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Production of High-Content Fructo-Oligosaccharides

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Abstract**—**Fructo-oligosaccharides (FOS) are produced from sucrose by *Aureobasidium pullulans* in yields between 40-60% (w/w). To increase the amount of FOS it is necessary to remove the small, non-prebiotic sugars, present. Two methods for producing high-purity FOS have been developed: the use of microorganisms able to consume small saccharides; and the use of continuous chromatography to separate sugars: simulated moving bed (SMB). It is herein proposed the combination of both methods. The aim of this study is to optimize the composition of the fermentative broth (in terms of salts and sugars) that will be further purified by SMB. A yield of 0.63 $g_{FOS}.g_{Sucrose}^{-1}$ was obtained with *A. pullulans* using low amounts of salts in the initial fermentative broth. By removing the small sugars, *Saccharomyces cerevisiae* and *Zymomonas mobilis* increased the percentage of FOS from around 56.0% to 83% (w/w) in average, losing only 10% (w/w) of FOS during the recovery process.

Keywords**—**Fructo-oligosaccharides, microbial treatment, *Saccharomyces cerevisiae*, *Zymomonas mobilis*.

I. Introduction

IN the recent years the adoption of healthier lifestyles is being encouraged, and the demand of food with functional properties is increasing. Fructo-oligosaccharides (FOS) are non-digestible sugars, known to prevent and treat gastrointestinal disorders due to their prebiotic activity [1]. They have been used as low-calorie substitutes for sugar in dietetic and diabetic food and have also great technological properties as improving the organoleptic quality and shelf-life of the products [2], [3].

Industrially, FOS have been produced through sucrose fermentation by microorganisms' fructosyltransferases enzymes (FTase), extracted from, for example, *Aureobasidium* sp. or *Aspergillus* sp. Industrially the process involves two stages, one for the production of enzymes followed by the other for the FOS synthesis by the extracted enzymes [4]. A more economical and fast process is being recently applied for FOS synthesis, involving a single step where the whole microorganisms' cells, suspended or immobilized, are used [5]. Maximum theoretical FOS yield achieved by fermentation process is however still limited due to glucose inhibition. By using *Aureobasidium pullulans* cells, a maximum of 64% was achieved [6], while for the two-stages fermentation, this yield varies between 40 to 60% based on the initial sucrose concentration [7].

During FOS synthesis by microbial enzymes, non-oligosaccharides as fructose, glucose and sucrose are released in the mixture, decreasing the prebiotic activity of the final mixture. To include FOS produced into different dietetic and diabetic related foods, a downstream purification step is needed.

Ultra or nanofiltration, activated charcoal systems, microbial treatment and ion-exchange chromatography are some of the techniques more used for sugars separation [8], [9]. At industrial scale, chromatographic processes such as Simulated Moving Bed (SMB) have been employed, with success, in difficult separations of sugars [10]. Compared to other separation techniques, SMB has the advantage of operating in continuous mode using water as eluent [11], [12]. FOS have been successfully separated from smaller sugars, when using cationic resins such as Diaion UBK535Ca in calcium form [10], [13]. Since these resins have cations as functional groups, it is important to demineralize the liquid mixture before feeding it to the SMB plant to avoid the ionic exchange between the mixture and the adsorbent.

Obtaining high-purified FOS through SMB is stated in only few reports and it is still a challenge, mainly due to the physicochemical similarities between the different oligosaccharides and small saccharides [8]. In this context, different authors studied the impact of the continuous removal of the small saccharides from the medium during FOS synthesis [14], either using an extracted β-fructofuranosidase for FOS synthesis and *Pichia pastoris* cells for removing the small sugars [15], or using a mixture of enzymes, one FOS producer and other able to consume small sugars without FOS hydrolyze activity [14], [16], [17]. In this work it is proposed the use of the whole cells of *A. pullulans* to produce FOS and *Saccharomyces cerevisiae* and *Zymomonas mobilis* able to ferment glucose, fructose and sucrose into alcohols and organic acids, with ethanol as the primary product [18], [19].

The present study is divided in two main tasks: 1) The optimization of salt composition in the fermentative broth for FOS production by *A. pullulans* cells; 2) The use of two different microorganisms, namely *S. cerevisiae* or *Z. mobilis*, for non-oligosaccharides reduction in the fermentative mixture. The process consists in two series fermentation where first FOS are produced by *A. pullulans* and secondly small sugars are reduced by *S. cerevisiae* or *Z. mobilis*.

