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Microbial co-culturing strategies for fructo-oligosaccharide production

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ABSTRACT

Fructo-oligosaccharide (FOS) mixtures produced by fermentation contain large amounts of non-prebiotic sugars. Here we propose a mixed culture of *Aureobasidium pullulans* and *Saccharomyces cerevisiae* cells to produce FOS and consume the small saccharides simultaneously, thereby increasing FOS purity in the mixture. The use of immobilised *A. pullulans* in co-culture with encapsulated *S. cerevisiae*, inoculated after 10 h fermentation, enhanced FOS production in a 5 L bioreactor. Using this strategy, a maximal FOS concentration of 119 g L^{-1}, and yield of 0.59 g_{FOS} $g_{sucrose}^{-1}$, were obtained after 20 h fermentation, increasing FOS productivity from about 4.9 to 5.9 g_{FOS} L^{-1} h^{-1} compared to a control fermentation of immobilized *A. pullulans* in monoculture. In addition, the encapsulated *S. cerevisiae* cells were able to decrease the glucose in the medium to about 7.6% (w/w) after 63 h fermentation. This provided a final fermentation mixture with 2.0% (w/w) sucrose and a FOS purity of over 67.0% (w/w). Moreover, a concentration of up to 58.0 g L^{-1} of ethanol was obtained through the enzymatic transformation of glucose. The resulting pre-purified FOS mixture could improve the separation and purification of FOS in downstream treatments, such as simulated moving bed chromatography.

Introduction

Fructo-oligosaccharides (FOS) are synthesised through the trans-fructosylation of sucrose by enzymes from microorganisms such as *Aureobasidium pullulans* [1]. Fermentation is conventionally conducted with purified enzymes, however, the use of microorganisms containing the respective enzymes has been shown to provide increased FOS yield, without needing the enzyme production and purification steps [2,3]. The main drawbacks of FOS production by fermentation are the low yields and low purity of the FOS mixtures achieved. The glucose released during FOS synthesis inhibits the fructosyl-transferring reaction which, together with the enzymatic hydrolysis of FOS, occurs throughout the fermentation process and reduces the FOS yield and productivity [3,4]. Fermentation mixtures contain not only FOS, but also other sugars, such as fructose, glucose and residual unreacted sucrose, which reduce the prebiotic functionality of the FOS mixture achieved.

Several downstream processes have been explored to purify FOS mixtures after fermentation, including ultra- and nano filtration, activated charcoal systems, microbial treatment, simulated moving bed (SMB) and ion-exchange chromatography [3,5–8]. While ultra- and nano filtration membranes can be used to permeate glucose, but not sucrose and FOS [7], activated charcoal can physically adsorb FOS in a reversible process based on van der Waals forces [6]. Microbial treatments are also used to reduce small saccharides in FOS mixtures [5] and ion exchange resins can be used as adsorbents in SMB systems, to separate carbohydrates based on their molecular differences rather than their macroscopic properties [8]. However, FOS separation from other saccharides remains challenging since the physicochemical properties of sugars are very similar, particularly sucrose (GF) and kestose (GF_2), which only differ by one fructose moiety. Thus, lowering the amount of sucrose in the mixture, increasing the purity of the FOS mixtures themselves or reducing the amount of salts and other by-products of fermentation, may result in higher efficiency of these downstream treatments [3,9].

Other strategies used to increase the purity of FOS are based on the removal of non-oligosaccharide sugars from the FOS mixtures during fermentation. Glucose reduction from the fermentative broth also decreases the inhibitory effects, resulting in both increased FOS production yields and increased FOS purity. Small sugars conversion results in

Abbreviations: FOS, fructo-oligosaccharides; SMB, simulated Moving Bed; AP, *Aureobasidium pullulans*; SC, *Saccharomyces cerevisiae*; IT, inoculation time; AP_f, free *A. pullulans*; AP_e, encapsulated *A. pullulans*; AP_i, immobilised *A. pullulans*; SC_f, free *S. cerevisiae*; SC_e, encapsulated *S. cerevisiae*; YGC_a, yeast extract glucose agar; YGC_b, yeast extract glucose broth; RPF, reticulated polyurethane foam; FFase, fructofuranosidade; FTase, fructosyltransferase

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other secondary metabolites, such as ethanol, sorbitol and carbon dioxide, which are easy to separate from the mixtures. Two different approaches have mainly been used: (i) systems consisting of two mixed enzymes, *e.g.* β-fructofuranosidase with glucose oxidase [4,10,11]; (ii) co-culture systems with two microorganisms, one FOS-producer and the second a small saccharides consumer: *Aspergillus japonicus* and *Pichia pastoris* [12], *A. japonicus* and *Pichia heimii* immobilised in calcium-alginate beads [13], and *Aspergillus ibericus* and *S. cerevisiae* YIL162 W (with the gene responsible for sucrose hydrolysis disrupted) [14]. In recent work, a co-culture of *A. pullulans* with *Saccharomyces cerevisiae* was tested; the yeast was able to reduce the amount of small sugars in the mixture, although the amount of FOS produced also decreased [3].

The immobilisation of microbial cells for FOS production has shown to improve the thermal, chemical and shear stress force resistance of the biocatalysts [15]. Moreover, it enables cell recovery, allowing them to be reused in repeated FOS production cycles, improving the economic impact of the global process. Entrapment, microencapsulation, or binding to a solid carrier and cross-linking of enzyme aggregates, are some of the immobilisation strategies already used in FOS production processes [15]. Microbial whole cells have been entrapped in sodium alginate [5,16] or chitosan [1] beads, or immobilised in carriers of a different nature, such as agro-industrial wastes [17,18], lignocellulosic materials [19], and synthetic materials, including polyurethane foams, sponges, scourers and fibres [17,20]. In another study by our team, polyurethane reticulated foam was selected from a screening of different types of carrier, to immobilise whole cells of *A. pullulans* [17]. Cell immobilisation resulted in an increase of FOS concentration (15%), purity (8%) and yield (12%) (w/w) as compared to the fermentation run with free cells. The reduction of the enzymatic inhibition by glucose [21], the efficient transfer of nutrients and oxygen within the medium [22] and the hydrodynamic condition variations [23] induced by the immobilisation procedure, are some of the explanations reported for the improvement of the FOS production process. The results obtained in the previous reports instigated optimisation of the fermentation condition of the co-culture system, in order to improve overall FOS production and purification.

