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Research Article

One-Step Ultrafiltration Process for Separation and Purification of a Keratinolytic Protease Produced with Feather Meal

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A purification technique to obtain keratinolytic proteases produced by *Bacillus* sp. P45 in a medium containing chicken feather meal as substrate is presented. The experiments were carried out in a dead-end ultrafiltration unit, and the influence of the membrane cutoff, pH of enzymatic extract, and operating pressure on the purification of keratinase were studied. The one-step ultrafiltration process with the membrane molecular mass cutoff of 10 kDa at pH 8.0 and operating pressure of 0.147 MPa showed an enzyme recovery of 87.8% and a 4.1-fold purification factor. It is showed that ultrafiltration could be potentially used in the purification of keratinases.

1. Introduction

Brazil is a major producer of poultry meat globally, generating tons of organic waste such as viscera, feet, bones, feathers, and blood that are not exploited for human consumption. These organic by-products are mostly used to prepare animal feed or soil fertilizers though methods of incineration in the disposal of such waste are still applied in some locations [1]. Chicken feathers represent about 10% of the waste disposed of by the poultry industry. Feathers are essentially composed of keratin (about 90% w/w), a protein of difficult degradation due to the presence of strong chemical bonds in the polypeptide chain as disulfide bridges, hydrogen, and hydrophobic interactions that hinder their rapid degradation in the environment [2–4].

The recovery of keratin poses a great challenge to the poultry industry. An alternative to recycle these keratinous materials is the bioconversion into products with higher added value by specific microorganisms producing keratinolytic proteases. These proteases, named keratinases, are often serine or metalloproteases capable of degrading keratinous wastes [5, 6]. Alkaline keratinases are produced by several bacterial species, including *Bacillus licheniformis* [7–9], *Kocuria rosea* [10], *Streptomyces* sp. [11], and even fungi such as *Aspergillus niger* [12]. A keratinase-producing bacterium, *Bacillus* sp. P45, was isolated from the intestine of the Amazon basin fish *Piaractus mesopotamicus* [4]. *Bacillus* sp. P45 efficiently degraded feather keratin during submerged cultivations, producing extracellular keratinolytic enzymes.

This type of enzyme has gained biotechnological interest for use in the fertilizer, detergents, and cosmetic industries, and also for the potential use of keratinous residues to produce biohydrogen and biogas [1, 3, 13]. Furthermore, keratinolytic proteases can be applied to obtain animal feed rich in amino acids. The enzymatic hydrolysis preserves essential amino acids such as methionine, lysine, and tryptophan and avoids the formation of nonnutritive amino acids such as lanthionine and lysinoalanine [14]. New applications have also been developed

such as prion degradation for prevention of mad cow disease [3], biodegradable plastic manufacturing, and keratin peptide production [14, 15].

The feasibility of an industrial application of keratinases resides initially in obtaining the enzyme from a viable source, such as poultry feathers, and its purification using a simple protocol. Several techniques have been studied to purify microbial keratinases, including ammonium sulfate precipitation, solvent precipitation, ultrafiltration, ion exchange chromatography, gel filtration chromatography, hydrophobic interaction chromatography, and hydroxyapatite chromatography [5, 7–9, 11, 16]. However, those studies employ purification processes with sequences of different techniques in order to obtain highly purified enzyme preparations needed to characterize the enzyme. In those protocols, the membrane separation has been applied only as a concentration step. For industrial applications, high degrees of purity are often not required. Thus, it is necessary to study industrially applicable techniques to purify microbial keratinases, reducing the costs of this process. In addition to scale-up problems, which limit protein production levels, the traditional techniques such as chromatography require complex instrumentation support to run efficiently and yield low throughput of product at an extremely high cost. Hence, a separation technique that can provide high productivity and purity at the same time at low process cost would certainly be beneficial to the biotechnology industry [17].