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The nutritional needs of *S. cerevisiae* and *Z. mobilis* were taken in consideration and the fermentative broth composition was also optimized for these cultures.

II. Materials and Methods

A. Microorganisms and Culture Conditions

Aureobasidium pullulans strain was kept on Petri plates containing Czapeck Dox Agar (Oxoid, UK) medium at 4ºC and was monthly subcultured. The spores suspension was prepared by growing the fungus in Petri plates at 28ºC and after 5 days spores were scraped down from the plates using a 0.1% (w/v) solution of Tween 80 (Panreac, AppliChem, Spain). The suspension was diluted to a concentration of 1 x 10^7 spores.mL^{-1}, based on Newbauer chamber counts.

Saccharomyces cerevisiae 11982 and *Zymomonas mobilis* ATCC 29191 strains were grown in YEG (yeast extract-glucose) culture medium, containing 5 g.L^{-1} yeast extract and 20 g.L^{-1} glucose (both from Fluka, Germany), during 24 hours, at 30ºC and 150 rpm agitation. The strains were maintained by transferring every month to fresh YEG agar plates and stored at 4ºC after incubation at 30ºC for 5 days.

B. Experimental Design for Fermentation Broth Optimization

The salt composition of the *A. pullulans* fermentation broth was optimized. The impact of the reduction of two relevant salts, $NaNO_3$ and KH_2PO_4, frequently used in increased concentrations, was studied using the Response Surface Method (RSM). Eleven independent assays were performed, regarding the impact on the maximization of FOS production.

Superior, central and inferior concentrations were considered: 5.00, 12.50 and 20.00 g.L^{-1} for $NaNO_3$ and 4.00, 6.00 and 8.00 g.L^{-1} for KH_2PO_4. Significant positive effects were considered for the reported *p-values* lower than 0.05. The statistical experimental design was generated and evaluated using the JMP^{TM} – The Statistical Discovery Software.

C. Shaken-Flasks Fermentations for FOS Production

Erlenmeyer flasks of 100 mL with test tube aluminum caps were used. An aliquot of 1 mL of *A. pullulans* spores suspension with 1 x 10^7 spores.mL^{-1}, was transferred to 50 mL of fermentation medium, containing: 200 g.L^{-1} sucrose, 0.5 g.L^{-1} KCl, 0.35 g.L^{-1} K_2SO_4, 0.5 g.L^{-1} $MgSO_4.7H_2O$, 0.01 g.L^{-1} $FeSO_4.7H_2O$, and optimized concentrations of $NaNO_3$ and KH_2PO_4. All salts were obtained from VWR (Belgium). Chemicals used were of analytical grade, except sucrose used for FOS synthesis, which was a commercial sugar obtained by Raffinerie Tirlemontoise, S.A., Belgium.

The pH of the culture medium was adjusted for 5.5 before inoculation and fermentations were performed at 28°C with 150 rpm agitation. Several samples were taken at different points in time to evaluate sugars profile.

D. Bioreactor Fermentations for FOS Production

An aliquot of *A. pullulans* spores suspension with 1 x 10^7 spores.mL^{-1} was transferred to 100 mL of inoculum medium containing 100 g.L^{-1} sucrose and the same salt concentrations as the ones used in shaken-flask fermentations for FOS production. The inoculum was grown at 28°C and 150 rpm and transferred after 3 days to a 5 L bioreactor – BIOSTAT® B module (Sartorius, Germany), using a working volume of 3 L of culture medium (200 g.L^{-1} sucrose and the same salt concentrations as the ones used in the inoculum). Fermentations were carried out at 32°C and 385 rpm with a fixed pH of 5.5.

E. Non-Oligosaccharides Removal

The ability of two microorganisms, *S. cerevisiae* and *Z. mobilis*, for mono- and disaccharides removal, was evaluated, using a two-stage process. FOS were synthesized in a first fermentation by *A. pullulans*, in a 5 L bio-reactor. The synthesis reaction was stopped in the maximum FOS concentration point and the biomass removed by filtration with cellulose acetate filters (VWR, Belgium) with a cut-off of 0.2 μm.

