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Food Chemistry 107 (2008) 112–119
Food Chemistry
www.elsevier.com/locate/foodchem

Pectin hydrolysis in a free enzyme membrane reactor: An approach to the wine and juice clarification

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Received 3 May 2007; received in revised form 21 June 2007; accepted 18 July 2007

Abstract

In this paper, a long-term investigation of the enzyme hydrolysis of pectin, using a free enzyme membrane reactor (FEMR), is presented. Production of crude enzymes (polygalacturonase and pectin lyase), in *Aspergillus niger* CECT 2088 cultures grown on an agricultural subproduct (apple pomace) as a low cost inducer, was also studied. Observing the retention of induced pectinases by the ultrafiltration membranes, the best FEMR configuration was achieved with the membrane of 10,000 nominal molecular weight limit (NMWL), since the biocatalyst was retained with no loss of enzyme activity. The monitoring of pectin hydrolysis in the FEMR was carried out by determining the viscosity and amount of reducing groups in both reaction mixture and permeate. A viscosity reduction of 88%, below the initial value, was reached in the reaction mixture only after 0.25 h of operation, while a gradual release of reduced sugars, in both reaction mixture and permeate, was detected. The FEMR was maintained in operation for 15 days, achieving an excellent catalytic efficiency.

Keywords: Apple pomace; *Aspergillus niger*; Free enzyme membrane reactor; Induction; Pectinases; Pectin hydrolysis

1. Introduction

Pectinases are responsible for the degradation of long and complex molecules called pectins that occur as structural polysaccharides in the middle lamella and the primary call walls of young plant cells. These enzymes have been extensively studied because of their applications in food technology, e.g., in the extraction and clarification processes during the manufacturing of juice and wine (Fogarty & Kelly, 1983; Kashyap, Vohra, Chopra, & Tewari, 2001; Van Rijssel, Gerwig, & Hansen, 1993). Although many yeasts, some bacteria and a large variety of filamentous fungi are known to synthesize pectinases, the preferred industrial choices are fungi of the genus *Aspergillus* (Nair, Rakshit, & Panda, 1995; Rombouts & Pilnik, 1980), particularly *Aspergillus niger*. Apart from the question of enzyme effectiveness, the choice of *Aspergillus* enzymes is also determined by the acceptability requirements of the food processing industry (Bailey & Pessa, 1990; Pariza & Foster, 1983). The synthesis of pectic enzymes is reported to be induced by either pectin and its derivatives or by complex fibre forms, such as beet-pulp or wheat bran (Naidu & Panda, 1998; Solis-Pereira, Favela-Torres, Viniegra-Gonzalez, & Gutierrez-Rojas, 1993). The idea of using cheaper raw materials for enzyme production is an important parameter in useful technological development (Panda, Nair, & Prem Kumar, 2004). In this context, the use of apple pomace is one of the options. Results from some earlier studies have indicated a possibility that apple pomace appears to be an inducer for pectolytic enzymes in *A. niger* A163 by solid state bioprocessing (Berovic & Ostroversnik, 1997).

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doi:10.1016/j.foodchem.2007.07.057

Traditional technology for the clarification of juices and wine is characterized by pure enzyme utilization (enzyme is used only once) (Grzeskowiak-Przywecka & Slominska, 2007). An alternative method for the conventional batch reactor is a free enzyme membrane reactor (FEMR). This approach is based on the difference in molecular weight between the enzyme and the hydrolysis products. The soluble enzyme is confined in the retentate side of the membranes where it is in contact with the substrate (which is retained as well), whereas the product is small enough to permeate through the membrane (Guadix, Camacho, & Guadix, 2006). One of the main advantages of this configuration is that it makes recovery and reuse of enzyme possible. Moreover, product inhibition can be reduced significantly by the use of an FEMR configuration (Rodriguez-Nogales, Ortega, Perez-Mateos, & Busto, 2005). An additional advantage of the continuous recycling membrane reactor is its high efficiency and low labour cost. The major problems in the application of a membrane reactor are large decreases in permeate flux due to fouling and concentration polarization (Grzeskowiak-Przywecka & Slominska, 2007), although the use of hydrolytic enzymes helps to reduce these effects.

