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Solid-liquid extraction of cellulases from fungal solid-state cultivation in a packed bed bioreactor

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Abstract−Cellulases are enzymes required for the production of second-generation ethanol (E2G) via biochemical route. The current paper reports the development of an apparatus for solid-liquid extraction of cellulases from solid-state fermentation (SSF) carried out in a packed bed bioreactor (PBB), operated as batch and as semicontinuous. The case study was the cultivation of *Myceliophthora thermophila* I-1D3b on sugarcane bagasse (SCB) and wheat bran (WB) (7 : 3 w/w). The current work integrates the PBB to the first downstream step for recovering the enzymes produced by SSF. The substrate was inoculated and packed into the modules that composed the PBB. The fermentation occurred at 45 °C and air was supplied with flow rate of 350 L/h. At the end of the cultivations, each module was placed in an extraction column, a dynamic closed system in which distilled water was circulated and made to percolate the cultivated material. Variables tested were volume of water per mass of substrate, water flow rate and time of percolation. Higher contact time (120 min) and higher flow rate (2.4 m^3/h) allowed us to recover up to 85% of total enzyme activity by percolation. Lower volume (20 mL/g) provides higher titer extract. The apparatus showed to be promising for SSF downstream, mainly for semicontinuous operation of PPBs.

Keywords: Fixed Bed, Downstream, Cellulolytic Enzymes, Bioethanol, Biorefineries

INTRODUCTION

From the global concern about the greenhouse effect, the development of renewable energy matrices becomes strategic [1,2]. In Brazil, the concept of a biorefinery emerges as that of an industry focused on the production of liquid biofuels, including first generation ethanol (E1G, obtained from the fermentation of juices of sugarcane and of saccharin sorghum) and second generation ethanol (E2G, obtained from agricultural waste and lignocellulosic biomass). In parallel, other high value-added biomolecules (such as enzymes, pigments and many other organic compounds) are produced, assuring the economic viability of the biorefineries [3].

Hydrolysis of lignocellulosic materials for E2G production can be performed chemically or enzymatically. The enzymatic route is more environmentally friendly, given the enzyme-substrate specificity and the employment of milder reaction conditions [2]. However, the high cost of the commercial enzymes limits the feasibility of the process [4]. The in-house production of such enzymes in a parallel process performed inside the biorefinery can overcome this drawback and practically guarantee the self-sufficiency of this industry, concerning E2G biofuel production [5].

In this context, solid-state fermentation (SSF) appears as a promising alternative biotechnological process to produce enzymes required by E2G biochemical route, mainly cellulases. Additionally, SSF can be performed with different combinations of substrates, microorganisms and operational conditions. Besides cellulases, other high-added value biomolecules can be obtained. Therefore, biorefineries can explore such biomolecules for self-supply (enzymes and organic acids, for instance) or for commercialization (pigments, gums, biosurfactants, nanocellulose, and others). Moreover, by-products of the biorefinery can be used as substrates [3,6].

SSF may be defined as the growth of microorganisms on wet solid particles [7]. Due to the lower water activity in SSF systems in comparison to traditional submerged fermentation (SmF), the microorganisms that most adapt to SSF are filamentous fungi. Among them, thermophilic fungi stand out for producing thermostable enzymes, interesting for industrial applications [8].

Meanwhile, despite its excellent application potential, SSF is not yet fully established as a successful industrial alternative, mainly due to the lack of available and optimized industrial equipment. Also, SmF is already available at industrial level for many products, making it more difficult for SSF to compete with similar results in most products. Fundamentally, the solution to this impasse depends on engineering studies, including modeling, simulation and experimentation, ranging from flask scale tests to bench and pilot scales, focusing both on bioreactor development and on proposing new strategies for performing either up or downstream operations [9].

Fixed beds are the focus of the current paper because the non-

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movement of the particles makes them ideal bioreactors for shear stress sensitive fungi [9]. Fixed bed bioreactors in which the air is blown through the bed are called packed-bed bioreactors (PBBs). A typical PBB is a column in which a perforated plate at the bottom supports the bed of substrate, while air is continuously blown and it percolates the bed. There are several recent investigations on the production of bioproducts by SSF in PBBs, mainly enzymes production [10].

Another great challenge for engineering is to make SSF processes continuous, gaining higher visibility for industrial application, due to advantages like reduced dead-time (load and cleaning) and possibility of using smaller equipment for upstream and downstream [11]. An alternative is a modular PBB in which it is possible to add and to remove modules along the cultivation, setting a semicontinuous operation. This idea was proposed by Mitchell et al. [11], who simulated thermal profiles and found that overheating would be avoided in this mode of operation.

Another aspect that calls attention is the proper recovering of the product of interest from the cultivated material. At the end of the cultivation, the fermented material must go through several steps so that the enzymes secreted can be extracted or recovered. This recovering involves operations like solid-liquid extraction, agitation, filtration and centrifugation [12].

Many factors may influence the efficiency of recovering bioproducts from SSF by solid-liquid extraction, according to specific characteristics of the interesting biomolecule, of the substrate and of the conditions employed in the operation [12]. In the majority of SSF processes, the cultivated material is not directly applied, as it is the case of enzyme preparations for industries. Hence, it is necessary to add a solvent to the solid substrate, usually water or a buffer solution, in order to provide a final liquid extract with the desired catalytical activity. Factors like solvent type, relation of volume of solvent per mass of solid cultivated and time and velocity of agitation can affect the yield of the solid-liquid extraction. Therefore, the study of the effect of these factors can lead to more efficient extractions, which is especially important when SSF processes are intended to be scaled-up for bench, pilot and industry. Once the enzyme extract is obtained, additional unit operations will be required to increase enzyme concentration or purity, depending on the application.

On all the above, the current paper has been focused on enzyme extraction, an operation that succeeds SSF in PBBs. The aims were to build an apparatus for the leaching operation and to evaluate the effect of variables involved on the solid-liquid extraction of cellulolytic enzymes produced by a thermophilic fungus cultivated in SSF in a modular PBB operated as batch and as semicontinuous. Different combinations among the levels of the variables (volume of water per mass of substrate, water flow rate and time of circulation of water) were tested according to a 2^k experimental design.

MATERIALS AND METHODS

1. Substrate and Microorganism

The substrate for cultivation was composed of sugarcane bagasse (SCB) and wheat bran (WB) (7 : 3 w/w). Wheat bran (WB) was acquired from a local retailer (Cerealista Alvorada, São Carlos, SP, Brazil). Sugarcane bagasse (SCB) was kindly provided by Usina Santa Cruz, São Martinho Group (Américo Brasiliense, SP, Brazil). SCB was oven dried at 50 °C until constant weight and then sieved using 4- and 3-mm sieves. The material that passed through the 4 mm sieve and that was retained by the 3 mm sieve was used for the cultivation. Both substrates were packed in polyethylene bags and kept under refrigeration at 4 °C until used.

The microorganism used was *Myceliophthora thermophila* I-1D3b, a fungal strain isolated from piles of SCB in an ethanol plant at Olímpia, SP, Brazil. The strain was stored in test tubes containing sloped agar-Sabouraud-dextrose, submerged in mineral oil and kept in a cold room at 5 °C. The strain belongs to the collection of the Laboratory of Microbiology and Applied Biochemistry of Institute of Biosciences, Letters and Exact Sciences, São Paulo State University (IBILCE/UNESP).