Thus, the main goal of the present work is the optimisation of the FOS production process using a co-culture of immobilised *A. pullulans* and *S. cerevisiae*. Cells of the former were used free, encapsulated in calcium-alginate beads, or immobilised in reticulated polyurethane foam, while *S. cerevisiae* was used free or encapsulated in calcium-alginate beads. In a first step, the influence of cell immobilisation and the time of the inoculation of *S. cerevisiae* on FOS production was evaluated using a full factorial design, with assays performed in shake flasks. As a second step, the process was validated in a 5 L bioreactor.

Materials and methods

Experimental design and statistical analysis

A full factorial design was applied to evaluate the impact of using different cell immobilisation strategies on FOS production by *A. pullulans* (FOS producer) with *S. cerevisiae* (small sugars consumer) in co-culture. Fermentations were conducted using a completely randomised 3 × 6 factorial design arrangement of fermentation culture conditions. Two independent factors (or variables) were studied: a) the *A. pullulans* immobilisation strategy (AP), and b) the *S. cerevisiae* immobilisation strategy (SC), with different inoculation times (IT). For the two factors evaluated, the levels considered were: (i) three category levels for the AP cells: free (AP_f), encapsulated in calcium-alginate beads (AP_e) and immobilised in a reticulated polyurethane foam carrier (AP_i); (ii) six category levels were used for SC cells inoculated at a defined time: free cells (SC_f) inoculated at 0, 10 or 20 h of fermentation (SC_fIT_0, SC_fIT_{10}, and SC_fIT_{20}, respectively), and encapsulated in calcium-alginate beads (SC_e) inoculated at 0, 10 and 20 h of fermentation (SC_eIT_0, SC_eIT_{10}, and SC_eIT_{20}, respectively). FOS production, purity, yield and productivity (dependent variables) were evaluated for each condition. Fermentations were carried out in shake flasks and assays were performed in duplicate. A total of 36 combinations were generated.

The statistical application JMP®12.2.0 – *The Statistical Discovery Software* was used for the experimental design and the regression analysis of the experimental data. A second-order model was fitted to the experimental results with a multiple regression analysis for each response variable studied, namely FOS concentration, purity, yield and productivity. The models were simplified by eliminating the terms without statistical significance. The effect of each independent variable on the chosen dependent variables was evaluated for each model. The quality of the fitted model was statistically verified by the magnitude of the R^2 coefficient of determination, and its statistical significance was evaluated by the *F*-test analysis of variance (ANOVA). The coefficients of the response surface were evaluated using the student's *t*-test.

Preparation and cultivation of microorganisms

The FOS-producing strain used was *Aureobasidium pullulans* CCY 27-1-94. The fungal cells were smeared on Czapeck Dox Agar (Oxoid, UK) plates and grown for 5 d at 28 °C A suspension containing 9.0×10^7 spores mL^{-1} was prepared by scraping and diluting the spores from the plates, using 0.1% (w/v) Tween 80 (Panreac, AppliChem, Spain). The cell concentration of the starting suspension was adjusted based on microscope counting with a Neubauer chamber. A *Saccharomyces cerevisiae* 11,982 strain was used to reduce the small sugars of the fermentative broth. The assays conducted with the yeast were prepared by transferring an aliquot of cells from a YGC_a (yeast extract glucose agar) plate (5 g L^{-1} yeast extract, 20 g L^{-1} glucose and 15 g L^{-1} agar (Fluka, Germany)), grown for 3 d at 30 °C, to 100 mL of YGC_b (yeast extract glucose broth) (5 g L^{-1} yeast extract, 20 g L^{-1} glucose). The cell suspension was grown for 24 h at 30 °C under 150 rpm agitation. Cells were counted with the Neubauer chamber and the concentration was adjusted to 9.6×10^6 cells mL^{-1} (corresponding to an optical density at 620 nm (OD_{620}) of 1) (Spectrophotometer Anthos 2010 Standard, Biochem).

Cell encapsulation in calcium-alginate beads

A solution of 3% (w/v) calcium-alginate (Sigma-Aldrich, USA), sterilised at 121 °C for 15 min, was prepared. Suspensions with a 5:5 (v/v) ratio of calcium alginate and *A. pullulans* spores (9.0×10^7 spores mL^{-1}) or *S. cerevisiae* cells (9.6×10^6 cells mL^{-1}) were prepared and lightly shaken in a beaker. The mixture containing the cells/spores in calcium alginate was added dropwise to a 2% (w/v) $CaCl_2$ (Sigma-Aldrich, USA) solution using a 100 mL syringe, with a 0.9 × 70 mm (20 G × 2 2/3″) needle, under a flow-rate of 1.33 mL min^{-1}. Beads were hardened for 1 h in the $CaCl_2$ solution and rinsed with sterile water before storage. *A. pullulans* was only used encapsulated in the assays conducted in shake flask (according to the experimental design conditions), while *S. cerevisiae* was used encapsulated in both shake flask and bioreactor fermentation assays (according to the fermentation conditions selected from the experimental design).

Preparation of reticulated polyurethane foam carrier

Reticulated polyurethane foam (RPF) (SKTfilter, China), with a porosity of 1 mm, was used to immobilise *A. pullulans*. The carrier was treated according to the methodology of [17]. Briefly, the foam was cut into 1 cm^3 pieces, boiled for 10 min and washed 3 times with distilled water, dried overnight at 60 °C and sterilised at 121 °C for 15 min before use. The ratio of RPF volume per shake flask or bioreactor total volumes was previously optimized. FOS production and cell immobilisation ability were evaluated using different RPF mass and sizes (data not shown in the manuscript) and the most efficient conditions were reproduced in the present study. Cells were immobilised in the RPF *in situ*

Table 1
Full factorial design experimental and predicted responses.

