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RESEARCH PAPER

Successive membrane separation processes simplify concentration of lipases produced by *Aspergillus niger* by solid-state fermentation

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Abstract In this study, we developed a simplified method for producing, separating, and concentrating lipases derived from solid-state fermentation of agro-industrial residues by filamentous fungi. First, we used *Aspergillus niger* to produce lipases with hydrolytic activity. We analyzed the separation and concentration of enzymes using membrane separation processes. The sequential use of microfiltration and ultrafiltration processes made it possible to obtain concentrates with enzymatic activities much higher than those in the initial extract. The permeate flux was higher than 60 L/m^2 h during microfiltration using 20- and 0.45-μm membranes and during ultrafiltration using 100- and 50-kDa membranes, where fouling was reversible during the filtration steps, thereby indicating that the fouling may be removed by cleaning processes. These results demonstrate the feasibility of lipase production using *A. niger* by solid-state fermentation of agro-industrial residues, followed by successive tangential filtration with membranes, which simplify the separation and concentration steps that are typically required in downstream processes.

Keywords Agro-industrial residues · *Aspergillus niger* · Lipase · Microfiltration · Ultrafiltration

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Introduction

Currently, the production of enzymes is improving rapidly due to the better understanding of the structure of specific enzymes, the increased use of the enzymes, and optimization of production and purification processes. The industrial applications of enzymes are growing due to the development of new technologies, the use of genetic engineering in production, and the need for new fields of application. The global market for industrial enzymes was estimated at US$ 4.2 billion in 2015, and it is expected to reach US$ 5.8 billion in 2020 [1].

Lipases are enzymes that can catalyze the partial or total hydrolysis of triglycerides to yield free fatty acids, monoglycerides, and diglycerides, and they are also active in esterification, transesterification, and interesterification in water-restricted environments. Lipases are employed in different industrial sectors, such as the production of food, detergents, textiles, pulp and paper, grease, oil, biodegradable polymers, pharmaceuticals, cosmetics, teas, biosensors, leather, and biodiesel, as well as in diagnostic tests, medical applications, and sewage treatment, as reviewed previously [2, 3].

Microbial enzymes are preferred for industrial applications because of lower production times, ease of genetic manipulation, simple scale-up and purification, specificity, and stability. Industrial lipases are mainly produced by filamentous fungi, particularly *Aspergillus, Rhizopus, Penicillium, Mucor, Geotrichum*, and *Fusarium*. However, the increased industrial use of enzymes demands lower production costs. Strategies to meet this need include the selection of new microorganisms for production and the use of inexpensive culture media. In particular, the use of solid-state fermentation for the production of fungal lipases is preferable, because this process can

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utilize agricultural residues in the culture medium, such as soybean meal, mustard seed, peanuts, walnuts, rice bran, barley bran, castor beans, and sugar cane bagasse [4].

After producing the enzyme, it is necessary to separate and purify the product, which are the biggest challenges in the area of separation processes because of the unique characteristics of the particles that need to be concentrated. Industrial enzymes are typically produced using submerged or solid-state fermentation, followed by the traditional methods of enzyme recovery (centrifugation, pre-filtration, and chemical precipitation). However, centrifugation increases the temperature and causes protein denaturation, as well as preventing the separation of proteins with low densities. The conventional dead-end filtration method has a low yield due to cake formation early in the process, which prevents the remainder of the operation. Precipitation involves the use of chemical agents which increase the cost and the amount of waste [5].

The separation of components from a mixture is an important unit operation. Various technologies are available for separation processes, which are based on the physical and chemical properties of the mixture. A method that is becoming increasingly attractive is separation by membranes via tangential filtration because of the emergence of new types of membranes, as well as the modification of process parameters by varying the pressure, shear rate, temperature, and electrostatic charge of the system [6]. Membrane separation technologies have several advantages, such as energy savings, selectivity, the separation of labile compounds, simplicity of operation, and scale-up. The major applications of membrane separation processes include sterilization, clarification, cell concentration, protein concentration and fractionation, solvent recovery during oil extraction, wastewater treatment, enzyme purification, desalination, and various other applications [7].

Although cross-flow filtration processes with membranes have various advantages, it is necessary to perform a thorough assessment of the process parameters that influence membrane fouling to optimize the permeate flux, and thus the effective concentration of the enzyme. The use of sequential separation processes (microfiltration and ultrafiltration) can reduce the number of downstream stages, thereby minimizing the production costs. The industrial production of enzymes is growing significantly, and thus, improvements are required in the efficiency of cultivation and the purification processes involved in production [8]. In the present study, we aimed to produce lipases from agricultural residues using solid-state fermentation with filamentous fungi and to concentrate these enzymes via sequential membrane separation processes.

Materials and methods

Microorganism and inoculum preparation

The fungus used in this study was *A. niger*, which was previously isolated by Colla et al. [9] from diesel-contaminated soil and selected as lipase producer during solid-state fermentation. The microorganism was submitted for genetic identification at the Center for Nuclear Energy in Agriculture (CENA) from the University of São Paulo (Brazil). The sequences were compared with 18 S rRNA data obtained from GenBank (http://www.ncbi.nlm.nih.gov), and the fungus was identified as *A. niger* DAOM (100% identity, GenBank accession number: KC545858.1).

The microorganism was kept in inclined tubes with potato dextrose agar (PDA) under refrigeration at 4 °C with periodic replications every 3 months. The inoculum was prepared by inoculating *A. niger* into 1 L Erlenmeyer's flasks containing 30 mL of solidified PDA medium, followed by incubation at 30 °C for 7 days. A spore suspension was obtained by adding 20 mL of 0.1% Tween 80 to the inoculum after incubation and by scraping the spores with a Drigalski spatula [9].

Lipase production by solid-state fermentation

To produce lipase, solid-state fermentation was performed in polypropylene trays (30 cm × 35 cm) containing 250 g of the medium (dry basis). The culture medium employed was prepared according to the procedure described with 85% wheat bran and 15% rice husk [9]. The bran was used as a source of nutrients and support, while the husk was used to increase the porosity of the medium and to facilitate oxygen transfer. The culture medium was autoclaved at 121 °C for 20 min and then added to saline solution with 2% soybean oil as an inducer of lipase production. The moisture content was adjusted to 65% by adding sterile distilled water. The *A. niger* spore solution was inoculated at a final spore concentration of 10^7 spores per g of substrate. The trays were covered with hydrophobic acrylic fabric and incubated at 30 °C for 6 days (144 h), before enzyme extraction prior to the membrane separation processes. The enzymes were extracted as described [9], using a ratio of 1 g of fermented medium relative to 10 mL of sodium phosphate buffer 0.1 mol/L (pH 7.0), followed by agitation at 160 min^{-1} for 30 min at 37 °C. The enzyme extracts were stored in a freezer (−18 °C) until the microfiltration and ultrafiltration processes.

