenmeyer versus bioreactor), a higher rotation was used in the assays conducted in the bioreactor to guarantee a better aeration of the medium, since the fungus is an aerobic microorganism (200 rpm instead of 150 rpm used in the shaken flask assays). Actually, this was the main reason why agitation was not included as variable in the experimental design for the optimization of FOS production in shaken flasks. This parameter will have to be further optimized at the bioreactor scale taking into account its mixture and agitation patterns. During submerged cultivation in stirred fermenter, the agitation intensity has been reported to influence the fungal morphology and the specific energy dissipation rate (Cui, van der Lans, & Luyben, 1997). The pellet may be damaged by the impellers of the reactor, contributing to free filamentous mycelia and reseed their growth, what ultimately can result in an increase of FOS production by the enzymes being produced by the highest biomass level.

A broad range of FOS production yields has been reported for other *Aspergillus* strains, varying from 0.20 to 0.60 ($g_{FOS}.g_{initial\ sucrose}^{-1}$) (Dominguez et al., 2013). *A. flavus*, *A. japonicus, A. niger* and *A. oryzae* are between the *Apergillus* strains reported as FOS producers. The FOS production yield depends not only on the strain used, but also on the

production process employed. Predominantly, FOS have been produced in a two-stage process using isolated enzymes produced by submerged fermentation (Sangeetha et al., 2005). Herein, FOS were produced using the whole cells of the *A. ibericus* avoiding the enzyme purification step. Production yields up to 0.64 ± 0.02 $g_{FOS}.g_{initial\ sucrose}^{-1}$ were achieved. The process proved to be not only faster but also more economical as higher FOS production yields have been attained. Similarly, high production levels of FOS were obtained in our recent work conducted with the whole cells of *A. pullulans* (0.63 $g_{FOS}.g_{initial\ sucrose}^{-1}$) (Dominguez et al., 2012; Nobre et al., 2016) and *A. japonicus* (0.61 $g_{FOS}.g_{initial\ sucrose}^{-1}$) (Mussatto, Aguilar, Rodrigues, & Teixeira, 2009). The use of one-step fermentation has been reported also for other microorganisms, although with yields much lower than the ones herein reported, such as *Microbacterium paraoxydans* with 0.38 $g_{FOS}.g_{initial\ sucrose}^{-1}$ (Ojha, Rana, & Mishra, 2016) and *Penicillium expansum* with 0.58 $g_{FOS}.g_{initial\ sucrose}^{-1}$ (Prata, Mussatto, Rodrigues, & Teixeira, 2010).

The *A. ibericus* was studied for the production of FOS since a positive reaction was obtained in preliminary tests made in agar plates with this fungus while screening for transfructosylation activity (Dominguez et al., 2006). The main goal of the current work was to determine the potential for FOS production of this new isolate (*A. ibericus*) and to chemically characterize the FOS mixture produced. The best temperature and pH conditions for the production of FOS by this organism were established.

Although the production yields obtained were considerably high as compared to other works (Dominguez et al., 2013), we believe that further optimizations are still required whenever scaling-up the bioprocess to bioreactors, namely in what concerns agitation rates and dissolved oxygen, which could possibly increase further the production yields. Moreover, alternative culture media based on food industry by-products and residues may also be considered to optimize the FOS production yields and to improve the bioprocess sustainability. Additionally, downstream techniques could be employed for FOS purification such as Simulated Moving Bed chromatography or microbial treatment (Nobre, Teixeira, et al., 2015).

3.3. HPLC characterization of the FOS produced by A. ibericus

Fig. 3 shows the chromatographic profile obtained for a sample of the fermentation conducted with the *A. ibericus* as compared to the standard FOS from Wako.

The HPLC chromatogram analysis revealed that *A. ibericus* was able to produce three different oligosaccharides with retention times of 9.521 (DP2), 12.176 (DP3) and 15.920 min (DP4). The retention times were identical with that of the standards 1-kestose (9.560 min), nystose (12.191 min) and 1^F-fructofuranosylnystose (15.886 min) confirming the production of these three FOS by the *A. ibericus*. The three other peaks obtained earlier for the fermentative sample represent fructose (5.252 min), glucose (6.112 min) and sucrose (7.153 min).

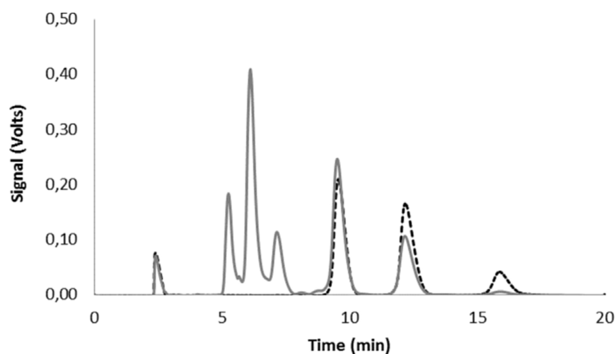

Fig. 3. HPLC chromatogram obtained for a sample of the fermentative broth from the *Aspergilus ibericus* culture (grey line) and the standard kestose, nystose, and 1^F-fructofuranosylnystose from Wako (dash black line). Samples were eluted at 1 mL.min^{-1} and 25 °C in a Prevail Carbohydrate ES column.