Assays	Strategy	FOS (g L^{-1})		% FOS (w/w)[a]		Yield (g_{FOS} $g_{sucrose}^{-1}$)		Q_p (g_{FOS} L^{-1} h^{-1})	
		Exp.[b]	Pred.[c]	Exp.[b]	Pred.[c]	Exp.[b]	Pred.[c]	Exp.[b]	Pred.[c]
A1/A13	$AP_iSC_eIT_{20}$	93.1 ± 8.3	93.1	50.0 ± 0.1	56.2	0.47 ± 0.04	0.47	1.41 ± 0.20	0.87
A2/A4	$AP_eSC_fIT_0$	5.7 ± 0.4	5.6	5.3 ± 0.8	11.6	0.02 ± 0.01	0.02	0.09 ± 0.01	0.63
A3/A8	$AP_iSC_fIT_{10}$	52.8 ± 0.7	52.7	32.2 ± 1.6	38.5	0.26 ± 0.01	0.26	0.84 ± 0.01	0.3
A5/A30	$AP_fSC_fIT_{20}$	110.8 ± 5.8	110.8	52.4 ± 6.1	58.7	0.50 ± 0.05	0.50	2.19 ± 0.07	2.2
A6/A10	$AP_fSC_eIT_0$	9.4 ± 1.7	9.4	7.3 ± 1.0	13.5	0.04 ± 0.01	0.04	0.15 ± 0.04	0.16
A7/A23	$AP_iSC_fIT_{20}$	108.7 ± 6.8	108.7	58.7 ± 2.6	64.9	0.44 ± 0.03	0.44	1.58 ± 0.10	1.04
A9/A11	$AP_fSC_eIT_{10}$	60.3 ± 5.6	60.3	39.7 ± 2.3	46.0	0.25 ± 0.01	0.25	0.96 ± 0.09	0.96
A12/A20	$AP_eSC_fIT_{20}$	73.3 ± 6.9	73.3	49.2 ± 0.5	55.5	0.28 ± 0.01	0.28	1.07 ± 0.10	1.61
A14/A27	$AP_iSC_fIT_0$	18.2 ± 9.0	13.0	15.2 ± 7.2	19.3	0.08 ± 0.04	0.05	0.25 ± 0.12	0.25
A15/A29	$AP_fSC_fIT_{10}$	47.4 ± 0.2	47.3	33.0 ± 2.5	39.3	0.21 ± 0.03	0.21	0.71 ± 0.02	0.71
A16/A36	$AP_eSC_eIT_0$	12.7 ± 0.5	12.6	10.0 ± 2.0	16.3	0.06 ± 0.01	0.06	0.19 ± 0.02	0.74
A17/A33	$AP_iSC_eIT_{10}$	131.9 ± 5.9	131.9	54.9 ± 1.3	61.2	0.47 ± 0.02	0.47	2.64 ± 0.12	2.1
A18/A21	$AP_eSC_fIT_{10}$	36.0 ± 1.8	38.9	23.3 ± 6.1	29.6	0.14 ± 0.01	0.15	0.57 ± 0.03	1.11
A19/A34	$AP_fSC_fIT_0$	13.0 ± 0.5	13.0	13.0 ± 3.7	19.3	0.05 ± 0.02	0.05	0.25 ± 0.08	0.25
A22/A35	$AP_eSC_eIT_{10}$	31.0 ± 11.0	30.9	21.7 ± 6.1	27.9	0.12 ± 0.05	0.12	0.44 ± 0.14	0.98
A24/A31	$AP_iSC_eIT_0$	8.7 ± 0.1	8.7	8.4 ± 0.2	14.7	0.03 ± 0.01	0.03	0.17 ± 0.00	0.00
A25/A28	$AP_fSC_eIT_{20}$	96.3 ± 2.9	96.2	48.2 ± 0.1	54.5	0.48 ± 0.02	0.48	2.03 ± 0.04	2.03
A26/A32	$AP_eSC_eIT_{20}$	88.5 ± 5.7	88.5	50.2 ± 2.2	56.5	0.41 ± 0.02	0.41	1.29 ± 0.09	1.83

AP_f – free *A. pullulans*; AP_e – encapsulated *A. pullulans;* AP_i – immobilised *A. pullulans.* SC_f – free *S. cerevisiae*; SC_e – encapsulated *S. cerevisiae;* IT_0, IT_{10} and IT_{20} – Inoculation time after 0, 10 and 20 h of fermentation. FOS – Fructo-oligosaccharides; Q_p – Productivity.

[a] On a dry weight basis.

[b] Exp.: experimental value (average ± standard deviation).

[c] Pred.: model-predicted value.

in the fermentation medium at 25 °C, by natural adsorption and entrapment. The immobilisation was initiated after the direct contact of the cells with the RPF pieces, *i.e.* after transferring the spores to the fermentation medium with the carrier in shake flask, or after transferring the inoculum to the bioreactor containing the carriers in bioreactor, and occurred throughout the fermentation process.

Shake flask fermentations

The experimental design assays were performed in shake flasks and carried out under the same conditions as used previously [17]. A volume of 100 mL of *A. pullulans* fermentation medium, containing 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} $FeSO_4.7H_2O$, was transferred to a 500 mL Erlenmeyer flask. The pH of the culture medium was adjusted to 5.5 at the beginning of the fermentations, which were performed at 32 °C in an orbital shaker under 150 rpm agitation, for 63 h. *S. cerevisiae* was added to this fermentation medium, after supplementation with yeast extract (5 g.L^{-1}), to boost the development of yeast cells [3].