Some works have focussed on pectin hydrolysis using membrane reactors with free enzymes. Alkorta, Garbisu, Llama, and Serra (1995a, 1995b) studied the reduction in viscosity of pectin solutions catalyzed by pectin lyase from *Penicillium italicum* in a membrane reactor, maintained in operation for 8 h, achieving a viscosity reduction of 60% after the first 30 min. Belafi-Bako, Eszterle, Kiss, Nemestothy, and Gubicza (2007) found that a flat sheet membrane bioreactor designed for the hydrolysis of pectin by a polygalacturonase from *A. niger* worked with excellent stability for more than 50 h. However, reports were not found concerning pectin hydrolysis in a membrane reactor with free enzyme for a long-term period.

Therefore, the aim of this work was to study pectin hydrolysis by fungal pectinases using an FEMR as a prior step for the further application of this design to juice and wine clarification. First, some results regarding the effect of apple pomace concentration on the production of pectinases in *A. niger* cultures were considered. Then, the membrane module was characterized and the stability of the fungal pectinases in the FEMR studied. Finally, the hydrolytic process was carried out and its performance monitored for a long-term period.

2. Materials and methods

2.1. Cell growth and enzyme production

In order to produce the enzymes, 100 ml of mineral medium (3.0 g $NaNO_3$; 1.02 g $MgSO_4 \cdot 7H_2O$; 0.5 g KCl; 1 g K_2HPO_3; 0.018 g $FeSO_4 \cdot 7H_2O$ per litre of Milli-Q water) was transferred into 250 ml Erlenmeyer flasks and supplemented with 0.2% peptone, then autoclaved for 30 min at 121 °C. Later, 2.5 ml of a suspension with about 10^7 *A. niger* CECT 2088 (strain obtained from the official Spanish Culture Collection) spores (counted in a Thoma chamber) was inoculated into the medium adding, as carbon source, apple pomace, previously autoclaved, at 3.6% and 1.7% (w/v) (Rodriguez-Nogales et al., 2005). The cultures were run for 5–7 days at 30 °C without agitation.

2.2. Isolation of microbial enzymes

The cultures were filtrated through a glass fibre filter type A/E (Millipore®). The cell-free filtrate was concentrated by tangential ultrafiltration using a Minitan® system (Millipore®) equipped with 10,000 NMWL membranes (PTGC OMP04 Polysulfone). The enzyme activity of this extramycelial fraction was systematically assayed (Rodriguez-Nogales et al., 2005).

2.3. Enzyme assays

Polygalacturonase activity (endo- and exo-enzyme) was assayed by measuring the released reducing groups, using the Nelson–Somogyi method (Nelson, 1944; Somogyi, 1952). The reaction mixture, containing 1 ml of 0.5% (w/v) polygalacturonic acid (Sigma) in 0.1 M acetate buffer at pH 4.2, 4 ml of 0.1 M acetate buffer at pH 4.2 and 1 ml of crude enzyme, was incubated at 30 °C for 1 h. One unit of enzyme activity (U) was defined as the amount of enzyme releasing one μmol of galacturonic acid, per minute.

Pectin lyase activity was determined spectrophotometrically by monitoring the absorbance, at 235 nm for 120 s (Albersheim & Killias, 1962), of the reaction mixture containing 2.25 ml of 0.5% (w/v) citrus pectin in 0.1 M citrate/phosphate buffer at pH 6.0 and 0.75 ml of crude enzyme. Pre-incubations were carried out at 40 °C for 15 min, starting the reaction by adding pectin. One unit of enzyme activity (U) was defined as the amount of enzyme releasing one μmol of unsaturated methyl-oligosaccharides per minute, based on the molar extinction coefficient of 5500 $M^{-1}\,cm^{-1}$ of the unsaturated products.

