Production of Fructooligosaccharides by *Aspergillus phoenicis* Biofilm on Polyethylene as Inert Support

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Summary

Aspergillus phoenicis biofilms on polyethylene as inert support were used to produce fructooligosaccharides (FOS) in media containing 25 % (*m/V*) of sucrose as a carbon source. The maximum production of total FOS (122 mg/mL), with 68 % of 1-kestose and 32 % of nystose, was obtained in Khanna medium maintained at 30 °C for 48 h under orbital agitation (100 rpm). At high concentrations of sucrose (30 %, *m/V*), the recovery of FOS was higher than that observed at a low concentration (5 %, *m/V*). High levels of FOS (242 mg/mL) were also recovered when using the biofilm in sodium acetate buffer with high sucrose concentration (50 %, *m/V*) for 10 h. When the dried biofilm was reused in a fresh culture medium, there was a recovery of approx. 13.7 % of total FOS after 72 h of cultivation at 30 °C, and 10 % corresponded to 1-kestose. The biofilm morphology, analyzed by scanning electron microscope, revealed a noncompact mycelium structure, with unfilled spaces and channels present among the hyphae. The results obtained in this study show that *A. phoenicis* biofilms may find application for FOS production in a single-step fermentation process, which is cost-effective in terms of reusability, downstream processing and efficiency.

Key words: fructooligosaccharides, kestose, nystose, *Aspergillus* sp., biofilm

Introduction

The functional food industry has received considerable attention in recent years, moving billions of dollars in the global market. These foods can be made using different ingredients with functional characteristics, such as dietary fibre, sugar alcohols, prebiotics, probiotics and oligosaccharides (*1*). Oligosaccharides are very important in the dietary carbohydrate market, especially fructooligosacharides (FOS) (*2*), which are relatively sweet and therefore may be used as sweeteners, replacing sucrose in a number of food and pharmaceutical applications. FOS can be obtained from many kinds of plants like wheat, barley, asparagus and Jerusalem artichokes, in which they are found at low concentrations. They are known for their ability to improve health conditions by stimulating the growth of bifidobacterium microflora in the human colon, suppressing putrefactive pathogens that can cause diarrhoea, and decreasing serum cholesterol concentration (*3*). In addition, FOS are not metabolized in the human small intestine, due to the absence of an enzyme to hydrolyze their glycosidic linkages, which makes their energy value theoretically lower than that of sucrose (*4,5*). Consequently, FOS are currently added to pastry, confectionery and dairy products as functional food ingredients to improve consumers' health. The safety of the

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consumption of FOS has been documented in various studies (*5*).

Many microorganisms produce FOS, particularly bacteria and fungi (*2*), such as *Xanthophyllomyces dendrorhous* (*6*), *Aspergillus japonicus* (*7*), *Aspergillus oryzae* CFR 202 (*8*), and more recently, *Rhizopus stolonifer* (*9*). The fructosyltransferase (FTase) is responsible for microbial production of FOS from sucrose, starting with the synthesis of 1-kestose (GF$_2$), followed by 1-nystose (GF$_3$), and lastly 1-fructofuranosyl nystose (GF$_4$) (*2*). Generally, FTase from fungi is obtained by submerged or solid-state fermentation and is employed to produce FOS (*2*). However, a new fermentation process, *i.e.* biofilm fermentation, has been developed to produce biotechnologically attractive molecules, combining the advantages of solid-state fermentation with high productivity (*10*). The development of filamentous fungi in the natural environment occurs in intimate association with the substrate, which seems to be relevant for some important processes such as spore adhesion, germination and elongation of hyphal tubes to constitute the mycelium. During the formation of a biofilm on a polymeric inert support, the same processes occur, and the observed phenotypes result from differential gene expression, compared to free living conditions (*11*). In this context, the aim of this work is to study FOS production using *A. phoenicis* biofilms on polyethylene support as an alternative methodology to improve their production.

Materials and Methods

Microorganism

The filamentous fungus *Aspergillus phoenicis* (FJ810504) was isolated from sugar cane bagasse in São Paulo State, Brazil (*12*), identified by the André Tosello Foundation, Campinas, SP, Brazil, and maintained on slants of 4 % oatmeal (Quaker Brazil Ltda., São Paulo, Brazil) at 4 °C.

