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Separation of Cells and Proteins from Fermentation Broth in a Shear-Enhanced Cross-Flow Ultrafiltration Module as the First Step in the Refinement of Lactic Acid

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Abstract

A shear-enhanced, cross-flow ultrafiltration module was used to separate cells and proteins from the fermentation broth. Three (fermented) media were studied: rich medium, rich medium with hydrolytic enzymes added after fermentation, and wheat flour hydrolysate. To find a membrane with as high a flux as possible, but still capable of separating cells and proteins from the lactic acid containing broth, the performance of three hydrophilic membranes of varying cutoffs (10,000, 20,000, and 30,000) and one hydrophobic membrane (cutoff 25,000) was investigated. The proteins produced by the lactic acid bacteria during fermentation and the hydrolytic proteins were retained by the hydrophilic membrane with a cutoff of 20,000, whereas wheat flour proteins were detected in the permeate. In the permeates from the hydrophobic membrane (cutoff 25,000), almost no proteins were detected. The flux of the whole-wheat flour hydrolysate was significantly lower than that of rich medium, for both the hydrophilic and the hydrophobic membranes. The flux was, in all cases, higher for the hydrophilic membrane (12–85 $L/[m^2 \cdot h]$, depending on which medium was treated) than for the hydrophobic one (8–45 $L/[m^2 \cdot h]$), even though the nominal cutoffs of the hydrophobic and hydrophilic membranes were almost the same. However, the difference in flux was smaller when the whole-wheat flour hydrolysate was processed (12 vs 8 $L/[m^2 \cdot h]$) than when the rich medium was processed (85 vs 45 $L/[m^2 \cdot h]$). Protein retention was higher for the hydrophobic membrane than for the hydrophilic membrane (cutoff 20,000) owing to blocking of the pores by proteins adsorbed on to the hydrophobic membrane surface.

Index Entries: Lactic acid; membrane; separation; ultrafiltration; wheat flour.

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Introduction

Lactic acid is an industrially important organic acid used in various applications, e.g., as a preservative in the dairy industry and for the production of cosmetics *(1,2)*. Another product that has attracted considerable interest during recent years is the polymer polylactic acid (PLA), which can be made using lactic acid as a monomer. PLA is biodegradable, which means that polymers made of L-lactic acid are useful in medicine and the pharmaceutical industry for sutures and as a coating for controlled-release drugs *(1,3)*. The degradation product of the polymer is L-lactic acid, which occurs naturally in the human body and is not harmful to humans *(1)*. D-lactic acid, on the other hand, is harmful, so this isomer must be avoided. PLA may also be useful in packaging materials, but the cost of producing the monomer needs to be lowered.

There are two main methods of producing lactic acid: organic synthesis and fermentation *(3,4)*. At present, organic synthesis is the most widely used method. However, the increasing cost of petroleum and increasing environmental concern have made the fermentation method more interesting *(3,5)*. Fermentation is a quite inexpensive technique for producing lactic acid, especially when waste products, such as sweet whey and wheat-bran extract, can be used as substrates *(6,7)*. Unfortunately, the refinement of organic acids from fermentation broths is quite expensive, owing to the low concentration and the presence of other molecules, such as proteins, produced during fermentation *(8)*. To increase the concentration of the acid in the fermentation broth, the pH in the broth is kept constant by adding a base, yielding the salt of lactic acid (lactate) as the product *(9)*. Lactate also inhibits the production of lactic acid, but only at very high concentrations *(10)*. The salt is then converted to the acid, usually by adding a stronger acid, e.g., sulfuric acid. One problem with this technique is that a waste stream of chemicals, e.g., gypsum, with little or no value is produced *(9,13)*. Another problem is that common chemical separation processes, such as distillation, can not be used to concentrate the acid because lactic acid has a high boiling point and polymerizes at elevated temperatures *(9,11,12)*. Results from the refinement of lactic acid with extraction, with both organic solvents and aqueous two-phase systems, and chromatography techniques have been published *(14–19)*. All these techniques have the disadvantages of being expensive, producing waste streams, being unspecific and/or being difficult to use in the large-scale production of lactic acid *(5,17,20)*. In the present study, membrane filtration was investigated as it has low energy consumption, has low running costs, and is easy to scale up and to use in continuous processes *(12)*.

The aim of this investigation was to find a membrane with as high a flux as possible, but still capable of separating cells and proteins from the lactic acid containing broth. The investigation presented in this article is part of a research project of which the aim is to produce lactic acid at a low cost, and with a minimum of energy consumption, so that the polylactic

acid polymer can compete with synthetic plastics. To make the process as environmentally friendly as possible, the raw material must be renewable to avoid a net contribution of carbon dioxide to the atmosphere when the plastic is destroyed. In this study, L-lactate is produced, as the only isomer, in a fermentation step in which *Lactococcus lactis* ferments wheat starch *(21)*. Wheat starch was chosen because it is used industrially for the production of ethanol and is therefore well documented. Wheat starch is hydrolyzed to glucose in two steps: liquefaction and saccharification. Usually, both hydrolysis steps are performed before fermentation, but in this study saccharification was performed simultaneously with the fermentation stage *(22)*. Since rich medium (salts and nutritive substances) is often easier to work with, the characterization of the flux dependence on the cutoff was evaluated using this type of medium.

