One-step co-culture fermentation strategy to produce high-content fructo-oligosaccharides

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ABSTRACT

An integrated process enabling the simultaneous production and purification of fructo-oligosaccharides (FOS) was explored. A co-culture fermentation with *Aspergillus ibericus* (used as FOS producer strain) and *Saccharomyces cerevisiae* YIL162 W (for small saccharides removal) was optimized. Inoculation conditions of *S. cerevisiae*, fermentative broth composition, temperature and pH were optimized by experimental design. Yeast extract concentration and temperature were the most significant variables affecting FOS purity. Co-culture fermentations with simultaneously inoculation of the strains, run under 30 °C, initial pH 6.0 and 17 g L⁻¹ yeast extract led to FOS mixtures with 97.4 ± 0.2% (w/w) purity. The fermentations conducted in bioreactor, at a 0.8 vvm aeration rate, yielded 0.70 ± 0.00 g_{FOS}·g_{initial GF}⁻¹ at 45 h fermentation, with a FOS content of 133.7 ± 0.1 g L⁻¹. A purity of FOS up to 93.8 ± 0.7% (w/w) was achieved. The one-step fermentation proved to be efficient, economical and fast.

1. Introduction

Growing consumer awareness on high nutritional value ingredients and their impact in health has been raising the interest in functional food. Among functional food, fructo-oligosaccharides (FOS) are increasingly popular fulfilling all the criteria to be considered prebiotics (Nobre, Cerqueira, Rodrigues, Vicente, & Teixeira, 2015). They are not hydrolyzed by the human digestive system but are completely fermented selectively by health-promoting colonic microbiota, bifidobacteria and lactobacilli (Nobre, Sousa et al., 2018), to short-chain fatty acids, inhibiting the growth of harmful microorganisms, and stimulating the immune system, reducing liver toxins, and aiding the absorption of certain minerals (Younis, Ahmad, & Jahan, 2015).

FOS have been industrially produced via sucrose fermentation by several purified microorganism's enzymes, in a two-step bioprocess including 1) microorganism growth fermentation and enzyme production and 2) FOS synthesis with the purified enzymes. Microorganisms belonging to *Aspergillus*, *Penicillium* and *Aureobasidium* genera are the most reported for FOS production (Maiorano, Piccoli, Da Silva, & De Andrade Rodrigues, 2008; Nascimento, Nobre, Cavalcanti, Teixeira, & Porto, 2016). During the fermentation process, a high amount of glucose is generated, which induces enzyme activity inhibition, leading to low sucrose conversion yield (0.55–0.60 g_{FOS}·g_{sucrose}⁻¹) and more than 10 % of unreacted sucrose (Nishizawa, Nakajima, & Nabetani, 2001;

Sangeetha, Ramesh, & Prapulla, 2005). Consequently, FOS mixtures obtained at the end of the fermentation have only around 60% purity, thus preventing their inclusion in diabetic and dietetic food.

Numerous studies have been focused on the development of downstream treatments to purify FOS from sugar mixtures, such as the use of membrane techniques, chromatographic methods and microbial treatment using successive purification fermentations (Nobre, Teixeira, & Rodrigues, 2012; Nobre, Suvarov, & De Weireld, 2014; Nobre et al., 2016; Nobre, Teixeira, & Rodrigues, 2015; Suvarov, Kienle, Nobre, De Weireld, & Vande Wouwer, 2014; Yang, Wang, Teng, & Zhang, 2008). Alternatively, FOS have been simultaneously produced and purified by mixtures of enzymes e.g. β-fructofuranosidase and glucose oxidase or by cell systems (Jung, Kim, Jeon, & Lee, 1993; Sheu, Lio, Chen, Lin, & Duan, 2001; Sheu, Duan, Cheng, Bi, & Chen, 2002; Sheu, Chang, Wang, Wu, & Huang, 2013; Yun & Song, 1993). However, the use of free enzymes presents some practical limitations such as low stability, discontinuous production and limited reusability (Mouelhi, Abidi, & Marzouki, 2016). These drawbacks make the whole-cell process a lot more appealing as it does not require enzyme purification from the microorganism cells and therefore, the fermentations are conducted in a one-step bioprocess. Production yields up to 0.64 g_{FOS}·g_{initial GF}⁻¹ were achieved using whole-cells of *Aspergillus ibericus* (Nobre, Alves Filho et al., 2018) and *Aureobasidium pullulans* (Dominguez et al., 2012; Nobre et al., 2016).

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Our recent work, using a one-step process with a co-culture of *A. pullulans* with *Saccharomyces cerevisiae* clearly showed the potential of the yeast to reduce the glucose present in the broth. However, due to microorganisms' competition by the substrate, the fermentation yields obtained were slightly lower than the ones obtained using a single *A. pullulans* fermentation (Nobre et al., 2016). To overcome this effect, the use of a *S. cerevisiae* strain, with the gene responsible for sucrose hydrolysis disrupted, is herein proposed. FOS production and purification were optimized as an integrated process using whole-cells of both *A. ibericus* fungi for FOS production and *S. cerevisiae* YIL162 W yeast for FOS purification. Co-culture conditions such as, initial yeast concentration, inoculation time, fermentative broth composition, temperature and pH were firstly optimized and finally, the process was scaled-up to a bioreactor.

2. Material 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 in Czapeck Dox Agar (Oxoid, UK) plates from frozen glycerol stock solutions. After 7-day grown at 30 °C, spores were scrapped from the plates with a 0.1 % (w/v) solution of Tween 80 (Panreac, AppliChem, Spain) to prepare a concentrated spore suspension. The suspension spore concentration was determined using an improved *Neubauer* chamber, and afterwards adjusted to 1×10^8 spores. mL⁻¹. The spores' solution was used to inoculate the shake-flask assays and the bioreactor inoculum.