Concentration of lipases using membrane separation processes

The enzyme concentration assays were performed in a membrane module made of acrylic. The system comprised a PVC tank to house the feed solution, a valve to control the feed, a module for the membrane (measuring 14.5 cm long × 8.5 cm wide × 8.7 cm high), a peristaltic pump (Masterflex®, model 77200-52), and a valve to control the pressure, manometers, and interconnector hoses (Fig. 1). The maximum operating pressure of the system was 300 kPa. The module was designed for use with flat membranes measuring 12.5 cm long × 6.0 cm wide, thereby presenting a filtration area of 0.006 m^2 (60 cm^2). Five different flat membranes were used sequentially. The two microfiltration membranes were hydrophilic and made of cellulose acetate, where the pore diameter was 20 μm for the first membrane (Whatman®, Grade 4) and 0.45 μm for the second (Millipore®, model HAWP04700). The three hydrophilic ultrafiltration membranes were made of polyethersulfone. The first (Omega®, model OM100150) had a molecular weight cutoff (MWCO) of 100 kDa, the second (Omega®, model OM050150) had a MWCO of 50 kDa, and the third (GE Osmonics®, YMPWSP3001 model) had a MWCO of 20 kDa.

The enzyme extract was first passed through a microfiltration membrane with pores of 20 μm to remove any suspended material. This permeate was then subjected to a second round of microfiltration using a membrane with pores of 0.45 μm to separate particles > 0.1 μm from the fermentation extract. The filtration system operated at 25 °C with a fixed flow rate of 2 L/min and a transmembrane pressure of 20 kPa for the first microfiltration step and 98 kPa for the second microfiltration step. The membranes were immersed in deionized water at room temperature for 24 h before use. After each microfiltration step, the protein level and hydrolytic activity were determined in the permeates and the retentates. The permeate from the second microfiltration step was stored in a refrigerator (4 °C) for subsequent ultrafiltration. Three sequential separation steps using ultrafiltration membranes (MWCO of 100, 50, and 20 kDa) were performed to fractionate the enzyme extract. The permeate from each filtration step was used as the feed solution for the next process. The system operated at 25 °C with a fixed flow rate of 1 L/min and a transmembrane pressure of 98 kPa for the first and second ultrafiltration steps and 196 kPa for the third ultrafiltration step. The membranes were immersed in ethanol for 24 h and then in deionized water at room temperature for 24 h to allow membrane conditioning. After each ultrafiltration step, the protein level and hydrolytic activity were determined in the permeate and the retentate. To evaluate the feasibility of concentrating the enzymatic extract directly using one single membrane, an attempt was first carried out, using a 20 kDa ultrafiltration membrane and the initial enzymatic extract as feed.

The different enzymatic extracts obtained from the membrane separation processes (permeates and retentates) were also analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

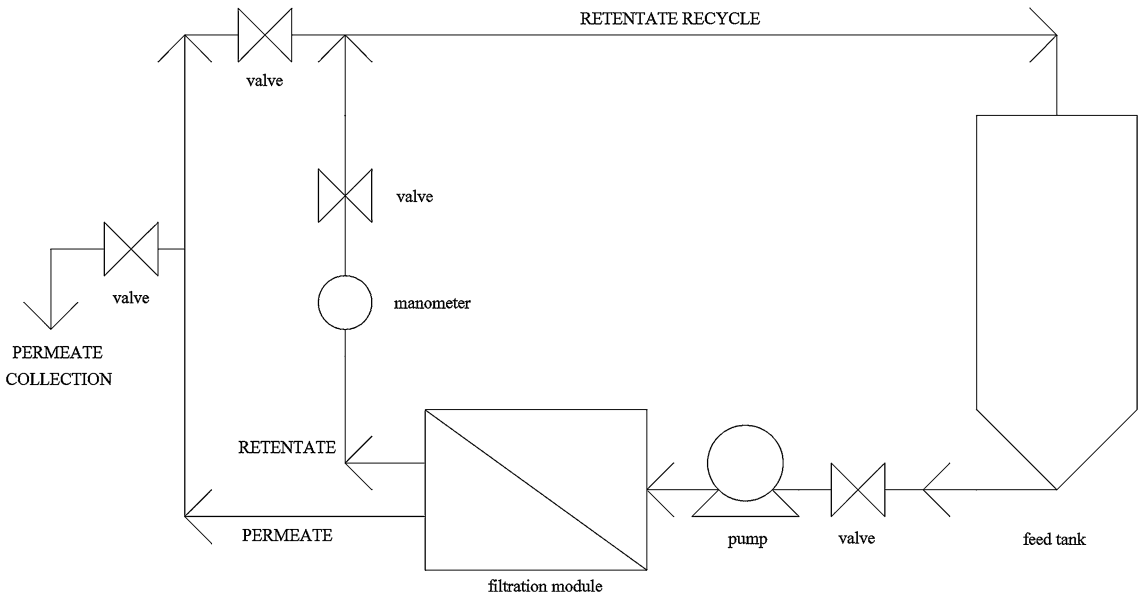

Fig. 1 Schematic diagram of the membrane filtration set

Determination of enzymatic activity levels

The hydrolytic activity and specific enzymatic activity of the lipases were determined in triplicate using the method proposed by Burkert et al. [10], which is based on using sodium hydroxide to titrate the free fatty acids released by the action of lipase from the enzyme extract on triacylglycerols in olive oil emulsified by gum arabic. Emulsion without enzyme was employed during assay as control. One unit of hydrolytic activity was defined as the amount of extract that released 1 µmol of fatty acid per minute per mL of enzyme extract (1 U/mL = 1 µmol/min mL) under the test conditions. One unit of specific enzymatic activity was defined as the amount of extract that released 1 µmol of fatty acid per minute per mg of enzyme (1 U/mg = 1 µmol/min mg) under the test conditions.