The filtered broth was used for the subsequent fermentation and inoculated with 1 mL of *S. cerevisiae* or *Z. mobilis* cells (with an optical density of 1). Yeast extract was added to the second fermentation to obtain a final concentration of 5 g.L^{-1}. Shaken-flask fermentations were carried out at 30°C and 150 rpm agitation with an initial pH of 5.5.

F. Sugars Analysis

Samples were analysed by HPLC (Jasco) equipped with a refractive index detector working at 30ºC and a Prevail Carbohydrate ES 5u column (5 μm, 25 x 0.46 cm length x diameter) (Alltech). A mixture of acetonitrile (HPLC Grade, Carlo Erba, France) in pure-water (70:30 v/v), and 0.04% of ammonium hydroxide (HPLC Grade, from Sigma, Germany) was used as mobile phase. Elution was conducted at 1 mL.min^{-1} flow rate and room temperature [20], [21]. The chromatographic signal was recorded and further integrated using the Star Chromatography Workstation software (Varian, USA).

FOS standards, namely 1-kestose (GF_2), nystose (GF_3) and 1-fructofuranosylnystose (GF_4) were acquired from Wako (*Chemicals GmbH*, Japan). Sucrose (GF) and fructose (F) standards were obtained from Merck (USA) and glucose (G) from VWR (Belgium). All chemicals were of analytical grade.

G. Statistical Analysis

Fermentation experiments were carried out in triplicate. Statistical data analysis was performed using analysis of variance and Tukey's HSD test at a 5% level of significance.

TABLE I
RESPONSE SURFACE METHOD FOR STUDYING THE IMPACT OF THE INITIAL FERMENTATION BROTH COMPOSITION: THE EXPERIMENTAL CONDITIONS AND RESPONSES OBTAINED DURING SALT OPTIMIZATION

	A	B	Fermentation time (h)	FOS ($g.L^{-1}$)	%FOS (w/w)	Yield ($g_{FOS}.g_{Sucrose}^{-1}$)	Q_p ($g_{FOS}.L^{-1}.h^{-1}$)
A1	5.00	8.00	47.83	101.32	48.90	0.54	2.12
A2	20.00	4.00	53.50	91.51	43.50	0.46	1.71
A3*	12.50	6.00	53.50	103.67	48.10	0.53	1.94
A4	5.00	4.00	47.83	95.42	50.80	0.50	1.99
A5	5.00	6.00	53.50	95.05	49.60	0.48	1.78
A6	12.50	8.00	53.50	101.85	49.80	0.51	1.90
A7*	12.50	6.00	47.83	104.31	50.60	0.53	2.18
A8	20.00	6.00	47.83	105.68	49.30	0.53	2.21
A9	12.50	4.00	53.50	105.61	49.70	0.53	1.97
A10	20.00	8.00	53.50	103.00	47.00	0.52	1.93
A11*	12.50	6.00	53.50	99.89	49.00	0.50	1.87

A – $NaNO_3$ ($g.L^{-1}$); B – KH_2PO_4 ($g.L^{-1}$); *Central points

III. RESULTS AND DISCUSSION

A. Fermentation Broth Composition Optimization for FOS Production

A microbial treatment to increase the amount of FOS in relation to other sugars in FOS mixtures obtained by fermentation is proposed in the present study. Mixtures are intended to be further fed in SMB chromatographic plant for refined purification. A two-stage fermentation strategy is herein proposed using a strain able to consume the remaining small sugars from the FOS mixtures, synthesized by fermentation with *A. pullulans.*

To avoid the cation exchange between the liquid mixture and the adsorbent, the effect of decreasing salt amount in the fermentative broth composition for FOS production was evaluated. Different concentrations of two salts normally present in higher concentrations in the fermentative broth composition, adapted from [6], $NaNO_3$ and KH_2PO_4, were tested. Results obtained for the study of the impact of different initial salt concentrations identified by an experimental design are shown in Table I.

Based on the statistical analysis, the tested concentrations of $NaNO_3$ (5.00, 12.50 or 20.00 $g.L^{-1}$) and KH_2PO_4 (4.00, 6.00 or 8.00 $g.L^{-1}$) did not affect significantly FOS production ($p< 0.05$).