Free and encapsulated *A. pullulans* (AP_f and AP_e) was inoculated into the fermentation medium (100 mL) to a concentration of 9×10^5 spores mL^{-1} at the beginning of fermentation. In AP_i fermentations, 1 g of RPF was added to the flask, corresponding to 8.0 ± 1.0% (v/v) of the total flask volume, before adding the free cells. Free or encapsulated *S. cerevisiae* (SC_f and SC_e) were inoculated after 0, 10 and 20 h, such as to achieve 9.6×10^4 cells mL^{-1} after addition. The selection of the inoculation times of the yeast was based in the results obtained in our previous study, conducted with a mono-culture of free *A. pullulans* [3]. Since maximal FOS production was obtained at 20 h fermentation, the three inoculation times selected were specifically at or before 20 h, namely 0, 10 and 20 h. Several samples were collected at different fermentation times for sugar concentration profile determination. A commercial sucrose ("Grand Pont", Raffinerie Tirlemontoise, S.A., Belgium) was used for the FOS synthesis. All the other reagents used were analytical grade (VWR, Belgium).

Bioreactor fermentations

The co-culturing strategies performed in shake flasks with the best performances for FOS production were selected and reproduced in a 5 L bioreactor. Fermentations were carried out in a 3 L working volume using a fermentation medium with the same salt concentration as above and the same methodology previously used [3]. The fermentation broth, containing 10 g of RPF (corresponding to 6.6 ± 0.9% (v/v) of the total bioreactor volume), was inoculated with 100 mL of *A. pullulans* inoculum (AP_i). A *S. cerevisiae* suspension with free or encapsulated cells (SC_f and SC_e), was transferred (after 10 or 20 h) into the bioreactor fermentative broth at a concentration of 9.6×10^4 cells mL^{-1} after inoculation. A control fermentation with immobilized *A. pullulans* (AP_i), without *S. cerevisiae*, was also carried out. Several samples were collected at different fermentation times to determine the sugar concentration profile.

Sugar analysis

Samples collected during fermentation were analysed by HPLC (Jasco, France) equipped with a refractive index detector (working at 30 °C) and a Prevail Carbohydrate ES 5 µm column (5 µm, 25 × 0.46 cm length × diameter) (Alltech, USA). A mixture of acetonitrile (HPLC Grade, Carlo Erba, France) in pure-water (70:30 v/v), containing 0.04% ammonium hydroxide in water (HPLC Grade, from Sigma, Germany) was used as a mobile phase. Eluent was eluted at a flow-rate of 1 mL.min^{-1} at room temperature [24]. The chromatographic spectra were analysed using Star Chromatography Workstation software (Varian, USA). FOS standards were acquired from Wako (Chemicals GmbH, Japan), sucrose and fructose from Merck (USA), and glucose from VWR (Belgium).

Results and discussion

Statistical significance of the applied fermentation strategies

Here a fermentation strategy has been proposed using a mixed culture of *A. pullulans* and *S. cerevisiae* to achieve high-content FOS production, with reduction of non-oligosaccharide sugars in the

mixture. The full factorial design resulted in 36 assays for the maximisation of FOS concentration, purity, yield and productivity. The fermentation conditions proposed are shown in Table 1. The statistical analysis for each response was performed using a standard least squares analysis.

Experimental and predicted values were similar for the responses studied (Table 1). Regression analysis showed that the models used for the maximisation of FOS concentration ($R^2 = 0.99$), purity ($R^2 = 0.98$), yield ($R^2 = 0.99$) and productivity ($R^2 = 0.99$) achieved a very satisfactory fitting. The analysis of variance for the experimental results obtained in the full factorial experiments, maximising FOS concentration, purity, yield and productivity, are summarised in Supplementary Tables A1, A2, A3 and A4, respectively. The positive or negative coefficient, and the *t*-value, elucidate the impact on the response for each studied factor. Significant factors, with a *p*-value ≤ 0.001, were used to represent the simplified models for the maximisation of each response variable, as summarised in Eqs. (1)–(4).

$$[\boldsymbol{FOS}]\ (\boldsymbol{g.\ L^{-1}}) = 55.4 - 14.3\,AP_e + 13.5\,AP_i - 43.2\,SC_fIT_0 - 10.1\,SC_fIT_{10} + 42.2\,SC_fIT_{20} - 45.2\,SC_eIT_0 + 19.0\,SC_eIT_{10} + 37.2\,SC_eIT_{20} - 14.8\,AP_fSC_eIT_{10} - 15.0\,AP_iSC_eIT_0 + 44.0\,AP_iSC_eIT_{10} + 16.6\,AP_eSC_eIT_0 - 29.2\,AP_eSC_eIT_{10} \quad (1)$$

$$\%\ \boldsymbol{FOS}\ (\boldsymbol{w_{FOS}.\ w_{Total\ sugars}^{-1}}) = 31.8 - 5.2\,AP_e + 4.7\,AP_i - 20.6\,SC_fIT_0 + 21.6\,SC_fIT_{20} - 23.2\,SC_eIT_0 + 7.0\,SC_eIT_{10} + 17.6\,SC_eIT_{20} + 11.4\,AP_iSC_eIT_{10} - 11.9\,AP_eSC_eIT_{10} \quad (2)$$

$$\boldsymbol{Yield}\ (\boldsymbol{g_{FOS}.\ g_{Sucrose}^{-1}}) = 0.241 - 0.069\,AP_e + 0.053\,AP_i - 0.187\,SC_fIT_0 + 0.164\,SC_fIT_{20} - 0.198\,SC_eIT_0 + 0.212\,SC_eIT_{20} + 0.076\,AP_fSC_fIT_{20} + 0.138\,AP_iSC_eIT_{10} + 0.080\,AP_eSC_eIT_0 - 0.092\,AP_eSC_eIT_{10} \quad (3)$$

$$\boldsymbol{Q_p}\ (\boldsymbol{g_{FOS}.\ L^{-1}.\ h^{-1}}) = 0.94 + 0.11\,AP_f + 0.21\,AP_i - 0.33\,AP_e - 0.74\,SC_fIT_0 - 0.23\,SC_fIT_{10} + 0.68\,SC_fIT_{20} - 0.76\,SC_eIT_0 + 0.41\,SC_eIT_{10} + 0.64\,SC_eIT_{20} + 0.47\,AP_fSC_fIT_{20} - 0.25\,AP_iSC_fIT_{20} - 0.50\,AP_fSC_eIT_{10} + 0.34\,AP_fSC_eIT_{20} + 1.08\,AP_iSC_eIT_{10} - 0.38\,AP_iSC_eIT_{20} + 0.35\,AP_eSC_eIT_0 - 0.58\,AP_eSC_eIT_{10} \quad (4)$$

