Eur Food Res Technol (2012) 235:13–22
DOI 10.1007/s00217-012-1728-5

ORIGINAL PAPER

Production of fructooligosaccharides and β-fructofuranosidase by batch and repeated batch fermentation with immobilized cells of *Penicillium expansum*

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Received: 25 January 2012 / Revised: 20 March 2012 / Accepted: 25 March 2012 / Published online: 20 April 2012

Abstract The production of fructooligosaccharides (FOS) and β-fructofuranosidase (FFase) by immobilized cells of *Penicillium expansum* was evaluated. In an initial stage, different low-cost materials including synthetic fiber, polyurethane foam, stainless steel sponge, *loofah sponge*, and cork oak were tested as carrier for the fungus immobilization. Additionally, the influence of the inoculum age (1 or 3 weeks) on cells immobilization, FOS and FFase production was also verified. Synthetic fiber and polyurethane foam were the best materials for *P. expansum* immobilization (2.21 and 1.98 g/g carrier, respectively) and FOS production (120.3 and 104.8 g/l), and gave also high results of FFase activity (23.01 and 32.42 U/ml). Then, the production of FOS and FFase by repeated batch fermentation with *P. expansum* immobilized on synthetic fiber was studied, aiming to improve the batch fermentation results. The results obtained in this stage were very promising with FOS yields of 87, 72, and 44 %, in the 3 initial cycles (60 h), respectively; the FFase activity was constant throughout the process (6 cycles, 96 h). Repeated batch fermentation with immobilized cells of *P. expansum* was found as being a technology with great potential for FOS and FFase production on industrial scale.

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Keywords Fructooligosaccharides · β-Fructofuranosidase · Immobilized cells · *Penicillium expansum* · Repeated batch

Introduction

Fructooligosaccharides (FOS) are fructose oligomers that cannot be hydrolyzed by the gastrointestinal enzymes [1] and that are regarded as prebiotic since they selectively stimulate the growth and/or activity of bifidobacteria and lactobacilli [2, 3], which are the microorganisms responsible for promoting benefits for the human's health. Therefore, it has been observed in the past few years a growing demand on FOS utilization by the food industry.

FOS are mainly composed of 1-kestose, 1-nystose, and 1-β-fructofuranosyl nystose, which can be found in many fruits and vegetables, although in very low concentrations to exert any beneficial effect, and its production is limited by seasonal conditions [1, 4]. Thus, extraction procedures to obtain these compounds are not viable, and the FOS production on an industrial level is required. On industrial scale, FOS are produced either from sucrose by transfructosylation with fructosyltransferase enzymes or from inulin by controlled enzymatic hydrolysis with endoinulinases [5, 6]. The companies that commercially manufacture FOS were summarized in a recent study [6]. FOS have been produced from sucrose by Beghin-Meiji Industries, France (Actilight), Cheil Foods and Chemicals Inc., Korea (Oligo-Sugar), GTC Nutrition, USA (Nutra-Flora), Meiji Seika Kaisha Ltd., Japan (Meioligo), and Victory Biology Engineering Co. Ltd., China (Prebiovis scFOS), while the commercial production of FOS from inulin has been done by the companies Orafti Active Food Ingredients, USA (Raftilose), Beneo-Orafti, Belgium (Orafti), Cosuera Group

Warcoing, Belgium (Fibrulose), and Jarrow Formulas, USA (Inulin FOS).

When comparing the two different processes for FOS production (from sucrose or inulin), the transfructosylation process is considered to have a greater potential because it is possible to synthesize FOS oligomers of defined chain length or to obtain desired composition mixtures by controlling the reaction time [5]. Fructosyltransferase enzymes can be derived from plants, bacteria, or fungi, the last having higher transfructosylating activity [1, 7]. However, the yields of transfructosylation reactions are usually low (55–60 %) [1], and the development of a more economically viable process for the production of FOS is desired. Although many studies have been carried out to attain this purpose, up to date, no relevant results capable of replacing the industrial process were obtained, and the search for new methods to improve the FOS yield is still a big challenge.

Our research group has been focused in finding an efficient process for FOS and β-fructofuranosidase (FFase, enzyme with hydrolysis and transfructosylation activity) production able to be used in industrial scale [8–10]. Although several *Aspergillus japonicus* strains have been reported as potentially adequate for industrial production of FOS [11], *Penicillium expansum* was recently found as being a fungal strain of great potential for FOS production from sucrose [12]. Despite the fact that the utilization of immobilized microorganisms as biocatalysts for the production of antibiotics, organic acids, and enzymes has been already extensively studied, there are few reports on the use of these systems for FOS production, and there is not any published study about the FOS production by immobilized *P. expansum*.