To be used in the experiments, *M. thermophila* culture was picked on Erlenmeyer flask or thick-walled polyethylene bags containing agar-Sabouraud-dextrose (SDA, HiMedia Laboratories Pvt. Ltd., Mumbai, India), which were kept in a Bio-Oxygen Demand (BOD) culture chamber for 48 h at 45 °C. The agar surface was then scraped and the spores were dispersed in a nutrient salt solution composed of 0.35% (w/v) $(NH4)_2SO_4$, 0.3% (w/v) KH_2PO_4, 0.05% (w/v) $MgSO_4 \cdot 7H_2O$, 0.05% (w/v) $CaCl_2$ and 0.1% (v/v) Tween 20 at pH 5.0 [13]. A Neubauer hemocytometer was used to count the spores, and the suspension concentration for inoculum was set at approximately 10^7 spores per gram of initial dry solid substrate.

2. Cultivation in Packed-bed Bioreactor

The cultivation took place within a stainless steel PBB like the one used by Perez et al. [14]. Air was provided by a radial compressor, filtered to retainment of oil and filth, and conditioned regarding temperature and relative humidity (saturated by bubbling through a water column at 45 °C). Airflow rate was set with the help of a rotameter as 350 L/h.

The PBB was composed of modules with 13 cm of internal diameter and 10 cm of length. All individual modules were jacketed (double wall), through which water was circulated throughout at the same temperature as of the inlet air, 45 °C. Cultivations were performed in both batch and semicontinuous modes of operation.

In batch operation, the total bed length of four fermentative modules (positions 3 to 6 at Fig. 1(a)) was kept throughout the process. In semicontinuous operation (Fig. 1(b)), bed length varied along the transient period of the process, in which it was added, successively, one module per day for four days. After this transient period, bed length remained constant due to the removal of one module (the one that reached four days of residence in the column) per day, followed by the addition of a new module containing fresh inoculated substrate. The movement of the remaining modules along the column (positions 3 to 6) was descending, and this cultivation lasted 16 days in total.

The PBB, vertically oriented, was composed of one entrance module (module 1 in Figs. 1(a) and 1(b)), one exit module (module 8 in Figs. 1(a) and 1(b)), two auxiliary modules (2 and 7 in Figs. 1(a) and 1(b)) and, finally, the fermentative modules, in which spores of *Myceliophthora thermophila* were inoculated in the substrate SCB : WB (7 : 3 w/w) and moisturized with nutrient salt solution up to 75% (wet basis moisture content) [14]. On the bottom of each mod-

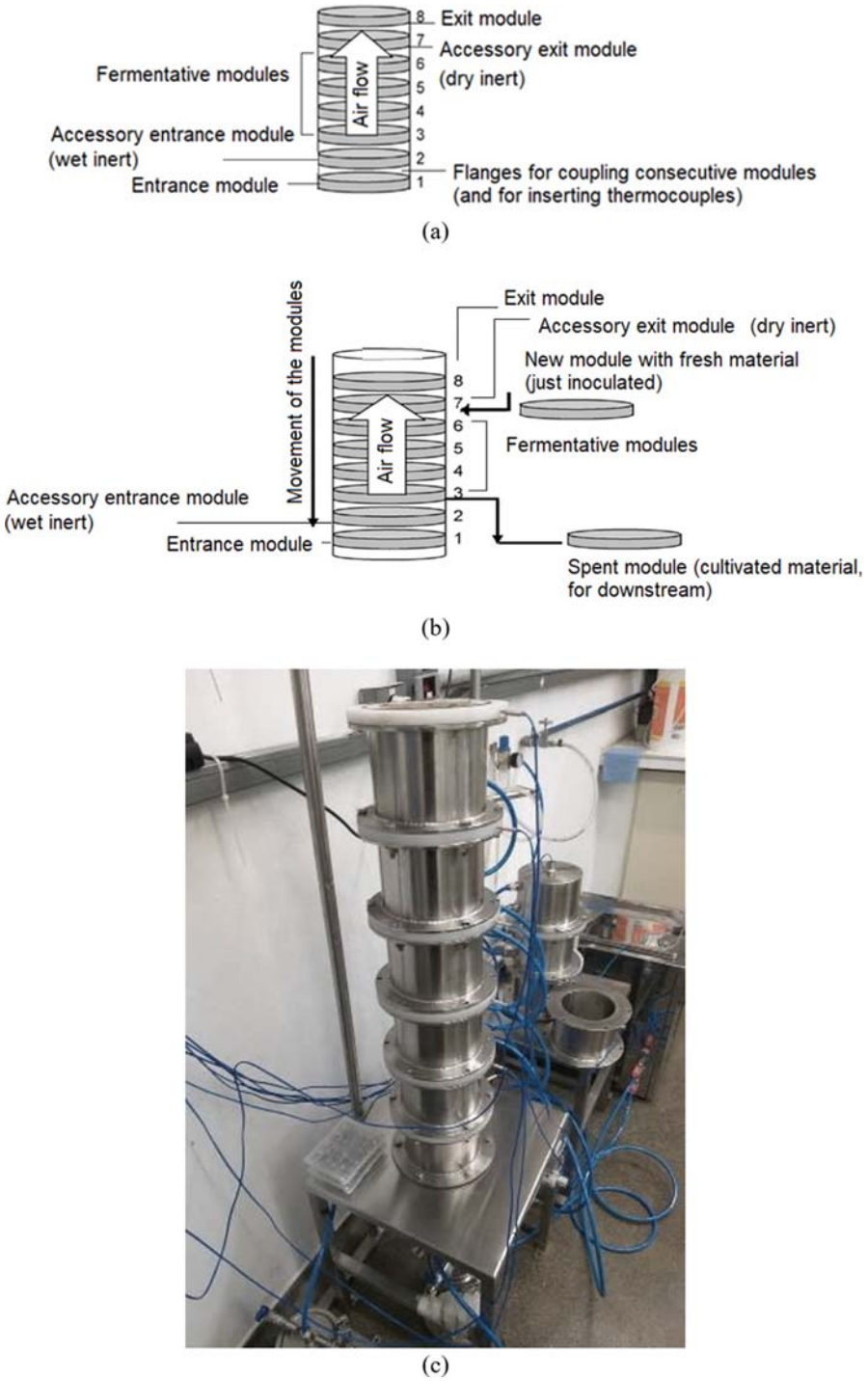

Fig. 1. Schematic representations of the PBB: (a) Batch operation; (b) pseudo continuous operation; (c) photograph of the apparatus.

ule was a perforated plate, avoiding the loss of packed material during the movement of the modules in the semi continuous operation.

The superficial velocity of the air was fixed at 7 mm s^{-1} and was kept constant along the fermentation, independent of the length of the PBB. The ideal temperature for growth and endoglucanase production by *M. thermophila* (45 °C) was established for the percolating saturated air and for the water flowing by the jacket [13,14].

Entrance and exit modules have the same dimensions as the intermediary ones, but for its conical shape inside. To establish the flow pattern of the air, percolated end-to-end through the PBB, the entrance module (position 1 on Figs. 1(a) and 1(b)) was filled with 3 mm glass beads. Exit module (position 8 on Figs. 1(a) and 1(b)) was empty.