The immobilisation of *A. pullulans* (AP_i) resulted in a significant positive effect on the responses studied, namely FOS concentration, purity, yield and productivity, for which the *p*-value was < 0.01. Conversely, *A. pullulans* encapsulated in calcium-alginate beads (AP_e) resulted in a negative effect on FOS production (with the same significance level). The inoculation of free or encapsulated *S. cerevisiae* cells after 20 h (SC_fIT_{20} and SC_eIT_{20}) had a positive effect in all the response variables studied, which was also verified when the they were inoculated at 10 h fermentation (SC_eIT_{10}), although, this contribution was only significant for FOS production and purity. Inoculation of free *S. cerevisiae* at IT = 10 h (SC_fIT_{10}) provided a negative and statistically significant effect on FOS production ($p < 0.01$). For all the response variables, the simultaneous inoculation of both microorganisms at the beginning of fermentation (IT_0), provided a significant negative effect (*p*-value < 0.0001) (Supplementary Tables A1–A4).

Optimisation of FOS production using different immobilisation strategies in shake flasks

The use of immobilised *A. pullulans* in co-culture with encapsulated *S. cerevisiae,* inoculated after 10 h ($AP_iSC_eIT_{10}$), resulted in higher levels of FOS concentration (131.9 ± 5.9 g L^{-1}), purity (54.9 ± 1.3% (w/w)), yield (0.47 ± 0.02 $g_{FOS}\ g_{Sucrose}^{-1}$) and productivity (2.6 ± 0.1 $g_{FOS}\ L^{-1}\ h^{-1}$) in assays A17/A33 (Table 1). Similar FOS purity was obtained in $AP_iSC_fIT_{20}$, 58.7 ± 2.6% (w/w) (assays A7/A23). FOS yield was likewise high in $AP_fSC_fIT_{20}$, namely 0.50 ± 0.05 $g_{FOS}.g_{Sucrose}^{-1}$ (assays A5/A30). The lowest responses were found using encapsulated *A. pullulans* (AP_e). The use of *S. cerevisiae* free or encapsulated (SC_f and SC_e), did not provide a positive or negative impact on FOS production. In general, *S. cerevisiae* inoculation after 20 h fermentation (SC_fIT_{20} and SC_eIT_{20}), provided better results compared to IT_0 or IT_{10}. Inoculation of the yeast at the beginning of the immobilised *A. pullulans* fermentation, $AP_iSC_fIT_0$ (assays A14/A27) or $AP_iSC_eIT_0$ (assays A24/A31), sharply decreased the maximal levels achieved for FOS concentration (18.2 ± 9.0 and 8.7 ± 0.1 g.L^{-1}), purity (15.2 ± 7.2 and 8.4 ± 0.2% (w/w)), and productivity (0.3 ± 0.1 and 0.2 ± 0.1 $g_{FOS}.L^{-1}.h^{-1}$) (Table 1).

An improvement in the global fermentation process has been reported when carrying out FOS production in shake flasks, while using immobilised whole cells in RPF carriers. A maximal FOS concentration of 110.3 g L^{-1}, with a yield of 0.6 $g_{FOS}\ g_{Sucrose}^{-1}$ was reported for fermentations run with a monoculture of *A. japonicus* cells immobilised in RPF, based on an initial sucrose concentration of 200 g.L^{-1} [19]. A maximal FOS concentration of 108.2 ± 8.8 g.L^{-1} with a purity of 43.2 ± 0.4% (w/w), yield of 0.52 ± 0.05 $g_{FOS}\ g_{Sucrose}^{-1}$ and productivity of 4.3 ± 1.5 $g_{FOS}\ L^{-1}\ h^{-1}$ was obtained for fermentations run with a monoculture of *A. pullulans* immobilised in RPF carriers [17]. In the present work, using a co-culture strategy, a higher purity of FOS was reached with similar amount of FOS produced, but the FOS yield and productivity achieved were slightly lower.

Taking into consideration the results obtained in shake flasks, it is possible to summarise that in order to improve FOS parameters of concentration, purity, yield and productivity, immobilised *A. pullulans* should be used in co-culture with encapsulated *S. cerevisiae*, inoculated after 10 or 20 h, or with free *S. cerevisiae* inoculated after 20 h. In this context, the fermentation strategies selected to scale-up production in a bioreactor were: $AP_iSC_fIT_{20}$, $AP_iSC_eIT_{20}$ and $AP_iSC_eIT_{10}$.

FOS production in a bioreactor

Fig. 1 shows FOS production and sucrose consumption kinetics throughout the fermentations carried out in a bioreactor for the co-culture strategies selected. The control fermentation, conducted with a mono-culture of *A. pullulans* immobilised in RPF (AP_i) is also shown. FOS synthesis and sucrose consumption profiles were identical for all co-culture fermentations. The monoculture AP_i achieved higher amounts of FOS in the final hours of fermentation, with a higher amount of residual sucrose (squares), compared to the co-culture strategies. Sucrose consumption was faster using co-culture strategies, suggesting that both microorganisms were simultaneously using the substrate available in the medium. In this fermentation, the concentrations of immobilized and free cells were, respectively 2.4 ± 0.2 $g_{immob\ cells}\ g_{carrier}^{-1}$ and 0.5 ± 0.1 g L^{-1}.