The present study deals with the first step in the downstream processing in which the cells and the larger proteins are separated from the broth. Separation was performed by ultrafiltration in a shear-enhanced, cross-flow module. The fouling susceptibility of the membranes was investigated using membranes with different hydrophobic/hydrophilic properties. The advantage of hydrophilic membranes is the low protein binding tendency whereas the chemical stability of hydrophobic membranes often is better. Flux and retention were investigated using membranes with different cutoffs to establish which cutoff was required for a satisfactory separation of cells and proteins from the lactic acid containing broth.

Materials and Methods

Lactic Acid Production

The lactic acid–producing bacteria *L. lactis* spp. *lactis* ATCC 19 435 was grown on two media: rich medium and wheat flour hydrolysate. The rich medium consisted of 5 g/L yeast extract, 5 g/L peptone, 1 g/L casamino acids, 2.5 g/L K_2HPO_4, 2.5 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, and 70 g/L glucose *(23)*. The wheat flour hydrolysate was prepared by mixing wheat flour (250 g/L) with water. The wheat flour mixture was heated to 50°C and 67 µL/L of the enzyme α-amylase Termamyl 120 L (Novo Nordisk, Bagsvaerd, Denmark) were added. The mixture was heated to 90°C and kept at this temperature for 50 min, while the liquefaction occurred. The hydrolysate was then cooled to 30°C and transferred to the fermentor where 1200 µL/L of the enzyme mixture (α-amylase and amyloglucosidase) SAN Super 240 L (Novo Nordisk), 5g/L yeast extract, and the inoculum were added *(22)*.

The inoculum was prepared by taking a colony of *L. lactis* grown on an M17 agar in a Petri dish and transferring it to a test tube with 5 mL of M17 (Merck, Darmstadt, Germany). The test tube was incubated overnight at 30°C, and the mixture was then transferred to a 1-L Erlenmeyer flask containing 800 mL of rich medium. The flask was incubated for 12 h and the cells were then transferred to the fermentor. The inoculation procedure

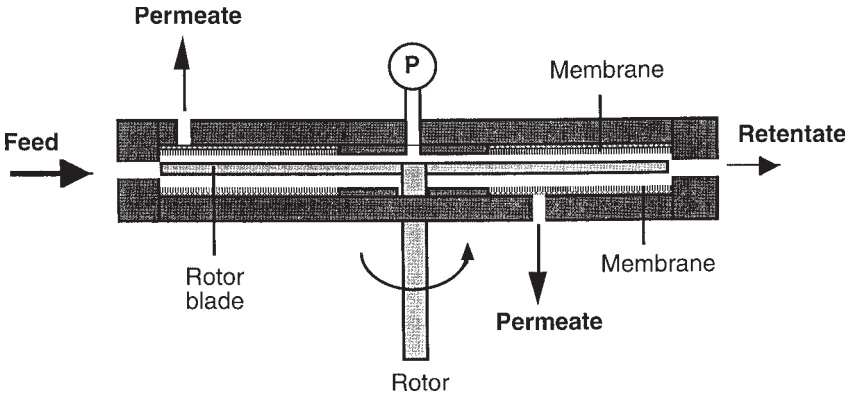

Fig. 1. The Flootek shear-enhanced membrane module.

was performed in the same way for both the rich medium fermentation and the wheat hydrolysate fermentation.

The lactic acid was produced in a 22-L fermentor (model NLF22, Bioengineering, Wald, Switzerland) with baffles. The temperature was 30°C, the stirring speed was 300 rpm, and the pH was maintained at 6.0 by adding 30% (w/v) NaOH. The addition of base was about 2–3.5 L, depending on which medium was fermented.

Ultrafiltration

Ultrafiltration was performed in a shear-enhanced, cross-flow, lab-scale ultrafiltration module (CR filter from Flootek, Malmö, Sweden) with recirculation of the retentate. The module was equipped with two membranes: one above and one below the rotor blade (*see* Fig. 1). The membrane area of each membrane was 0.05 m^2. Four membranes (Hoechst, Wiesbaden, Germany) were compared: PES25, C10, C20, and C30. PES25 is a polyethersulfone membrane (hydrophobic) with a cutoff of 25,000. C10, C20, and C30, with cutoffs of 10,000, 20,000, and 30,000, respectively, are all made of regenerated cellulose, which is a hydrophilic material. All experiments were performed under the same conditions: temperature 30°C, rotor speed 800 rpm, and a transmembrane pressure of 0.8 bar. The permeate was withdrawn continuously. The limited amount of fermentation broth (17 L) and the dead volume of the ultrafiltration equipment limited the volume reduction to 70%. The volume reduction is the ratio of the volume of the permeate withdrawn and the initial volume of the fermentation broth. Samples, of both the retentate and the permeate, were withdrawn at regular volume-reduction intervals during the entire concentration process.

To evaluate the influence of the hydrolytic enzymes on the flux, a small, flat, cross-flow membrane module with a membrane area of 95.4×10^{-4} m^2 was used. In this module, only one membrane at a time can be used (in this case a C20 membrane). Both the permeate and the retentate were

recirculated to maintain the concentration in the feed at a constant level. The hydrolytic enzymes were added to a salt solution (2.5 g/L K_2HPO_4, 2.5 g/L KH_2PO_4, pH set to 6.0 with HCl), and the solution was then treated in the module for 5–8 h. The pressure in the module was 0.8 bar and the temperature 30°C.