Ultrafiltration has been widely used for protein concentration and separation because of the lower complexity compared to the previously mentioned purification techniques [18]. The major advantage of the ultrafiltration processes over a conventional bioseparation processes is the high product throughput. However, in spite of widespread use of ultrafiltration in processes such as diafiltration and concentration, the potential for its use in protein fractionation has not been exploited in the biotechnology industry [17]. Nevertheless, there are studies that show that ultrafiltration can be applied to purification of enzymes, obtaining high yields and product purity at the same time [19–21].

This study provides a potential one-step purification process for obtaining a microbial keratinase. Furthermore, this process adds value to the most problematic by-product of the poultry industry. In the present research, the use of an industrially applicable technique for the purification of the keratinase obtained from *Bacillus* sp. P45 using feathers was studied. The enzyme was concentrated and purified by ultrafiltration. This approach allows obtaining a purified keratinase in a single step.

2. Materials and Methods

2.1. Microorganism and Inoculum Preparation. *Bacillus* sp. P45 (GenBank accession number AY962474) was maintained at 4°C on brain heart infusion (BHI) agar plates. For inoculum preparation, *Bacillus* sp. P45 was inoculated on BHI plates and incubated at 30°C for 24 h. The cultures were gently scraped from the agar surface, added to a sterile NaCl solution (8.5 g/L), and mixed until a homogeneous suspension with an optical density of 0.5 at 600 nm was obtained [22].

Table 1: Experimental design 2^3 in coded levels (real values).

Trial	MWCO (kDa)	pH	Pressure (MPa)
1	−1 (10)	−1 (7.0)	−1 (0.147)
2	+1 (30)	−1 (7.0)	−1 (0.147)
3	−1 (10)	+1 (8.0)	−1 (0.147)
4	+1 (30)	+1 (8.0)	−1 (0.147)
5	−1 (10)	−1 (7.0)	+1 (0.245)
6	+1 (30)	−1 (7.0)	+1 (0.245)
7	−1 (10)	+1 (8.0)	+1 (0.245)
8	+1 (30)	+1 (8.0)	+1 (0.245)

MWCO: molecular weight cutoff.

2.2. Submerged Cultivation. The enzyme was produced by submerged cultivation as described by Daroit et al. [22] using the culture medium composed of (g/L) feather meal (50) and NH_4Cl (5.25) prepared in a mineral medium (NaCl (0.5), K_2HPO_4 (0.3), and KH_2PO_4 (0.4)). In Erlenmeyer flasks (250 mL) containing 50 mL of medium, the initial pH was adjusted to 7.0 before sterilization by autoclaving at 121°C for 15 min. Cultures were initiated with 1% (v/v) inoculum. The growing conditions were 30°C and 125 rpm for 48 h. At the end of the cultivation, the supernatant was separated by centrifugation (5.000 ×g for 20 min), obtaining the crude enzyme extract.

2.3. Ultrafiltration (UF). Experiments were conducted in a dead-end ultrafiltration unit with a working volume of 160 mL stirred by a magnetic bar suspended down to 5 mm of the membrane. The module was equipped with a regenerated cellulose membrane (Millipore) having a total filtering area of 19.63 cm^2. A new membrane was used for each experiment, and two different sizes were used with molecular weight cutoff of 10 kDa and 30 kDa. The system was pressurized with compressed nitrogen, and the temperature was kept at 15°C to avoid enzyme denaturation. Prior to the ultrafiltration process with crude enzyme extract, a flux of water was passed through the membrane. A volume of 40 mL of crude enzyme extract was added, and the process was stopped when the volumetric concentration factor reached the value of 4 [21]. The enzyme activity and the protein content of the feed (input crude extract), retentate, and permeate were assayed at the end of each experiment.

2.4. Concentration and Purification of Keratinase by Ultrafiltration. The influence of the operating pressure, the pH of the enzyme extract, and the molecular weight cutoff (MWCO) during the ultrafiltration process was studied by a 2^3 experimental design, totaling eight experimental trials carried out in duplicate. The responses evaluated were enzyme recovery and purification factor. The statistical analysis of the experimental design was performed using analysis of variance (ANOVA) with a confidence level of 95%. Statistica 5.0 software (StatSoft Inc., USA) was used for the regression and graphical analysis of the data obtained by the experimental design. Table 1 presents the matrix of experimental design.