In average, the maximum concentration of FOS (102 ± 3 $g.L^{-1}$) was obtained at 54 ± 2 h of fermentation in shaken-flasks. Average fermentation yield was 0.52 ±0.01 g_{FOS}/g_{GF} with an amount of 49 ± 1% of FOS in total sugars. No statistical difference was found in FOS production when diminishing salts contained in the fermentative broth, the concentrations of 5.00 $g.L^{-1}$ $NaNO_3$ and 4.00 $g.L^{-1}$ KH_2PO_4, were used in the two-stage fermentations for FOS production. The decrease of the $NaNO_3$ and KH_2PO_4 amounts from 20 to 5 $g.L^{-1}$ and 7.89 to 4 $g.L^{-1}$, respectively, will have a positive impact in costs and time associated to demineralization procedures needed before feeding in the SMB plant. Also, the use of lower salt amounts reduces costs associated to the fermentation itself, resulting in an improved FOS producing process.

B. FOS Production in Bioreactor

Fermentations were scaled up to a 5 L bioreactor using a concentration of 5.00 $g.L^{-1}$ of $NaNO_3$ and 4.00 $g.L^{-1}$ of KH_2PO_4 since no statistical differences were found in FOS production when diminishing salts contained in the fermentative broth. Bioreactor fermentations results obtained are presented in Figs. 1 (a) and (b), and summarized in Table II.

TABLE II
BIOREACTOR FERMENTATION PARAMETERS USING THE OPTIMIZED FERMENTATION BROTH

	Average ± STD	[5]
Time (h)	20	43
FOS (%)	54.0 ± 1.6	NF
Yield (% $w_{FOS}/w_{Sucrose}$)	63.0 ± 3.2	64.1
Productivity (g/L.h)	4.8 ± 1.4	2.9
FOS (g/L)	118.6 ± 1.6	123.0
Yield ($g_{1\text{-}kestose}/g_{Sucrose}$)	0.378 ± 0.078	0.436
Yield ($g_{nystose}/g_{Sucrose}$)	0.231 ± 0.053	0.206

NF – Not found

The maximum concentration of FOS was verified after 20h of fermentation, where GF_2, GF_3 and GF_4 concentrations are 71.6±12.6, 43.8±11.2 and 3.4±22.4 $g.L^{-1}$, respectively (Fig. 1 (b)). In Fig. 1 (b) it is possible to observe that the maximal concentration of GF_2 is before 20h, and after this point, this sugars starts being transformed in GF_3, through transfructosylation reaction, according to (1) [22].

Under the optimized conditions used, the time needed to achieve the maximum FOS production was twice lower than in previous works, resulting in a process with much higher productivity (4.8 ± 1.4 compared to 2.9 $g_{FOS}.L^{-1}.h^{-1}$) (Table II).

$$GF_n + GF_n \rightarrow GF_{(n-1)} + GF_{(n+1)}, \; n=1, 2 \text{ or } 3 \qquad (1)$$

The yield and FOS concentration in bioreactor (Table II) are similar to those obtained in previous works using the whole cell of *A. pullulans* at analogous operational conditions of temperature, pH and agitation, though with higher concentrations of salts [6], [9]. Also, in the present work, a

yield of 63.0 ± 3.3% (w/w) of FOS was achieved, for a one-step fermentation, working with the whole cells.