S. cerevisiae strains used were acquired from EUROSCARF – European *S. cerevisiae* archive for functional analysis. Both yeasts, *S. cerevisiae* BY4741 (wild type) and *S. cerevisiae* YIL162 W (with the gene responsible for sucrose hydrolysis disrupted (*SUC2*)), were grown in YPD (10 g L⁻¹ yeast extract, 20 g L⁻¹ peptone, and 20 g L⁻¹ glucose) agar plates for 7 days. Colonies were further transferred to a YPD liquid culture medium, previously autoclaved at 121 °C for 15 min, and grown during 3 days at 30 °C and stored at 4 °C. The cells suspension was grown during 24 h at 30 °C and 150 rpm. Cells concentration was determined using an improved *Neubauer* chamber, and afterwards adjusted to an optical density of 1.0, measured at 620 nm in a Synergy HT Multi-Mode Microplate Reader (Bio-Tek Instruments, USA), corresponding to 6.5×10^7 cells mL⁻¹.

2.2. Shake-flask experiments

Shake-flasks of 250 mL covered with aluminum caps were used. Assays run before the culture media and operational conditions optimization were performed with 45 mL of the following fermentation medium: 5.0 g L⁻¹ yeast extract, 5.0 g L⁻¹ NaNO₃, 4.0 g L⁻¹ KH₂PO₄, 0.5 g L⁻¹ KCl, 0.35 g L⁻¹ K₂SO₄, 0.5 g L⁻¹ MgSO₄·7H₂O and 0.01 g L⁻¹ FeSO₄·7H₂O and 200 g L⁻¹ sucrose. Sucrose and FeSO₄·7H₂O solutions were sterilized by filtration (0.2 μm) and the other salt solutions were autoclaved at 121 °C for 15 min. The pH of the culture medium was adjusted to 6.2 before inoculation and assays were run at 30 °C and 150 rpm (Nobre et al., 2016; Nobre, Alves Filho et al., 2018).

A mono-culture fermentation was run with *A. ibericus* under the abovementioned conditions. Shake-flasks were inoculated with 1 mL of *A. ibericus* spore suspension solution (1×10^8 spores per mL). Co-culture fermentations were further conducted with both *A. ibericus* and *S. cerevisiae* YIL162 W strains. *S. cerevisiae* YIL162 W was inoculated with an optical density of 1.0, corresponding to 6.5×10^7 cells per mL. Inoculation conditions were firstly optimized as following a) two inoculation volumes tested, namely 1 and 3 mL; b) three inoculation times of the *S. cerevisiae* YIL162 W culture evaluated, namely at 0, 10 and 20 h of the *A. ibericus* fermentation.

Samples were taken at different fermentation time points for further

determination of sugar concentration.

2.3. Experimental design and data analysis

The fermentation broth composition from co-culture fermentations was optimized by experimental design to achieve maximal FOS purity. Additionally, the influence of pH and temperature conditions was also optimized.

The FOS purity was calculated according to the following equation:

$$FOS \text{ purity (\%)} = \frac{m_{FOS}}{m_{total \text{ sugars}}} \times 100 \quad (1)$$

where m_{FOS} represents the sum of the individual mass of FOS (kestose - GF₂, nystose - GF₃ and fructofuranosyl nystose - GF₄) and $m_{total \text{ sugars}}$ represents the sum of the total mass of sugars (FOS, sucrose, fructose and glucose).

An initial 2³ central composite design (CCD), with three central points, was used for fermentation broth composition optimization, in which the three independent variables, NaNO₃, KH₂PO₄ and yeast extract concentrations, were coded to dimensionless ones X_1 , X_2 and X_3 , respectively, accordingly to the following equation:

$$x_i = \frac{(X_i - X_0)}{\Delta X_i}, \quad i = 1, 2, \dots, k \quad (2)$$

where x_i is the coded value of the independent variable, X_i is the real value of an independent variable, X_0 is the real value of an independent variable at the central point and ΔX_i is the step change value.

Afterwards, for the operational conditions optimization, a 2² CCD, with three central points, was used, in which two independent variables, temperature and pH, were converted to dimensionless ones X_4 , and X_5 , respectively, accordingly to Eq. (2).

The medium component concentrations and operational conditions levels were established according to our previous work on FOS production maximization (Nobre et al., 2016; Nobre, Alves Filho et al., 2018). The range and levels of the independent variables studied is given in Table 1.

Experimental results were fitted with a second-order polynomial equation by multiple regression analysis (Eq. (3)). The quadratic mode for predicting the maximal FOS purity point based on the coded values of the independent variables (X_i), were expressed according to the following equation:

$$FOS \text{ purity (\%)} = \beta_0 + \sum_{i=1}^k \beta_i X_i + \sum_{i=1}^k \beta_{ii} X_i^2 + \sum_{i=1}^{k-1} \sum_{j=i+1}^k \beta_{ij} X_i X_j \quad (3)$$

where β_0 is the interception coefficient; β_i , β_{ii} and β_{ij} are the linear, squared and the cross-product coefficients, respectively; k is the number of factors.

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

Table 1

Experimental range and levels of the independent variables selected for the two designs, according to the central composite design (CCD).