Table 2 summarises the values obtained for the maximal FOS parameters using the different strategies. The percentage of each small saccharide in the mixture is also shown for the maximal FOS production time, and after 63 h of fermentation. The higher maximal FOS concentrations were obtained using immobilised *A. pullulans* in mono-culture, AP_i, 122 ± 4 g L^{-1}, and in co-culture with encapsulated *S. cerevisiae* inoculated at 10 h fermentation, $AP_iSC_eIT_{10}$, 119 ± 1 g L^{-1} (Table 2). For the co-culture fermentations where free ($AP_iSC_fIT_{20}$) and encapsulated ($AP_iSC_eIT_{20}$) *S. cerevisiae* was inoculated after 20 h, a

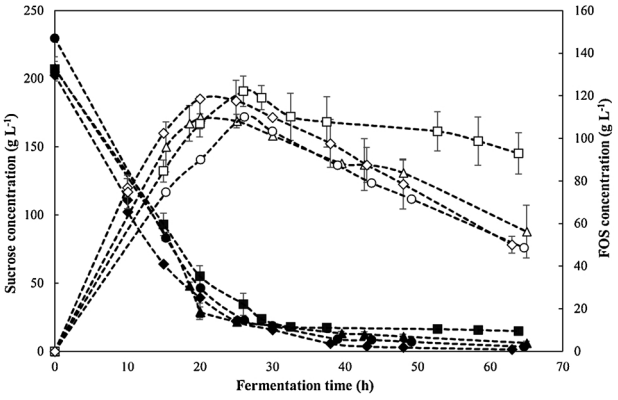

Fig. 1. Evolution of sugar concentration in each fermentation run performed in a bioreactor: sucrose (full symbols) and fructo-oligosaccharides (empty symbols); using a mono-culture of AP_i (squares) and co-cultures of $AP_iSC_fIT_{20}$ (circles), $AP_iSC_eIT_{20}$ (triangles), and $AP_iSC_eIT_{10}$ (diamonds).
AP_i – mono-culture of *A. pullulans* cells immobilised in reticulated polyurethane foam (RPF).
$AP_iSC_fIT_{20}$ – *A. pullulans* immobilised in RPF in co-culture with free *S. cerevisiae* inoculated after 20 h of fermentation.
$AP_iSC_eIT_{20}$ – *A. pullulans* immobilised in RPF in co-culture with *S. cerevisiae* encapsulated in calcium-alginate beads inoculated after 20 h of fermentation.
$AP_iSC_eIT_{10}$ – *A. pullulans* immobilised in RPF in co-culture with *S. cerevisiae* encapsulated in calcium-alginate beads inoculated after 10 h of fermentation.

slightly lower FOS concentration was obtained, 110 ± 1 g L^{-1} for both.

A longer fermentation time was needed to achieve maximal FOS concentration for strategies AP_i and $AP_iSC_fIT_{20}$ (25 h for both), compared to $AP_iSC_eIT_{20}$ and $AP_iSC_eIT_{10}$ (20 h for both). Subsequently, lower productivity levels were found for AP_i and $AP_iSC_fIT_{20}$ (4.9 ± 0.2 and 4.4 ± 0.1 g_{FOS} L^{-1} h^{-1}, respectively), compared to $AP_iSC_eIT_{20}$ (5.5 ± 0.1 g_{FOS} L^{-1} h^{-1}) and $AP_iSC_eIT_{10}$ (5.9 ± 0.1 g_{FOS} L^{-1} h^{-1}). Among the four strategies carried out in bioreactor, APi and APiSCeIT10 provided higher values for FOS concentration (122 ± 4 and 119 ± 1 g L^{-1}) and yield (0.59 ± 0.05 and 0.59 ± 0.01 g_{FOS} $g_{glucose}^{-1}$), as compared to the other two co-cultures, namely APiSCfIT20 (concentration: 110 ± 1 g L^{-1}; yield: 0.48 ± 0.01 g_{FOS} $g_{glucose}^{-1}$) and APiSCeIT20 (concentration: 110 ± 1 g L^{-1}; yield: 0.53 ± 0.01 g_{FOS} $g_{glucose}^{-1}$). Differences found were statistically significant (p-value < 0.05).

Monoculture AP_i provided similar maximal FOS concentration (122 ± 4 g L^{-1}) and yield (0.59 ± 0.05 g_{FOS} $g_{sucrose}^{-1}$), compared to that reported in our previous work (118.6 ± 1.6 g L^{-1} and 0.63 ± 0.03 g_{FOS} $g_{sucrose}^{-1}$, respectively), where free *A. pullulans* was used in mono-culture under the same fermentation conditions [3]. However, the time needed to achieve the maximal FOS production was lower using free *A. pullulans* cells, namely 20 h. The immobilisation of the cells seemed to increase the mass transference resistance resulting in a lower access of cells to the substrate. Consequently, for the fermentations run with free *A. pullulans*, slightly higher productivities were achieved (5.8 ± 0.2 g_{FOS} L^{-1} h^{-1}) compared to those achieved with immobilised cells (4.7 ± 0.9 g_{FOS} L^{-1} h^{-1}). Besides the lower productivities obtained when cells were attached to a carrier, this strategy allows the colonised carriers to be reused in successive batch or continuous fermentations, decreasing overall operational costs and time related to the maintenance and cultivation of the microorganisms.