3.4. NMR characterization of the FOS produced by A. ibericus

The prebiotic activity of sugars is mainly dependent on their chemical structure (Borromei et al., 2009). Composition of the oligosaccharides and linkages between the monomers may influence their digestion by the colonic microflora and by the salivary, pancreatic and brush border human enzymes (Suzuki, Tanaka, Amano, Asakura, & Muramatsu, 2004). Therefore, the chemical structure of the FOS mixture synthesised by *A. ibericus* in the present work was determined.

FOS were firstly purified up to 93% (w/w) in an activated charcoal column through selective desorption using ethanol concentration gradients to obtain the IB mixture (Nobre et al., 2012). The same procedure was used for the FOS synthesised by *A. pullulans* in our previous work (Nobre et al., 2016). Finally, FOS chemical structures were identified by NMR and compared with a commercially available Actilight FOS mixture and Wako FOS standards.

The NMR characteristics as ^{1}H and ^{13}C chemical shifts, correlations (coupling constants) and integration values obtained for the three samples (AP, IB and Actilight) were equal, and therefore, corresponding to FOS with same substitution pattern and degree of polymerization (DP). In addition, the FOS structures observed in the ^{1}H-^{13}C HSQC spectra from these three samples presented equal ^{1}H and ^{13}C interactions when compared to the ^{1}H-^{13}C HSQC spectrum from a mixture of standard FOS from Wako (Neuss, Germany) containing GF_2 (1-kestose), GF_3 (nystose), and GF_4 (1^F-fructofuranosylnystose).

In Fig. 4 it is possible to observe the similarity of the spin systems between the ^{1}H-^{13}C HSQC NMR spectra from the standard FOS from Wako (a) and the sample IB (b), since the other spectra (AP and Actilight) were equal. In addition, the signals in Fig. 4a were assigned and labeled according to the 1-kestose structure. The COSY and HSQC spectra permitted assigning the spin systems, while the connectivity was known from cross peaks in the HMBC spectrum (*data not shown*). Moreover, the complete hydrogen assignments were obtained by means of HMBC and ^{13}C NMR (Santos-Moriano et al., 2015).

The ^{1}H NMR spectra showed the presence of signals in the anomeric region between the chemical shifts δ 5.37–5.48 (duplets 3.9 Hz), others previously identified signals between δ 4.18–4.30 and δ 4.02–4.14, and those between δ 3.44 and δ 3.93 (Liao et al., 2011). The anomeric regions in ^{13}C NMR spectra were more complex presenting overlapping resonances between δ 105.8–106.6 and δ 94.8–95.5. Signals for FRU units were recognized by their downfield positions at δ 105.8–106.6, which present cross peaks with the units GLU H1 (δ 5.37–5.48) and FRU H1a and H1b (different conformations of FRU between δ 3.74–3.87). The connectivity between GLU and FRU units was determined from the existence of a cross peak between the H1 from GLU unit and the quaternary carbon C2 from FRU unit in the HMBC spectrum. The occurrence of a cross peak between H1 of the GLU unit and C2 of a FRU unit and the concomitant absence of correlations between quaternary C2 of FRU unit and H2, 3, 4 and/or 6 of the GLU unit favoured the position of the GLU unit in the beginning of the chain. Moreover, the presence of characteristic chemical shifts for C3 and C4 of the FRU units at δ 76.6–77.4 was also indicative of the connection between C1 and C2 of the FRU units. This observation combined with the DP estimated from the integration of the signals between δ 5.37–5.48 and those at δ 4.18–4.30 and δ 4.02–4.14 were in favour to the linear structure of the FRU units.

Furthermore, the spin systems of the three samples (AP, IB and Actilight) allowed establishing the presence of the main correlations of the compounds as: … H1 βFRU C2 → H1 βFRU C2 → H1 αGLU. The constant coupling ($J_{H\text{-}H}$) observed in Table 4 were obtained by the *J*-resolved and only approximated chemical shifts (δ) were obtained due to signals overlap spin systems.