Analysis

The concentration of lactic acid and by-products was measured with high-performance liquid chromatography (HPLC) in an Aminex HPX-87H column 300 × 7.8 mm (Bio-Rad, Richmond, VA) with a carrier fluid (0.5 m*M* H_2SO_4) flow rate of 0.4 mL/min. The detector used was an RID-6A refractive index detector (Shimadzu, Kyoto, Japan). The protein concentration was measured using the Bradford Coomassie Brilliant Blue method (Coomassie Protein Assay Reagent 23200, Pierce, Rockford, IL), and the size distribution of the proteins was measured using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad Laboratories) *(24)*. The molecular weight standard used contained the following proteins: phosphorylase b (mol wt 94,000), bovine serum albumin (mol wt 67,000), ovalbumin (mol wt 43,000), carbonic anhydrase (mol wt 30,000), soybean trypsin inhibitor (mol wt 20,100), and α-lactalbumin (mol wt 14,400) (Pharmacia Bio-tech, Sollentuna, Sweden). The cell growth in the rich medium was measured with optical density, but for the whole-wheat flour hydrolysate, the cell growth could not be detected owing to the very high particle content of the medium. Because of problems with keeping the permeate side of the membrane sterile, analysis for the presence of *L. lactis* was difficult to perform. In the ultrafiltration step, the cutoff of the membrane varied between 10,000 and 30,000. These membranes retain proteins with a size at least 10 times smaller than the size of the cells, which is why it was assumed that no cells passed through the membrane.

Cleaning Procedure

After filtration, the membranes were rinsed with deionized water until the retentate stream was clear. The membranes were then cleaned with three cleaning solutions: a 0.4% NaOH solution, a 0.4% alkaline cleaning agent (Ultrasil 10, Henkel, Germany), and a 0.5% enzymatic cleaning agent (Ultrasil 53, Henkel). The cleaning solutions were circulated in the system at 50°C for 20 min, and the system was then rinsed with deionized water at 20°C (5 × 5 L). The membranes were treated with a 1% citric acid solution after the enzymatic treatment to reduce the enzyme activity. The citric acid was then rinsed out of the system with deionized water (5 × 5 L). To evaluate the results of the cleaning procedure, the pure-water flux (PWF) was measured before and after cleaning the membrane, and this value was compared with the PWF value of the membrane before it was used to process the fermented medium.

Results and Discussion

Fermentation

Fermentation was allowed to proceed until all the sugar had been consumed, which is equivalent to cessation of lactic acid production. The fermentation time for the rich medium was 4 d and for the hydrolysate 7 d. The concentration of lactic acid in the broth after fermentation (calculated from the HPLC measurement of the salt) was almost 70 g/L for the rich medium and 140–160 g/L for wheat flour hydrolysate. Seventy grams/liter of glucose were added to the rich medium. In the case of whole-wheat flour, 50–60% of the wheat flour is starch, which is converted to glucose in the hydrolysis stage. This means that almost all the glucose is used for the production of lactic acid and that only a small amount is used for cell growth and the production of by-products. These results have also been shown in other investigations *(21)*.

Influence of Membrane Cutoff

The performance of the three hydrophilic membranes made of regenerated cellulose with cutoffs of 10,000 (C10), 20,000 (C20), and 30,000 (C30) was tested first. These experiments were performed with fermented rich medium without the addition of hydrolytic enzymes. The results of the performance of the three membranes are presented in terms of the flux and the concentration of proteins in both permeate and retentate, as a function of volume reduction.

The flux was almost the same for the C20 and the C30 membranes, whereas the flux of the C10 membrane was significantly lower, as shown in Fig. 2A. The fluxes of the C30 and C20 membranes were, on average, 70 L/[$m^2 \cdot h$], and the flux of the C10 membrane was 15 L/[$m^2 \cdot h$]. Only a small, if any, flux reduction with increasing concentration was observed. The variation in the flux with volume reduction for the C30 membrane is owing to the variation in temperature of the feed solution. During this experiment, the temperature varied between 30 and 45°C, whereas the temperature was kept constant at 30 ± 1°C in the other experiments.

The protein retention of the three membranes was almost the same. The protein concentration in the retentate and in the permeate from the C10 and C20 membranes is shown in Fig. 2B. No protein smaller than 20,000 Daltons could be detected when determining the molecular weight distribution in the fermentation broth by SDS-PAGE. As the protein retention was almost the same for the three membranes, the C20 membrane, with about the same flux as the C30 membrane and a five times higher flux than the C10 membrane, was chosen for further investigations.

The assumption made that all cells were retained by the membranes was correct. No proteins larger than 20,000 could be detected in the permeate from the membrane with a cutoff of 20,000.