For avoiding the drying of the first inoculated module, wet inert material was packed into the first accessory module (position 2 on Figs. 1(a) and 1(b)), placed immediately above the entrance mod-

ule. The accessory module placed just before the exit module was packed with inert dry material (position 7 on Figs. 1(a) and 1(b)), in order to absorb water drops possibly condensed due to lower temperatures of the walls of the exit module.

Upstream operations like sterilization, inoculation and packing were performed in batch for both batch and semicontinuous modes. On the semicontinuous operation, the substrate was inoculated just before to be packed within the module that would be coupled into the column. Previous to the cultivations, the modules were sterilized with 70% (v/v) ethanol. Substrate was sterilized by humid heat (121 °C, 1.1 atm, 20 minutes) within plastic bags containing the ideal amount to be packed within each module. The substrate was moisturized according to Perez et al. [14], followed by inoculation at the same plastic bag and hand mixing.

Between consecutive modules, a nylon flange was placed, through which sheathed T-type thermocouples (1.5 mm diameter) were inserted and whose tips were positioned at different axial positions along the bed, so that temperature profiles could be evaluated throughout the operation. The thermocouples were connected to a data acquisition system COMPAQ-DAQ (National Instruments, Austin, USA), managed by a LabView® routine (National Instruments, Austin, USA), and the signals were recorded and stored in a computer for further processing and analysis.

3. Extraction by Percolation

For the extraction experiments, a system was built using modules identical to the ones used for assembling the PBB for the cultivations. The extraction system consisted of one entrance module, one intermediary module (containing the material that came from the cultivation to be extracted) and one exit module. The entrance module was packed with 3 mm glass bead in order to disperse the flow of liquid over the full cross section of the bed. Perforated sieves were placed just before and after the bed of cultivated material in order to avoid that particles were dragged with the liquid flow.

The circulation of liquid was promoted by a submerged pump. Operational variables evaluated in the extraction tests were volume of water per mass of initial dry substrate, water flow rate and time of circulation, as stated in Table 1. Values of flow rate were checked with the help of graduated measuring cylinders and chronometer.

According to 2^k factorial design with central point, generated by software Chemoface v1.61® (Chemoface, UFLA, Brazil), 11 extractions were performed, according to Table 2. Same software was used for statistical analysis of the results.

Extracts P1 to P7 came from fermentative modules cultivated in the PBB operated as semicontinuous. Extracts B1 to B4 came from PBB operated as batch. At the end of the extractions, an aliquot was separated to be filtered and centrifuged (5 °C, 10,000 rpm, 15 min).

Table 1. Variables and levels tested in extraction experiments

Variables	Levels		
	−1	0	+1
Volume of water per gram of initial dry solid substrate (mL/g)	26	30	34
Water flow rate (L/h)	15	35	55
Time of circulation (min)	15	30	45

Table 2. Experimental design of the tests of extraction by percolation

Extraction code*	Ratio mL/g	Flow rate (L/h)	Time (min)	Residence time** (min)	Number of cycles***
SC1	34	55	15	1.4	10.4
SC2	34	55	45	1.4	31.1
SC3	26	55	15	1.4	10.4
SC4	26	55	45	1.4	31.1
SC5	26	15	15	5.3	2.8
SC6	26	15	45	5.3	8.5
SC7	34	15	15	5.3	2.8
B1	34	15	45	5.3	8.5
B2	30	35	30	2.3	13.2
B3	30	35	30	2.3	13.2
B4	30	35	30	2.3	13.2

*Extracts P1 to P7 came from fermentative modules cultivated in the PBB operated as semi continuous and extracts B1 to B4 came from PBB operated as batch.

**For residence time calculation, volume of a module of 1.3 L was considered.

***Number of cycles calculated by dividing time of circulation per residence time.

The soluble protein concentration was analyzed by Bradford method (20 μL sample+1 mL Bradford reagent, 5 min room temperature, absorbance at 595 nm). Standard curve was prepared with solutions of Bovine Serum Albumin (BSA) [15].

Endoglucanase activity was determined according to Ghose [16] and sugars released were quantified by DNS method [17]. Controls were prepared with the enzyme extract being added after the boiling step of the referred methodology in order to discount sugars possibly present in enzyme extracts prior to the reaction. One unit of enzyme activity (U) was defined as that amount which released 1 μmol of glucose per minute under the reaction conditions, by using glucose standard curve at 540 nm. To express enzymatic activity in U/gss (units per gram of initial dry solid substrate), the result of U/mL was multiplied by the relation of volume of liquid used per gram of substrate, for each extraction condition.

After extraction by percolation, the solid material was sent to conventional extraction in a mixing tank, in which the material was mixed with 10 mL of water per gram of initial dry solid substrate. These extracts were named residual and were subject to the same analysis as extracts obtained by percolation. Hence, it could be calculated the efficiency of the extraction by percolation, in terms of the percentage of mass of proteins and of endoglucanase activities recovered.

Based on the results of the experimental design, one more batch cultivation was performed in order to provide additional modules for improved extraction tests, aiming to increase enzymes recovered by percolation.

RESULTS AND DISCUSSION

1. Remarks on the Conditions Along the Cultivations

Fig. 2 presents thermal profiles along the processes in both modes

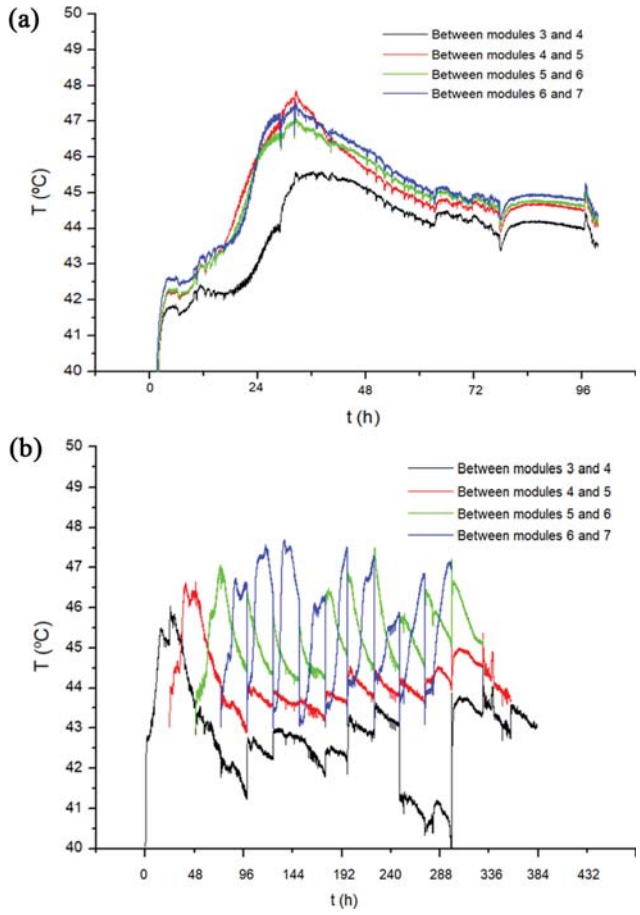

Fig. 2. Thermal profiles in the PBB of SSF: (a) Batch; (b) pseudo-continuous.

of operation. The maximum temperatures reached in batch and in semicontinuous operation were almost equal, 47.9 °C (Fig. 2(a)) and 47.7 °C (Fig. 2(b)), respectively.