Biofilms, culture conditions and FOS production

A. phoenicis biofilms were developed on polyethylene inert supports (2.3×2.3 cm) previously washed with commercial detergent and water, dried at 40 °C and sterilized by UV exposure for 15 min. The sterilized supports were immersed in an aqueous spore suspension (10^5 spores/mL) at 30 °C under agitation (50 rpm) for 2 h for spore adhesion to the support. The supports were then rinsed twice with distilled water for 30 min under agitation (50 rpm) in order to remove the unadhered spores, and those with the spores were transferred to 150-mL Erlenmeyer flasks containing 50 mL of Segato Rizzatti (SR) (*13*), Adams (*14*), Khanna (*15*) or Vogel (*16*) culture media supplemented with 20–25 % (*m/V*) of sucrose as carbon source for direct production of FOS. These concentrations were used only to select the best medium for FOS production. The cultures were maintained at 30 °C for 72 h under agitation (100 rpm). FOS production was tested also by the immersion of the biofilm obtained from Khanna medium for 72 h in 50 mL of 100 mM sodium acetate buffer, pH=4.5, containing 50 % of sucrose (*m/V*), under the same conditions as above, to optimize the FOS recovery at high sucrose concentration. The formation of FOS was monitored after different time intervals (2–24 h). The time course of FOS production (24–96 h) and the influence of different concentrations of sucrose (5–30 %, *m/V*) in Khanna medium were analyzed.

Enzymatic assay

The free enzyme activity was determined using 2 % sucrose as substrate in 100 mM sodium acetate buffer, pH=4.5, and the released reducing sugars were quantified using dinitrosalicylic acid (DNS), as described by Miller (*17*). One activity unit was defined as the amount of enzyme necessary to produce 1 μmol of reducing sugars per minute under the assay conditions.

The experiments were performed in triplicate and the values were expressed as mean values±S.D.

Biomass determination and biofilm reutilization

After incubation, the biofilm was removed from the medium, blotted between two filter paper sheets, and dried in a stove at 40 °C for 48 h. The dry biomass was estimated as the difference between the biofilm and the inert support mass, and expressed in grammes (g). In order to verify the possibility of biofilm reuse for FOS production, the dried biofilm was immersed in a fresh Khanna medium with 20 % sucrose (*m/V*) as carbon source and maintained at 30 °C for 72 h under agitation (100 rpm). After that, the biofilm was removed and FOS were quantified as described in the HPLC analysis section below.

Scanning electron microscopy (SEM)

A. phoenicis biofilms were dehydrated with increasing concentrations of ethanol in water (10–90 %, by volume) and finally with absolute ethanol, sprayed with colloidal gold and submitted to SEM analysis using a Zeiss equipment EVO50 (Carl Zeiss, Oberkochen, Germany) with IXRF Systems 500 digital processing. The inert support and the biofilm were also photographed with a Sony Cyber-shot W110 digital camera (Sony, Tokyo, Japan).

HPLC analysis

Samples of culture media containing fructooligosaccharides were analyzed in a Shimadzu (Kyoto, Japan) high-performance liquid chromatograph (HPLC) equipped with a refractive index detector and an EC 250/4.6 Nucleosil® 100 NH$_2$ column (30×0.75 cm, Sigma-Aldrich, St. Louis, MO, USA), maintained at 40 °C. The mobile phase was 82 % (by volume) acetonitrile and the standards employed were sucrose (Mallinckrodt, Inc., St. Louis, MO, USA), fructose and glucose (Merck, Darmstadt, Germany), and 1-kestose and nystose (Fluka Chemie, Steinheim, Germany).