Endopectinolytic activity was followed by a viscosimetric method according to Mutlu, Sarioglu, Demir, Ercan, and Acar (1999) with some modifications. The reaction mixture contained 9 ml of 1% (w/v) apple pectin solution in 0.1 M sodium acetate buffer at pH 4.8 and 1 ml of crude enzyme. The enzymatic reaction was stopped, after 15 min of shaking at 150 rpm at 40 °C, by adding 7 ml of 0.5 N HCl, and the viscosity of the mixture was determined. One unit (U) of enzyme activity was defined as the amount of enzyme that reduces the viscosity to one half of the initial value per min at 40 °C.

Enzyme production was expressed in units per millilitre of crude enzyme solution (U ml^{-1}).

2.4. Free enzyme membrane reactor experiments

Enzyme hydrolysis of apple pectin was carried out in the FEMR (Fig. 1). An ultrafiltration system equipped with Prep/Scale™ TFF spiral-wound polysulfone membrane

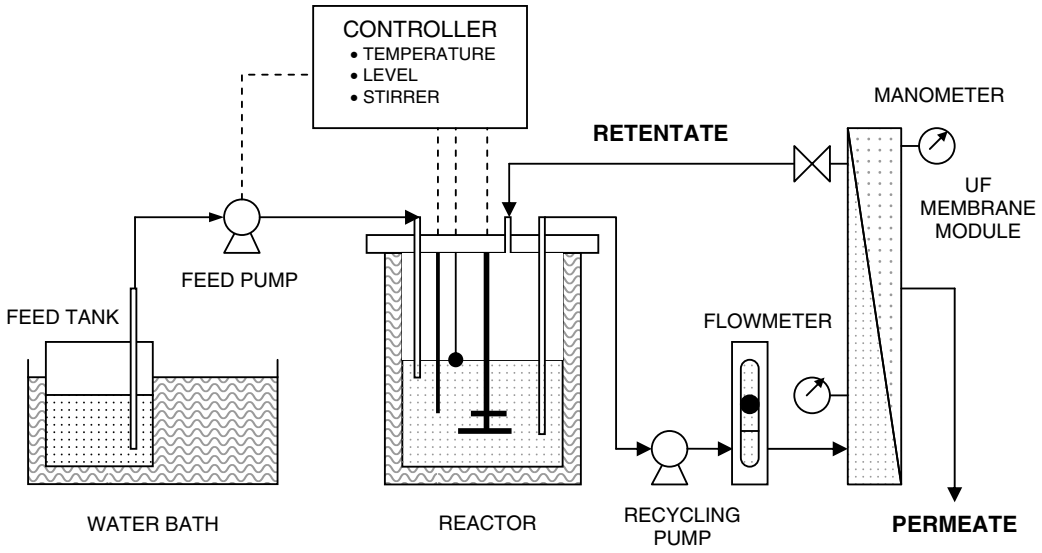

Fig. 1. Schematic diagram of the free enzyme membrane reactor.

(Millipore) of 10,000 and 50,000 nominal molecular weight limit (NMWL) and surface is of 0.23 m^2, coupled with a stirred tank reactor which served as a FEMR. The reactor volume (500 ml) was kept steady by means of an automatic level control (S196166, Carlo Gavazzi, Steinhausen, Switzerland), and the temperature and agitation of the reactor were automatically controlled by a Biostat® B microcomputer (Beckman Instruments, Fullerton, California, USA). Experiments in the FEMR were carried out by recirculating the reaction mixture from the reactor to the membrane module, using a peristaltic pump. A 1 l Erlenmeyer flask immersed in a water batch at 46 °C was used to feed new substrate to the reactor. The optimal conditions for the apple pectin hydrolysis were 23.3 substrate-to-enzyme ratio, pH 4.8 (0.1 M sodium acetate buffer) and 46 °C (Rodriguez-Nogales, Ortega, Perez-Mateos, & Busto, 2007). The pectin used was a highly methylated (70–75%) apple pectin (P8471; Sigma, St. Louis, MO). Operational conditions of the FEMR were 36 l h^{-1} recycling flow rate with a transmembrane pressure of 34.5 kPa, and using a reaction volume of 500 ml (Rodriguez-Nogales et al., 2005).