In batch (Fig. 2(a)), the peak of temperature was reached around 36 h from the beginning of the cultivation. It is also observed that first fermentative module of the PBB (temperatures registered in between positions 3 and 4 on Figs. 1(a) and 2(a)) is subject to slightly lower overheating when compared to the other fermentative modules (temperatures registered in between positions 4 and 5, 5 and 6 and 6 and 7 on Figs. 1(a) and 2(a)). This may be attributed to convective heat transport by ascendant end-to-end aeration trough the bed, while the first fermentative module is the one closer to the fresh air inlet.

In semicontinuous operation (Fig. 2(b)), the cultivation lasted up to 16 days (384 h). At the end of the cultivation, 12 fermented modules were provided. For extraction tests by percolation, only seven of them (removed after the column reached its fixed length) were used. With this, the experimental design was completed (adding the four modules from batch operation). It is important to mention that, in semicontinuous operation, the cultivations can last as long as the process engineer wishes.

In Fig. 2(b), it is possible to observe that, during an initial transient period, interruptions happened every 24 h for the addition of new inoculated modules. From 72 h, the PBB reached the height of four fermentative modules. From 96 h, interruptions continued to be carried out every 24 h, but then for the removal of the module closer to the air inlet (module 3 on Fig. 1(b)), that completed 96 h of residence in the column. Upon this removal, modules on positions 4, 5 and 6 on Fig. 1(b) were moved down, taking positions 3, 4 and 5, and a new module with fresh inoculated material was added on position 6 of Fig. 1(b). The cultivation proceeded successively like this up to 312 h. After that, the modules removed every 24 h were not replaced anymore. Last fermentative module was withdrawn after 384 h from the beginning of the cultivation.

This intermittent scheme of operation of the semicontinuous PBB explains the temperature drops every 24 h, as seen on Fig. 2(b), once the flows of percolating air and of cooling-water had to be shortly interrupted for the addition, removal and movement of the modules. The pattern that higher temperatures are registered at highest fermentative modules (positions 5-6 and 6-7 on Figs. 1(a) and 2(a)) continued to be observed. However, on the semicontinuous mode of operation with descending modules and ascending aeration, the second fermentative module (position 4 on Fig. 1(b)) kept its temperature between 43 and 45 °C along the cultivation, suggesting that module movement favored thermal homogeneity along bed height. The cyclic repetition of thermal profiles along the cultivation confirmed that the bed achieved steady-state after bed height was fixed. With this, identical profiles passed to be registered for a given position along the column. Hence, each module can be considered as a cluster traveling through the plug flow reactor.

Experimental data here obtained for this mode of operation of a PBB for SSF are unprecedented in the literature. By simulations, Mitchell et al. [11] reported predicted results for a PBB operated as semicontinuous for the cultivation of *Aspergillus niger* on wheat bran (maximum specific growth rate 0.236 h^{-1}). According to these authors, either keeping air temperature at 33 °C (given as 5 °C below the optimum temperature) in order to avoid overheating, the predicted data indicated that the temperature would reach 45 °C at the top of the PBB with end-to-end aeration in batch operation. On the other hand, simulations of the semicontinuous operation of the same system indicated that maximum temperature reached would decrease for 41 °C.

In the current paper, maximum temperatures were not that much different on the operations as batch (T_{max}=47.9 °C) or as semicontinuous (T_{max}=47.7 °C), probably due to both operational concerns and intrinsic microbial characteristics. Concerning operationality, Mitchell et al. [11] simulated very thin layers (1 cm) of a very large-diameter (1 m) PBB being moved every 1 h. However, these operational conditions are experimentally unpracticable, hence we used 10 cm layers being moved every 24 h. Simulation tests showed that the intervals between consecutive movement of the modules affects significantly on how much the maximum temperatures reached will decrease from batch to semicontinuous operation [18]. The longer the intervals, the higher the difference of maximum temperatures predicted for batch and for semicontinuous modes of operation of the PBB. This is one reason why our temperatures were not that much different for both modes of operation.

On the microbial aspects, the thermophilic fungus here chosen as case-study presents maximum specific growth rate 0.06 h^{-1} at 45 °C [9]. Hence, metabolic heat generation rate can be expected

as four-times lower than the one found when *A. niger* is cultivated. Moreover, the substrate used in the current paper is mostly composed of sugarcane bagasse, a fibrous material that provides high porosity to the packed bed. Therefore, conductive and convective heat dispersion is improved [19,20]. Additionally, the modules of our PBB have a jacket through which water flows continuously along the process at the ideal temperature. Once bed diameter was not that large (only 13 cm), heat transfer by conduction on radial direction must have favored the temperature maintenance close around the optimal one. On the other hand, as mentioned before, Mitchell et al. [11] simulated layers with 1 m diameter, within which heat removal by cooling jacket would become negligible [9].

Either in batch or in semicontinuous mode of operation, the substrate did not dry out along the process, according to measurements of the final moisture content of the cultivated material (data not shown). This can be attributed to the combination of three factors: i) the temperature increase within the PBB was very mild; ii) the flowing air entered saturated into the column; and iii) the inlet accessory module was effective in avoiding the flowing air becoming undersaturated. Moreover, although respiration gases (oxygen and carbon dioxide) measurements were not performed, it is possible to suppose the descendent movement of the modules on the semicontinuous mode of operation can favor an ideal oxygen supply. On this operation scheme, the module where microbial concentration is higher receives fresh air just entering the reactor, rich in oxygen and poor in carbon dioxide. As air flows in direction to upper modules, it became more concentrated in carbon dioxide, which is released along the column and transported by advection.

The semicontinuous mode of operation of the PBB here presented, unprecedented in an experimental context, can be applied for many other combinations of microorganism, substrate and product. Mainly when shear-stress sensitive microorganisms are employed, this mode of operation allows making SSF processes closer to a continuous regime, while still keeping the bed of particles unshaken.

Furthermore, the semicontinuous operation provides shorter process downtime, enables one to extend the PBB operation and allows the recovery of enzymes over a longer period. Additionally, in the current work, because only one module was driven for downstream steps per day, product withdrawn and extraction became easier and faster once enzyme recovery was performed throughout the cultivation. Hence, it is possible to say the semicontinuous operation improved overall PBB performance, since it behaved like a plug-flow reactor, with temperature and product concentration profiles being kept constant at a given position and avoiding either overheating or product heterogeneity.

As already mentioned, based on the results of the experimental design, one more batch cultivation was performed in order to provide additional modules for improved extraction tests. Thermal profiles are not shown for this extra cultivation, but all temperatures behaved like presented on Fig. 2(a), as well as there was no drying of the substrate.

2. Results of Extraction Experiments

Fig. 3 presents the percentage of total soluble proteins, comprising enzymes, recovered from modules containing the cultivated material by percolation (see conditions on Table 2) and respective residuals. As observed, the mass percentage of proteins recovered by percolation was always significantly higher than the residual one recovered by mixing after the percolation. In most of the tests, more than 60% of proteins were extracted by percolation, almost reaching 70% for lower ratio of volume of liquid per mass of initial dry solid material, higher flow rate and longer time, indicating the system was somehow efficient for solubilizing total proteins from the cultivations.