The efficiency of the process of concentration and purification of the enzyme keratinase by ultrafiltration was

evaluated through the enzyme recovery and purification factor. The enzyme recovery (%R) was obtained by the ratio between the total activity in the retentate and the total activity in the feed. The purification factor (PF) was calculated by dividing the specific activity of the enzyme in the retentate (U/mg) by the specific activity of the enzyme extract used in the feed (U/mg).

2.5. Enzyme Assay. Keratinase activity was monitored using the soluble substrate azocasein (Sigma, Saint Louis, USA) as described by Daroit et al. [4]. One unit (U) of protease activity was defined as the amount of enzyme that caused an increase of 0.1 absorbance unit at the defined assay conditions.

2.6. Total Protein Determination. Protein was determined by the method of Lowry et al. [23], using bovine serum albumin (BSA) as the standard.

3. Results and Discussion

3.1. Study of Flux with Variation of pH, Operating Pressure, and MWCO in the Ultrafiltration Process. During the trials, the flux behavior in the process of concentration and purification of keratinase was evaluated (Figures 1 and 2). In the ultrafiltration at pH 7.0 (Figure 1), it is possible to observe that the flux variation of trial 2 was 76.4 to 25 L/m^2·h with a reduction of 67.5% and an operating time of 0.6 h. Trials 5 and 6 showed a flux reduction of 65.1 and 64.4%, respectively. Trial 1 (10 kDa and 0.147 MPa) showed the smallest flux variation from 38.2 to 19.5 L/m^2·h with a reduction of 48.9% and an operating time of 0.73 h.

In Figure 2, the flux profiles at pH 8.0 show that the membrane cutoff and the operating pressure affect the permeate flux more than the pH—the change for pH 8.0 in trials 3, 4, 7, and 8 has no significant influence on the flux permeate, compared with the same conditions at pH 7.0. Trial 3 (10 kDa, 0.147 MPa, and pH 8.0) had the lowest percentage decrease of the flux with a value of 46.1% (39 to 21 L/m^2·h), almost the same as trial 1 (48.9%), which is performed with the same MWCO and operating pressure but with pH 7.0.

When the effect of the membrane cutoff on the permeate flux was analyzed, it was observed that, with the 30 kDa membrane, the reduction of the flux is larger than with the 10 kDa membrane at low pressures (0.147 MPa). This may be attributed to the fact that the extract contains peptides, amino acids, and other proteolytic enzymes with molecular mass below 30 kDa, which facilitates their initial transport through the membrane, thereby obtaining a higher initial flux of the process. However, due to the concentration polarization and fouling phenomena, the flow rate drops to values as low as those obtained with 10 kDa.

The flux reductions caused by the fouling and concentration polarization have long been recognized as major problems in the protein ultrafiltration. It could be seen that, at the beginning of the process, there is a rapid decline of the flux. After this initial period, there is a gradual decline of the

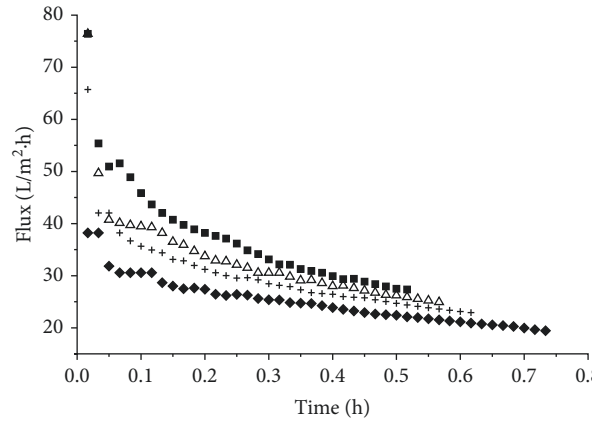

Figure 1: The flux permeate during the ultrafiltration process at pH 7.0: trial 1 (◆): 10 kDa and 0.147 MPa; trial 2 (Δ): 30 kDa and 0.147 MPa; trial 5 (+): 10 kDa and 0.245 MPa; trial 6 (⧏): 30 kDa and 0.245 MPa.