Experiments for the membrane characterization were carried out to determine the resistance, retention and flux of the membranes by using the "pure water flux" (hydraulic permeability) method for ultrafiltration membranes (Mulder, 1996). The operation conditions of runs were as follows: transmembrane pressure of 10–170 kPa, recycling flow rate of 36 l h^{-1}, and 25 °C. Enzyme retention experiments were conducted with 500 ml of enzyme solution (0.250 U ml^{-1} of endopectinases at pH 4.8) recirculating from the reactor to the membrane module at a transmembrane pressure of 34.5 kPa.

The reactor conversion was calculated by measuring the reaction mixture viscosity and expressed as follows:

$$X_\mu = \frac{\mu_0 - \mu_t}{\mu_0} \times 100$$

where μ_0 and μ_t are reaction mixture viscosity at initial and t incubation time, respectively (Rodriguez-Nogales et al., 2007).

2.5. Analytical assays

Cellular growth was determined by drying the mycelium overnight at 80 °C (retained in a cotton-glass filter) to constant weight (Alaña, Alkorta, Domínguez, Llama, & Serra, 1990). Protein was estimated in dialyzed cell-free samples by the method of Lowry, Rosebrough, Farr, and Randall (1951) with bovine serum albumin (BSA) as the standard. The release of reducing groups resulting from the enzymatic hydrolysis of apple pectin was quantified by the modified Nelson–Somogyi method (Nelson, 1944; Somogyi, 1952) using galacturonic acid as reference (Solis-Pereira et al., 1993). This analysis tests a measure of the activity of the depolymerising pectolytic complex. The viscosity of the reaction mixture was measured in a Cannon-Fenske 100 viscometer at 40 °C (Alaña et al., 1990) after adding 7 ml of 0.5 N HCl to the reaction mixture (9 ml).

2.6. Statistical analysis

All fermentations, hydrolysis experiments and assays were carried out in triplicate. At least three replicated samples were used in all analytical determinations. Mean values and standard deviations are shown in Figures.

3. Results and discussion

3.1. Production of fungal pectinases induced by apple pomace

One of the main agro-industrial by-products, abundantly produced in Europe, is apple pomace, from the apple juice and cider industries. Apple pomace, composed

of peel, seed and pulp, contains high levels of pectic substances and it could be used as a substrate for the biosynthesis of pectinases. Fig. 2 shows the time courses of extracellular polygalacturonase and pectin lyase production by *A. niger* cultures supplemented with apple pomace at 1.7% and 3.6% (w/v). Enzymatic activity increased slowly in the early stages of growth, and reached the highest level after 7–8 days. For the highest concentration of apple pomace, extramycelial polygalacturonase activity was not detected during the first four days. This behaviour was also observed for the pectin lyase biosynthesis on both concentrations of apple pomace. Under these conditions, it could be possible that the fungus did not secrete enzymatic activity until the simple carbon sources were consumed. These results are in agreement with those found by other authors (Solis-Pereira et al., 1993) in cultures of *A. niger* supplemented with pectin and sugars (sucrose and glucose). These authors attributed this behaviour to a substrate inhibition occurring during the early fermentation days as a result of the presence of sucrose and glucose.

The maximum levels of polygalacturonase were detected at 7 days in the medium enriched with apple pomace. Nevertheless, a rise of the concentration of this by-product decreased the production of pectin lyase, achieving the best levels of enzymatic activity at 7–8 days of incubation. However, at 7 days, a rise of apple pomace concentration caused an increase of the specific activity (expressed as U mg protein^{-1}) to 32% for pectin lyase. A high microbial growth was observed in the fungal cultures supplemented with this carbon source, giving values of biomass of 4–10 g l^{-1} (Table 1). Regarding the pH evolution, an acidification of the medium occurred, giving a value of about 3 after 4–5 days of incubation.

