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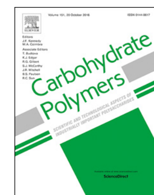

One-step co-culture fermentation strategy to produce high-content fructo-oligosaccharides

C. Nobre*, D.A. Gonçalves, J.A. Teixeira, L.R. Rodrigues

CEB - Centre of Biological Engineering, University of Minho, 4710-057 Braga, Portugal

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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).

* Corresponding author.
E-mail addresses: clarissenobre@deb.uminho.pt (C. Nobre), danielalexandra94@hotmail.com (D.A. Gonçalves), jateixeira@deb.uminho.pt (J.A. Teixeira), lrmr@deb.uminho.pt (L.R. Rodrigues).

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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. cerevisae* 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} $NaNO_3$, 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} $FeSO_4.7H_2O$ and 200 g L^{-1} sucrose. 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 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:

$$FOS\ purity\ (\%) = \frac{m_{FOS}}{m_{total\ sugars}} x100 \tag{1}$$

where m_{FOS} represents the sum of the individual mass of FOS (kestose - GF_2, nystose - GF_3 and fructofuranosylnystose - GF_4) and $m_{total\ sugars}$ represents the sum of the total mass of sugars (FOS, sucrose, fructose and glucose).

An initial 2^3 central composite design (CCD), with three central points, was used for fermentation broth composition optimization, in which the three independent variables, $NaNO_3$, KH_2PO_4 and yeast extract concentrations, were coded to dimensionless ones X_1, X_2 and X_3, respectively, accordingly to the following equation:

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

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 2^2 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:

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

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_2PO_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 (ºC)	X_4	28.0	32.0	37.0
	pH	X_5	5.0	6.0	7.0

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