Characterization of the membrane separation processes

The deionized water flux was measured to determine the hydraulic permeability of the five types of membranes. Samples were collected every 5 min during the process, where the transmembrane pressure (ΔP) increased throughout sampling. The permeate flux (J) was calculated by dividing the volume (V) of permeate collected by the area (A) of the membrane and by the time (t) of sampling (Eq. 1), while the hydraulic permeability (L_p) was determined by Eq. 2. The permeate fluxes were also measured during the sequential filtration steps for the enzymatic extracts and calculated according to Eq. 1:

$$J = \frac{V}{A \cdot t} \tag{1}$$

$$J = L_p \cdot \Delta P. \tag{2}$$

To assess the resistance of each sequential filtration process, the model of resistances arranged in series was used, as given by Eq. 3 [11], where R_{uf} is the total resistance of the fouled membrane, R_m is the intrinsic hydraulic resistance of the membrane, R_{if} is the resistance due to irreversible fouling, and R_{rf} is the resistance due to reversible fouling. The total resistance (R_t) was calculated according to Eq. 4, where μ is the permeate viscosity. The membrane resistance was determined based on filtration with water, the irreversible fouling resistance was calculated after filtration and washing with water, and the reversible resistance was determined by difference:

$$R_{uf} = R_m + R_{if} + R_{rf} \tag{3}$$

$$R_t = \frac{\Delta P}{\mu \cdot J}. \tag{4}$$

The retention (R) of the membrane was calculated according to Eq. 5 [12], where Par is the evaluated parameter (protein or hydrolytic activity). R was determined for each step in the sequential separation process:

$$R = \left(1 - \frac{\text{Par}_{\text{permeate}}}{\text{Par}_{\text{retentate}}}\right) \times 100. \tag{5}$$

The membranes used in separation processes were evaluated by scanning electron microscopy (SEM; VEGA 3LM, Tescan Orsay Holding) to monitor the morphology, and thus to observe changes in the structure before and after the procedures. Prior to the SEM analysis, samples of the ultrafiltration membrane were fractured in liquid nitrogen. The membranes were mounted on a stainless steel disk using double-sided carbon tape, before being subjected to sputter-coating with a thin gold film (300 Å) at 30 mA for 4 min using a metallizer (ES-Q150R, Quorum Technologies Ltd.).

Results

Lipases production by solid-state fermentation and concentration using membranes

The enzymatic activity of the extract was evaluated after 6 days (144 h) of the solid-state fermentation process using trays inoculated with *A. niger*. The results showed that the average hydrolytic activity was 20.24 U/mL, thereby indicating the ability of the fungus to produce lipases with hydrolytic activity from agro-industrial residues.

Sethi et al. [13] produced lipases with *A. terreus* using agro-industrial residues. The use of wheat bran in solid-state fermentation to obtain lipases yielded a hydrolytic activity of 50 U/mL after optimizing the fermentation parameters by combining different organic residues and supplying complementary nutrients for the fungus. This was also the case in the present study, where we prepared a medium with wheat bran and rice husk, which was supplemented with a mixture of micronutrients. The use of solid-state fermentation of organic residues is becoming important for reducing the production costs of enzymes. Coradi et al. [14] produced lipases by submerged fermentation and solid-state state fermentation using the fungus *Trichoderma harzianum*, where they found that the highest hydrolytic activity in the extract was obtained after submerged fermentation, but the cost of the carbon source was ten times higher due to the amount required, thereby increasing the cost of producing lipase by solid-state fermentation.

Our previous study [9] showed that the enzyme produced by this *Aspergillus* strain presented the maximum hydrolytic activity at pH 6.0 and 35 °C. The enzyme also

keeps 72% of residual activity after 1 hour of exposition at 90 °C, presenting a high thermostability. Moreover, lipase showed residual activity higher than 80% even in basic pH (between 8.0 and 10.0), which means that this enzyme might have applications in industrial processes that occur at higher temperatures and wide pH ranges.

The first attempt to concentrate the enzymatic extract using one single ultrafiltration membrane (20 kDa) was not successful. The pores were immediately blocked, and the permeate flux was zero. The system pressure would have to be increased too much to obtain a few drops of permeate, making the process impossible, technically and economically.

After the first unsuccessful filtration, the enzymatic extract was then concentrated through successive separation steps. Table 1 shows the hydrolytic activities of the samples at each step in the sequential filtration process and the protein levels. Both the protein content and the initial hydrolytic activity of the crude extract were not significantly different ($p > 0.05$) to those in the permeate from the first microfiltration, which can be explained by the retention of particles >20 µm, thereby yielding similar concentrations of small molecules, such as proteins in both samples (the retentate and permeate). The second stage (microfiltration with a 0.45-µm membrane) yielded the highest retention of protein (>60%), and the combination of this step with the subsequent ultrafiltration stage (100 kDa) retained over 90%. The retention of protein did not reach 100% at the 20 kDa ultrafiltration stage, which indicates that low-molecular weight peptides were collected in the permeate from the last stage.

Table 1 Protein concentration and enzymatic activities of the samples during each successive step using microfiltration and ultrafiltration membranes

Sample	Protein (mg/mL)*	HA (U/mL)*	$HA_{specific}$ (U/mg)*
Initial extract	$15.36^{b} \pm 0.68$	$20.24^{b} \pm 0.65$	$1.318^{cd} \pm 0.042$
Retentate (20 µm)	$14.26^{b} \pm 1.36$	$19.78^{b} \pm 1.95$	$1.387^{bc} \pm 0.137$
Permeate (20 µm)	$15.53^{b} \pm 1.30$	$19.32^{b} \pm 1.95$	$1.244^{cd} \pm 0.126$
Retentate (0.45 µm)	$26.25^{a} \pm 1.86$	$18.86^{b} \pm 0.65$	$0.718^{d} \pm 0.025$
Permeate (0.45 µm)	$10.06^{c} \pm 1.86$	$14.72^{b} \pm 0.65$	$1.423^{bc} \pm 0.065$
Retentate (100 kDa)	$17.19^{b} \pm 0.06$	$60.72^{a} \pm 3.90$	$3.532^{a} \pm 0.227$
Permeate (100 kDa)	$2.49^{d} \pm 0.19$	$4.60^{c} \pm 0.65$	$1.845^{bc} \pm 0.261$
Retentate (50 kDa)	$3.94^{d} \pm 0.00$	$7.82^{c} \pm 1.30$	$1.986^{b} \pm 0.330$
Permeate (50 kDa)	$2.54^{d} \pm 0.12$	0.00^{d}	0.00^{e}

HA hydrolytic activity, $HA_{specific}$ specific hydrolytic activity

*Results expressed as mean ± standard deviation. Means of same alphabet superscript within each column are not statistically significant