The use of immobilized cell systems is considered a more promissory and advantageous alternative when compared to free cell systems, for several reasons as follows: (i) It promotes the increase in biomass concentration that leads to an improvement in the process efficiency and productivity [13]; (ii) viscosity problems caused by certain fungi morphologies, responsible for mass transfer limitations, are eliminated [14]; (iii) it makes easier the biomass reutilization and separation from the fermented broth [15, 16]. There are several immobilization techniques, and the right choice of the immobilization method and the type of carrier are crucial factors for the development of an efficient system [8, 17]. Additionally, the fermentation operation mode also influences the process efficiency, with repeated batch being a well-known method for enhancing the productivity of microbial cultures [18–20]. The use of immobilized cell systems is particularly feasible for repeated batch fermentation because it facilitates the biomass recovery for use in the subsequent batches.

The present study evaluated the production of FOS and FFase by immobilized cells of *P. expansum*. Initially, five low-cost materials including synthetic fiber, polyurethane foam, stainless steel sponge, *loofah sponge*, and cork oak were evaluated, and the best material carrier for the cells immobilization, FOS and FFase production, was selected. Then, assays using the fungal strain immobilized in the selected material were carried out through repeated batch operation mode aiming to maximize the batch fermentation results.

Materials and methods

Microorganism and inoculum preparation

Penicillium expansum MUM 02.14 (from Micoteca of the Centre of Biological Engineering, University of Minho) was the fungus used in the experiments. The strain was maintained at 4 °C on plates containing potato dextrose agar (PDA, Difco). For the production of spores, it was grown on PDA medium, at 22–25 °C [12], during 1 or 3 weeks, except for the repeated batch fermentation assays, which used spores obtained after the strain cultivation during 1 week. The inoculum for use in the experiments was obtained by suspension of the produced spores in sterilized solution of 0.1 % (w/v) Tween 80 and adjustment to the desired concentration by counting in a *Neubauer* chamber.

Carriers' preparation

Three inorganic materials, such as synthetic fiber (SF), polyurethane foam (PF), and stainless steel sponge (SS) (all of them from Scotch Brite, 3 M Spain, SA), and two lignocellulosic materials, such as *loofah sponge* (LS), and cork oak (CO), were tested as carrier for the cells immobilization. To be used in the experiments, they were cut to particle sizes of approximately 3 × 3 mm (length × width), boiled for 10 min, washed three times with distilled water, dried at 60 °C, and sterilized at 121 °C for 20 min.

Media and fermentation conditions

Assays for the selection of the material carrier and evaluation of the inoculum age were carried out in 500-ml Erlenmeyer flasks containing 1 g of the sterilized immobilization carrier and 100 ml of culture medium with the following composition (% w/v): sucrose, 20.0; yeast extract, 2.75; $NaNO_3$, 0.2; $MgSO_4 \times 7H_2O$, 0.05; K_2HPO_4, 0.5; and KCl, 0.05. Steam sterilization of the medium was carried out at 112 °C for 15 min. Some fermentation assays were also performed without the carrier addition, in order to compare the performance between free and immobilized cells. All the flasks were aseptically inoculated with 1.0 ml

of a spore suspension containing 1.8×10^7 spores/ml and incubated in a rotary shaker at 25 °C and 160 rpm for 48 h. Cells were immobilized in situ in the flasks by natural adsorption through their direct contact with the carrier at the beginning of each fermentation run. To evaluate the influence of inoculum age, the experiments were conducted with *P. expansum* grown during 1 or 3 weeks in PDA medium at 22–25 °C. The pH of the media was not controlled during the experiments, being gradually decreased from 7.0 (at the beginning of the processes) to 6.0 (at the end of the fermentations). During the fermentations, samples were aseptically collected and filtered through 0.2-μm sterile cellulose membranes to remove any free cells from the liquid culture. The filtered fermentation broth was used for FOS, residual sugars, and FFase activity quantification.