3.2. Hydrolysis of pectin in a free enzyme membrane reactor (FEMR)

The hydrolysis of pectin was performed in an FEMR with the crude enzymes described above, and induced in the cultures of *A. niger* with 3.6% (w/w) apple pomace during 7 days at 30 °C. Under these culture conditions, the lyophilized exocellular fraction exhibited an endopectinase activity of 4.17 U g^{-1}. The use of this type of bioreactor permits a separation of the hydrolysis products with simultaneous recycling of nonhydrolyzed substrate and enzyme (Prazeres & Cabral, 1994). For observing the molecular weights of pectinases from *A. niger* (Burns, 1991; Lang & Dornenburg, 2000), ultrafiltration membranes of 10,000 and 50,000 NMWL were assayed.

To analyse the behaviour of the reactor, a characterization of both ultrafiltration membranes (10,000 and 50,000 NMWL) was performed, determining their hydraulic permeabilities and their enzyme retentions. To determine the membrane permeability, Milli-Q water at 25 °C was circulated in the membrane reactor at various transmembrane pressures (10–170 kPa) and the flux was measured. For these experiments, the transmembrane pressure was increased in steps, waiting for the permeate flux to stabilize before recording its value (Fig. 3). Only in the case of Milli-Q water did the permeate flux follow Darcy's law and was proportional to the transmembrane pressure. From the slope of flux versus transmembrane pressure line, the hydraulic membrane permeabilities were determined to be 92.7 and 143.2 l h^{-1} m^{-2} bar^{-1} for membranes with 10,000 and 50,000 NMWL, respectively. As expected, the hydraulic membrane permeability was higher for the membrane with the highest cut-off. Therefore, 0.2% and 0.8% (w/v) apple pectin solutions were circulated under similar conditions and the flux was measured. By increasing the transmembrane pressure of the system, the permeate flux obtained was also higher. However, in this case, the flux was not proportional to the pressure because the solute was retained, causing a fouling of the membranes. Application of membrane with 10,000 NMWL, at 100 kPa and for both solutions, allowed us to obtain about 60% decrease in the flux in comparison with the 50,000 NMWL membrane.

An ultrafiltration membrane suitable for pectin hydrolysis in an FEMR must be able to retain the enzyme. There-

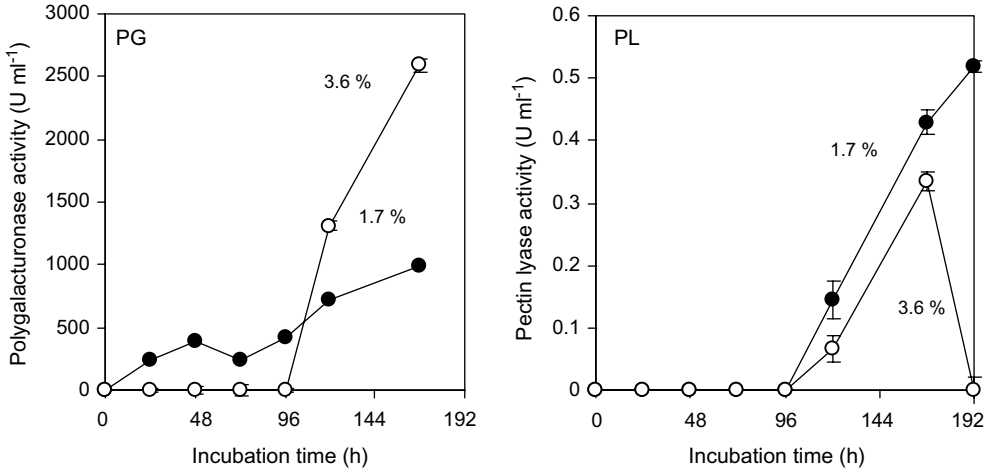

Fig. 2. Extramycelial polygalacturonase (PG) and pectin lyase (PL) induced in *A. niger* CECT 2088 cultures by apple pomace at different concentrations.