The hydrolytic activity of the initial extract did not differ ($p > 0.05$) from that in the permeates obtained from the first and second microfiltration stages. The retentate from the 100-kDa ultrafiltration stage yielded higher results in terms of both the hydrolytic activity and the specific activity, thereby indicating that the lipases produced by solid-state fermentation were retained when using this membrane to concentrate the enzymes. Some lipases permeated the membrane, but they were retained completely by the 50-kDa membrane. The permeate from this membrane had no hydrolytic activity, which indicates that the enzymes were retained by the preceding processes. In the second microfiltration stage (0.45 µm), the retention rate for lipases with hydrolytic activity was around 17%, because the volume concentration factor (VCF, determined as the ratio between the volume of feed at the beginning of each filtering stage relative to the volume of retentate after the process) used was 6. The ultrafiltration stage with the 100-kDa membrane yielded a retentate with a hydrolytic activity that was 4.3-fold higher than its feed, which demonstrates that the membrane separation process is a viable option for concentrating the lipases produced by fungi during solid-state fermentation of organic residues. The specific hydrolytic activity also increased 2.5 times after the completion of this stage of the process. The permeate obtained from ultrafiltration with the 100-kDa membrane had a lower enzymatic activity than the previous fractions, which showed that most of the lipase produced by the fungus was retained on the 100-kDa membrane. After the ultrafiltration step with the 50 kDa membrane, no hydrolytic activity was detected, although some protein molecules permeated the membrane. The retention of the hydrolytic activity was 22% (calculated by considering the relative ratio of the enzymatic activities in the permeate and the retentate) during the second microfiltration step. In the first ultrafiltration step, this ratio was 92%, but it reached 100% during the second ultrafiltration step, and thus the final process was unnecessary in terms of the retention of the enzymatic activity.

Bharti et al. [15] purified and characterized the lipase produced by *A. japonicus*, and found that it had an approximate molecular weight of 40 kDa. Fernández-Lorente et al. [16] purified lipases derived from different strains of *A. niger* with various adsorption methods and several bands were observed after the preliminary analysis of the initial crude extracts, which indicated the presence of proteins with different molecular weights. After purification, three lipases were identified with the following molecular weights: 31, 43, and 65 kDa, all of which had varying degrees of selectivity. Coradi et al. [14] produced lipases

using organic residues by solid-state fermentation with the fungus *Trichoderma harzianum*. Proteins, such as enzymes, have a complex chemical structure. According to Singh and Mukhopadhyay [4], most lipases have an average molecular weight between 20 and 80 kDa, although enzymes have also been reported with weights of 12 and 270 kDa. Normal lipases comprise 250–550 amino acid residues, and their structures include areas with greater or lesser affinity for membranes [4]. In addition, the fouling phenomenon due to pore blocking may have led to the retention of enzymes on the first membranes. In the present study, where we used hydrophilic polyethersulfone ultrafiltration membranes, high retention of molecules weighing <100 kDa occurred on the membrane with the same MWCO. It should be emphasized that the MWCO stated for ultrafiltration membranes generally results in the retention of about 90% of the particles larger than this value, but the membrane pores are not symmetric so many smaller or larger pores are present. Furthermore, the parameters stated by membrane manufacturers may yield different results depending on the method employed and the test conditions [17].

Silva et al. [18] concentrated lipases produced by *P. brevicompactum* via solid-state fermentation of agricultural residues. The optimization of the process yielded enzymes with a hydrolytic activity up to 88 U/g with castor meal and with an esterification activity of up to 244 U/g with babassu meal. However, Silva et al. [18] reported no correlation between the hydrolytic activity and the esterification activity, and the optimum results were obtained in different conditions. Rigo et al. [19] also found that the best conditions for obtaining lipases with hydrolytic activity were not necessarily the same as those for obtaining lipases with esterification activity. This finding is crucial for the production of enzymes by biotechnological processes, because enzymes can be produced by the same microorganism for different industrial applications simply by adjusting the growth conditions. In other studies related to the production of lipases with filamentous fungi, the enzymatic activity values were very different compared with those obtained in the present study. Thus, Almeida et al. [20] evaluated the influence of the culture medium on the production of lipase, initially by screening 90 different fungi. The fungus *A. niger* had a specific hydrolytic activity of 7.5 U, whereas other strains of *Aspergillus* sp. had activities up to 6.4 U. As described [18], the differences found in the previous studies in terms of the absolute enzymatic activity values may have varied because of the microorganism, the raw material, and production conditions, but also due to the experimental conditions employed in the subsequent analysis. Rodríguez-Fernández et al. [12] obtained phytase from *A. niger* by solid-state fermentation using a 10-kDa ultrafiltration membrane, where they achieved a VCF of 6 relative to the starting extract, while the protein concentration was increased and the enzymatic activity was 4.3 times higher. The retention rate was 99.95% and the yield indicated that 14% of the activity was lost during the process, probably due to the inherent shearing stress in the cross-flow filtration process. Rodríguez-Fernández et al. [12] emphasized that the presence of certain ions may have inhibited the enzyme activity, so filtration was useful for eliminating these ions to improve the enzyme activity level. Golunski et al. [21] concentrated inulinase produced by *Kluyveromyces marxianus* via solid-state fermentation using a 100-kDa ultrafiltration membrane, where the purification factor was a 5.5-fold increase with a yield of 86%. According to the authors [21], the variation in the enzymatic activity yield can be attributed to the possible elimination or concentration of inhibitors, which may remain trapped in solution or filtered, while enzyme losses may occur due to adsorption on the membrane surface as a function of fouling. Gottschalk et al. [22] used ultrafiltration processes with membranes made of polysulfone and cellulose acetate with MWCOs of 10, 20, and 50 kDa to concentrate lignin peroxidase produced by *Streptomyces viridosporus*. They obtained high retention rates (>96%) with the 10- and 20-kDa polysulfone membranes, whereas the 50-kDa membrane obtained a retention rate of 58%. The 20-kDa cellulose acetate membrane achieved 77% retention with a smaller decrease in the overall permeability, thereby suggesting the possibility of less adsorption and pore blocking with this membrane. The yields obtained relative to the enzymatic activity were 15, 52, and 74% using the 50-, 20-, and 10-kDa polysulfone membranes, respectively, and 67% using the 20 kDa cellulose acetate membrane. Krstic et al. [23] performed ultrafiltration to concentrate an endo-pectinase solution and different yields were obtained depending on the VCF used, with initial yields of about 33% for a VCF of 1.5 and 7% with a VCF of 3. After changing some of the filtration conditions, such as the pressure, temperature, agitation, viscosity, and process time, the yield increased slightly, reaching values of >90%, thereby demonstrating the feasibility of optimizing the process.