Fig. 4 present the percentage of relative endoglucanase activity (U/gss) recovered from the cultivated material by percolation and residual. In most cases, higher percentage of endoglucanase activity

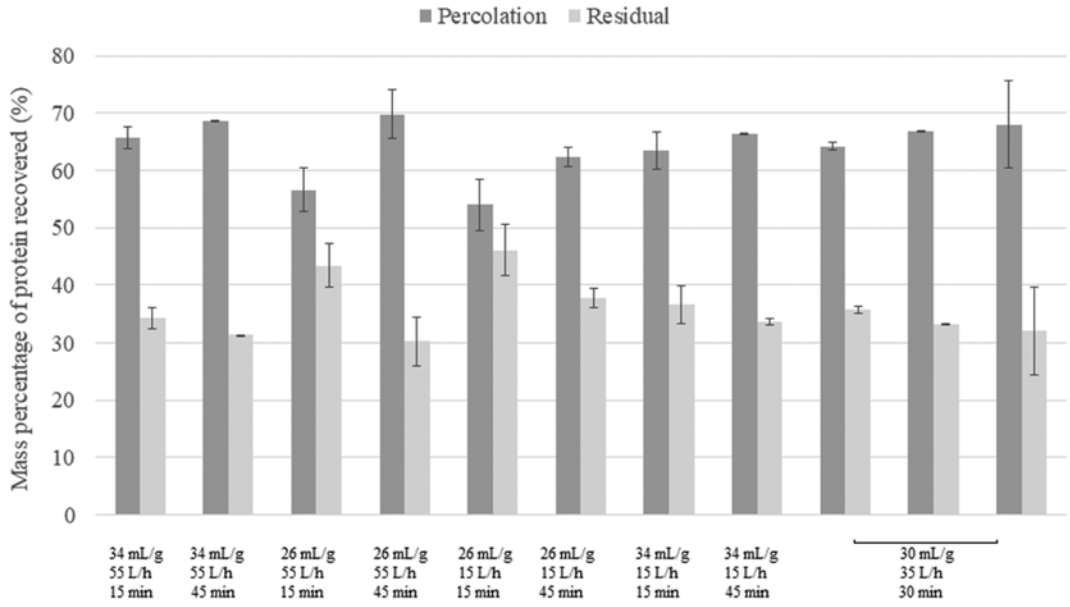

Fig. 3. Mass percentage of proteins recovered by percolation and residual, according to experimental design.

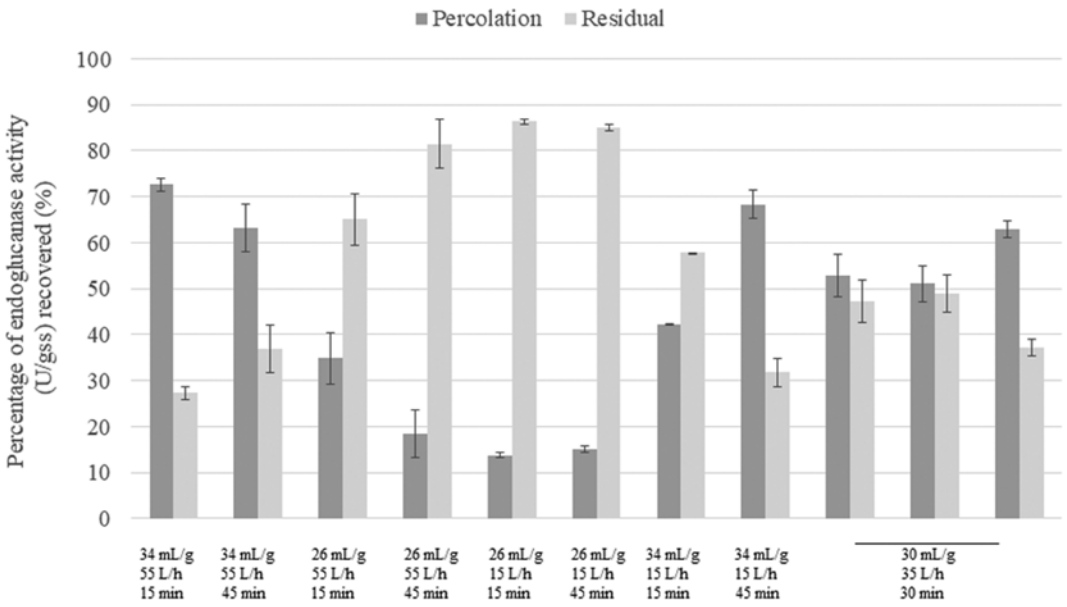

Fig. 4. Relative endoglucanase activities recovered by percolation and residual, according to experimental design.

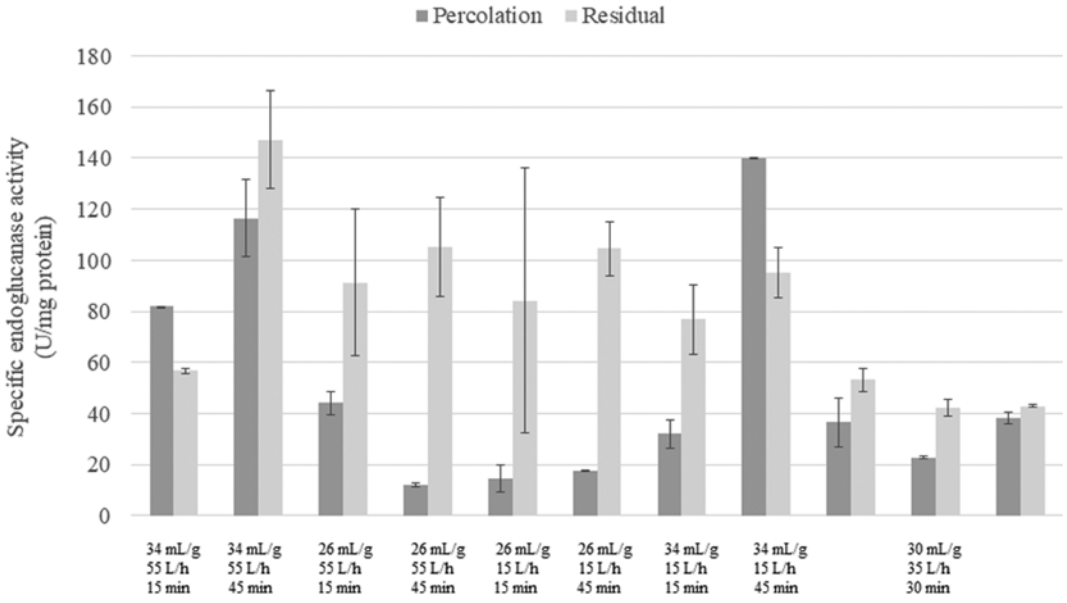

Fig. 5. Specific endoglucanase activity ($U/mg_{protein}$) of extracts from percolation and residual, according to experimental design.

remained on the residuals, suggesting low enzymatic activity being recovered by percolation. This result suggests that extraction by percolation will still be improved, aiming to increase the recovering of enzymatic activity by the automatized extraction system and, consequently, decreasing the residual enzyme activity on the percolated solid material. From Fig. 4, it seems that higher percentage of endoglucanase activity was recovered by percolation when the levels of all the variables were the highest ones, as for instance (34 mL/g, 55 L/h and 15 or 45 minutes). It is important to highlight that extracts from percolation were more diluted than the residual ones, once the volume of water needed to fill the system was higher than the minimal enough for a good mixing extraction.