	Independent variables	Symbol	Range and levels		
			−1	0	+1
Design 1	NaNO ₃ (g L ⁻¹)	X_1	5.0	10.0	15.0
	KH ₂ PO ₄ (g L ⁻¹)	X_2	4.0	6.0	8.0
	Yeast Extract (g L ⁻¹)	X_3	5.0	12.5	20.0
Design 2	T (°C)	X_4	28.0	32.0	37.0
	pH	X_5	5.0	6.0	7.0

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.

2.4. Bioreactor fermentation

The inoculum of *A. ibericus* was prepared in a 250 mL flask, containing 100 mL of fermentation medium composed of 100 g L⁻¹ sucrose and the optimized salt concentration. The inoculum medium was then seeded with 1 mL of the spore suspension solution (1×10^8 spores per mL) and grown for 5 days at 30 °C and 150 rpm. *S. cerevisiae* YIL162 W was grown in 150 mL of YPD, for 2 days, at 30 °C and 150 rpm of agitation. The optical density of the inoculum was then adjusted to 1.0 (6.5×10^7 cells per mL) before addition to the bioreactor. An inoculum of 300 mL of the *S. cerevisiae* YIL162 W was used.

Fermentations were carried out in a 3.75 L bioreactor (Autoclavable Benchtop Fermenter Type R'ALF, Bioengineering AG, Switzerland). A working volume of 1 L of medium was used, containing 200 g L⁻¹ sucrose and the previously optimized salt concentrations. The salts and yeast extract solutions were sterilized at 121 °C for 30 min, while sucrose and FeSO₄·7H₂O were sterilized by filtration (0.2 µm). All solutions were further added to the bioreactor under sterile conditions. Fermentations were conducted at 200 rpm under the operational conditions optimized by experimental design. The pH was controlled using ortho-phosphoric acid and ammonium hydroxide solutions. First fermentations were performed without supplementation of air and the last ones were supplied with a constant aeration rate of 0.8 vvm (selected based in experimental data obtained from fermentations previously conducted in the same bioreactor (data not shown)). Samples were collected during the fermentation for sugar concentration determination.

2.5. Sugars and ethanol analysis

Samples were analyzed by high-performance liquid chromatography (HPLC) for sugars and ethanol quantification. A HPLC (Jasco), equipped with a refractive index detector and a Prevail Carbohydrate ES column (5 µm, 25 cm × 0.46 cm length × diameter) (Alltech), was used. Samples were eluted with a mixture of acetonitrile in pure-water (70:30, v/v) and 0.04% of ammonium hydroxide in water (HPLC Grade, Sigma-Aldrich, Germany). The elution was conducted at a flow rate of 1 mL·min⁻¹ and 30 °C (Nobre et al., 2016). The chromatographic signal was recorded and further integrated using the Star Chromatography Workstation software (Varian, USA). FOS standards were acquired from Wako (Japan). Sucrose, fructose and ethanol standards were obtained from Merck and glucose from VWR (Belgium).

3. Results and discussion

3.1. FOS production by *A. ibericus* in mono-culture using shake-flasks

The combination of two microorganisms as a co-culture to increase the purity of the FOS mixture was proposed. Primarily, the fermentation profile of *A. ibericus* MUM 03.49 was studied to confirm its ability to produce FOS (Fig. 1(a) and (b)). A maximum fermentation yield of 0.652 ± 0.005 g_{FOS}·g_{initial GF}⁻¹ was obtained after 24 h of fermentation. A correspondent FOS concentration of 129.6 ± 2.4 g L⁻¹ was obtained at this time point, where GF₂, GF₃ and GF₄ concentrations were, respectively, 78.3 ± 4.7 , 49.3 ± 3.1 and 1.9 ± 0.4 g L⁻¹. After this time point, GF₂ was gradually converted into GF₃, and further the GF₃ was transformed into GF₄ through transfructosylation reactions. Also, a decrease of the total FOS concentration and an increase of the fructose

and glucose amounts in the medium was verified due to FOS hydrolysis.

During the first 10 h of fermentation, *A. ibericus* was adapting to the medium. Then, sucrose was quickly converted to FOS in a considerable rate until a maximal FOS concentration was reached (24 h). After this point, sucrose was consumed in a very slow rate due to the presence of high inhibitory amounts of glucose in the medium. At 60 h fermentation there was still 7.9 ± 0.4 g L⁻¹ of unreacted sucrose and an amount of 86.7 ± 3.0 g L⁻¹ of glucose. The glucose amount in the fermentation broth at the optimum point represented 26.2% of total sugars and the concentration of small sugars mixture in the medium was 85.1 ± 2.0 g L⁻¹. Thus, FOS were obtained with a purity of $60.4 \pm 0.4\%$ (w/w) and the productivity of the process was 5.4 ± 0.1 g_{FOS} L⁻¹ h⁻¹.

3.2. FOS production optimization

In this work, FOS were synthesised through transfructosylation of sucrose by the β -fructofuranosidase and β -fructotransferase enzymes produced by the *A. ibericus*. In the *A. ibericus* fermentation, a *S. cerevisiae* strain was co-cultured to remove the high amount of glucose released during the FOS production.

The *S. cerevisiae* genome contains seven unlinked loci that encode invertase. This enzyme, also known as β -fructofuranosidase or "sucrase" plays an important role in the sugar metabolism. Invertase catalyses the hydrolysis of the disaccharide sucrose producing the monosaccharides fructose and glucose. Although individual *S. cerevisiae* strains may carry a number and combination of SUC genes, the reference strain herein used encodes only for the SUC2 gene (*Saccharomyces* genome database www.yeastgenome.org). To avoid competition by the substrate (sucrose), a strain of *S. cerevisiae* with SUC2 gene deleted was used, namely the *S. cerevisiae* YIL162 W.