Figure 2 shows the SDS–PAGE results with the different fractions from the successive filtration processes, as well as the initial enzymatic extract and standard markers with molecular weights between 6.9 and 210 kDa. The initial enzymatic extract produced a high number of extended protein bands, which indicated that there was a very complex mixture of different proteins and/or enzymes. These results were compatible with those obtained for the initial samples of working processes involving the purification of enzymes [16]. However, some bands were more intense in the extract, where there were higher amounts of proteins with molecular weights between 40 and 80 kDa, which may have indicated the presence of lipases with different characteristics. This profile was replicated in both microfiltration

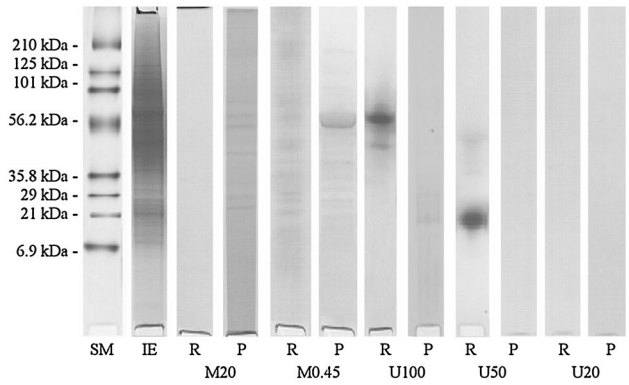

Fig. 2 SDS–PAGE showing the standard markers (SM) as well as the initial extract (IE) and the samples (*R* retentate and *P* permeate) obtained using *A. niger* after microfiltration (M20 and M0.45) and ultrafiltration (U100, U50, and U20) processes

stages, where we also observed the intensification of a band using the permeate from the second microfiltration step (0.45-µm membrane) and using the retentate from the first ultrafiltration step (100-kDa membrane). This component had a molecular weight of approximately 60 kDa, which indicated the presence of lipase, because greater amounts of proteins and higher enzymatic activity were found in the retentate from the first ultrafiltration step. The retentate of the second ultrafiltration step (50-kDa membrane) presented also a component which had a molecular weight of approximately 20 kDa.

Characterization of the sequential filtration processes

The analysis of the hydraulic permeability of the membranes used in this study are presented in Table 2, as well as the average permeate fluxes and working transmembrane pressures. The permeability decreased as the pore size increased, because the permeability is inversely proportional to the resistance of membrane filters. The results show that an increase in pressure in the range up to 100 kPa yielded an increase in the permeate flux of about 16,000 L/m^2 h for the 20-µm membrane, 4,500 L/m^2 h for the 0.45-µm membrane, 770 L/m^2 h for the 100-kDa membrane, 270 L/m^2 h for the 50-kDa membrane, and 25 L/m^2 h for the 20-kDa membrane.

The hydraulic permeability values supplied by the manufacturers of the membranes differed by approximately 50% compared with our results. These differences between the observed and stated values may be explained by variation in one or more of the following parameters: the membrane material, manufacturing conditions, and resulting structure of the membrane, as well as the characterization methods used by the manufacturers.

Figure 3 shows the permeate flux curves for the enzymatic extracts in the sequential processes using the ultrafiltration membranes. There was a sharp drop in the flow during the first minute of filtration, which was characteristic of the concentration polarization phenomenon. The first and the second ultrafiltration processes had average permeate flux values of 75 and 80 L/m^2 h, respectively, after the first minute of filtration, thereby leading to reductions of about 88 and 69%, respectively. The third ultrafiltration

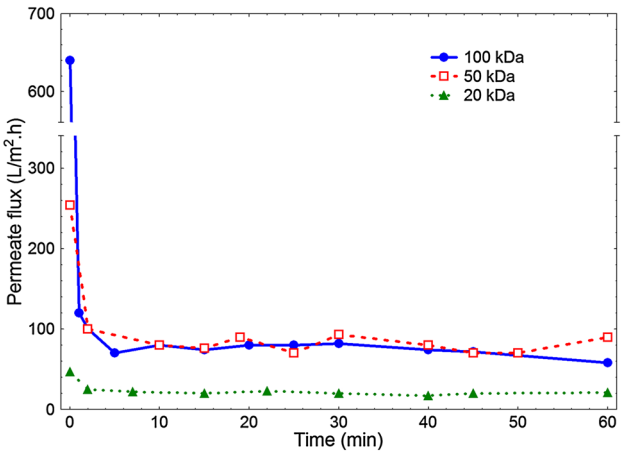

Fig. 3 Permeate fluxes of the enzymatic extracts during sequential ultrafiltration processes

Table 2 Hydraulic permeability of the membranes and permeate fluxes of the enzymatic extracts containing lipases during the successive filtration processes

Process	L_p (L/m^2 kPa h)	ΔP* (kPa)	r	J** (L/m^2 h)	ΔP*** (kPa)
Microfiltration (20 µm)	161.92	0 to 69	0.9273	3223.5 ± 95.5	20
Microfiltration (0.45 µm)	45.41	0 to 98	0.9195	60.9 ± 8.0	98
Ultrafiltration (100 kDa)	7.67	0 to 98	0.9761	74.4 ± 7.5	98
Ultrafiltration (50 kDa)	2.71	0 to 98	0.9860	79.9 ± 9.3	98
Ultrafiltration (20 kDa)	0.25	0 to 196	0.9937	20.4 ± 1.9	196

L_p hydraulic permeability, r correlation coefficient, J permeate flux, ΔP transmembrane pressure

*Variation in the transmembrane pressure to estimate the hydraulic permeability using water

**Permeate flux after 5 min of filtration using enzyme extracts (mean ± standard deviation)

***Transmembrane pressure used when filtering the enzyme extracts

Table 3 Concentration factors and permeate fluxes obtained in different processes involving concentration of proteins using membrane separation processes

Protein concentrated	Process used	Concentration factor obtained	Permeate flux	References
Lipase (*Aspergillus niger*)	Sequential micro- and ultrafiltration	3	Higher than 60 L/m^2 h	This study
Phytase (*Aspergillus niger*)	Ultrafiltration (10 kDa)	4.3	Lower than 18 L/m^2 h	Rodríguez-Fernández et al. [12]
Inulinase (*Kluyveromyces marxianus*)	Ultrafiltration (100 kDa)	5.5	Not evaluated	Golunski et al. [21]
Lignin-peroxidase (*Streptomyces viridosporus*)	Ultrafiltration (10 kDa)	10	Lower than 10 L/m^2 h after cleaning	Gottschalk et al. [22]
Endo-pectinase	Ultrafiltration (5 nm)	1.6	Around 30 L/m^2 h	Krstic et al. [23]
Phycocyanin (*Spirulina* sp.)	Sequential micro- and ultrafiltration	2	Around 27 L/m^2 h for optimum conditions	Chaiklahan et al. [26]
Ligninolytic enzymes (filamentous fungi)	Ultrafiltration (10 kDa)	>5	Not evaluated	Pant and Adoleya [29]

step resulted in a flow reduction of about 57% after the first minute of the process.