Specific enzymatic activities of the extracts from percolation and residuals, expressed as $U/mg_{protein}$, were calculated by dividing their endoglucanase activities ($U/mL_{extract}$) per their respective total soluble proteins concentration ($mg_{protein}/mL_{extract}$). Results are shown on Fig. 5. Despite the high data dispersion, residuals presented higher specific activity, suggesting these extracts were purer than the ones obtained from percolation. In most of the cases, low specific activity is related to low endoglucanase activity of the correspondent extract from percolation.

To confirm the effect of the operational variable, statistical analysis of the experimental design was performed for the three interesting response variables of the current paper. First, taking the percentage of proteins recovered by percolation, the pareto chart (Fig. 6(a)) shows that variables volume of water per gram of substrate

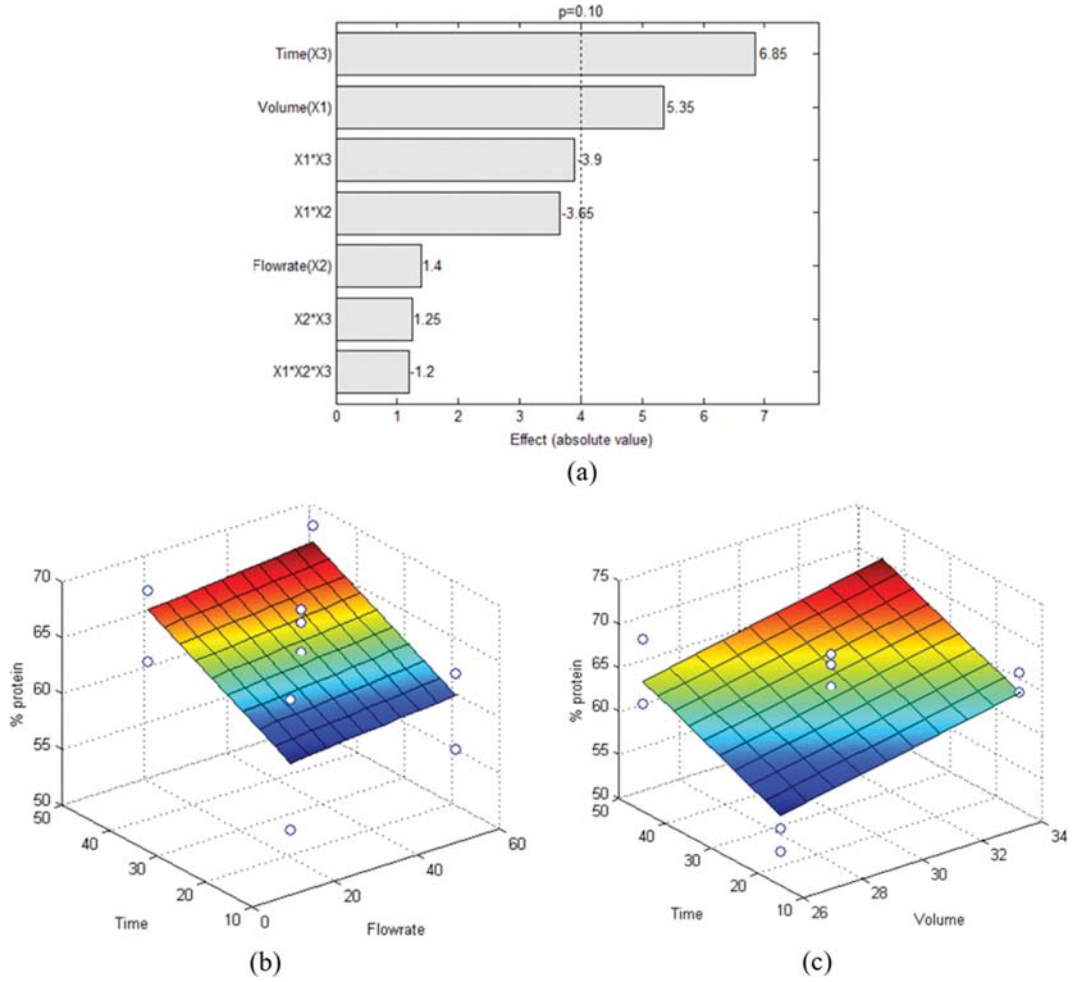

Fig. 6. Pareto chart (a) and response surfaces ((b) and (c)) for percentage of proteins recovered by percolation; in (b), volume is 30 mL per gram of initial dry solid substrate; in (c), flowrate is 35 L/h.

and time of circulation have a significant effect (p-values=0.06 and 0.04, respectively, with α-value=0.1) on the results. Complementing Fig. 6(a), response surfaces of percentages of proteins recovered by percolation are presented as a function of time and flow rate (Fig. 6(b)) and as a function of time and volume per gram (Fig. 6(c)). Even the flow rate has a non-significant statistical effect; response surface shows that the higher level of the three variables favored proteins extraction by percolation.

When the chosen response is the percentage of endoglucanase activity recovered by percolation, the pareto chart (Fig. 7(a)) shows that variable volume has a significant effect, as well as an interaction of variables time and flow rate (p-values=0.012 and 0.098, respectively, with α-value=0.1). Complementing Fig. 7(a), response surfaces of endoglucanase recovered by percolation are presented as a function of time and flow rate (Fig. 7(b)) and as a function of time and volume (Fig. 7(c)). The higher the volume, the higher the percentage of endoglucanase activity recovered by percolation (Fig. 7(c)). On the interaction effect (Fig. 7(b)), when airflow rate is higher, shorter time increases endoglucanase recovered; when airflow rate decreases, longer time improves enzyme activity recovered by percolation.

Finally, considering specific enzymatic activity as response variable, Fig. 8(a) shows that volume and time are the principal variables having a significant effect (p-values=0.0071 and 0.0416, respectively, with α-value=0.1), as well as interactions of volume and time (p=0.0189) and flow rate and time (p=0.0454). The highest the volume, the time and the flow rate increased the percentage of endoglucanase activity recovered by percolation (Figs. 8(b) and 8(c)).

According to Ichiba [21], who studied the recovering of proteins and endoglucanase activities by tank-mixing extraction (same procedure used for obtaining the residuals on the current paper), time played significant negative effect on proteins extraction, while volume presented a positive effect. This suggests that equilibrium is achieved in a short period, with possible re-adsorption occurring for longer times. The higher the volume, the higher the amount of proteins extracted; this suggests that lower volumes could not be enough to solubilize all proteins due to saturation effects. On endoglucanase activity recovered, either volume, time, and agitation speed had a significant positive effect. Specific activities were not significantly affected by the variables studied by Ichiba [21] in mixing-tank extraction.