Table 1
Evolution of pH, biomass and extramycelial protein content in *A. niger* CECT 2088 cultures supplemented with pomace apple at different concentrations

Induction time (h)	Culture pH		Biomass (g l^{-1})		Protein content (μg BSA ml^{-1})	
	1.7% (w/v)	3.6% (w/v)	1.7% (w/v)	3.6% (w/v)	1.7% (w/v)	3.6% (w/v)
0	5.36	4.73	0.00	0.00	n.d.	n.d.
24	4.54	4.41	0.88	2.40	598	493
48	3.10	3.95	3.19	4.33	512	164
72	2.87	3.19	3.40	5.75	418	534
96	2.84	2.61	4.41	6.49	388	220
120	2.88	3.06	4.28	7.06	539	435
168	3.11	2.72	n.d.[a]	8.67	570	306
192	3.49	3.24	5.48	9.91	518	328

[a] Not determined.

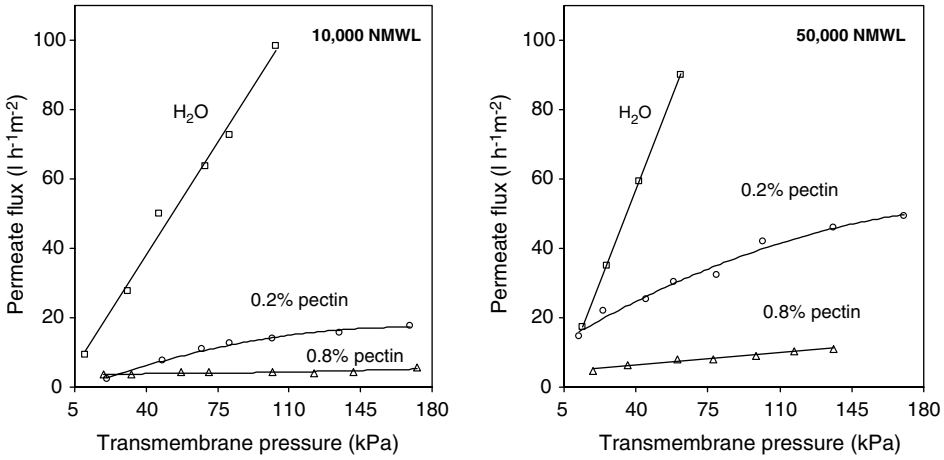

Fig. 3. Effect of the transmembrane pressure on the permeate flux of Milli-Q water and apple pectin solutions for 10,000 and 50,000 NMWL ultrafiltration membranes.

fore, the endopectinase activity in both reaction mixture and permeate was checked regularly for 90 min for both membranes (Fig. 4). The experiments carried out with the 10,000 NMWL membrane have given satisfactory retention of the enzyme activity, achieving retention values of about 98.8%. Only 1.2% of the initial enzyme activity was observed in the permeate. However, gradual drop of the enzyme activity was observed for the 50,000 NMWL membrane, at 90 min reaching 35.2% and 26.4% of the initial enzyme activity in the reaction mixture and permeate, respectively. From these results, a membrane with an NMWL of 10,000 was selected.

To study the pectin hydrolysis in the FEMR, the modification of the viscosity and the release of reducing sugars of samples withdrawn from both reaction mixture and permeate were evaluated for 2.5 h in a continuous mode (Fig. 5). The experiment was also carried out in a membrane reactor without adding the enzyme solution.