Since *S. cerevisiae* YIL162 W showed potential to increase FOS purity without competing with *A. ibericus* by the substrate, and subsequently increasing the amount of FOS produced, this strain was used in the following experiments (Supplementary material).

3.2.1. Optimization of the inoculum conditions

After demonstrating the potential of *S. cerevisiae* YIL162 W for purifying FOS by removing the monosaccharides from the mixture, without competition by the substrate, the co-culture with the *A. ibericus* was optimized. Firstly, the inoculated cell amount of *S. cerevisiae* YIL162W was selected, and secondly, the best time to inoculate the yeast in the ongoing *A. ibericus* fermentation was evaluated. Results obtained inoculating at the beginning of the *A. ibericus* fermentation an amount of 1 or 3 times more cells of *S. cerevisiae* YIL162 W, corresponding to an inoculation volume of 1 and 3 mL, respectively, are gathered in Table 2.

According to the statistical analysis, the amount of yeast inoculated did not affect significantly neither the FOS concentration (~ 122.5 g L⁻¹; $p = 0.756$) nor the yield of production (~ 0.61 g_{FOS} g_{initial GF}⁻¹; $p = 0.318$). On the other hand, the increase of the amount of cells inoculated significantly increased the purity of FOS obtained (from 84.9 ± 1.0 to $90.8 \pm 0.2\%$; $p = 0.048$), even if the time required to reach the optimal point was longer (52.5 to 68.5 h).

These preliminary results showed an increased FOS purity using the co-culture strategy as compared to the mono-culture ($p < 0.0003$), in which FOS were produced with a purity of $60.4 \pm 0.4\%$ (w/w). Regarding the time to achieve the maximum FOS concentration, it was found that the presence of the yeast seems to delay the fermentation as compared to the mono-culture experiments (optimum time was 24 h). On the other hand, the FOS concentration did not vary significantly between assays conducted by mono- or co-culture ($p > 0.062$), neither the yield while inoculating 3 mL of *S. cerevisiae* YIL162 W ($p = 0.068$). When inoculating 1 mL of the yeast, the yield was statistically lower ($p = 0.014$) as compared to assays in mono-culture. Since the aim of this study was to maximize the FOS purity, the inoculation of 3 mL of *S.*

Fig. 1. Shake-flask fermentation profiles of *Aspergillus ibericus*: (a) sucrose (●) and total fructo-oligosaccharides - FOS (■) concentration; (b) in primary axis, concentrations of: fructose - F (blue stripe bars), glucose - G (orange bars); kestose - GF₂ (yellow dotted bars), nystose - GF₃ (black bars) and fructofuranosylmaltose - GF₄ (green dotted bars); in secondary axis: Purity of fructo-oligosaccharides - FOS (○). The results correspond to the average $\pm$ standard deviation of triplicate independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

cerevisiae YIL162W in 45 mL of fermentation medium, at a 6.5×10^7 cells mL⁻¹ concentration was selected for the following experiments to be conducted in shake-flasks.

Taking into account the fermentation profile obtained for the monoculture of *A. ibericus*, together with the profile obtained when *S. cerevisiae* YIL162W was inoculated simultaneously with *A. ibericus*, two additional times for the inoculation of the yeast were tested, namely 4.5 h and 16.5 h. Results showed that the concentration of FOS ($p > 0.1$) and yield ($p > 0.09$) were not statistically influenced by the time at which *S. cerevisiae* YIL162W was inoculated. On the other hand, the purity of FOS significantly decreased when inoculating the yeast either

at 4.5 h ($p = 0.04$) or 16.5 h ($p = 0.001$). Although productivity increased when the time of yeast inoculation was 16.5 h ($p < 0.01$), the simultaneous inoculation of the fungi and *S. cerevisiae* YIL162W was found to be the best strategy towards the production of FOS with a higher degree of purity.

A considerable decrease of glucose amount in the medium from 56.3 ± 1.9 to 2.1 ± 0.9 g L⁻¹ was observed. The amount of fructose was also reduced from 10.4 ± 0.2 to 2.4 ± 0.4 g L⁻¹. *S. cerevisiae* preference by glucose over fructose is well known as it is considered a glucophilic yeast (Guillaume, Delobel, Sablayrolles, & Blondin, 2007). Consequently, although fructose is also expected to be consumed, the

Table 2

Fermentation results obtained for the variation of the inoculation time and volume of *Saccharomyces cerevisiae* YIL162 W inoculated in an ongoing fermentation by *Aspergillus ibericus* to produce fructo-oligosaccharides (FOS). The results correspond to the average $\pm$ standard deviation of duplicate independent experiments.

Inoculation Time	Inoculation Volume (mL) ^a	Time (h)	[FOS] (g L ⁻¹)	FOS purity (% (w/w))	Yield (g _{FOS} g _{initial GF} ⁻¹)	Productivity (g _{FOS} L ⁻¹ h ⁻¹)
0	1	52.5	123.0 $\pm$ 4.0	84.9 $\pm$ 1.0	0.59 $\pm$ 0.02	2.34 $\pm$ 0.07
0	3	68.5	122.0 $\pm$ 0.2	90.8 $\pm$ 0.2	0.62 $\pm$ 0.02	1.78 $\pm$ 0.00
4.5	3	45.0	115.3 $\pm$ 1.7	80.0 $\pm$ 0.4	0.56 $\pm$ 0.08	2.56 $\pm$ 0.05
16.5	3	19.5	100 $\pm$ 13	53.2 $\pm$ 3.8	0.49 $\pm$ 0.06	5.16 $\pm$ 0.67

^a A suspension of 6.5×10^7 cells mL⁻¹ was used.

glucose is firstly exhausted from the medium. Hence, a discrepancy between the amount of glucose and fructose consumption during fermentation was expected.