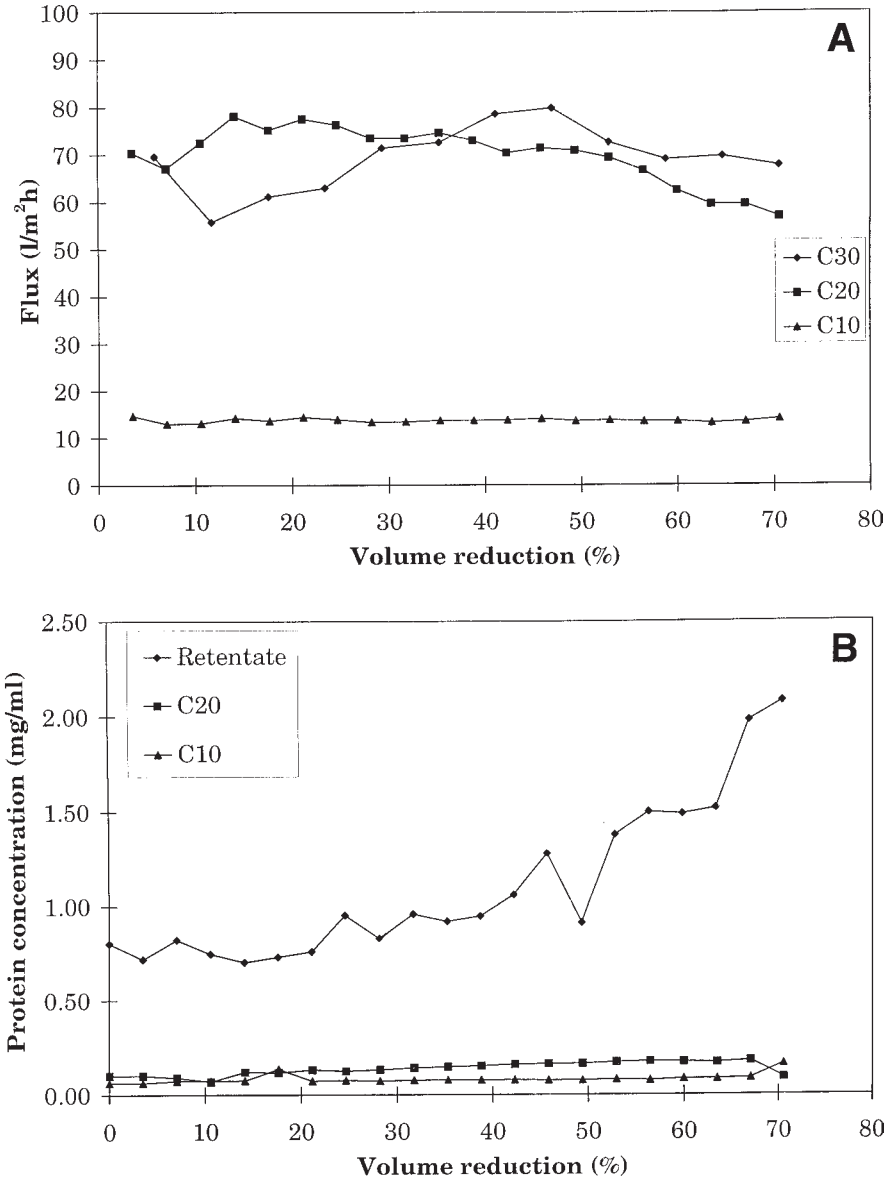

Fig. 2. **(A)** The influence of the volume reduction on the flux of the three hydrophilic membranes using fermented rich medium without hydrolytic enzymes; **(B)** the protein concentration in the retentate and in the permeates from the C10 and C20 membranes.

Influence of Membrane Material

To study the influence of the hydrophobic/hydrophilic properties of the membrane material on membrane performance, a comparative study of the hydrophilic C20 and the hydrophobic PES25 membranes was performed. Although the nominal molecular weight cutoff of the PES25 membrane is higher, the flux of the C20 membrane was almost twice as high as that