Repeated batch fermentation experiments were also carried out in 500-ml Erlenmeyer flasks containing 1 g of the sterilized immobilization material and 100 ml of culture medium (the same previously described). All the flasks were inoculated with 1.0 ml of a spore suspension containing 1.8×10^7 spores/ml and incubated in a rotary shaker at 25 °C and 160 rpm for 96 h. After the initial 36 h (duration of the first cycle), the fermented broth was replaced by fresh medium each 12 h until completion of a total of 6 cycles (96 h of fermentation). Since the cells were mostly immobilized in the support material, the fermented broth separation could be easily done by decantation. Samples were collected at the end of each cycle, filtered through 0.2-μm sterile cellulose membranes, and the fermentation broth was used for FOS, residual sugars, and FFase activity quantification.

Analytical methods

Concentration of free and immobilized cells was determined at the end of the fermentations. Free cells concentration (g/l) was determined after drying at 105 °C until constant weight. The difference between membrane weight and membrane with filtered cells gave the mass of cells that were not able to immobilize into the carrier. The concentration value was calculated considering the volume of filtered fermentation medium. Immobilized cells (g/g carrier) were determined after washing the carriers three times with distilled water and drying at 105 °C until constant weight. The biomass dry weight was calculated from the difference between the mycelium plus carrier and the carrier itself.

FOS (1-kestose, 1-nystose, and 1-β-fructofuranosyl nystose) and residual sugars (sucrose, fructose, and glucose) were analyzed using high-performance liquid chromatography (HPLC) [9] on an equipment LC-10 A (Jasco, Japan) with a Prevail Carbohydrate ES column (5 μm, 250 × 4.6 mm, Alltech) at room temperature and a refractive index detector. The response of the refractive index detector was recorded and integrated using the Star Chromatography Workstation software (Varian). A mixture of acetonitrile and 0.04 % ammonium hydroxide in water (70/30 v/v) was used as mobile phase at a flow rate of 1.0 ml/min. Before injection, the samples were filtered through 0.2-μm filters and diluted with Milli-Q water when needed. The sugars and FOS concentrations were determined from standard curves made with known concentrations of each compound. The yields of total FOS produced (sum of 1-kestose, 1-nystose, and 1-β-fructofuranosyl nystose) were calculated taking into account the initial sucrose concentration ($Y_{P/St}$, g/g) and the sucrose consumed along the fermentation ($Y_{P/Sc}$, g/g). FOS volumetric productivity (Q_P, g/l.h) was calculated by the ratio between the total FOS produced (g/l) and fermentation time (h).

The β-fructofuranosidase (FFase) activity was determined by quantification of the glucose released from sucrose, as previously described [9]. The reaction mixture contained 100 ml of the crude FFase extract, 300 mmol of sucrose and 50 mmol of sodium acetate buffer (pH 5.0) in a total volume of 1 ml. After incubation for 20 min at 30 °C, the reaction was stopped by heating for 5 min at 100 °C. After cooling, the amount of glucose released into the supernatant was measured by HPLC (see above). One unit (U) of the FFase activity was defined as the amount of enzyme required to release 1 μmol of glucose per min, from sucrose under the above conditions.

Photographs by scanning electron microscopy (SEM) of synthetic fiber, polyurethane foam, and stainless steel sponge were obtained from a Leica Cambridge S360 microscope. Before examination, samples were dried and sputter-coated with gold, and maintained under vacuum conditions. Each sample was observed with magnifications of 20-fold and 150-fold.

Statistical analysis

All the fermentation experiments were conducted at least in duplicate, and average values are reported. The results were statistically analyzed by using the *Tukey's* range test, where a p value of less than 0.05 was regarded as significantly different. Statgraphics Plus for Windows, version 4.1, was the software used for data analysis.

Results and discussion

Influence of inoculum age on cell immobilization, FOS production and enzymatic activity

Obtaining an inoculum in the exponential growth phase is essential for a fast microorganism growth after inoculation

in the fermentation medium. Cultures below this phase or beyond it could lead to lag phases of undefined lengths and the production of secondary metabolites non-essential to cell growth [21, 22]. For this reason, the influence of inoculum age (1 or 3 weeks) on *P. expansum* immobilization, FOS and FFase production was evaluated in a first step. Statistical analysis of the obtained data (Table 1) revealed no significant differences (at 95 % confidence level), neither on immobilization nor on FOS production, when *P. expansum* cultivated during 1 or 3 weeks was used as inoculum. The only difference verified concerning the two inoculum age was related to the FFase activity in the presence of SF, where the highest values of enzymatic activity were obtained for the 3-week inoculum. Nonetheless, this high FFase activity value was not a synonym of higher FOS production, suggesting that this enzyme was with an elevated hydrolysis activity (U_h), since it presents both transfructosylating and hydrolyzing activities (U_t and U_h, respectively) [23]. Regarding the fermentative parameters ($Y_{P/St}$, $Y_{P/Sc}$, and Q_P), only the FOS volumetric productivity (Q_P) on PF showed statistically significant difference between the experiments carried out with the inoculum grown for 1 or 3 weeks. Despite these two differences shown by the statistical analysis, it can be concluded that 1 week is an enough time for the inoculum cultivation to attain high cells immobilization and FOS production by *P. expansum*. One week was also considered the best inoculum age for the cultivation of other microorganisms [24–26].