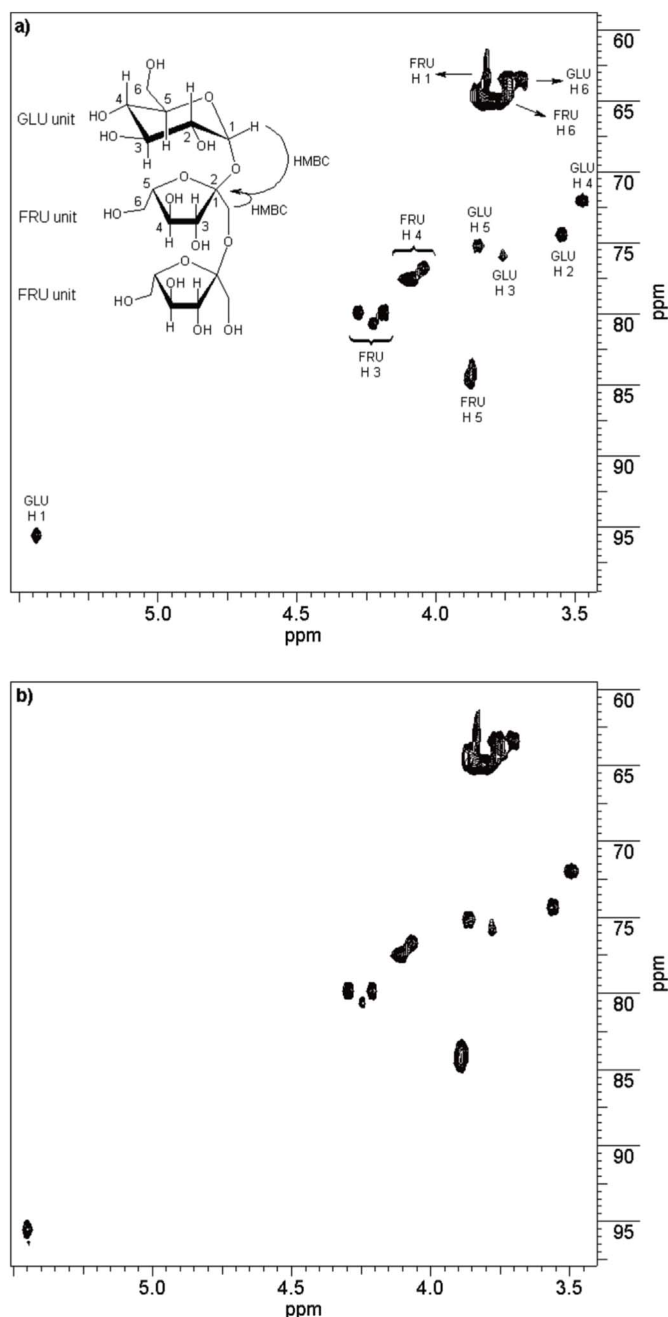

Fig. 4. Comparison between the ^{1}H-^{13}C HSQC NMR spectra from the samples: a) standard FOS sample from Wako supplier (GF_2, GF_3, and GF_4); b) FOS sample produced by *Aspergillus ibericus*.

4. Conclusions

A temperature of 37 °C and a pH 6.2 were established by experimental design as the optimal fermentation conditions to produce FOS by *A. ibericus*. Experiments run in bioreactor in a one-stage process, using the whole cells of the microorganism, resulted in a FOS production of 0.64 ± 0.02 $g_{FOS}.g_{initial\ sucrose}^{-1}$ at 38 h of fermentation, with a content of 118 ± 4 $g.L^{-1}$ in FOS and purity of 56 ± 3%. The FOS produced were identified by NMR and HPLC as 1-kestose, nystose, and 1^{F}-fructofuranosylnystose.

The results gathered in this study highlight the possibility of using the fungus *A. ibericus* MUM 03.49 as a prebiotic producer at a large scale.

Table 4
Chemical shifts (in ppm) of ^{1}H and ^{13}C, and constant coupling (in Hz) of all fructo-oligosaccharides (FOS) present in the four samples under study, namely: (IB) the FOS mixture produced in this work by *Aspergillus ibericus*; (AP) a FOS mixture produced by *Aureobasidium pullulans* CCY 27-1-94, Actilight mixture, and FOS from Wako.

Unit/Carbon	δ ^{1}H	multiplicity[a]; *J* in Hz	δ ^{13}C
GLU C1	5.37–5.48	*d* 3.9	94.8–95.5
GLU C2	3.53–5.48	*dd* 3.9, 10.0	73.5–74.6
GLU C3	3.73–3.79	*m*	75.0–76.0
GLU C4	3.45–3.51	*m*	71.6–72.7
GLU C5	3.82–3.88	*m*	74.8–75.9
GLU C6	3.80–3.89	*d* 10.4	63.4–64.5
FRU C1	3.67–3.71	*s*	62.8–63.8
FRU C2	–	–	105.8–106.6
FRU C3	4.18–4.30	*d* 8.6	79.1–80.7
FRU C4	4.02–4.14	*m*	76.3–77.8
FRU C5	3.84–3.90	*m*	83.5–84.6
FRU C6	3.74–3.87	*m*	64.6–65.8

[a] *s* – singlet; GLU - glucose; FRU - fructose; *d* – doublet; *dd* – doublet of doublets; *m* - multiplet.

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