A reduction of sucrose amount in the fermentation medium, at the optimal point, was also observed (from 18.4 ± 0.7 to 7.8 ± 1.1 g L⁻¹), thus meaning that the enzyme was more active in the hydrolysis and transfructosylation of the sucrose. This might be explained due to the glucose removal of the medium and consequent decrease of the enzyme inhibition by this sugar. Therefore, an increase of ~30% in purity (from 60.4 ± 0.4 to $90.8 \pm 1.6\%$ (w/w)) was obtained. The concentration of GF₂, GF₃ and GF₄ in the medium at the optimum point was 73.7 ± 6.4 , 45.0 ± 5.8 and 3.4 ± 0.8 g L⁻¹, respectively. Compared to the FOS production by the mono-culture of *A. ibericus*, a slight increase of the GF₄ and a reduction of the GF₂ and GF₃ concentrations was observed, although with no statistical significance, suggesting that the transfructosylation activity of the enzyme increased.

Based on these results, in the following assays, *S. cerevisiae* YIL162 W was always inoculated at the same time as *A. ibericus*.

3.2.2. Optimization of the fermentative broth composition

A first factorial design was performed to identify salts and yeast extract (YE) concentrations on the fermentation broth that lead to higher purity of FOS in the mixtures. Seventeen independent assays were performed at 30 °C (X₄) and initial pH 6.2 (X₅). FOS purity determined on each assay is shown in Table 3.

Purity in FOS was higher than 70% for all the conditions tested, meaning that the co-culture improved the purity in FOS, as compared to the mono-culture ($60.4 \pm 0.4\%$ (w/w)), independently on the fermentation broth composition tested. Nevertheless, several assays obtained purities higher than 91% (w/w). The highest purity was obtained when the co-culture was cultivated in a fermentation broth with the lowest concentration of NaNO₃ (5 g L⁻¹) and average concentration of both KH₂PO₄ (6 g L⁻¹) and YE (12.5 g L⁻¹).

Given the differences observed in the results, a statistical analysis was carried out aiming at identifying which independent variable significantly influences the purity of FOS. The statistical significance of NaNO₃, KH₂PO₄ and YE on the purity of FOS is given in Table 4. Neither the concentration of NaNO₃ nor KH₂PO₄ affected significantly the FOS percentage in the range of concentrations tested given that $p > 0.05$. On the other hand, the YE concentration presents a statistically

Table 3

Experimental runs using coded levels of NaNO₃, KH₂PO₄ and yeast extract according to the 2³ central composite design (CCD) and FOS percentage obtained under those conditions.

Runs	Independent Variables			FOS purity (% (w/w))		
	X ₁	X ₂	X ₃	Experimental	Predicted	Residues
1	-1	-1	-1	72.86	74.50	-1.64
2	-1	-1	+1	95.90	94.77	1.13
3	-1	+1	-1	72.32	72.43	-0.11
4	-1	+1	+1	94.21	96.19	-1.98
5	+1	-1	-1	78.91	77.37	1.54
6	+1	-1	+1	92.92	93.26	-0.34
7	+1	+1	-1	71.08	72.66	-1.57
8	+1	+1	+1	93.22	92.03	1.19
9	-1	0	0	97.23	94.62	2.61
10	+1	0	0	93.16	93.98	-0.82
11	0	-1	0	94.50	95.19	-0.69
12	0	+1	0	96.02	93.54	2.48
13	0	0	-1	78.48	76.70	1.78
14	0	0	+1	96.53	96.52	0.01
15 ^a	0	0	0	94.38	95.56	-1.18
16 ^a	0	0	0	94.57	95.56	-0.99
17 ^a	0	0	0	94.15	95.56	-1.41

X₁, X₂ and X₃ being the coded values of NaNO₃, KH₂PO₄ and yeast extract, respectively.

^a Central Points.

Table 4

Statistical analysis of the central composite design (CCD) performed for the optimization of the fermentative broth composition. The variables studied include NaNO₃, KH₂PO₄ and yeast extract concentration (Design 1) and temperature and pH (Design 2). The response variable was FOS purity.

Variables and interactions		Estimated effects	Standard Errors	t-value	p-value
Design 1					
X ₁	X ₁	-0.644	1.431	-0.450	0.666
	X ₁ ²	-2.522	2.765	-0.912	0.392
	X ₂	-1.649	1.431	-1.152	0.287
	X ₂ ²	-2.392	2.765	-0.865	0.416
	X ₃	19.823	1.431	13.850	0.000*
	X ₃ ²	-17.903	2.765	-6.475	0.000*
	X ₁ X ₂	-1.325	1.600	-0.828	0.435
	X ₁ X ₃	-2.193	1.600	-1.370	0.213
	X ₂ X ₃	1.743	1.600	1.089	0.312
Design 2					
X ₄	X ₄	-27.165	1.830	-14.842	0.000*
	X ₄ ²	-26.452	2.817	-9.391	0.000*
	X ₅	-1.186	1.830	-0.648	0.546
	X ₅ ²	4.328	2.817	1.537	0.185
	X ₄ X ₅	-4.237	2.242	-1.890	0.117

X₁, X₂, X₃, X₄ and X₅ are the coded values of NaNO₃, KH₂PO₄, yeast extract concentration, Temperature and pH, respectively.

* Significant influence at 95% confidence level.

significant effect on the FOS purity. The positive effect of YE means that an increase of the YE concentration will lead to a higher FOS percentage. The interaction between the studied variables were not significant at a 95% confidence level.