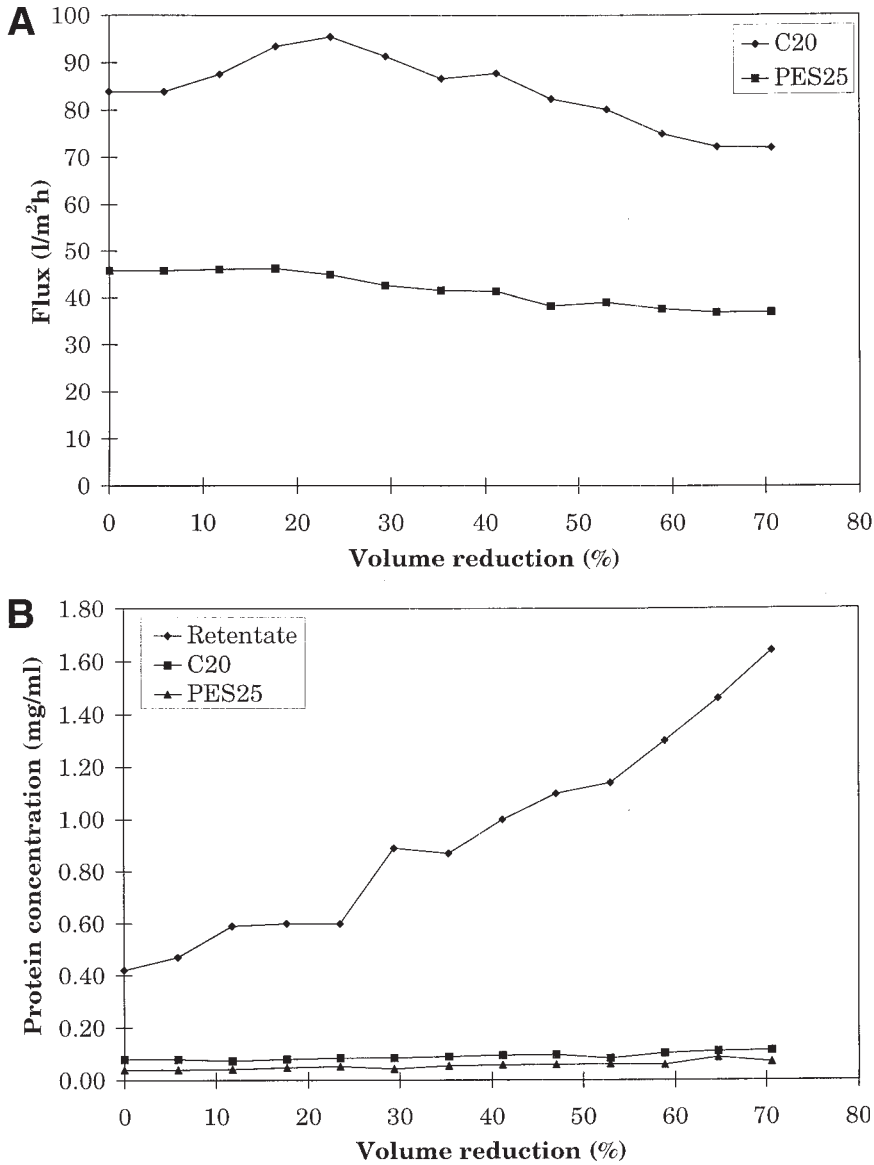

Fig. 3. **(A)** The influence of the volume reduction on the flux of the hydrophilic C20 and the hydrophobic PES25 membrane using fermented rich medium without hydrolytic enzymes; **(B)** the protein concentration in the retentate and in the permeate from the two membranes.

of the PES25 membrane (*see* Fig. 3A). The protein concentration in the permeate from the two membranes was almost the same, as shown in Fig. 3B.

Influence of Fermentation Medium

The relative fluxes (i.e., the ratio between the flux of the solution and the PWF) of the C20 and PES25 membranes were 0.85 (85/100) and 0.75

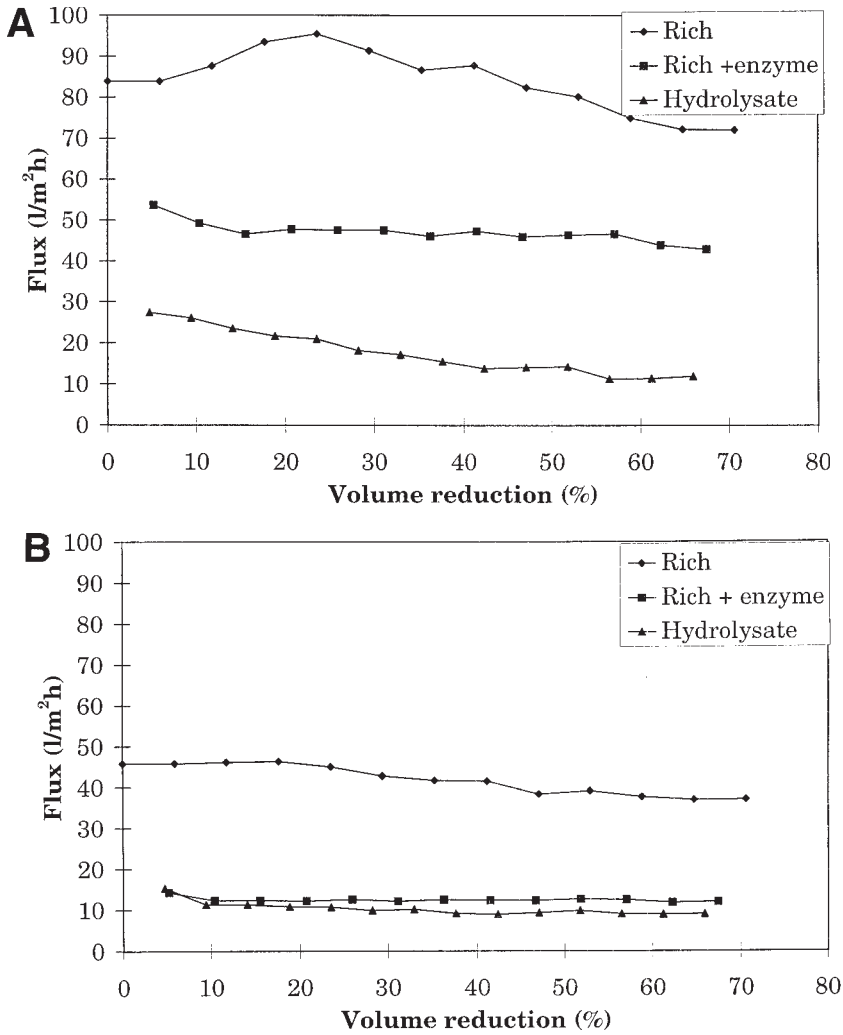

Fig. 4. The influence of volume reduction on the flux of **(A)** the C20 and **(B)** the PES25 membrane, using fermented rich medium with and without hydrolytic enzymes and whole-wheat hydrolysate.