Selection of the carrier for immobilization and FOS production by *P. expansum*

Free and immobilized cell concentrations of *P. expansum* on the five different carriers are represented in Table 2. It can be noted that the fungus had a higher adhesion into the inorganic carriers (SF, PF and SS) than in the lignocellulosic materials (LS and CO). In addition, in these three cases the fermentation media were clearer since they presented less suspended free cells, while the media where the immobilization was inefficient (LS and CO) had more free cells in suspension. Besides immobilizing in a larger extent, the total cell growth in the media containing inorganic carriers was higher (Table 2), which is in agreement with Cohen [13] who refers that immobilization increases biomass growth, when compared to the medium containing only free cells in suspension. Despite the similarity to the free cells medium (which gave 14.08 g cells/l), cell growth on the media containing SS and CO did not occur at the same extent as those verified on media with SF or PF. On the other hand, the lower total growth was observed on medium containing LS (7.36 g/l), which could be related to a possible release of toxic compounds from the carrier and inhibition of these compounds on the microorganism growth.

In a general form, FOS production was maximal at 48 h of fermentation, with the exception of the media containing CO and without carrier (free cells) that had a similar performance with the maximum FOS production occurring at 36 h (Fig. 1). This similar behavior could be due to the fact that the medium containing CO presented more free cells in suspension (11.31 g/l) than the media containing the other carriers. Therefore, bioconversion of sucrose into FOS occurred at the same extent as in the medium containing only free cells, which gave 14.08 g/l of total cells concentration.

Immobilization results, FOS production, and FFase activity by *P. expansum* immobilized on different carriers are presented in Table 3, which also shows the statistically significant differences between samples, at 95 % confidence level, according to a multiple comparison analysis. According to this analysis, the inorganic carriers SF and PF gave similar fermentation results for all the analyzed parameters, as well as verified for the pair of lignocellulosic carriers (LS and CO).

Concerning the cell immobilization, SF and PF gave the highest results, being different ($p < 0.05$) from those achieved with the other carriers. This result suggests a good potential of applicability of SF or PF in repeated batch or continuous fermentation processes. Despite the fact that SS had attached less cells than these two inorganic carriers (difference significant at 95 % confidence level), the obtained immobilization results were even better than those attained for the lignocellulosic carriers (LS and CO). SS has been reported as an excellent material for the immobilization of *Trametes hirsuta* during the laccase production for the degradation of textile dyes [17]. In the present work, this material also revealed ability to immobilize the fungus *P. expansum* during FOS production. Nevertheless, its results were not as high as the ones obtained with SF and PF, probably due to its smooth surface (Fig. 2e, f), which was not so adequate for the fungus attachment. According to Kosaric and Blaszczyk [27], microorganisms tend to form thin layers on smooth surfaces, being easily destroyed by the normal agitation conditions. Conversely, the great immobilization results achieved with SF and PF carriers can be related to their rough and porous structures (Fig. 2a–d), which, besides increasing the surface area, allow the microorganisms to attach more firmly, since the environment becomes more hydrodynamically stable. This, consequently, reduces the detachment of immobilized cells by hydraulic shearing forces [13, 28], which was corroborated by the clear fermentation media obtained for the SF and PF materials. Similar results were achieved by Guimarães et al. [29], who verified that the porous structures of PF and SF gathered the best colonization characteristics/

Table 1 Multiple comparison analysis of inoculum age influence (1 or 3 weeks) on cells immobilization, fructooligosaccharides (FOS) production, β-fructofuranosidase (FFase) activity, FOS yields and productivity, by *P. expansum* cells immobilized on different carriers