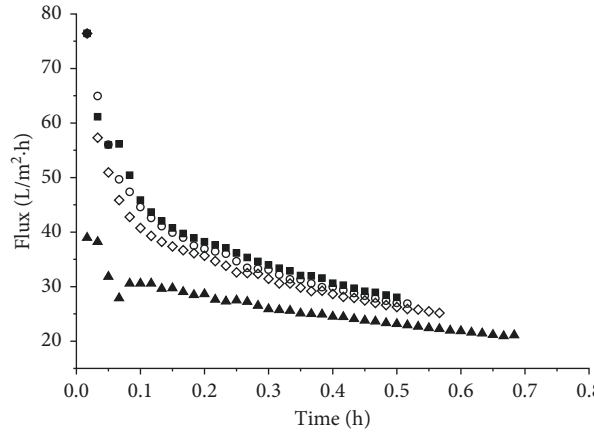

Figure 2: The flux permeate during the ultrafiltration process at pH 8.0: trial 3 (▲): 10 kDa and 0.147 MPa; trial 4 (◊): 30 kDa and 0.147 MPa; trial 7 (○): 10 kDa and 0.245 MPa; trial 8 (■): 30 kDa and 0.245 MPa.

flux that occurs due to the formation of incrustations of proteins into the membrane surface forming the effect of concentration polarization and the fouling phenomena. The proteins that are retained by the membrane can form a gel layer on the membrane surface, which acts as a second dynamic membrane, increasing protein retention. Other authors who used the ultrafiltration process for concentration and separation of proteins [17, 21, 24] observed the same behavior. During the process of concentration and purification of a pretreated protease from the tuna spleen extract, a decline of 59% was obtained in the flux [25], which is close to values obtained in this work.

However, at higher pressures (0.245 MPa), the difference on the flux reduction between the 10 kDa and 30 kDa membranes is less pronounced. In fact, higher operating pressures provided higher initial flow rates in the process. This higher pressure may have decreased the effect of concentration polarization; thus, the process with the 10 kDa membrane achieved initial fluxes close to the experiments with the 30 kDa membrane.

Table 2: Responses of 2^3 experimental design.

Trial	%R predicted	%R experimental	Relative standard (%)	PF predicted	PF experimental	Relative standard (%)
1	80.5	79.3	−1.5	3.8	3.4	−11.2
2	40.1	41.3	3.0	2.3	2.1	−11.4
3	86.7	87.8	1.3	3.8	4.1	7.8
4	46.2	44.6	−3.7	2.3	1.9	−23.2
5	82.9	77.3	−7.3	3.7	3.6	−2.2
6	62.7	62.2	−0.9	3.4	3.5	1.7
7	82.9	82.4	−0.7	3.7	3.7	0.5
8	68.9	69.4	0.7	3.4	3.4	−1.2

$n = 2$; %R: enzyme recovery; PF: purification factor.

Table 3: Analysis of variance for enzyme recovery and purification factor.

	Factor	Sum of square	Degrees of freedom	Mean squares	$F_{\text{calculated}}$	$F_{\text{tabulated}}$
%R ($R = 0.99$)	Regression	4175.8	4	1043.9	300.8	3.4
	Residue	38.2	11	3.5		
	Total corrected	4213.9	15			
PF ($R = 0.84$)	Regression	5.3	3	1.8	9.00	3.5
	Residue	2.2	12	0.2		
	Total corrected	7.5	15			