The decrease in flow is attributed to two main mechanisms: pore blocking, which is responsible for the initial decline in the flow, and cake formation, which is responsible for the gradual long-term decline. Various factors can affect the decline in flux, and the ideal is to achieve an equilibrium position at the working pressure to avoid excessive fouling of the membrane. All the sequential microfiltration and ultrafiltration processes achieved permeate streams of >20 L/m^2 h, where these values are considered suitable for use in industrial processes, such as the concentration of juice [24]. The viability in any concentration process using membranes depends fundamentally on the conditions involved in the process, such as the properties of the membrane, membrane-solute affinity, the solution temperature, and transmembrane pressure [25].

Chaiklahan et al. [26] used sequential microfiltration and ultrafiltration processes to separate and concentrate phycocyanin, which has a molecular weight between 44 and 260 kDa, from aqueous extracts of microalgae. They detected no reduction in the permeate flux when using a 5 µm polypropylene microfiltration membrane, but with a 0.2 µm polyvinylidene fluoride membrane, the flux was reduced by approximately 90%. When ultrafiltration was performed with polyethersulfone membranes of 50, 70, and 100 kDa, the permeate flux reduced less, where it was maintained at ≥50 L/m^2 h. A transmembrane pressure of 138 kPa with a 50-kDa membrane induced gel or cake formation on the membrane surface, thereby resulting in the retention of protein and phycocyanin, which reduced the permeate stream. Therefore, the pressure was halved to minimize the effect of fouling. Bacchin et al. [27] showed that one of three outcomes can occur depending on the relationship between the solute and the size of the pore: adsorption by the pores, where the solute is much smaller than the pore; pores blocking when the sizes are similar; or gel formation and the subsequent formation of a cake layer when the solute is much larger than the pore. During the microfiltration process, there is a tendency toward innermost fouling (adsorption) by proteins, whereas pore blockage occurs during ultrafiltration, thereby explaining the phenomenon observed in this study. Chaiklahan et al. [26] also showed that there was a loss of phycocyanin in each filtration step, which reached 25% during microfiltration, but the use of sequential microfiltration (5- and 0.2-µm membranes) and ultrafiltration (50-kDa membrane) processes is technically feasible, and it may incur lower costs than purification processes involving precipitation and chromatography.

After the washing process with water, the 20-µm microfiltration membrane and 100-kDa ultrafiltration membrane yielded considerable flow recoveries (74% and 88%, respectively). It should be noted that washing with water was only performed for about 10 min to remove fouling deposits, which characterized the phenomena of concentration polarization and cake formation. Although the flow recovery was lower with the other membranes, it should be emphasized that industrial processes involves cleaning using chemical detergents, oxidants, and enzymes to remove particles that adhere more strongly to the membrane.

Peeva et al. [28] separated proteins (albumin and myoglobin) by tangential ultrafiltration with normal and modified polyethersulfone membranes. A flow recovery of around 50% was obtained using 10-, 30-, and 50-kDa polyethersulfone membranes after three cleaning steps with water and NaOH, where the flux recovery was lower than that in the present study, even without using detergents. Peeva et al. [28] noted the effects of attraction and repulsion forces between proteins and membranes only at the

beginning of the filtration process, and after several minutes, the membrane had similar rejection and fluxes even at different pH values due to cake formation and electrostatic attraction between the proteins themselves and the dense cake layer, which reduced the selectivity of the process.

Table 3 presents the summarized results obtained in different studies involving concentration of proteins using filtration processes. It is observed that the studies which presented the highest concentration factors also presented lower permeate fluxes, or have this important parameter not evaluated.

Table 4 shows the overall resistance to the permeate flux, as well as the resistance of the membrane, the resistance of irreversible fouling (deposition on the membrane by adsorption), and the resistance of reversible fouling (deposition and/or concentration polarization). This evaluation of the resistances during the different stages was used to determine the percentage contribution of each step to the permeate flux reduction. The membrane resistance increase as the pore size (or MWCO) decreased. The resistance of the 0.45-µm microfiltration membrane was 4.6-fold lower than that of the 100-kDa ultrafiltration membrane, while the resistance of the latter membrane was 27.2-fold higher than that of the 20-kDa ultrafiltration membrane. The resistance results for the membranes were consistent with the hydraulic permeability results, which decreased as the pore size decreased, because the resistance is inversely proportional to permeability.

The resistances in the first and second ultrafiltration processes were smaller than those in the second microfiltration stage, because this process retained particles that would have caused greater resistance in the subsequent processes, especially due to complete pore blockage and subsequent cake formation. These results demonstrate the advantage of performing sequential filtration with a gradual decrease in the pore membrane size to avoid excessive increases in resistance during the process when an ultrafiltration membrane is used directly, which would also reduce its working life. The irreversible resistance characterized by inner pore blockage or by irreversible adsorption on the membrane was higher during the second microfiltration stage and in the third ultrafiltration step. The internal blocking of pores in the 20-kDa membrane can be explained by the presence of small peptides during this step, which were adsorbed onto the internal structure of the membrane. The reversible resistance characterized by the layer responsible for the polarization concentration and subsequent cake formation was about 1000 times lower during the first microfiltration stage, because this process involved the lowest decrease in flow compared with the others due to the size of the pores (20 µm). The four sequential filtration processes had reversible resistances ranging from 2.1×10^{12} to 4.8×10^{12} m^{-1}, thereby indicating that the majority of the membrane fouling can be removed by simple cleaning processes. Brião and Tavares [30] evaluated the pore blocking mechanism during ultrafiltration of dairy effluent. The filtration of this effluent containing an abundance of protein components with high-molecular weights showed that particles that blocked the pores externally could be removed using a simplified washing processes, thereby allowing the reuse of the membrane.