A viscosity reduction of 88% was reached after only 0.25 h of reaction. By contrast, the viscosity of the reaction mixture without enzyme increased linearly with the operation time, whereas the permeate viscosity was constant for both configurations. The amount of reducing sugars in the reaction mixture and permeate linearly increased during the first hour in the FERM and then there was a less

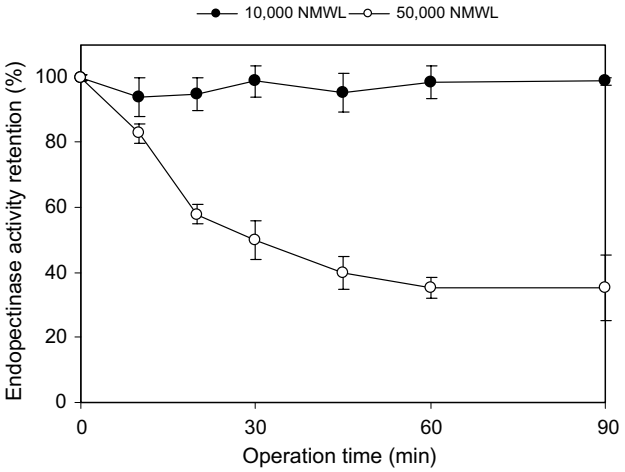

Fig. 4. Influence of membrane pore size (NMWL) on endopectinase activity retention.

intense increase. However, the reducing sugar content remained at the same level in the membrane reactor without enzymes, about 0.34 and 0.51 mg ml^{-1} in the permeate and the reaction mixture, respectively. An increase in the concentration of the reducing sugars is related to an increase in hydrolysis of the pectin and a reduction in the molecular weight (Szaniawski & Spencer, 1997).

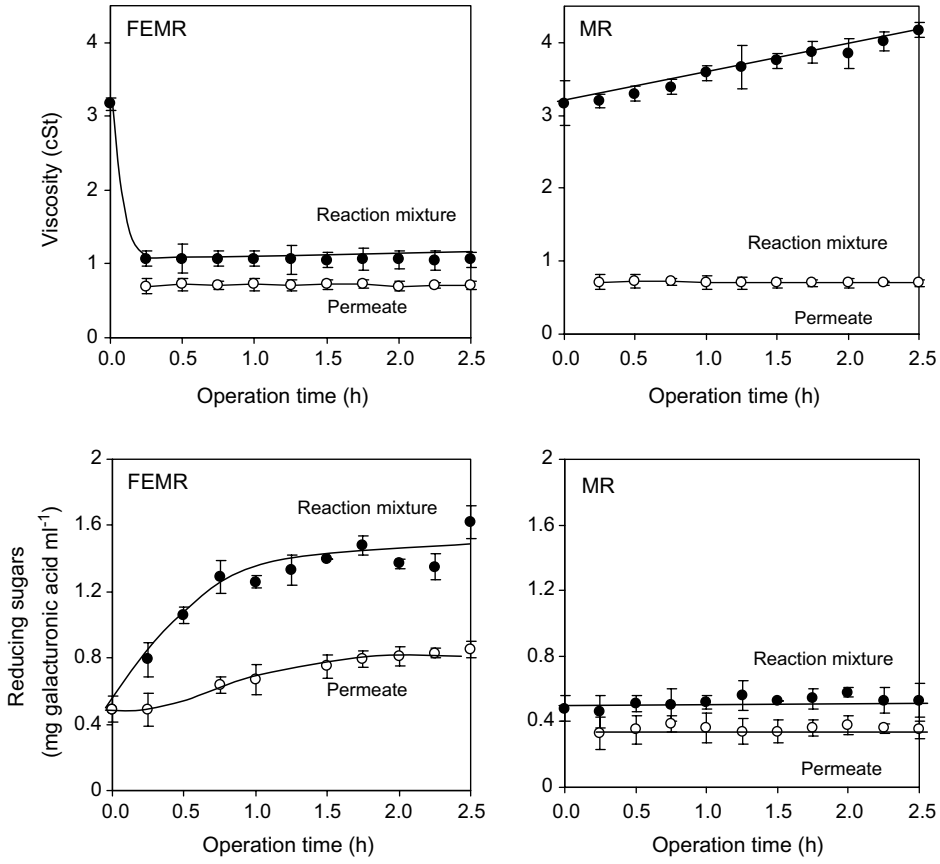

Fig. 5. Evolution of viscosity and concentration of reducing sugars in the free enzyme membrane reactor (FEMR) and the membrane reactor (MR).