The effect of the independent factors on FOS purity can be better visualized by examining the surface plot. The three-dimensional response surface curves (Fig. 2(a) and (b)) clearly show that the percentage of FOS was not significantly affected neither by NaNO₃ nor KH₂PO₄, while for yeast extract, an optimum point was found after which higher or lower concentration values did not improve the FOS purity. Yeast extract is a source of nitrogen, soluble B vitamins, amino acids and co-factors, which are essential for the growth of various microorganisms.

Experimental values were further fitted to a second order equation obtained by multiple regression analysis (Eq. (3)). Both *F*-test and ANOVA analysis were used as significance criteria for the fitted model. The calculated *F*-value (31.49) was higher than the listed one (*F*_{9,7} = 3.68) thus, the model was considered statistically significant at 95% confidence level. The model explained 97.6% of the dependent variable's variability (*R*² = 0.976) with a good adjusted determination coefficient (*R*_{adjusted}² = 0.945). Based on the *R*² value determined, the model was considered accurate leading to a good prediction performance.

The simplified model proposed after elimination of the statistically insignificant terms is given in Eq. (4):

$$FOS \text{ purity (\%)} = 38.22 + 5.24[YE] - 0.16[YE]^2 \quad (4)$$

The values predicted by the model are presented in Table 3 along with the values observed experimentally. The central composite design (CCD) and regression analysis were effective in identifying the optimal conditions that maximize the percentage of FOS as a good agreement between the predicted and the experimental values was found.

Within the range of concentrations studied, the following optimal concentration values were obtained by the model for FOS purity maximization: 8.2 g L⁻¹ of NaNO₃, 5.9 g L⁻¹ of KH₂PO₄ and 16.8 g L⁻¹ of YE. Under these conditions the model predicted a FOS purity of 98.4% (w/w). In order to validate the fitted model, three independent fermentations were conducted under the optimized conditions in shake-flasks (Fig. 3).

A FOS purity of $97.4 \pm 0.2\%$ (w/w) was obtained after 68.5 h, with a FOS content of 116.1 ± 6.0 g L⁻¹ and a FOS production yield of

Fig. 2. Response surface of the central composite design (CCD) for the optimization of the fructo-oligosaccharides purity (% FOS (w/w)) as function of the NaNO_3 , KH_2PO_4 and yeast extract (YE) concentration (g L^{-1}) conditions.

Fig. 3. Fermentation profile in shake-flasks under the optimized conditions (8.2 g L^{-1} of NaNO_3 , 5.9 g L^{-1} of KH_2PO_4 and 16.8 g L^{-1} of yeast extract). Small sugars concentration (white bars); total FOS concentration (black bars); sucrose concentration (○); Purity of fructo-oligosaccharides - FOS (% (w/w)) (□); Sugars concentration in the primary axis and % of FOS in the secondary axis. The results correspond to the average $\pm$ standard deviation of triplicate independent experiments.

$0.56 \pm 0.03 \text{ g}_{\text{FOS}} \text{ g}_{\text{initial GF}}^{-1}$. The concentration of fructose and glucose on the mixture at the optimum point were 0.23 ± 0.06 and $0.24 \pm 0.04 \text{ g L}^{-1}$, respectively. Moreover, the amount of sucrose was only $2.6 \pm 0.3 \text{ g L}^{-1}$. The FOS percentage found experimentally is in good agreement with the predicted one, confirming once more the significance of the model.

3.2.3. Optimization of the operational fermentation conditions

A second factorial design was assessed to evaluate the effect of temperature and pH variation on the purity of the FOS achieved. Eleven experiments were run using the broth composition optimized in the first design (X_1 : 8.2 g L^{-1} of NaNO_3 , X_2 : 5.9 g L^{-1} of KH_2PO_4 and X_3 : 16.8 g L^{-1} of YE) and the respective FOS purities obtained are shown in Table 5.

The experimental values were fitted on a second-order equation obtained by multiple regression analysis (Eq. (3)). The F test and ANOVA analysis showed that the model was statistically significant at 95% confidence level. The calculated F -value (62.69) was significantly higher than the listed one ($F_{5,5} = 5.05$). The coefficient of determination was also considerably high ($R^2 = 0.984$) with a good adjusted determination coefficient ($R_{\text{adjusted}}^2 = 0.920$) ensuring a good adjustment of the quadratic model. Given that, the FOS purities prediction of the proposed model was considered accurate in the studied experimental region.

Table 5

Experimental runs using coded levels of Temperature (X_4) and pH (X_5) according to the 2^2 central composite design (CCD) and FOS purity obtained under those conditions.

Runs	Independent Variables		FOS purity (% (w/w))		
	X_4	X_5	Experimental	Predicted	Residues
1	−1	−1	92.41	92.33	0.08
2	−1	0	92.10	91.69	0.42
3	−1	+1	94.88	95.38	−0.50
4	0	−1	92.11	94.09	−1.98
5 ^a	0	0	92.21	91.33	0.88
6 ^a	0	0	93.22	91.33	1.89
7 ^a	0	0	91.38	91.33	0.04
8	0	+1	92.08	92.90	−0.83
9	+1	−1	71.30	69.40	1.90
10	+1	0	61.30	64.52	−3.22
11	+1	+1	65.30	63.98	1.32

^a Central Points.

Table 5 shows the effect of pH and temperature on the purity of FOS achieved in the fermentation mixtures obtained at the optimal point. Based on the statistical analysis, in the range of the tested concentrations, only the temperature (both the linear and the quadratic terms) affected significantly the purity of FOS attained. The negative effect associated means that a decrease of the temperature will lead to higher FOS purity. Both pH and the interaction between the studied variables were not significant at a 95% confidence level.