Carrier/inoculum age[a]	Immobilized cells ($g/g_{carrier}$)[b]		FOS (g/l)		FFase activity (U/ml)		$Y_{P/St}$ (g/g)[c]		$Y_{P/Sc}$ (g/g)[d]		Q_P (g/l h)[e]	
	Average	Difference	Average	Difference	Average	Difference	Average	Difference	Average	Difference	Average	Difference
SF/1	2.21	0.38	120.3	22.0	23.01	−20.87*	0.60	0.11	0.66	0.11	2.51	−0.22
SF/3	1.83		98.3		43.88		0.49		0.55		2.73	
PF/1	1.98	0.92	104.8	−16.8	32.42	−16.42	0.53	−0.09	0.62	−0.19	2.19	−1.19*
PF/3	1.06		121.6		48.84		0.61		0.81		3.38	
LS/1	0.21	−0.12	133.1	25.8	23.56	−36.44	0.66	0.12	0.72	0.13	2.78	0.54
LS/3	0.33		107.3		59.99		0.54		0.59		2.24	
CO/1	0.17	0.02	111.8	6.6	51.18	14.25	0.56	0.03	0.63	−0.12	3.11	0.19
CO/3	0.15		105.2		36.94		0.53		0.75		2.92	
SS/1	1.29	0.61	130.1	4.2	28.37	−41.98	0.65	0.02	0.71	−0.09	2.71	−0.79
SS/3	0.69		125.9		70.35		0.63		0.80		3.49	

[a] *SF* synthetic fiber, *PF* polyurethane foam, *LS loofah sponge, CO* cork oak, *SS* stainless steel sponge. Inoculum age: weeks

[b] Immobilized cells were determined by dry weight

[c] $Y_{P/St}$: FOS yield concerning the total initial sucrose concentration

[d] $Y_{P/Sc}$: FOS yield concerning the consumed sucrose

[e] Q_P: FOS volumetric productivity

* Denotes a statistically significant difference at 95 % confidence level

Table 2 Concentration of cells in the free form and immobilized on different carriers, after 48 h of sucrose fermentation by *P. expansum*

Carrier	Immobilized cells (g/g carrier)	Free cells (g/l)	Total cells[a] (g/l)
Synthetic fiber—SF	2.21	0.96	23.06
Polyurethane foam—PF	1.98	0.61	20.41
Stainless steel sponge—SS	1.29	0.49	13.39
Loofah sponge—LS	0.21	5.26	7.36
Cork oak—CO	0.17	11.31	13.01

Free and immobilized cells were determined by dry weight

[a] Free cells + immobilized cells on the 100 ml of fermentation medium

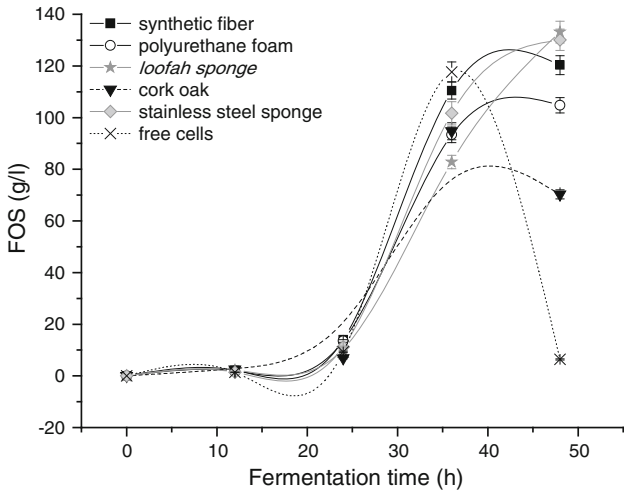

Fig. 1 Fructooligosaccharides (FOS) production by *P. expansum* immobilized (or not) on different materials

properties for the attachment of the *Phanerochaete chrysosporium* mycelia, when compared to immobilization on SS.

When comparing all the studied materials, lignocellulosic carriers had the poorest performance regarding cell immobilization of *P. expansum*. In a similar way, during the production of xylanase by immobilized cells of *Bacillus pumilus*, the inorganic carriers (SF and PF) were also more efficient when compared to the natural ones (cotton and silk) [30]. The low immobilization results verified on cork oak could be explained by its contents of extractives (responsible for its permeability properties) and/or phenolic compounds (character toxic for the microorganisms) [31], which might had interfered in the adhesion capacity of the *P. expansum* cells into the carrier. On the other hand, the low immobilization results observed for LS could be related to the simple pre-treatment performed (only boiling). Coelho [32] reported that LS treatment with vapor altered the material's structure and favored the immobilization of *Candida guilliermondii*.