Pirota et al. [12] also studied enzyme recovery from SSF using

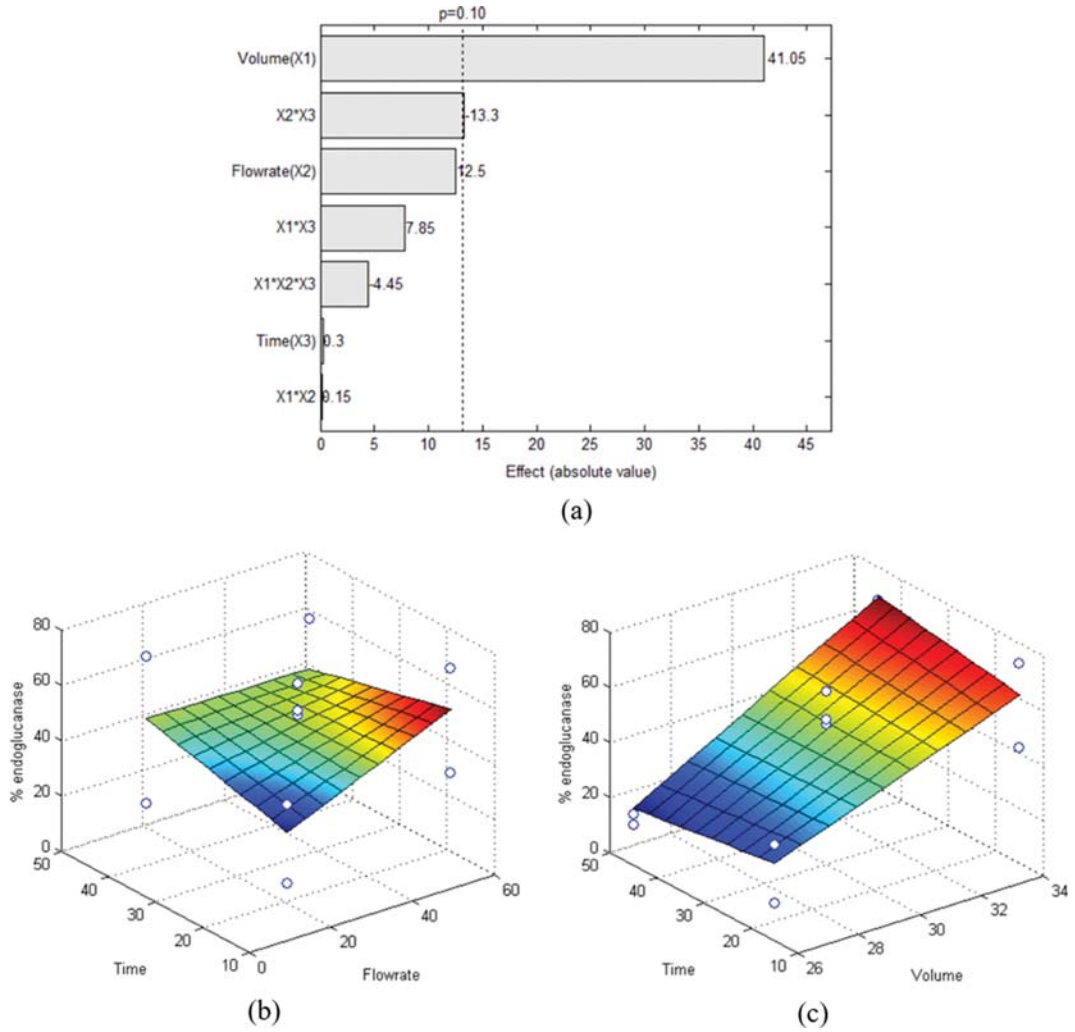

Fig. 7. Pareto chart (a) and response surfaces ((b) and (c)) for percentage of endoglucanase activity recovered by percolation; in (b), volume is 30 mL per gram of initial dry solid substrate; in (c), flowrate is 35 L/h.

0.2 mol/L acetate buffer at pH 4.8 for tank-mixing extraction. They applied an experimental design methodology to investigate the effects of the operational parameters solid to liquid ratio (1 : 3, 1 : 6 and 1 : 9), stirring rate (80, 120 and 160 rpm), and temperature (10, 22 and 35 °C) on the recovery of endoglucanases produced by *Aspergillus niger* cultivated under SSF. Authors found that only the solid to liquid ratio had a significant effect on endoglucanase extraction. Highest endoglucanase recovery was achieved using 9 mL/g during 10 minutes agitation. They also performed sequential extraction experiments, by means of which they concluded that most of the enzyme was recovered during the first extraction.

Therefore, both extraction methods responded similarly to variable volume, but time had a different meaning on mixing-tank or in a percolating column. For percolation, another time related variable that appeared is the residence time, a variable that relates the packed-bed volume to the liquid flow rate. From Table 2, it is possible to infer that time of circulation varied from 3 to 30 times the residence time, meaning the liquid percolated the cultivated material this number of times. Fig. 4 slightly suggests that longer times of residence decrease the recovering of endoglucanase activity by percolation. This agrees with the findings of Ichiba [21] if we consider time of residence in extraction by percolation equivalent to time of contact in tank-mixing extraction. Aiming to improve the yields of protein and endoglucanase activity recovered by percolation, additional tests were carried out using higher levels of flow rate and time of circulation.

3. Improved Results of Extractions by Percolation

Fig. 9 shows the results of improved extraction by percolation. In first improved test, 20 mL per gram of initial dry solid substrate percolated the packed bed for 120 min at 60 L/h (residence time 1.3 min, 90 cycles). In these conditions, more than 60% of proteins (Fig. 9(a)) and around 75% of endoglucanase activity (Fig. 9(b)) were recovered by percolation; consequently, specific activity of the extract from percolation was 75% higher than the one of the extract from tank-mixing (Fig. 9(c)). On second improved extraction, 27 mL per gram of initial dry solid substrate percolated the packed bed for 30 min at 2,400 L/h (residence time 2 seconds, 900 cycles). At these conditions, nearly 80% of proteins (Fig. 9(a)) and around 85% of endoglucanase activity (Fig. 9(b)) were recovered by percolation; consequently, specific activity of the extract from percolation