The model equation attained, after elimination of the statistically insignificant terms is given in Eq. (5), where T represents the temperature:

$$\text{FOS purity (\%)} = -363.36 + 33.69T - 0.53T^2 \quad (5)$$

Predicted values by the model and the values experimentally observed were in good agreement, as shown in Table 5.

The optimal temperature found by the model to enhance FOS purity with the co-culture was 30°C . *A. ibericus* MUM 03.49 is able to grow between 25 – 37°C with good FOS production above 30°C and maximal production at 37°C (Nobre, Alves Filho et al., 2018). The optimum grow conditions for the *S. cerevisiae* spp. used is between 25 – 30°C , as indicated by the strain source (EUROSCARF). Accordingly, 30°C is a good compromise to guarantee the growth of both species in co-culture.

The pH showed no significant influence on the purity of FOS achieved within the range studied. Thus, since maximal FOS production

Fig. 4. Sugars profile obtained in fermentations run in bioreactor at 30 °C and pH 6.0 with (solid line) and without aeration (dash line). Sucrose (▲) and fructo-oligosaccharides - FOS (○) concentration in the primary axis and FOS percentage (●) in the secondary axis. Results correspond to the average of three independent experiments $\pm$ standard deviation.

with *A. ibericus* is obtained with a pH around 6.0, the following experiments run in the bioreactor were performed at this fixed pH (Nobre, Alves Filho et al., 2018).

3.3. FOS production in bioreactor

After the optimization of the fermentation culture medium and operational conditions in shake-flask, the fermentation was scaled-up to a 3.75 L bioreactor. A fermentation broth composed by 200 g L⁻¹ sucrose, 0.5 g L⁻¹ KCl, 0.35 g L⁻¹ K₂SO₄, 0.5 g L⁻¹ MgSO₄·7H₂O, 0.01 g L⁻¹ FeSO₄·7H₂O, 8.0 g L⁻¹ NaNO₃, 6.0 g L⁻¹ KH₂PO₄ and 17.0 g L⁻¹ YE was used, and the experiments were run under controlled temperature and pH of 30 °C and 6.0, respectively. Fig. 4 shows the concentration profile of the sucrose consumed and the respective FOS produced, as well as the purity of FOS obtained for the experiments run.

At first, assays were run without aeration since *A. ibericus* is able to produce good FOS yields without extra air supply when grown in mono-culture under similar conditions, namely same bioreactor shape, agitation impellers and speed (Nobre, Alves Filho et al., 2018). Under these conditions, FOS purities of 92.5 $\pm$ 0.5% were attained at 141 h fermentation. The concentration of FOS in the mixture was 108.9 $\pm$ 6.6 g L⁻¹, with 50.3 $\pm$ 7.8 GF₂, 54.2 $\pm$ 2.0 GF₃ and 4.4 $\pm$ 0.9 g L⁻¹ GF₄. Small saccharides represented only 7.5% of the total sugars, in a concentration of 8.8 $\pm$ 1.0 g L⁻¹. A FOS fermentation yield of 0.59 $\pm$ 0.03 g_{FOS}·g_{initial GF}⁻¹ was obtained with a productivity of 0.77 $\pm$ 0.04 g_{FOS} L⁻¹·h⁻¹.

The maximum concentration of GF₂ was obtained at 72 h, with further conversion into GF₃ and GF₄ through the transfructosylation reaction. Maximum FOS concentration of 114.0 $\pm$ 7.5 g L⁻¹ was obtained earlier, at 117 h, although with a slightly inferior purity of FOS of 89.3 $\pm$ 2.2%.

As compared to the assays run with the mono-culture, great purities of FOS were achieved since the monosaccharides decreased from 85.1 $\pm$ 2.0 g L⁻¹ to 8.8 g L⁻¹ $\pm$ 2.0 g L⁻¹. Regarding the FOS produced, a 15 g L⁻¹ decrease was found in the maximal amount obtained. On the other hand, the time needed to achieve maximal production was much higher when using the co-culture. The presence of the yeast seems to delay the fermentation, although the yeast is not competing by the substrate, which can be observed by the similar amount of FOS produced in mono- and co-culture. Consequently, the fermentation productivity also decreased substantially. The rate of sucrose consumption/conversion is less affected when using the *S. cerevisiae* BY4741 wild type as shown in Fig. S1. Accordingly, in a work conducted with a co-culture of *A. pullulans* and *S. cerevisiae*, the sucrose consumption profile obtained was very similar, as well the maximum FOS production time (Nobre et al., 2016).

Considering that *Aspergillus* species are mostly aerobic moulds (Hall & Denning, 1994) and *S. cerevisiae* grow either in aerobic or anaerobic

conditions, even if they cannot truly grow under strictly anaerobic conditions (Walker & Stewart, 2016), an aeration of 0.8 vvm was supplied to the fermentation medium. The provided oxygen enhanced the strains growth and consequently, the sucrose conversion rate (Fig. 4). Most probably, when inoculating *S. cerevisiae* YIL162 W simultaneously with *A. ibericus* in the bioreactor, the oxygen transfer rate decreased to a critical value that inhibited the strains growth. In the shake-flasks experiments this effect was less evident since both agitation and head-space are different, resulting in fermentation times lower than 48 h to obtain maximal FOS production.