Concerning the maximum FOS production yield ($Y_{P/St}$), volumetric productivity (Q_P), and enzymatic activity of FFase, the obtained results did not differ at 95 % confidence level for the different carriers used (Table 3). Therefore, the next step of this study consisted in a comparison between FOS production results by free cells and immobilized in SF or PF (the most suitable materials for *P. expansum* immobilization) (Table 4). When statistically compared, the results obtained for these three fermentation media did not show differences at 95 % confidence level. However, maximum FOS production in free cells medium was achieved after 36 h of fermentation, while 48 h were necessary to obtain the maximum production in media containing cells immobilized in SF or PF, suggesting that microenvironmental conditions of immobilized cells may differ from free cells [33]. FOS production by free or immobilized cells did not differ also in terms of proportion of 1-kestose (GF2), 1-nystose (GF3), and 1-β-fructofuranosyl nystose (GF4) in the final mixture. Fermentation with free cells gave a final FOS mixture containing 72.1 % GF2, 26.4 % GF3 and 1.5 % GF4, while fermentation with cells immobilized in SF or PF yielded FOS mixtures containing 74.2 % GF2, 22.9 % GF3 and 2.9 % GF4; and

Table 3 Multiple comparison analysis of cells immobilization results, fructooligosaccharides (FOS) production and β-fructofuranosidase (FFase) activity, obtained by *P. expansum* immobilized on different carriers (inoculum age of 1 week)

Carrier	Immobilization (g/g carrier)[I]	FOS (g/l)	FFase activity (U/ml)	$Y_{P/St}$ (g/g)[II]	Q_P (g/l h)[III]
SF	2.21^{c}	120.3^{ab}	23.01^{a}	0.60^{ab}	2.51^{ab}
PF	1.98^{c}	104.8^{a}	32.42^{a}	0.53^{a}	2.19^{a}
SS	1.29^{b}	130.1^{b}	28.37^{a}	0.65^{ab}	2.71^{abc}
LS	0.21^{a}	133.1^{b}	23.56^{a}	0.66^{b}	2.78^{bc}
CO	0.17^{a}	111.8^{ab}	51.18^{a}	0.56^{ab}	3.11^{c}

Same letters denote no statistically significant differences between samples, at 95 % confidence level

SF synthetic fiber, *PF* polyurethane foam, *SS* stainless steel sponge, *LS loofah sponge, CO* cork oak