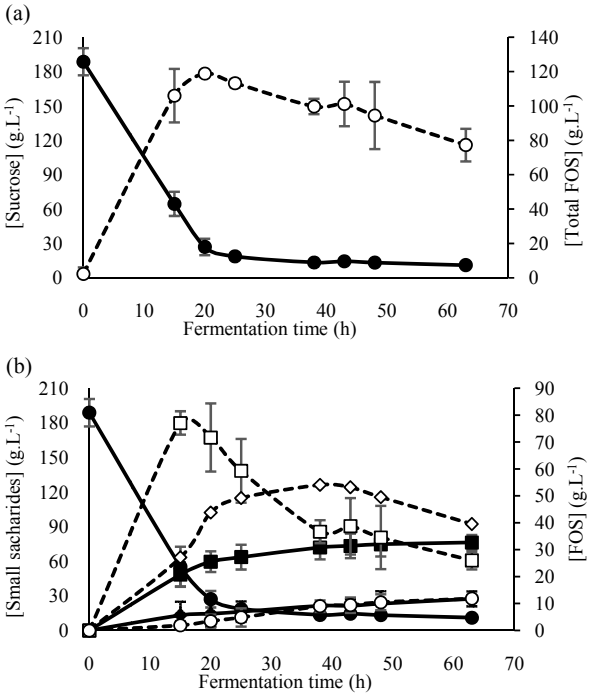

Fig. 1 Bioreactor fermentation profiles under the optimized fermentation broth: (a) - ● Sucrose, ○ Total FOS; (b) – Sugars profile: ● Sucrose; ■ Glucose; ♦ Fructose; □ GF_2; ◊ GF_3; and ○ GF_4. Full and dashed lines correspond to primary and secondary axes, respectively

C. Non-Oligosaccharides Removal by Two-Stage Fermentation

S. cerevisiae and *Z. mobilis* can be conveniently used to remove mono- and disaccharides that constitute by-products found in enzymatic preparations of FOS. On the other hand, β-linked saccharides, such as 1-kestose (GF_2), nystose (GF_3) and fructo-furanolysnystose (GF_4), are not hydrolyzed. A two-stage fermentation strategy was evaluated to increase the percentage of fructo-oligosaccharides in relation to other sugars in a mixture. FOS were firstly produced by *A. pullulans* in bioreactor (using the optimized medium) and then purified in a second fermentation through small saccharides reduction by *S. cerevisiae* and *Z. mobilis*.

The FOS synthesis fermentation with *A. pullulans* was stopped at 20 h fermentation, where the maximal concentration of FOS was achieved, as shown in Fig. 1 (a) and Table II. After *A. pullulans* removal, a concentrated solution of yeast extracted was added to the fermentative broth to enrich the nutritional composition of the medium, allowing *S. cerevisiae* or *Z. mobilis* growth (since both strains showed to not grow in the final mixture produced from *A. pullulans* containing FOS and a high concentration of glucose). These second fermentations were performed in 100 mL shaken flasks containing 50 mL of filtrated medium where 5 $g.L^{-1}$ were added.

Fig. 2 presents the results obtained for the second fermentation with *S. cerevisiae* (full bar and line) and *Z. mobilis* (dashed bar and line). Comparing the performance of both strains, no statistical differences were found. Both strains could remove small saccharides (SGF) including sucrose, glucose and fructose (light grey bars), without decreasing the total amount of FOS until 25h of fermentation. After this point a slight decrease of the FOS concentration (dark grey bars) occurs, around 10% until 43h of the second fermentation, as FOS begin to be hydrolysed. On the other hand, the percentage of FOS increases around 13% during time, while the small saccharides are being consumed by both *S. cerevisiae* and *Z. mobilis.*

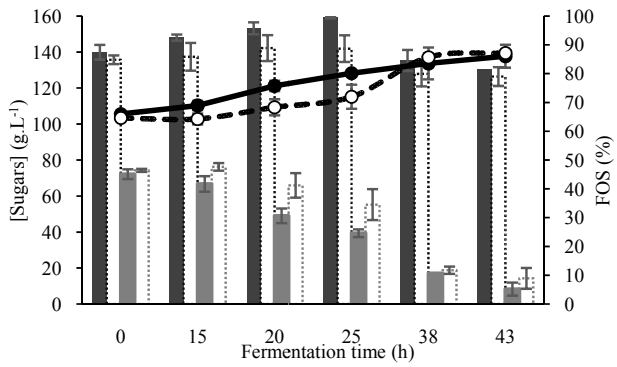

Fig. 2 FOS production profiles through second fermentation serie carried out in shaken-flasks using *S. cerevisiae* (full bars and symbol) and *Z. mobilis* (empty bars and symbol): FOS concentration (dark grey bars); SGF concentration (light grey bar); and % FOS (black lines). FOS and SGF correspond to primary axe and % FOS to secondary