(45/60), respectively, in the experiments illustrated in Fig. 3. The higher flux reduction of the PES25 membrane is probably owing to protein adsorption on the hydrophobic membrane surface *(25)*. However, the flux reduction was quite modest for both membranes. To study the influence of protein adsorption on membrane flux, the enzyme mixtures (Termamyl 120 L and SAN Super 240 L), used during the hydrolysis of the starch in the wheat flour, were added to the rich medium. The enzymes were added to the rich medium after fermentation so that they would not affect the fermentation step.

A severe flux decline was observed when the rich medium containing enzymes was treated, as shown in Fig. 4A,B. The relative fluxes of the C20

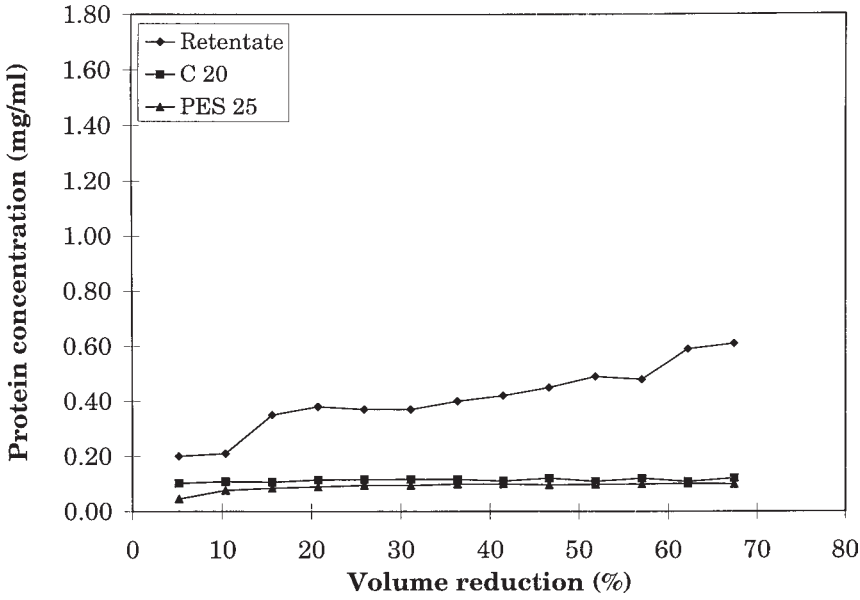

Fig. 5. The protein concentration in the retentate and in the permeates from the two membranes during concentration of the fermented rich medium with enzymes.

and PES25 membranes were 0.45 and 0.2, respectively. The concentration of proteins in the permeates (*see* Fig. 5), was approximately the same as when treating the rich medium without hydrolytic enzymes. However, the protein concentration in the retentate from the rich medium with enzymes was lower than that in the rich medium (*see* Fig. 3B). This is owing to the fact that two fermentation broths are never exactly alike regarding composition, or to adsorption of the enzymes to the membrane, or to the cells. The enzymes can adsorb to the cell surface and are then separated from the broth together with the cells when they are removed by centrifugation before the analysis of the samples. The flux of the membrane is reduced owing to a more dense filter cake, caused by the higher complexity of the medium.

Treatment of Whole-Wheat Hydrolysate

When the whole-wheat flour hydrolysate was ultrafiltered, the flux reduction of the C20 membrane was quite pronounced, whereas the flux of the PES25 membrane was about the same as when treating rich medium with the addition of hydrolytic enzymes. The flux of the C20 membrane also decreased as the concentration in the feed solution increased (*see* Fig. 4A). At the end of the concentration period, the flux of the two membranes was almost the same.

Protein retention was still high, as can be seen in Fig. 6. However, the whole-wheat flour hydrolysate contained small proteins with molecular

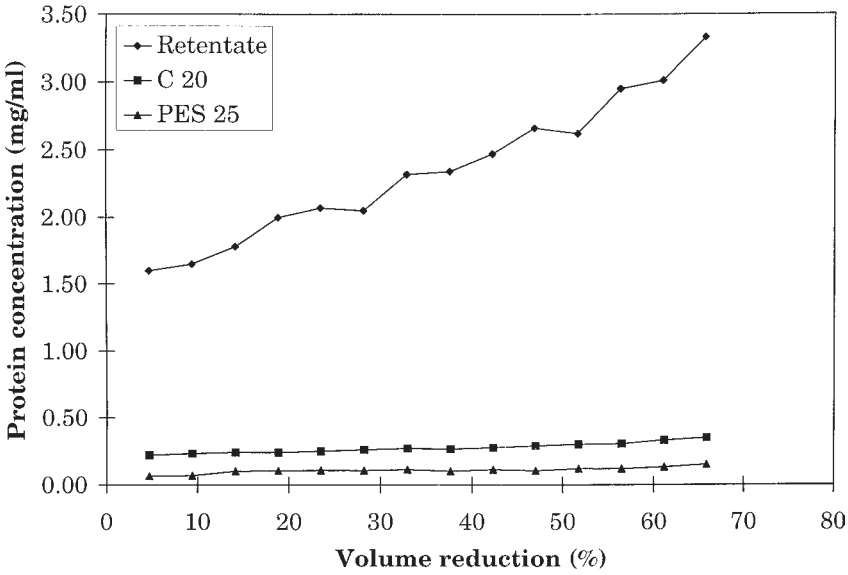

Fig. 6. The protein concentration in the retentate and in the permeate from the two membranes during the concentration of whole-wheat flour hydrolysate.