[I] Immobilized cells were determined by dry weight

[II] $Y_{P/St}$: FOS yield concerning the initial sucrose concentration

[III] Q_P: FOS volumetric productivity

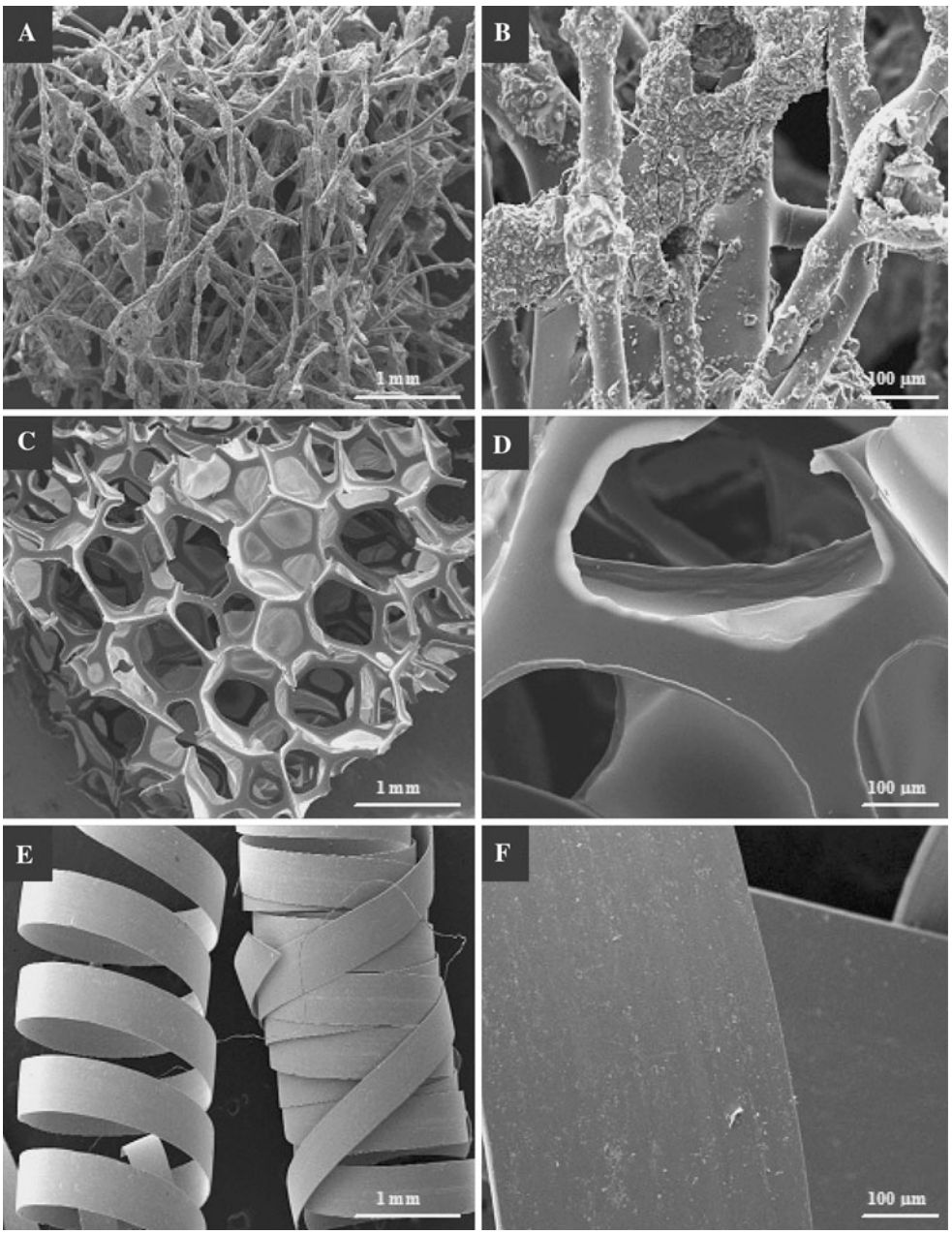

Fig. 2 Scanning electron microscopy (SEM) of synthetic fiber (**a**, **b**), polyurethane foam (**c**, **d**), and stainless steel sponge (**e**, **f**) samples. Magnification: 20-fold (*scales* of 1 mm) and 150-fold (*scales* of 100 µm)

67.3 % GF2, 30.0 % GF3 and 2.7 % GF4, for SF and PF, respectively. In brief, it can be concluded that FOS production by cells immobilized on SF or PF was very efficient, allowing the reproduction of the results obtained in the free cells medium. This is advantageous for their application in repeated batch fermentation processes.

Repeated batch fermentation assays

The FOS yield and productivity obtained during the repeated batch fermentations are presented in Table 5. The maximum FOS production (194.9 g/l) was reached at the end of the 1st cycle (36 h), giving a productivity (Q_P) of 5.41 g/l h. This condition gave also small quantities of fructose and glucose as by-products of the reaction, with glucose concentration being 15 % higher than fructose's, which denotes fructose use in FOS production. After replacing the fermentation medium, it was observed a reduction in FOS production of approximately 13 % by the end of the second cycle (48 h), even though they were still produced with high yields. Until the third cycle, *P. expansum* was capable of producing FOS with yields similar

Table 4 Fructooligosaccharides (FOS) production and β-fructofuranosidase (FFase) activity by free or immobilized cells of *P. expansum*

Fermentative parameters[a]	Immobilized cells		Free cells
	SF	PF	
FOS maximum (g/l)	120.3	104.8	117.7
$Y_{P/St}$ (g/g)	0.60	0.53	0.58
$Y_{P/Sc}$ (g/g)	0.65	0.62	0.68
Q_P (g/l h)	2.51	2.19	3.25
FFase maximum activity (U/ml)	23.01	32.42	41.15

SF synthetic fiber, *PF* polyurethane foam

[a] $Y_{P/St}$: FOS yield concerning the initial sucrose concentration, $Y_{P/Sc}$: FOS yield concerning the consumed sucrose, Q_P: FOS volumetric productivity

Table 5 Results achieved during the repeated batch fermentation for fructooligosaccharides production by *P. expansum*

Cycle (h)	$Y_{P/St}$ (g/g)	$Y_{P/Sc}$ (g/g)	Q_P (g/l h)
1st (36)	0.87	0.99	5.41
2nd (48)	0.72	0.77	3.42
3rd (60)	0.44	0.46	1.66
4th (72)	0.27	0.29	0.87
5th (84)	0.07	0.07	0.17
6th (96)	0.04	0.04	0.09