In Fig. 3 it is possible to observe the evolution of the sugars throughout the fermentation time. Both oligosaccharides, GF_2 and GF_3 verify an increase until 25h of fermentation, meaning that, after filtration, the *A. pullulans* enzymes released in the broth proceeded the synthesis activity and sucrose hydrolysis. After 38h of fermentation in FOS-containing medium, kestose (GF_2) slightly decreased from 45.4±1.3 to 43.9±1.9 $g.L^{-1}$ and from 44.0±0.8 to 37.1±7.2 $g.L^{-1}$ as well as nystose (GF_3) that decreased from 45.1±1.5 to 39.4±5.2 $g.L^{-1}$ and 44.7±0.5 to 37.8±6.2 $g.L^{-1}$, with *S. cerevisiae* and *Z. mobilis,* respectively. On the other hand, for the same interval, there was a small increase on the concentration of fructofuranosylnystose in *S. cerevisiae* fermentation from 4.1±0.2 to 8.3±3.2 $g.L^{-1}$, and remained constant for *Z. mobilis* fermentation (around 3.0 $g.L^{-1}$). So, the small decrease on the total FOS produced concerns to the proportional reduction of GF_2 and GF_3 in the medium to produce GF_4 by (1).

In relation to glucose, a considerable decrease was verified in the medium from 46.6±1.0 to 1.9±0.5 $g.L^{-1}$ and from 48.0±0.6 to 4.3±0.5 $g.L^{-1}$, in the presence of *S. cerevisiae* and *Z. mobilis,* respectively, and fructose kept constant during time. This highlights the preference for consuming glucose compared to fructose by both strains. At the same time, there

was a reduction of the sucrose in the medium, which was residual after 38h of fermentation (around 2 $g.L^{-1}$).

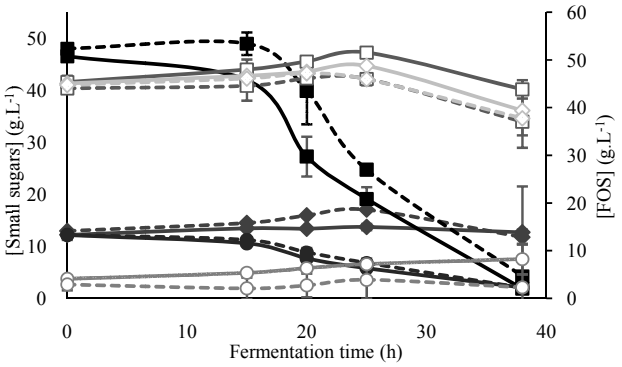

Fig. 3 Sugars profile for the second fermentation serie carried out in bioreactor with *S. cerevisiae* (full lines) and *Z. mobilis* (dashed lines): ■ Fructose; ● Glucose; ♦ Sucrose; □ GF_2; ○ GF_3; and ◊ GF_4

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The reduction of the small saccharides, sucrose and glucose, in the mixture by the two strains used, explains the increasing of the percentage of FOS obtained using the two-stage fermentation strategy.

The introduction of the second fermentation as a strategy for FOS production and purification modified the sugars composition of the mixture: % fructose (7.4 to 11.7 and 7.9 to 12.4), % glucose (28.1 to 1.7 and 29.1 to 4.5), % sucrose (7.4 to 1.9 and 7.4 to 2.2), % GF_2 (27.4 to 40.5 and 26.7 to 38.9), % GF_3 (27.2 to 36.4 and 27.1 to 39.6) and % GF_4 (2.5 to 7.5 and 1.8 to 2.4) in the presence of *S. cerevisiae* and *Z. mobilis,* respectively. According to the final mixture composition it is also expected an improvement of the SMB separation by enhancing the resolution of the chromatographic separation of FOS and small saccharides, based on their molecular weight: F > G > GF > GF_2> GF_3> GF_4 [10].

IV. Conclusion

The production and purification of FOS through a microbial treatment for further purification by SMB was proposed in the present work. The minimization of the salt amount in the fermentative broth did not affect the FOS production and showed to be a successful approach for a less expensive and more efficient process. The use a two-stage fermentation strategy increased the percentage of FOS in the mixture to 85 and 81%, in the presence of *S. cerevisiae* and *Z. mobilis*, respectively. The final mixture contained residual concentrations of small saccharides, namely glucose and sucrose in the presence of both strains, showing their similar performance under the same fermentation conditions in shaken flask.

The use of the two-stage fermentation strategy showed to be an interesting approach for FOS production and purification as it increased the percentage of FOS and reduced the percentage of small sugars in the final mixture, which can contribute for the improved efficiency of the purification process of FOS by SMB.

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