Figure 4 shows the percentage contribution of each type of resistance to the different filtration processes. In the first microfiltration stage, the main type of resistance (64.5%)

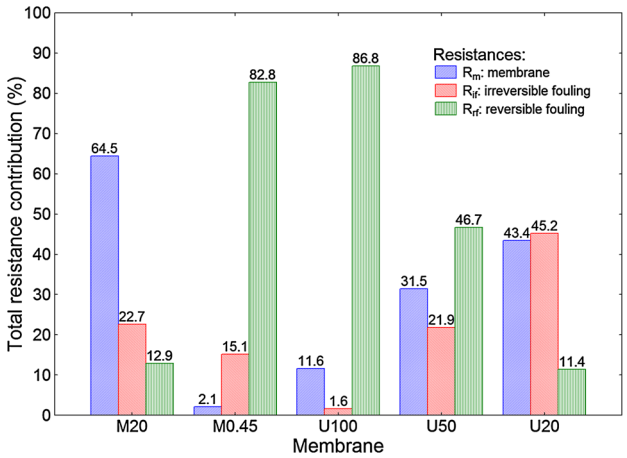

Fig. 4 Percentage contributions of the different types of resistances (membrane, irreversible fouling, and reversible fouling) during the microfiltration (M20 and M0.45) and ultrafiltration (U100, U50, and U20) processes

Table 4 Resistances calculated for the microfiltration and ultrafiltration processes using the extracts containing lipases produced by *A. niger* via solid-state fermentation

Process	R_t (10^{12} m^{-1})	R_m (10^{12} m^{-1})	R_{if} (10^{12} m^{-1})	R_{rf} (10^{12} m^{-1})
Microfiltration (20 µm)	0.022	0.014	0.005	0.003
Microfiltration (0.45 µm)	5.797	0.120	0.878	4.800
Ultrafiltration (100 kDa)	4.745	0.552	0.075	4.118
Ultrafiltration (50 kDa)	4.418	1.390	0.966	2.063
Ultrafiltration (20 kDa)	34.612	15.023	15.636	3.953

R_t total resistance, R_m membrane resistance, R_{if} irreversible fouling resistance, R_{rf} reversible fouling resistance

was membrane resistance, which can be explained by the small amount of suspended particles with sizes similar to the pore size, i.e., 20 µm. The two subsequent filtration steps (0.45-µm and 100-kDa membranes) comprised more than 80% of the resistance as a function of reversible fouling (particles that adsorbed onto the membrane surface caused concentration polarization and the particles subsequently formed a cake), which is characteristic of filtration processes, where the particle sizes are similar or slightly larger than the pore sizes. Although the permeate flow dropped sharply during these processes, the particles in the deposit could be removed simply by washing with water, thereby allowing the membrane to be reused. In the process using the 20-kDa membrane, almost 90% of the resistance was caused by irreversible fouling, which is more difficult to remove, where it is characterized by the presence of particles smaller than the pore and with greater affinity for the membrane, i.e., small peptides and amino acids in the hydrophilic polyethersulfone membrane. During the filtration of the enzymatic extract, we showed that it is not necessary to perform this last step, because the lipases are separated and concentrated in the previous stages. Although the 20-kDa membrane presented an irreversible fouling, it was concluded that the enzyme had been retained by the previous ultrafiltration membrane and the last separation step was not necessary for the process. The elimination of the last ultrafiltration step makes the process less onerous in terms of the filtration time and the subsequent cleaning of the membrane, thereby resulting in a less costly procedure.

Hwang and Chiang [31] separated proteins and polysaccharides by tangential filtration using membranes with different morphologies, where they compared 0.1-µm membranes made of cellulose esters, polyvinylidene fluoride, and polycarbonate. The permeate flux was 30% higher with the polyvinylidene fluoride membrane, where its cake resistance (reversible) was similar to that of the polycarbonate membrane but higher than that of the cellulose esters membrane. The results showed that the protein molecules formed deposits on the surface of the membrane, whereas, the polysaccharides were adsorbed onto the membrane and the inner walls of the pores. The resistance to irreversible fouling by the cellulose ester membrane was three times higher than that of the other membranes when the filtration process was performed at a pressure of 60 kPa and a feed flow of 0.5 m/s, which was attributed mainly to the rough and spongy structure of the cellulose ester membrane, with larger pores on the surface. The results obtained [31] are similar to those obtained in the present study with the 0.45-µm cellulose acetate membrane, which had high reversible resistance, where this was greater than the irreversible resistance and much larger than the membrane resistance.

Micrographs of the surface of the 0.45-µm membrane before and after performing the process (Fig. 5) show that this microfiltration membrane had a porous structure with a rough surface. Hwang and Chiang [31] also characterized the morphology of their microfiltration membranes, and they showed that the structures of the polyvinylidene fluoride, polycarbonate, and cellulose ester membranes

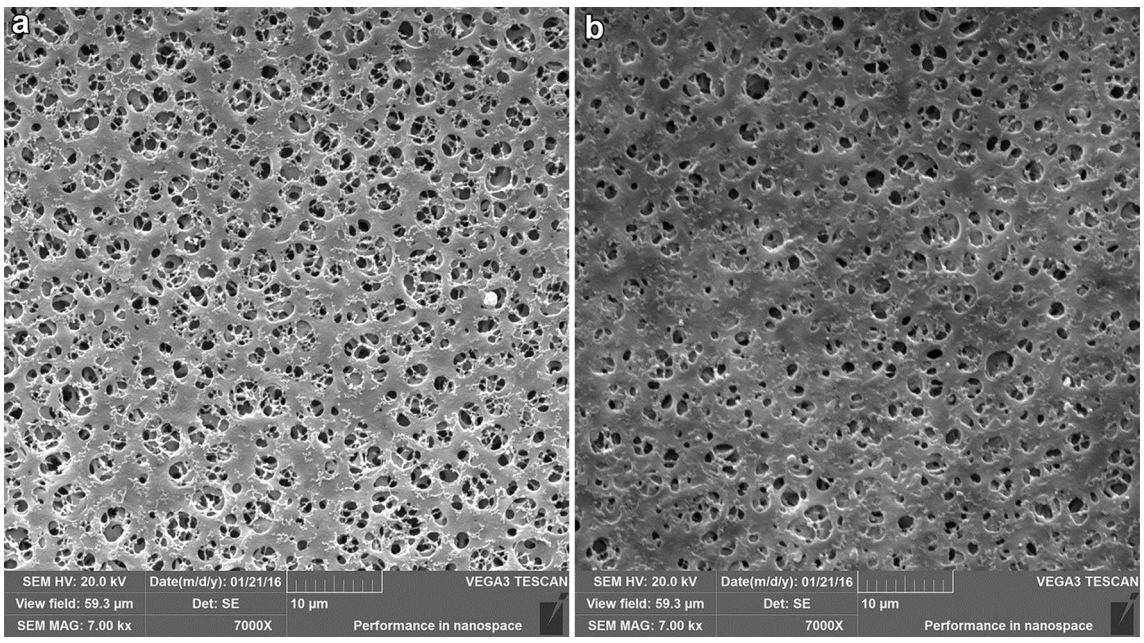

Fig. 5 Scanning electron microscopy images of the surface of the 0.45-µm microfiltration membrane used in the process: **a** before filtration and **b** after filtration

were different on both the surface and in cross section. The structure of their cellulose esters membrane was very similar to that found in our study, because the microfiltration membranes were made of the same material. A reduction in the number of microfiltration membrane pores after the process was also clearly visible in our study, particularly the entrances of the pores closest to the surface. Particle deposition and the consequent reduction in the number of pores were also analyzed visually by Rosas et al. [32], who evaluated the fouling of a 0.22-μm polyethersulfone microfiltration membrane after filtering a bacterial suspension.