3.2. Concentration and Purification of Keratinase by Ultrafiltration. Table 2 shows the values of the parameters evaluated during keratinase purification and the relative standard deviations with the predicted and experimental values obtained in the trials. The recovery of the enzyme keratinase was in the range of 41.3 to 87.8% and a purification factor between 1.9- and 4.1-fold. It was possible to verify that the increase of the membrane cutoff from 10 to 30 kDa decreases the recovery of enzyme and consequently the purification factor. Trial 3, with a membrane cutoff of 10 kDa, pH 8.0, and pressure of 0.147 MPa, provided the highest enzyme recovery (87.8%) and purification factor (4.1-fold). The membrane cutoff, the operating pressure, and the interaction between these two variables had significant effect on the two responses, enzyme recovery and purification factor; the pH, on the other hand, had a significant effect on the purification factor only. ANOVA was carried out using Fisher's statistical test (Table 3) for the validation of the empirical model obtained for the PF and recovery of the enzyme. Correlation coefficients (R) of 0.99 and 0.84 were obtained for the %R and PF, respectively. In addition to this, the $F_{\text{calculated}}$ value was 3 times higher than the $F_{\text{tabulated}}$ value for the PF and 90 times higher for the %R, showing that the model fitted the data satisfactorily and was considered predictive for both responses. Equations (1) and (2) represent empirical models codified for recovery (%R) and purification factor (FP), respectively.

$$\%R = 68.11 - 13.62 * \text{MWCO} + 3.08 * \text{pH} + 4.735 * P + 6.60 * \text{MWCO} * P, \quad (1)$$

$$\text{PF} = 3.31 - 0.42 * \text{MWCO} + 0.25 * P + 0.3 * \text{MWCO} * P, \quad (2)$$

where %R is the enzyme recovery, PF is the purification factor, MWCO is the molecular mass cutoff, and P is the operating pressure.

Figures 3 and 4 show the contour plots obtained from the empirical models for better understanding of the interaction of the variables pH, pressure, and MWCO and to obtain the best conditions for keratinase concentration and purification.

Analyzing the interaction of pH and pressure on enzyme recovery, it is possible to verify that when a 10 kDa membrane was employed with higher values of pH (between 7.5 and 8.0) and pressure between 0.196 and 0.147 MPa, the enzyme recovery increased (up to 80%), as shown in Figure 3. When a 30 kDa membrane was used, the effect of pressure on recovery values was even more pronounced. This is probably attributed to the formation of fouling that favors the retention of the enzyme.

With respect to the purification factor, the pH of the enzyme extract did not affect this response significantly, not generating the contour curve. The effect of pH is more related to the enzyme activity—keratinases usually have a great stability in neutral and alkaline pH [2, 14]. Only the MWCO and operating pressure had an influence on the purification factor (Figure 4). It was observed that higher purification factor values were obtained using lower MWCO values with operation pressures between 0.147 and 0.196 MPa.

Evaluating the influence of the MWCO, a negative effect was observed for both responses (recovery and purification factor). In other words, when the smallest MWCO is used (10 kDa), the highest values of recovery and purification factor are obtained. An MWCO of 30 kDa decreased the enzyme recovery by 27.2%. This can be attributed to the molecular mass of the enzyme under study. According to Daroit et al. [26], the molecular mass of keratinase from *Bacillus* sp. P45 is approximately 26 kDa, and thus, higher MWCO favors the passage of the enzyme to permeate, decreasing the recovery of the enzyme.

Despite the enzyme having a molecular mass of 26 kDa, the 30 kDa membrane was tested because it was expected that the concentration polarization and fouling phenomena could favor the retention of the enzyme due to formation of