Results and Discussion

Morphological pattern of the biofilms

The morphological pattern of *A. phoenicis* biofilms can be observed in Fig. 1. After 2 h of contact with the polyethylene support, the spores were efficiently adhered, and after 72 h of fermentation, the mycelia completely covered the support (Figs. 1b and 1c). The first step in the adhesion of the spores to the substrate depends on the production of adhesive substances to form a pad beneath
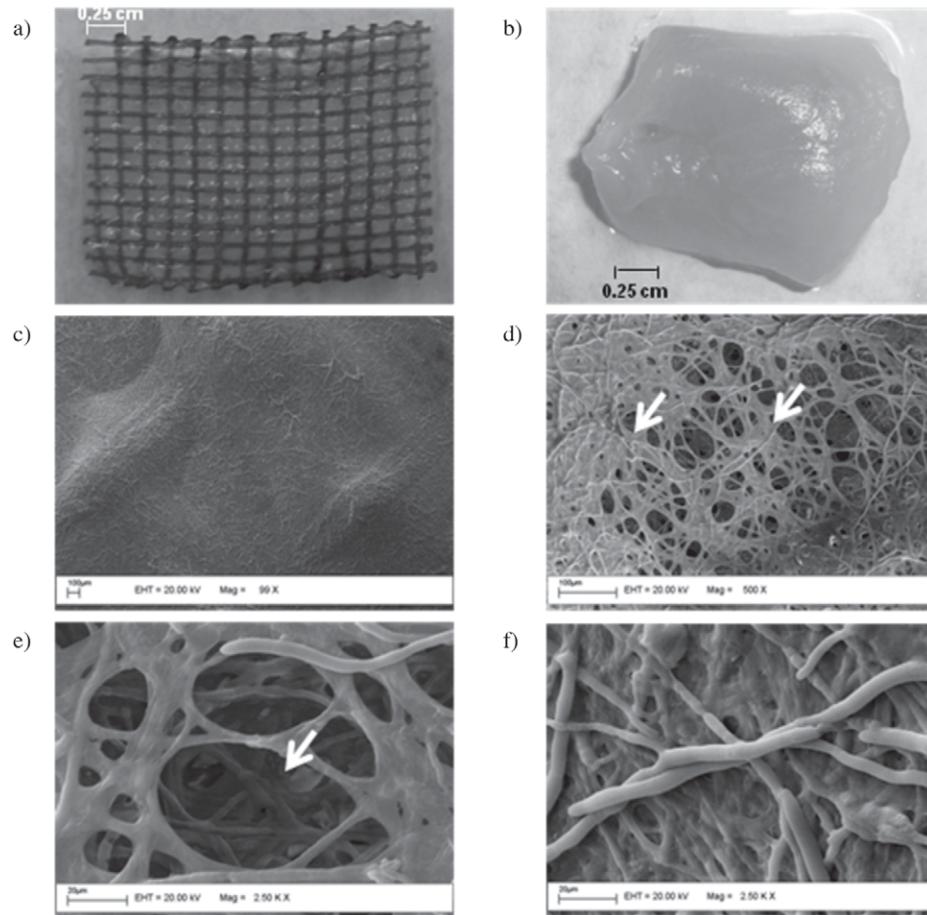
Fig. 1. Polyethylene inert support (a) used to produce *A. phoenicis* biofilm (b). SEM microphotographs showing the morphological pattern of the biofilm structure (c) with the spaces and channels among the hyphae (d and e) and hyphal elongation (f) after 72 h of fermentation

the spores (*18*). The results of SEM analysis showed that the morphology of the mycelium was not compact, and unfilled spaces and channels were observed among the hyphae (Fig. 1d), most likely helping the aeration and amplifying the contact area of the cells with the medium. This phenomenon was also observed by Villena and Gutiérrez-Correa (*18*) in *Aspergillus niger* biofilms. In addition, the hyphal elongation process was clearly observed in *A. phoenicis* biofilm.

### Production of FOS by *A. phoenicis* biofilms in different media

The production of FOS was performed using different culture media (Table 1) supplemented with 25 % of sucrose (*m/V*) as carbon source and the free enzymatic activity was estimated to be (1.5±0.1) U/mL. Different proportions of 1-kestose, nystose and traces of fructofuranosylnystose were detected by HPLC in all analyzed media (Fig. 2). Among all tested media, larger amounts of FOS were obtained in Khanna medium, yielding 132 mg/mL, of which 71 % corresponded to 1-kestose and 29 % to nystose, with a production ratio of 1014 mg/mL per g of biofilm biomass. However, the highest levels of 1-kestose were obtained in Vogel medium, with 1628 mg/mL per g of biofilm biomass (86 % of total biomass), around 2.5 times higher than observed in SR medium, despite the low production of nystose (262 mg/mL per g

of biofilm biomass). Moreover, in Vogel medium the FOS production ratio (1890 mg/mL per g of biofilm biomass) was higher than that observed in Khanna medium. In addition, the concentration of residual sucrose in Vogel medium was higher, compared to the other media, which could justify the low concentrations of nystose. 1-Kestose, a product of the condensation of sucrose and fructose catalyzed by a FTase, is a precursor for the production of nystose. Thus, 1-kestose was apparently not efficiently used for this purpose in Vogel medium. Furthermore, the hydrolysis step was not limiting, as may be seen from