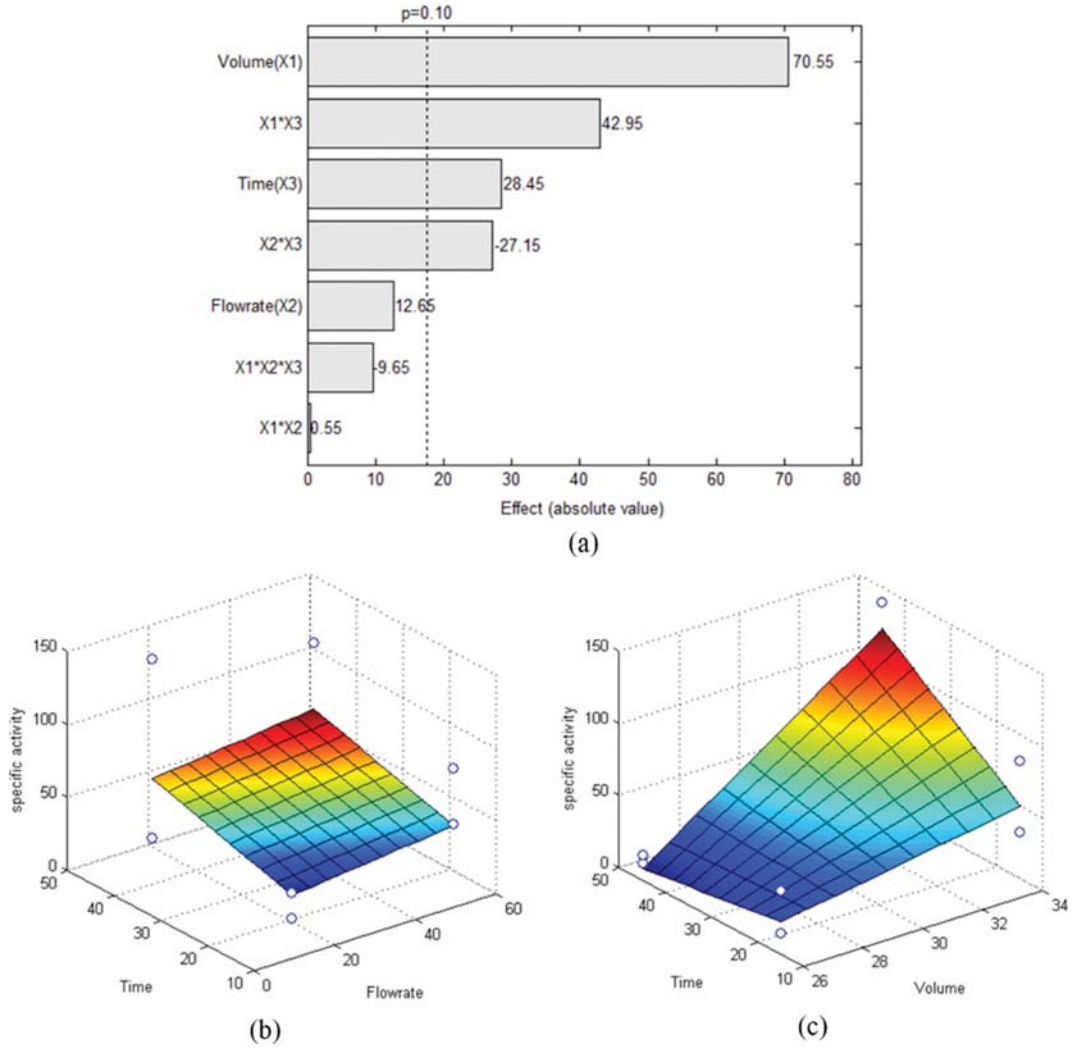

Fig. 8. Pareto chart (a) and response surfaces ((b) and (c)) for specific endoglucanase activity recovered by percolation; in (b), volume is 30 mL per gram of initial dry solid substrate; in (c), flowrate is 35 L/h.

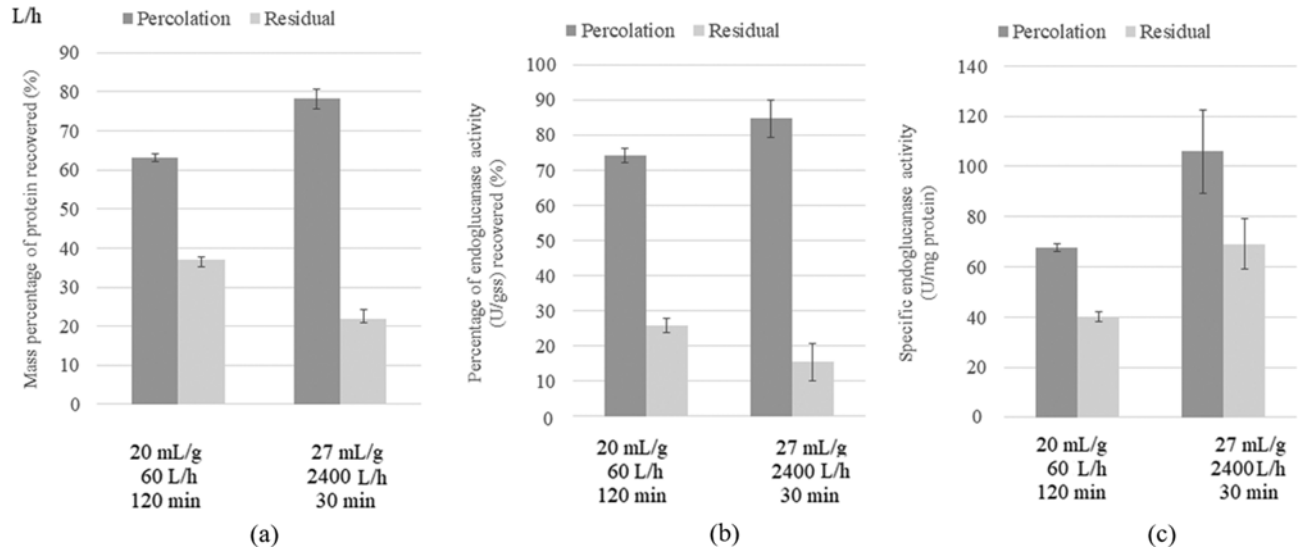

Fig. 9. Mass percentage of protein (a), relative endoglucanase activities (b) and specific activities (c) recovered by percolation and residual, for the improved extractions.

was nearly 60% higher than the one of the extract from tank-mixing (Fig. 9(c)).

Comparing both improved extractions, a higher flow rate provided better results than a much longer time, both with intermediary volume of water per gram of solid cultivated. Volume was not increased to avoid that final extracts from becoming very diluted. According to Zanelato et al. [13], 20 mL of water per gram of initial dry solids is enough for a good mixing-tank extraction, with no saturation of the solution with those enzymes. Hence, the increase of this volume to 27 mL per gram was probably not determinant for increasing the endoglucanase activity recovered by percolation. The same seems to be true for longer time, once endoglucanase activity recovered by percolation in P1 (34 mL/g, 60 L/h, 15 min, see Fig. 5(a)) was almost the same than recovered by 20 mL/g, 60 L/g, 120 min. On the other hand, the increase of water flow rate significantly improved the percentage of endoglucanase recovered by percolation. According to Fenila and Shastri [22], cellulolytic enzymes produced in SSF are strongly tight with the cellulosic substrate. Therefore, higher flow rate is needed to elute these enzymes from the cultivated porous medium.

Aiming at a more concentrated final extract, a last extraction test was performed for four modules cultivated with *M. thermophila*, using flow rate 2,400 L/h for 30 minutes and 27 mL/g. Fresh distilled water was used for percolation of the first module only; for next modules, the same liquid that percolated the previous modules was circulated. Results indicated this strategy was not suitable for extraction by percolation. Although there was an increase in the protein concentration of the extract between the leaching of each further module, the increase in protein concentration in the extract decreased over the modules. Protein mass obtained from the first module (approximately 3 g) was nearly double the mass obtained from percolation of the second module, addressing a decrease in the efficiency of the process, probably due to liquid saturation. Moreover, the recovered enzymatic activity remained almost constant between the first and the last two modules percolated, making the purity of the endoglucanase to be reduced, reinforcing that the percolated liquid may have reached a concentration such that the removal of enzymatic material was no longer possible. Therefore, although the final extract was a little more concentrated, the extraction efficiency decreased, hence enzymes from last modules would be wasted, if this operational condition were applied.

CONCLUSIONS

This paper reports the experimental results of enzymes production by SSF in a PBB operated in batch and semi continuously, coupled to a system built for performing the first downstream step for recovering the bioproduct of interest. The system was designed, built and tested according to an experimental design. When operated under improved conditions of water flowrate, time and volume for extraction, the system allowed recovery up to 85% of the total enzyme activity by percolation, dispensing manipulation of the cultivated material. Hence, the proposed integration of cultivation in a PBB operated as semi continuous and extraction by percolation can be considered as a promising strategy for enzyme production by SSF inside biorefineries.

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CONFLICT OF INTEREST

No conflict of interest declared.

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