When introducing air in the bioreactor, the fermentation time required to reach maximal FOS production decreased noticeably from 117 h to 45 h, which improved considerably the process productivity to 3.0 $\pm$ 0.0 g_{FOS} L⁻¹·h⁻¹. Besides, the amount of FOS produced, as well as the fermentation yield, increased significantly to 133.7 $\pm$ 0.1 g L⁻¹ and 0.70 $\pm$ 0.00 g_{FOS} g_{initial GF}⁻¹, respectively. *S. cerevisiae* YIL162W reduced the amount of monosaccharides to residual values of 1.1 $\pm$ 0.4 g L⁻¹ fructose and 2.2 $\pm$ 0.9 g L⁻¹ glucose. The glucose amount reduction had an effective impact on the activity of the β -fructofuranosidase enzyme from the *A. ibericus*, thus raising the FOS fermentation yield to values even greater than the ones obtained in mono-culture. FOS fermentation yields herein obtained are noticeably higher than the ones reported for other microorganisms (Dominguez, Rodrigues, Lima, & Teixeira, 2013; Ganaie, Lateef, & Gupta, 2014; Mano et al., 2018). Moreover, since the microorganisms whole-cells are used as an alternative to the isolated enzymes, which is the methodology predominantly used by other reports, the overall process of FOS production is much faster (Sangeetha, Ramesh, & Prapulla, 2005). The use of the whole-cells avoids the enzyme purification step, shorten the process from two-stages to one-stage.

The sugar mixtures collected achieved a maximal purity in FOS of 93.8 $\pm$ 0.7% at 53 h fermentation. At 45 h, fermentation mixtures contained 92.6% $\pm$ 1.0% of pure FOS. The main small sugar in the mixture was sucrose with a concentration of 7.4 $\pm$ 2.1 and 4.9 $\pm$ 2.6 g L⁻¹ for 45 and 53 h, respectively. Sucrose was still being converted to FOS and decreased to residual values after this fermentation time. However, FOS also began to be hydrolysed resulting in a decrease of the total FOS amount (Figs. 4 and 5).

Individual sugar profiles vary along the fermentation time (Fig. 5). Samples collected between 45 and 53 h were richer in GF₂, while for 70 and 75 h the main sugar present was GF₃. Since the purity of the mixture does not vary significantly during this time period (92.8 $\pm$ 0.8%), through the selection of a different harvesting fermentation time, a variety of mixtures containing different ratios of oligosaccharides may be obtained.

Fig. 5. Sugars profile obtained in fermentations run in bioreactor at 30 °C and pH 6.0 with aeration. In the primary axis concentrations of: fructose - F (blue bars), glucose - G (orange bars), sucrose - GF (dotted bars), kestose - GF₂ (yellow bars), nystose - GF₃ (black bars) and fructofuranosylnystose - GF₄ (green bars); in the secondary axis: % of fructo-oligosaccharides - FOS (○). Results correspond to the average of three independent assays $\pm$ standard deviation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

An amount of $23.8 \pm 1.7 \text{ g L}^{-1}$ of ethanol was produced from glucose through *S. cerevisiae* YIL162W fermentation at the maximum FOS production time. The concentration of ethanol progressively increased after 75 h fermentation up to $35.5 \text{ g L}^{-1} \pm 2.7 \text{ g L}^{-1}$.

The integrated production and purification process using co-cultures was previously explored by our group using *A. pullulans* and *S. cerevisiae* (Nobre et al., 2016). However, since the yeast strain did not have the SUC2 gene disrupted, there was competition by the substrate leading to lower fermentation yields and the purity only increased up to 58%.

Sheu et al. (2002) used a cell system consisting in mycelia of *A. japonicus* or *A. pullulans* with a living *Gluconobacter oxydans*. The system worked in continuous mode for 7 days, using a microfiltration module to discharge the FOS produced. Although more than 80% of the sugars achieved were FOS, still 5–7% glucose and 8–10% sucrose on a dry weight basis remained in the harvested mixtures (Sheu et al., 2002).

In another cell system, consisting in *A. japonicus* and *Pichia heimi* cells immobilized in calcium alginate beads, FOS were produced in three-steps using three tanks-in-series run in continuous mode. In the first two tanks, FOS were produced by *A. japonicus* cells and purified by *P. heimi* cells to 70.2% (first tank) and 95.6% (second tank). The third tank was used just to increase the purity of FOS up to 98.2% by *P. heimi*. One gram of sucrose yielded 0.62 g of FOS (Sheu et al., 2013). Compared to the cell system, our work using the living free microorganisms instead of immobilized cells that worked as enzymes bags, yielded higher values of FOS ($0.70 \pm 0.00 \text{ g}_{\text{FOS}} \text{ g}_{\text{initial GF}}^{-1}$) and similar purities of the FOS produced were achieved ($93.8 \pm 0.7\%$) using only one tank reactor. Therefore, the results gathered showed that the integrated system herein proposed allows achieving high FOS content with great purities using a one-step bioprocess.

4. Conclusions

An integrated system allowing the simultaneous production and purification of FOS was explored. A co-culture of *A. ibericus* and *S. cerevisiae* YIL162 W showed to maximize the amount and purity of the FOS mixtures produced. Under optimized conditions a yield of $0.70 \pm 0.00 \text{ g}_{\text{FOS}} \text{ g}_{\text{initial GF}}^{-1}$ was obtained at 45 h fermentation, with a content of $133.7 \pm 0.1 \text{ g L}^{-1}$ in FOS. FOS mixtures with purities up to $93.8 \pm 0.7\%$ were obtained.

In one single fermentation a high FOS content was produced, demonstrating that the system herein optimized is efficient, time-saving and economically attractive as compared to the conventional two-step bioprocess.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.carbpol.2018.08.051>.

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