Table 1. Production of FOS by *A. phoenicis* biofilms in different culture media

Culture medium	Biofilm biomass/g	γ(saccharide)/(mg/mL)			
		sucrose	glucose	1-kestose	nystose
Adams	0.12	28.1±4.8	81.8±10.1	75.6±7.6	35.2±3.1
Khanna	0.13	38.0±6.5	75.8±9.4	93.2±9.3	38.6±3.4
SR	0.10	21.4±3.7	62.1±7.7	64.8±6.4	35.9±3.2
Vogel	0.06	62.7±10.7	88.8±11.0	97.7±9.7	15.7±1.4

Sucrose was added to the media at 25 % and maintained at 30 °C for 72 h. The values are the mean of 3 independent experiments±standard deviation. The free enzyme activity was similar in all media and it was estimated as (1.5±0.2) U/mL
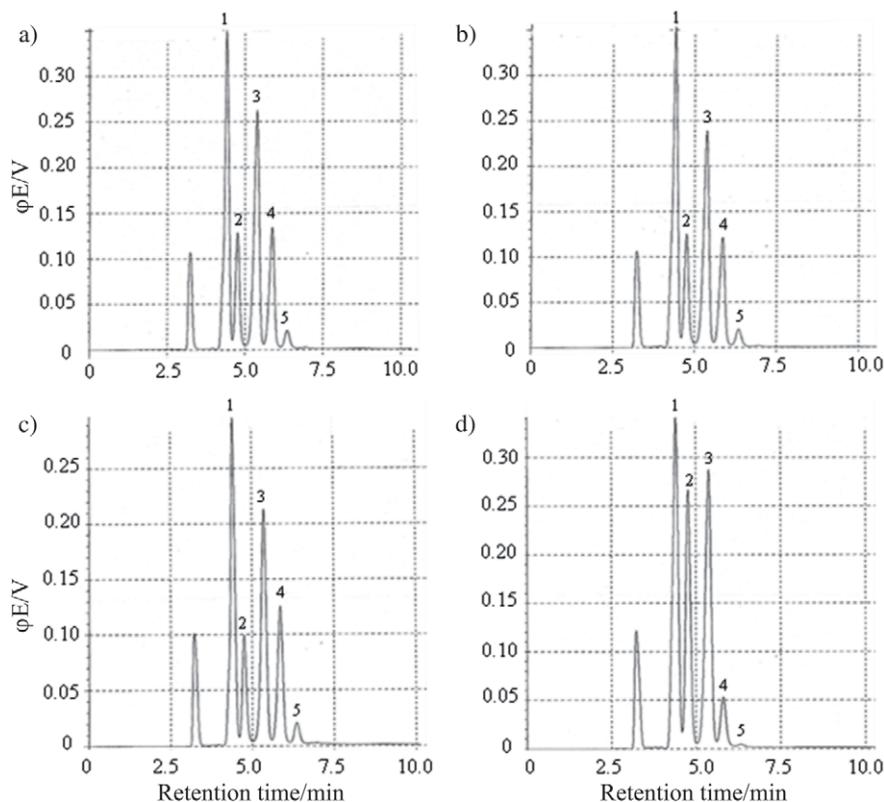
Fig. 2. HPLC profile of the saccharides in: a) Khanna, b) SR, c) Adams and d) Vogel media after 72 h of *A. phoenicis* biofilm fermentation. Peaks: monosaccharides (1), residual sucrose (2) and fructooligossacharides 1-kestose (3), nystose (4) and fructosylnystose (5)

glucose concentration, while the conversion steps from 1-kestose to nystose and from nystose to fructofuranosylnystose were limiting. These facts may be attributed to the presence of a FTase with a higher specificity for sucrose compared to 1-kestose, resulting in preferential catalysis of the condensation reaction, and thus in l-kestose accumulation. However, the existence of two enzymatic forms, F1, highly efficient in the production of 1-kestose and nystose, and F2, with a small degree of transfructosylation activity, has recently been shown by Kurakake *et al.* (*19*) in *A. oryzae* KB.

Production of FOS using other synthetic materials colonized by *A. japonicus* has also been reported (*20*). However, the support colonization was conducted in the culture medium, and thus the production was influenced by the metabolic action of both immobilized and free cells. In this study, only immobilized cells (biofilm) were used for FOS production, since the adsorption of spores to the support was conducted in an aqueous solution and not in the culture medium. This procedure not only aids cell separation but also the recovery of products (FOS) from fermentation broth.