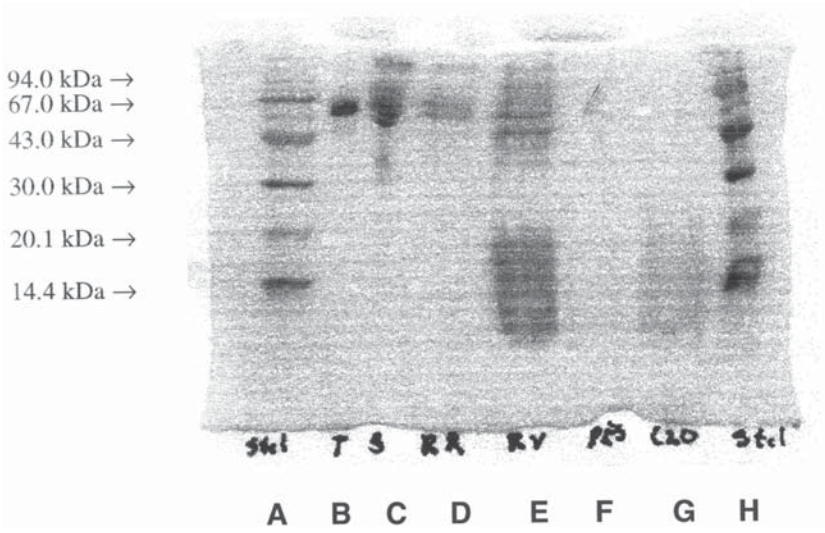

Fig. 7. The SDS-PAGE gel. Wells from the left: **(A)** standard, **(B)** Termamyl 120 L, **(C)** San Super 240 L, **(D)** retentate from rich medium with hydrolytic enzymes, **(E)** retentate from whole-wheat flour hydrolysate, **(F)** permeate from the PES25 membrane, **(G)** permeate from the C20 membrane, and **(H)** standard. The two permeates are from the experiments with the whole-wheat hydrolysate.

weights <20,000, and these proteins were detectable in the permeate from the C20 membrane, but not in the permeate from the PES25 membrane, as shown in Fig. 7.

The higher protein retention of the PES25 membrane, compared with the C20 membrane, is probably owing to the adsorption of proteins on to the membrane surface and in the pores of the hydrophobic membrane, thereby hindering the passage of smaller proteins.

The retention, R, is defined as

$$R = \left(1 - \frac{C_P}{C_F}\right) \cdot 100\%$$

where C_p and C_f are the concentration in the permeate and in the feed, respectively.

The retention of sodium lactate was <13% in all experiments. The highest retention, 12%, was observed for the wheat flour hydrolysate. For the rich medium with hydrolytic enzymes, the retention was 7%, whereas there was no detectable retention of sodium lactate for rich medium with no enzymes added.

Influence of Hydrolytic Enzymes on Membrane Flux

As the hydrolytic enzymes had a marked negative influence on the flux of both the C20 and the PES25 membranes (although it was more pronounced for the PES25 membrane), the influence on the flux of the enzymes themselves was studied. Figure 8 shows the relative flux as a function of time when treating a solution of constant enzyme concentration and one in which the concentration was increased stepwise. In these experiments, only SAN Super 240 L was used because it was found that the small concentration of Termamyl 120 L (67 µL/L) had no influence on the membrane flux (data not shown). The concentration used in the constant concentration experiment, 1200 µL/L San Super 240 L, was the same as the initial concentration in the fermented whole-wheat flour hydrolysate. When the concentration was increased stepwise, the final concentration was 4800 µL/L.

The membrane flux was only slightly affected by the enzyme concentration when there were no cells present, as shown in Fig. 8B. The decrease in flux of the membranes when both cells and proteins are present is most probably owing to a more dense filter cake.

Conclusion

Ultrafiltration has been found to be a suitable method for separating cells and larger proteins from a fermentation broth in which lactic acid has been fermented on hydrolyzed wheat flour. To our knowledge, there are no previous data on the ultrafiltration of fermented wheat flour hydrolysate in the literature. However, the use of PES membranes for the separation of cells and proteins in the production of lactic acid from whey permeate has been reported *(25)*. Compared with the results of that study, the fluxes found in the present study are lower, but that is because of a lower nominal membrane cutoff and higher protein content in the feed.