$Y_{P/St}$: FOS yield concerning the total initial sucrose concentration, $Y_{P/Sc}$: FOS yield concerning the consumed sucrose, Q_P: FOS volumetric productivity

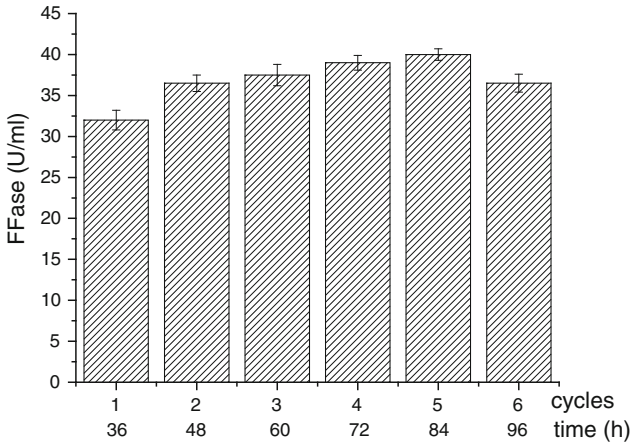

Fig. 3 β-Frutofuranosidase (FFase) activity during the repeated batch fermentation with *P. expansum* immobilized on synthetic fiber

to the ones attained at an industrial scale (55–60 %) [1]. After that period, FOS production (and consequently the $Y_{P/S}$ and Q_P results) decreased more sharply, reaching similar values at the two last cycles (86–96 h), lower than 15 g/l. This can be due to an accumulation of dead cells, along the 96 h of process, caused by the biomass reuse [34]. Another possible explanation is the accumulation of secondary products resulting from cellular metabolism, such as ethanol or acetic acid (not quantified in this work), which could negatively influence the subsequent fermentation cycles. Cell wash with water before its reuse in the next cycle could be a solution for this problem [35].

Unlike the FOS production, FFase activity was practically constant throughout the fermentation process (Fig. 3) that is a very interesting result due to the industrial importance of this enzyme. Similar results were obtained by Mussatto et al. [9] during the FOS production by *A. japonicus* in repeated batch fermentations, where the synthesis of this oligosaccharide decreased along the cycles, while the FFase activity remained constant. Nevertheless, the FOS production by repeated batch fermentation with *P. expansum* (present study) gave better results than our previous study of FOS production by repeated batch fermentation with *A. japonicus* immobilized on SF [9], revealing the greater ability of *P. expansum* for use on the production of FOS.

Concerning the cell growth, it was observed a gradual growth of cells as a dense layer on the surface of the carrier, which covered the entire material at the end of the fermentation. It merits emphasizing that the reuse of the immobilized cells allowed eliminating the time required for the fungus growth (7 days), raising the FOS productivity, as a consequence. In other words, it requires 9 days (7 for cell growth and 2 for fermentation) to achieve the maximum values of FOS and FFase by *P. expansum* by the traditional batch fermentation; that is, 27 days would be required to carry out three individual batches, while only

9.5 days were enough to perform three successive cycles under repeated batch fermentation conditions.

Conclusions

Penicillium expansum can be successfully immobilized in synthetic fiber or polyurethane foam and used for the production of FOS and FFase by fermentation. The use of this immobilized strain in repeated batch fermentations was demonstrated to be a process with great potential for application at an industrial scale, since substantial increases in the FOS yield and productivity were achieved (when compared to the values obtained under batch fermentation conditions) probably due to a reduction in substrate inhibition on transfructosylation process, and the FFase activity was obtained at elevated levels during a long fermentation time (6 cycles, 96 h). These results are very promising and contribute for the development of a more efficient industrial process for FOS and FFase production.

It is worth mentioning that although several *P. expansum* strains have been reported as being producers of patulin, there is not any published study mentioning the production of this mycotoxin by *P. expansum* MUM 02.14, and it is well known that the production of this toxin varies to each strain and with the conditions used for cultivation. This is an important aspect to be taken into account considering the use of this fungal strain for the production of FOS, which are compounds used for food purposes.

Acknowledgments The financial support from FCT, the Portuguese Foundation for Science and Technology (research grant SFRH/BPD/38212/2007) is gratefully acknowledged.

Conflict of interest The authors declare that they have no conflict of interest.

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