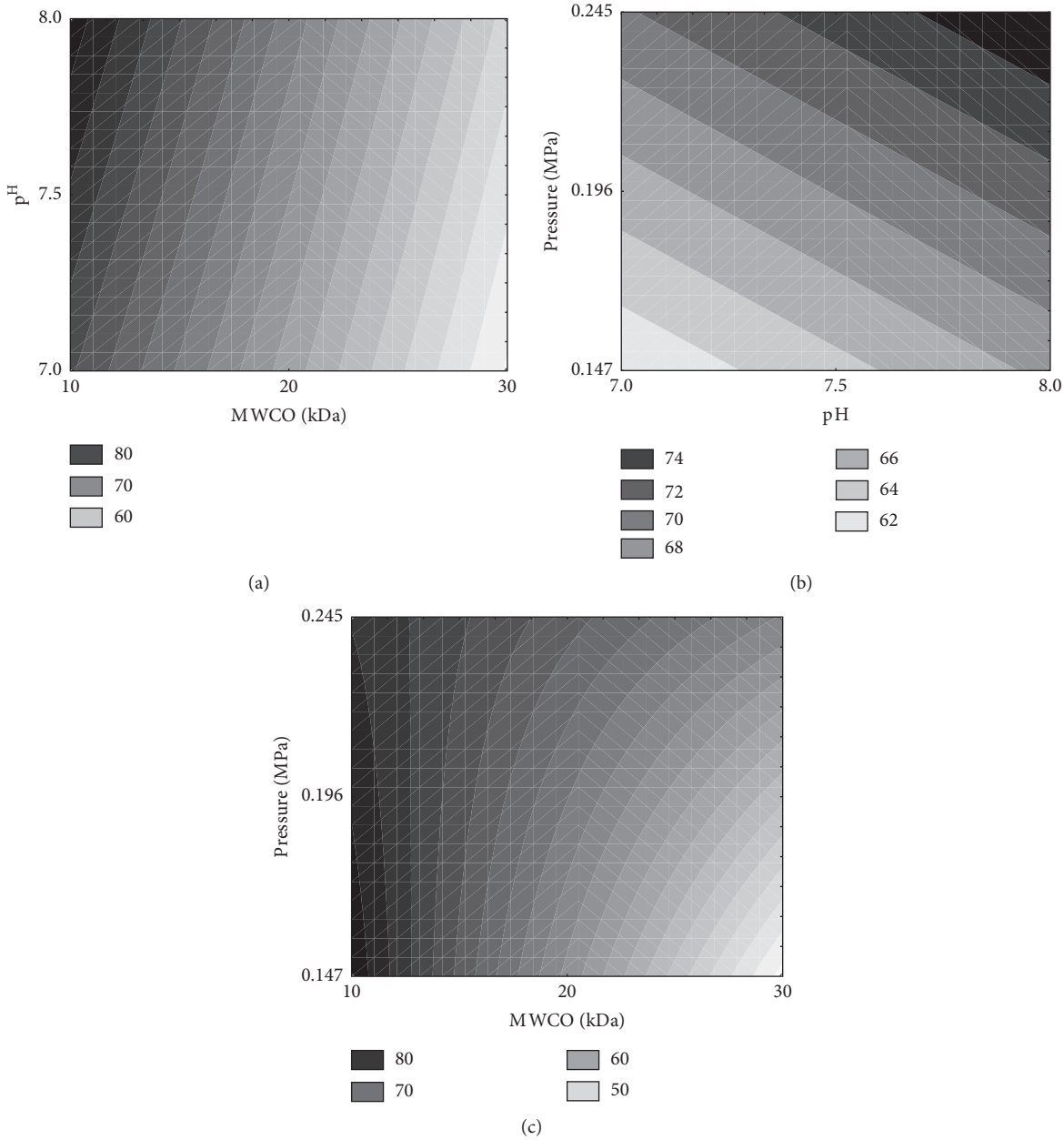

Figure 3: Contour curves for the recovery of enzyme as a function of (a) pH and MWCO, (b) pH and the operating pressure, and (c) MWCO and operating pressure.

the gel layer without major losses in the flux since it is a membrane with a larger pore diameter. Actually, a higher flow was observed, but the retention of the keratinase was much less efficient compared to the 10 kDa membrane. Therefore, the enzyme permeated the membrane of 30 kDa resulting in a smaller recovery and purification factor.

In relation to the pressure, it can be verified that lower pressures and MWCO provide values of higher purification factors. Furthermore, membranes of lower molecular mass cutoff with high values of pH and low operation pressures provide higher recovery values. Analyzing the flux behavior, at higher pressures (0.245 MPa), the reduction of the flux was more pronounced in the best condition (10 kDa membrane and pH 8) compared with lower pressures (0.147 MPa).