SEM images of cross sections of the ultrafiltration membranes are shown in Fig. 6, which indicate that all had asymmetric structures. The presence of a support that provides mechanical strength to the membrane is evident, as well as a skin layer that ensures selectivity. The 100-kDa membrane contained macrovoids in the sublayer, which could reduce the resistance to permeation. In addition, the thick spongy structure is responsible for high strength, which reduces the permeate flux.

After the separation processes, the 50- and 20-kDa ultrafiltration membranes exhibited a higher degree of compaction due to fouling by smaller particles adsorbed internally on their inner walls. The molecular adsorption on the pore walls resulted in a reduction in the pore size with a consequent increase in fouling resistance, thereby lowering the permeate flux, as mentioned above. The morphology of the polyethersulfone ultrafiltration membranes used in the present study is consistent with the results obtained by Mozia et al. [33] and Arahman et al. [34], who also observed that the morphology of this type of membrane can be modified by the addition of polymeric additives to increase the macrovoids or to change the hydrophilicity of the membrane. We also observed that the polyethersulfone membranes used in the present study had a different structure compared with ultrafiltration membranes made of polysulfone [35], polystyrene [36], ceramics [37], polyamide and polysulfone

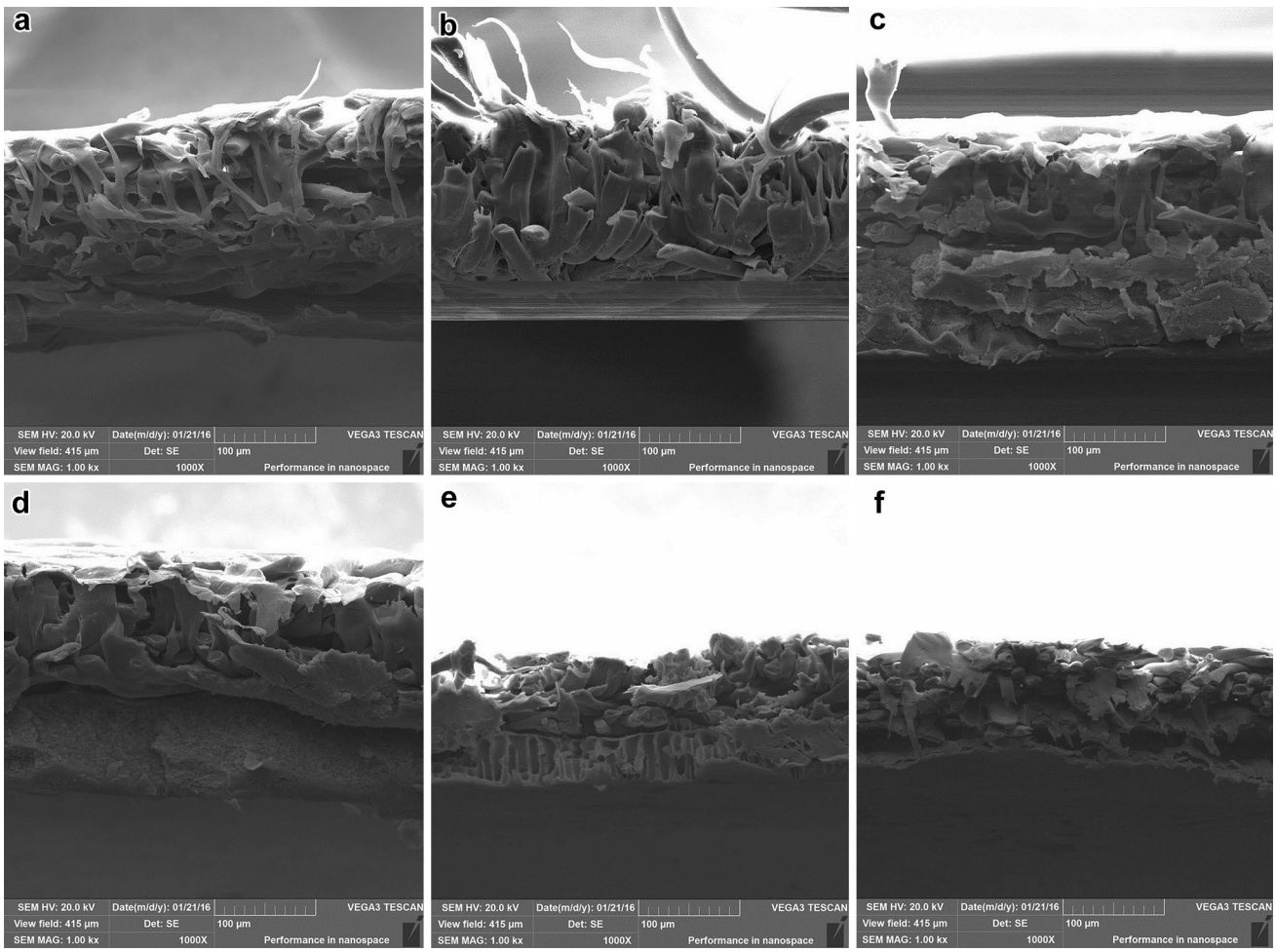

Fig. 6 Scanning electron microscopy images of cross sections of the ultrafiltration membranes before and after use in the processes, respectively: 100 kDa (**a, b**), 50 kDa (**c, d**), 20 kDa (**e, f**)

[38], polyamide [39], and polyethylene terephthalate [40], thereby indicating possible differences in their selectivity and permeation characteristics.

These observations are important when selecting the membranes used for separation and the concentration of proteins. The differences between these membranes, even when they were made of similar materials, are attributable to differences in the manufacturing methods employed. The interactions between the material being filtered and the membrane can also be modified chemically and/or physically even after the manufacture of the membrane, thereby changing the hydrophilicity and selectivity properties compared with the original membrane [41, 42]. Thus, there is an infinite range of possibilities for further research related to the actual use of membranes in various fields.

In conclusion, the combination of successive membrane separation processes using a tangential filtration module with microfiltration and ultrafiltration membranes allowed higher permeate fluxes than in direct ultrafiltration. Moreover, we obtained concentrated extracts with higher hydrolytic activities than those obtained in the initial extract, as well as reversible fouling, thereby demonstrating the feasibility of the successive concentration of lipases produced by *A. niger* via solid-state fermentation of agro-industrial residues, such as wheat bran and rice husk.

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Compliance with ethical standards

Human participants This article does not contain any studies with human participants or animals performed by any of the authors.

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

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