The production of FOS was also studied using biofilms obtained from Khanna medium in 100 mM sodium acetate buffer (pH=4.5) containing 50 % of sucrose (*m/V*). The total quantity of FOS increased as a function of reaction time. When the reaction was conducted for 2 h, around 72.5 mg/mL of FOS were produced, while 242 mg/mL (1.6 % of nystose and 98.4 % of 1-kestose) obtained after a 10-hour reaction, represented an improvement of 3.3-fold in FOS production. However, when the reaction was performed for 25 h, FOS concentration was reduced to

133.5 mg/mL, indicating their hydrolysis, which was confirmed by the increase in glucose and fructose concentrations. Transferase activity may be attributed mostly to an enzyme located in the cell membrane, and high concentrations of sucrose (≥50 %, *m/V*) have been used for FOS production for industrial purposes. Our results clearly demonstrate that *A. phoenicis* biofilms are effective for FOS production with this high sucrose concentration.

Influence of time course and sucrose concentration on FOS production

The production of FOS by *A. phoenicis* biofilm was influenced by the time of fermentation (Fig. 3a) and the maximum level of FOS (130 mg/mL) was obtained after 48 h of incubation. During the first 48 h of reaction, 1-kestose concentration increased, reaching its maximum at 120 mg/mL, while that of nystose remained practically constant. After 48 h, however, the concentration of 1-kestose decreased, while that of nystose increased up to 72 h. Considering all the period of analysis, sucrose concentration decreased, while glucose concentration increased, indicating the efficiency of both hydrolytic and transfructosylation activities of the enzyme.

The production of FOS in two steps has been described: (*i*) initially, the FTase is produced in culture medium by the microorganism, and after that (*ii*) the reaction is carried out using the FTase and high concentrations of sucrose. Using this process, maximum production of FOS by *A. japonicus* was reached within 8 h of the reaction, using the β-fructofuranosidase obtained after 96 h of cultivation under submerged fermentation (*7*), totalizing 104 h to complete the global process for FOS production.

This two-step process was also used by Hidaka *et al.* (*21*) with *A. niger* ATCC20611. Furthermore, using recycling cultures of *A. oryzae* CFR 202, the yield of FOS was maximum at the end of the second recycling (3 steps), totalizing 96 h (*8*). Fernández *et al.* (*22*) conducted the cultivation of *Aspergillus* sp. 27H for 72 h to obtain the FTase and after that, the enzyme was used in the reaction medium for more than 1 h to produce FOS. On the other hand, the production of FOS by *A. phoenicis* biofilm may be carried out directly from the culture media (one step), reducing the total time required. Reduction of process time for FOS production using synthetic materials as supports was also observed by Mussato *et al.* (*20*). High productivity in short time is very interesting and desirable for industrial applications.

The production of FOS increased as a function of sucrose concentration in the culture medium (Fig. 3b), being maximal (190 mg/mL) with 30 % sucrose. High concentration of sucrose favoured the conversion of 1-kestose to nystose, reaching a FOS concentration 10-fold higher than that observed in the presence of 5 % sucrose. Previous results have shown that transglycosylation does not occur using the released enzyme at low concentrations of sucrose. Increased production of FOS by the enzymes produced by *A. niger*, *A. awamori* and *Saccharomyces cerevisiae* was also dependent on higher sucrose concentrations (*21*). It has been reported that both β-D-fructofuranosidases (with hydrolytic and high transfructosylating activities) and FTase (with only transfructosylating activity) are able to produce FOS (*7,8*). The transfructosylating activity of β-D-fructofuranosidases is only observed at high sucrose concentration (≥20 %, *m/V*). Interestingly, FOS were obtained with *A. phoenicis* biofilms at low concentration of sucrose (5 %, *m/V*), indicating the presence of a FTase, which acts on sucrose by cleaving the β-2,1 linkage, transferring the fructosyl group to an acceptor molecule such as sucrose, releasing glucose (*23*). It is possible that the enzyme immobilization could aid the transfructosylation activity at low concentration of sucrose.