Ultrafiltration has been applied in the purification of keratinases only intended to concentrate the enzyme for the next purification step [7, 8, 27–29]. However, it is observed that the values found in the cited works are lower than those found in this study that use just one step. Radha and Gunasekaran [27] produced a keratinase by a recombinant *Bacillus megaterium*. During the purification process, the ultrafiltration step (MWCO of 10 kDa) achieved a purification factor of 2.3-fold with a recovery of 73.5%. Lin et al. [7] purified and characterized a keratinase isolated from a feather-degrading culture medium inoculated *with Bacillus licheniformis* PWD-1.

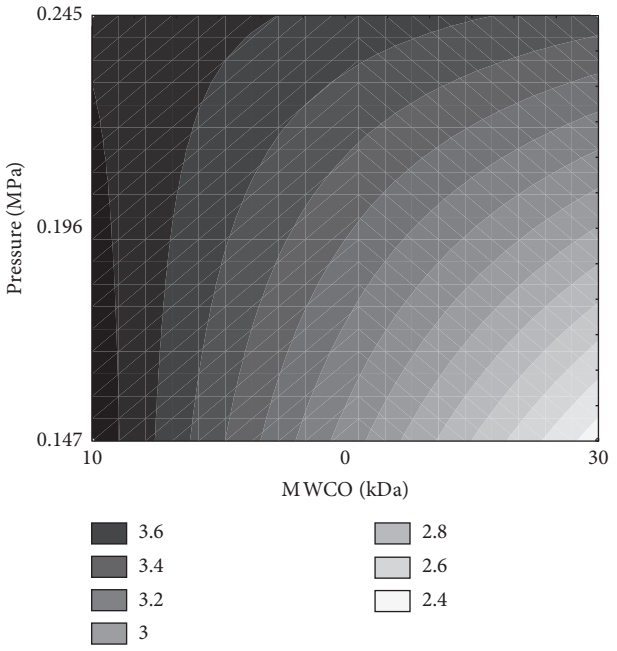

Figure 4: Contour curve for purification factor as a function of operating pressure and MWCO.

The ultrafiltration step (MWCO of 10 kDa) achieved a purification factor of 1.8-fold and recovery of 69.3%. In the study of Allpress et al. [28], the extracellular keratinase produced by *Lysobacter* NCIMB 9497 was purified for further characterization. In the ultrafiltration step (MWCO of 10 kDa), a purification factor of 2-fold was obtained.

Cheng et al. [8] and Suntornsuk et al. [29] also used a membrane MWCO of 10 kDa for keratinase concentration. Cheng et al. [8] characterized the keratinase produced by *Bacillus licheniformis* PWD-l produced with feather powder. Prior to the purification process, the enzyme was concentrated using a spiral cartridge concentrator with a MWCO membrane of 10 kDa. Suntornsuk et al. [29] isolated and determined the properties of a keratinase produced by a thermotolerant feather-degrading bacterial strain from Thai soil. For its purification, the enzyme was firstly concentrated by a 10 kDa MWCO membrane.

In this study, an ultrafiltration process was developed in the purification of keratinase from *Bacillus* sp. P45 in a single step. A system with an operating pressure of 0.147 MPa, membrane MWCO of 10 kDa, and pH of 8.0 provided a 4-fold purification factor without major recovery losses. Thus, it is possible to reduce the purification steps through an optimized ultrafiltration method for purification of this enzyme. The ease of operation and high efficiency makes the ultrafiltration process an interesting alternative in the purification of keratinases, which can be used in the degradation of resistant materials in effluents or even for applications in industry cleaning products like detergents. Besides, the enzyme has potential importance for production of protein hydrolysates [3].

4. Conclusions

The ultrafiltration system formed by the membrane with the molecular weight cutoff of 10 kDa, an operating pressure of 0.147 MPa, and a pH of 8.0 provided a recovery of 87.8% and 4.1-fold purification factor of the enzyme keratinase from *Bacillus* sp. P45. The ultrafiltration process is positioned as a potential alternative to be used for the industrial concentration and purification of keratinase from *Bacillus* sp. P45 in just one step. Furthermore, this process adds value to the most problematic by-product of the poultry industry, chicken feathers.

Data Availability

There are no supplementary data or materials for this article. However, the data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

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