The co-culture strategies where yeast cells were added after 20 h ($AP_iSC_fIT_{20}$ and $AP_iSC_eIT_{20}$) yielded lower FOS production performances. The diminution of FOS production when using the mixed cultures has already been reported in previous studies. It was suggested [3,25] that this could result from competition of both microorganisms for the substrate sucrose. They also suggest that this could be because of the presence of yeast extract in the medium, added to boost *S. cerevisiae* growth, as this can decrease fructofuranosidase (FFase) and fructosyltransferase (FTase) activities and, as a consequence, decrease FOS production [3,25]. Besides the typical decrease of FOS production using co-culture strategies, the inoculation of encapsulated *S. cerevisiae* cells at 10 h of *A. pullulans* fermentation improved FOS concentration, purity, yield and productivity, with similar values to those obtained in the mono-cultures using immobilised (APi) and free *A. pullulans* [25]. This suggests that the yeast cells exclusively consumed the glucose in the medium, without affecting sucrose transformation into FOS. The glucose produced as a result of the transfructosylation activity of FFase is simultaneously metabolised by *S. cerevisiae* to produce carbon dioxide and ethanol. As a result, besides having lower FOS concentration, yield and productivity, a higher FOS purity is reached at the end of the process (63 h fermentation), namely 66.6 ± 2.0% (w/w). It was reported that the metabolic behaviour of the yeast strains depends on the sugar composition in the mixture [26]. Thus, in a medium with a low glucose level, *S. cerevisiae* expresses the SUC2 gene, encoding invertase, which is responsible for the hydrolysis of sucrose into glucose and fructose [26]. On the other hand, in a medium with high glucose, the SUC2 is repressed and the yeast starts consuming mainly glucose instead of sucrose. In order to better understand the impact of both microorganisms on the fermentation mixture, the use of a yeast strain with a repressed SUC2 gene, such as *S. cerevisiae* YIL162 W (as reported by [14]), was suggested [26].

After 63 h of co-culture APiSCeIT10, an ethanol concentration of about 58 ± 5 g L^{-1} was obtained as a result of glucose transformation. The presence of this additional added value compound also decreases FOS purity in the final mixture, although, it can be easily recovered from the fermentation medium through reduced pressure distillation or

Table 2
Fructo-oligosaccharides production using the different fermentation strategies.

Fermentation	Strategy	Time (h)[a]	FOS (g L^{-1})	Yield (g_{FOS}·$g_{Sucrose}^{-1}$)	Q_p (g_{FOS}·L^{-1} h^{-1})	% FOS (w/w)[b]	% F (w/w)[b]	% G (w/w)[b]	% GF (w/w)[b]
Mono-culture	AP_i	25[c]	122 ± 4	0.59 ± 0.05	4.9 ± 0.2	53.5 ± 1.9	4.9 ± 0.8	26.6 ± 1.4	15.0 ± 2.6
Co-culture	$AP_iSC_fIT_{20}$	25[c]	110 ± 1	0.48 ± 0.01	4.4 ± 0.1	57.2 ± 1.1	7.5 ± 1.5	23.4 ± 3.7	11.8 ± 2.3
	$AP_iSC_eIT_{20}$	20[c]	110 ± 1	0.53 ± 0.01	5.5 ± 0.1	52.9 ± 1.4	6.6 ± 0.7	26.7 ± 0.2	13.8 ± 0.9
	$AP_iSC_eIT_{10}$	20[c]	119 ± 1	0.59 ± 0.01	5.9 ± 0.1	54.9 ± 0.2	4.2 ± 0.5	22.7 ± 0.9	18.3 ± 1.0
		63	50 ± 3	0.25 ± 0.02	0.8 ± 0.1	67.0 ± 2.0	23.4 ± 2.1	7.6 ± 0.1	2.0 ± 0.1

F – Fructose; G – Glucose; GF – Sucrose; FOS – Fructo-oligosaccharides; Q_p – Productivity.
$AP_iSC_fIT_{20}$ – *A. pullulans* immobilised in RPF in co-culture with free *S. cerevisiae* inoculated after 20 h of fermentation.
$AP_iSC_eIT_{20}$ – *A. pullulans* immobilised in RPF in co-culture with *S. cerevisiae* encapsulated in calcium-alginate beads inoculated after 20 h of fermentation.
$AP_iSC_eIT_{10}$ – *A. pullulans* immobilised in RPF in co-culture with *S. cerevisiae* encapsulated in calcium-alginate beads inoculated after 10 h of fermentation.
[a] Fermentation time.
[b] On a dry weight basis.
[c] Fermentation time for maximal FOS production.

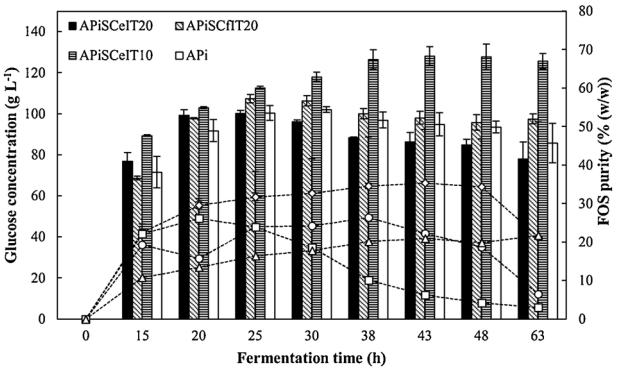

Fig. 2. Evolution of glucose concentration (lines) and purity of fructo-oligosaccharides (bars) in fermentation runs performed in a bioreactor using: a mono-culture of AP_i (control) (squares); and co-cultures of $AP_iSC_fIT_{20}$ (circles), $AP_iSC_eIT_{20}$ (triangles), and $AP_iSC_eIT_{10}$ (diamonds).
AP_i – mono-culture of *A. pullulans* cells immobilised in reticulated polyurethane foam (RPF).
$AP_iSC_fIT_{20}$ – *A. pullulans* immobilised in RPF in co-culture with free *S. cerevisiae* inoculated after 20 h of fermentation.
$AP_iSC_eIT_{20}$ – *A. pullulans* immobilised in RPF in co-culture with *S. cerevisiae* encapsulated in calcium-alginate beads inoculated after 20 h of fermentation.
$AP_iSC_eIT_{10}$ – *A. pullulans* immobilised in RPF in co-culture with *S. cerevisiae* encapsulated in calcium-alginate beads inoculated after 10 h of fermentation.

a simple evaporation at moderate temperatures, before FOS purification [13].