Reutilization of the dried biofilm

The possibility of reusing the catalysts employed for the production of biotechnologically interesting molecules is very attractive since it may reduce the process costs. Therefore, the production of FOS using dried biofilms was analyzed (Fig. 4a). When the dried biofilm was reused in a fresh culture medium, there was a recovery of approx. 13.7 % of total FOS after 72 h, where 10 % cor-

Fig. 4. a) Dried *A. phoenicis* biofilms obtained at different times of cultivation and b) production of FOS 1-kestose, nystose and fructofuranosylnystose (GF4) by the reutilization of the dried biofilm after 72 h of cultivation in Khanna medium with 20 % sucrose (*m/V*) at 30 °C
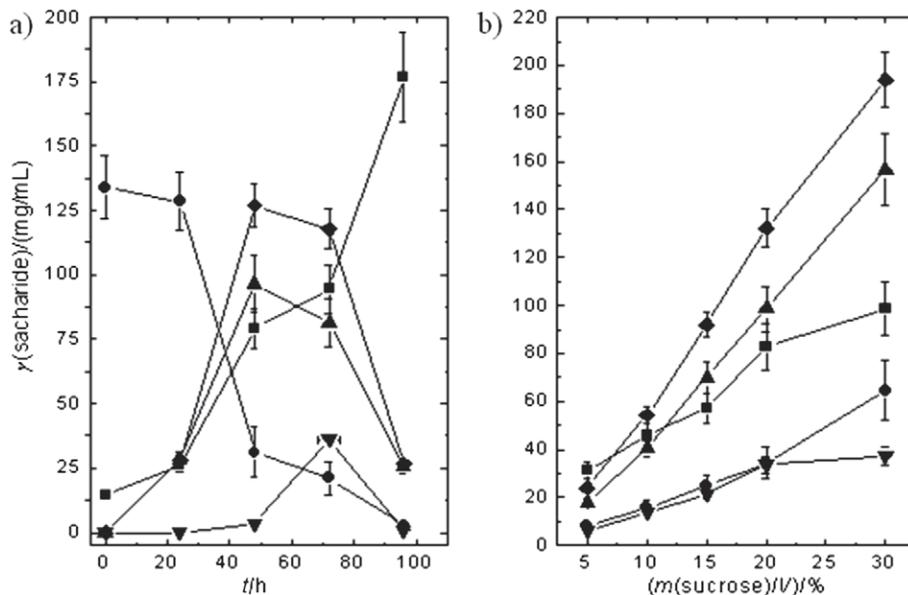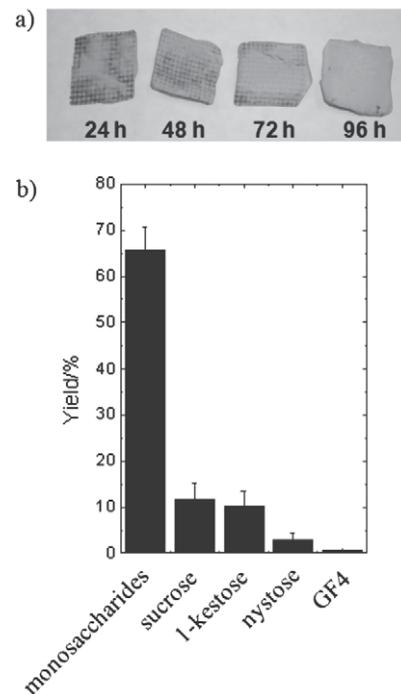

Fig. 3. Time course using 20 % sucrose (*m/V*) (a) and the influence of sucrose added to Khanna medium (b) on the production of FOS 1-kestose and nystose by *A. phoenicis* biofilms for 72 h. (●) sucrose, (■) glucose, (▲) 1-kestose, (▼) nystose, (◆) total FOS

responded to 1-kestose (Fig. 4b), despite the 46 % of recovery obtained in the first fermentation. Nystose and fructofuranosylnystose were also obtained. The high concentrations of the obtained monosaccharides may be explained by the low recovery of FOS, since the enzyme could have reduced its FTase activity and/or enzyme production by the fungus could have been reduced after drying. However, the cells were able to recover their turgor pressure and consequently activate the metabolic pathways related to FOS production. Therefore, it is possible to imagine a scenario where the reuse of biofilms on inert supports might be an interesting option, both in batch and continuous fermentation processes, especially in the former.

Conclusion

The use of biofilms has some advantages if compared to free cells such as, for instance, high cell concentration, which increases process efficiency, resistance to environmental modifications, cell stability and biofilm reutilization. The new system of fermentation, *i.e.* biofilm fermentation, for the production of FOS by the filamentous fungus *Aspergillus phoenicis* described herein proved to be very effective, and may represent an alternative to submerged fermentation system, resulting in high production. Moreover, the possibility of biofilm reutilization makes it viable to use this system in continuous processes in a bioreactor.

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