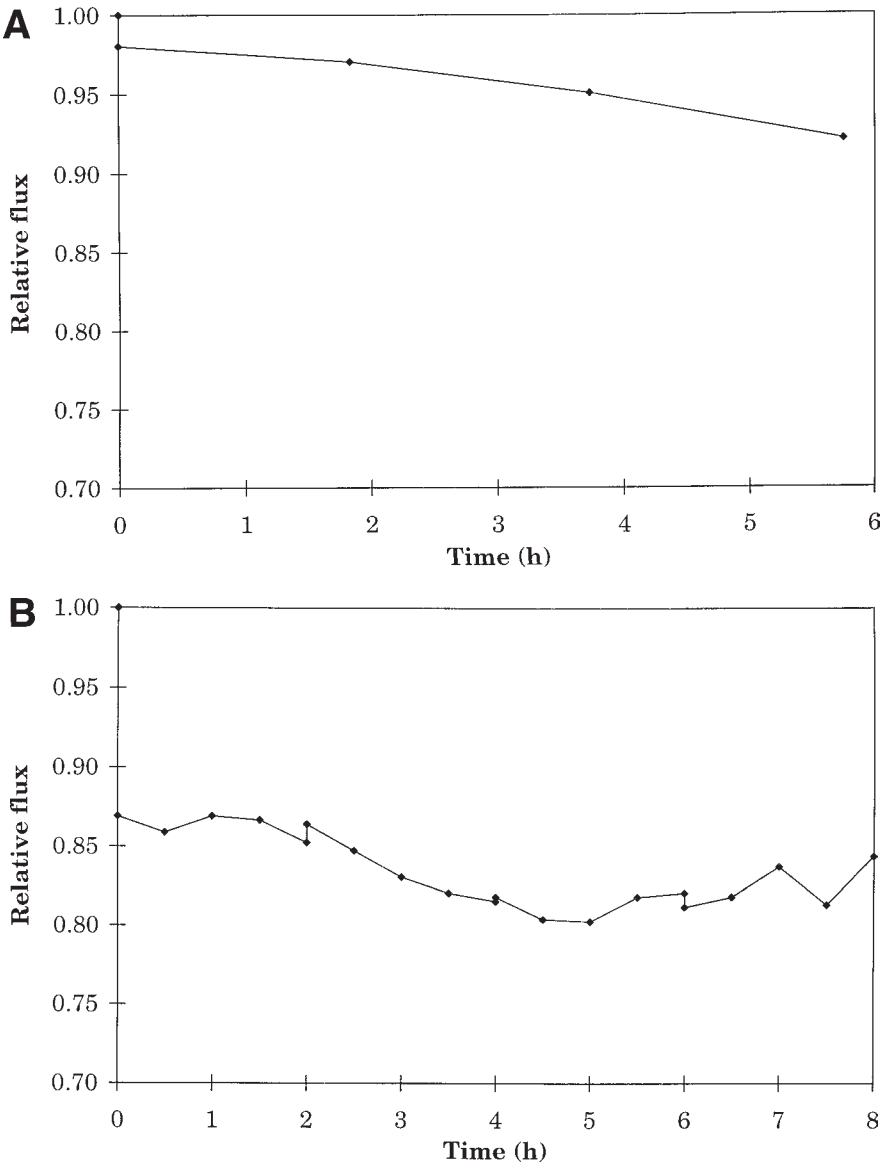

Fig. 8. The relative flux (J/J_{water}) as a function of time during ultrafiltration of the enzyme mixture SAN Super 240 L using a C20 membrane in the flat cross-flow ultrafiltration module. **(A)** Constant concentration of enzyme (1200 µL/L); **(B)** concentration increased stepwise (1200 µL/L SAN Super 240 L added at 0, 2, 6, and 8 h).

When fermented wheat flour hydrolysate was treated, the increase in protein concentration in the permeate was sharp for the C20 membrane, owing to a large amount of small proteins (mol wt < 20,000) in the feed. These small proteins can pass through the membrane and thus increase the concentration of proteins in the permeate. The concentration of proteins in the permeate from the PES25 membrane was lower than that in the perme-

ate from the C20 membrane, although the former has a higher nominal cutoff. This is probably because of a reduction in pore radius caused by a higher degree of protein adsorption in the pores of the hydrophobic membrane. The adsorption of proteins on to hydrophobic membranes is more pronounced than for hydrophilic membranes, and protein adsorption has been shown to lower membrane flux *(25)*. However, after cleaning, the PWF was completely recovered for all membranes, except for the C20 membrane after the treatment of the whole-wheat hydrolysate. The PWF after this experiment was only 70% of the initial value, whereas the PWF of the PES25 membrane, surprisingly enough, was totally restored.

When hydrolytic enzymes were added to the fermented rich medium, flux was reduced. During ultrafiltration of a salt solution (pH 6.0) containing only the hydrolytic enzymes, flux reduction was considerably less pronounced for the C20 membrane. The difference in flux between a medium with only cells and a medium with both cells and hydrolytic enzymes is owing to a more compact filter cake caused by a more complex medium. The filter cake becomes more dense when the flow channels between the cells in the cake are clogged with proteins.

To make the ultrafiltration step more efficient, we suggest that the cells should be separated from the broth before the broth is treated with ultrafiltration, either with microfiltration or centrifugation. A lower concentration of the wheat flour would also improve the separation. Also, a lower wheat flour concentration will probably improve the fermentation by reduction of product inhibition from high lactate concentrations *(10)*. However, this will increase the cost of subsequent concentration steps.

Acknowledgments

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