FOS purity in the bioreactor fermentation mixture

The sugar composition of the fermentation medium at the maximal FOS production time was similar for the mono- (AP_i) and co-culture strategies ($AP_iSC_fIT_{20}$, $AP_iSC_eIT_{20}$, $AP_iSC_eIT_{10}$) (see Table 2). At the maximal FOS production time, the average purities of fructose, glucose, sucrose and FOS determined were: 5.8 ± 1.6, 25.1 ± 2.4, 14.9 ± 3.1 and 54.7 ± 2.0% (w/w) respectively. Besides the similar values at the maximal FOS concentration time for the studied strategies, the time evolution of each sugar was different. The fructose percentage in the medium increased until the end of the fermentation process because of the hydrolytic activity of the fructofuranosidases. The time evolution of the FOS purity and glucose amounts for 4 fermentation strategies are shown in Fig. 2. For the mono-culture (AP_i), the glucose concentration continuously increased over the entire fermentation process, as a result of FFase and FTase activities, reaching a concentration higher than 74 $g.L^{-1}$ at 63 h. At this time, the composition in sugars determined was: 7.1 ± 0.3% (w/w) of sucrose, 9.8 ± 1.5% (w/w) of fructose and 38.8 ± 3.1% (w/w) of glucose.

In co-culturing strategies, a decrease in glucose percentage was observed throughout fermentations, particularly in the $AP_iSC_eIT_{10}$. In co-cultures $AP_iSC_fIT_{20}$ and $AP_iSC_eIT_{20}$, glucose concentration diminished after 38 and 48 h, respectively, showing that the encapsulation of the *S. cerevisiae* delayed the transformation of glucose to ethanol and carbon dioxide. On the other hand, in $AP_iSC_eIT_{10}$, glucose released to the medium occurred mainly after 25 h fermentation (Fig. 2). After 63 h, the sugar composition in this co-culturing fermentation was 2.0 ± 0.1% (w/w) of sucrose, 23.4 ± 2.1% (w/w) of fructose and 7.6 ± 0.1% (w/w) of glucose (Table 2). The percentages of sucrose and glucose in co-culture $AP_iSC_eIT_{10}$ were much lower compared to the mono-culture (APi). Sucrose reduction results not only from its consumption by the *S. cerevisiae*, but also by the recovery of the fructofuranosidase activity of the *A. pullulans* [12], while glucose is converted through glucose oxidase activity to ethanol and carbon dioxide [10,11]. Lower residual sucrose amounts can facilitate further purification steps. Sucrose is the sugar with chemical structure more similar to FOS, thus it is also the most difficult to remove using the traditional purification processes, such as nano-filtation and chromatography [6]. Using the APiSCeIT10 strategy, the residual sucrose at the end of fermentation diminished compared to a previous study where microorganisms were free in the culture and inoculated simultaneously [3].

The reduction of glucose and sucrose led to a purer FOS mixture with 67.0 ± 2.0% (w/w) of FOS, after 63 h fermentation. Statistical analysis showed that from 30 h fermentation, no significant improvements were found in FOS purity (p-value > 0.05), although the percentage of glucose and sucrose after 30 h fermentation significantly decreased (p-value < 0.05) in all co-culture fermentations. In this context, in order to facilitate the downstream purification, it would be more interesting to collect a FOS mixture in a fermentation time with lower amounts of glucose and sucrose than fructose, since the latter is easier to separate from FOS using liquid chromatography or nanofiltration processes [8].

In a previous report, *A. japonicus* and *P. heimii*, immobilized in calcium-alginate beads, were used to produce FOS, in a three tanks-in-series, run in continuous mode. As advantages, *P. heimii* used does not compete for the sucrose. FOS purities achieved in the first, second and third tanks were 70.2%, 95.6% and 98.2% (w/w), respectively [13]. In the present work, concerning only one tank reactor, similar values of purity were achieved as in the first tank, 67 ± 2% (w/w), using the APiSCeIT10 strategy.

Others have reported the use of a successive cultivation of *Pichia pastoris* in a FOS-mixture medium previously synthesized by extracellular β-fructofuranosidase from *Aspergillus japonicus* [12]. Results showed that glucose was completely exhausted and the final purity of FOS was about 93.8% (w/w). Although high values of FOS purity were achieved, a previous production and extraction of the enzymes is needed, and in addition the production and purification process is carried out separately in two-steps, which may increase the overall cost and time of the process, as compared to the integrated system presented here. In this context, we consider that $AP_iSC_eIT_{10}$ can be an interesting efficient strategy to obtain purer FOS mixtures in one single tank.

Conclusions

Different co-culturing strategies have been studied, using *A. pullulans* and *S. cerevisiae*, to increase purities of FOS mixtures produced. The strategies performed in shake flask that showed improved FOS concentration, purity, yield and productivity consisted of *A. pullulans* immobilized to polyurethane foam in co-culture with *S. cerevisiae*: i) free cells added after 20 h fermentation; ii) encapsulated in calcium-alginate beads inoculated after 10 h fermentation; and iii) encapsulated in Ca-alginate beads inoculated after 20 h fermentation. These strategies were tested in a bioreactor (5 L). Immobilised *A. pullulans* in co-culture with encapsulated *S. cerevisiae,* inoculated after 10 h fermentation, was the best approach to obtain purer FOS mixtures with high FOS concentration (˜ 119 g L^{-1}), good yield (˜ 0.59 g_{FOS} $g_{sucrose}^{-1}$) and FOS productivity (˜ 5.9 g_{FOS} L^{-1} h^{-1}). With this strategy, a final sugar mixture containing about 67% (w/w) FOS, 23% (w/w) fructose, 8% (w/w) glucose and 2% (w/w) sucrose was provided after 63 h fermentation. Furthermore, the glucose released was biotransformed by *S. cerevisiae* into ethanol, an added value by-product. These prepurified mixtures containing high levels of FOS may improve further separation and purification of FOS, since the amounts of sucrose and the other non-prebiotic small saccharides in the mixtures are almost residual.

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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.nbt.2019.01.009.

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