Fig. 6 shows the relationship between the reactor conversions (%) and reaction time. Maximum reactor conversion was established after only 0.25 h of reaction, achieving a good conversion (68%) during the 2.5 h of reaction time. The effect of the endopectinases on the permeate flux was also evaluated for both reaction configurations (with and without enzyme). Permeate flux obtained from the FEMR was 3.2 times higher than that obtained from the MR after 2.5 h of operation.

These results encouraged us to test the behaviour of the free enzyme reactor membrane for a long-term period (15 days). It was found that permeate flux decreased from 9.8 to 1.9 $l\,h^{-1}\,m^{-2}$ during the first day, whereas in the course of the following 14 days the flux obtained slightly decreased to 0.3 $l\,h^{-1}\,m^{-2}$. This is a typical behaviour for membranes which suffer the fouling process. The flux declines intensely during the first phase of the process and then decreases in a less intense way (Mannheim & Cheryan, 1990). Figs. 7 and 8 show the viscosity and reducing sugar evolution as a function of the operation time, respectively, for both permeate and reaction mixture. A drastic reduction in the viscosity of the reaction mixture viscosity was achieved in only 30 min. Then, the reaction mixture's viscosity was gradually increased, whereas the permeate's viscosity remained at the same level (0.6–0.7 cSt) in the course of 15 days of reaction time. On the other hand, the reducing sugars also increased in the permeate and the reaction mixture during the first day, reaching a steady-state after 1–2 days of reaction. Afterwards, a gradual decrease in the concentration of reducing sugars was observed for both streams, likely motivated by an enzyme deactivation. Lozano, Manjon, Iborra, Canovas, and Romojaro (1990) noticed a shear stress enzyme deactivation effect produced by tangential flow on a membrane reactor with immobilized pectinases after one operation day. Fane and Radovich (1990) reported that enzyme deactivation could be

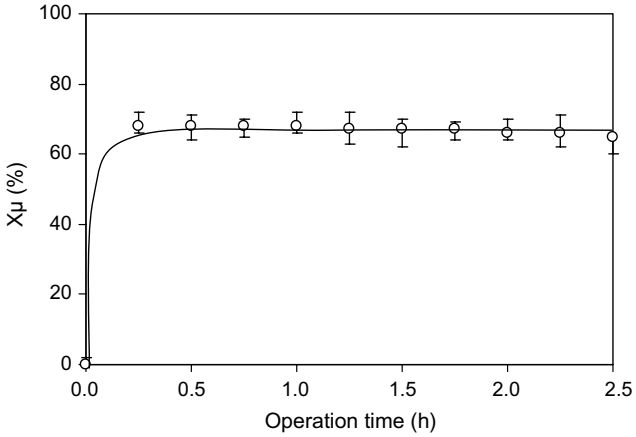

Fig. 6. Evolution of the reactor conversion (X_μ) in the FEMR. (Operation conditions: 23.3 substrate/enzyme ratio; 48 °C; pH 4.8; 36 $l\,h^{-1}$ recycling flow rate; 34.5 kPa transmembrane pressure).

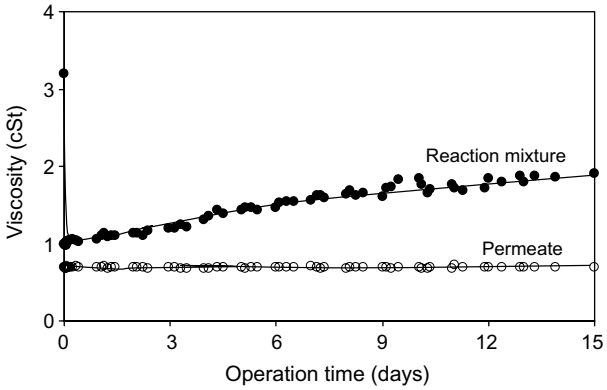

Fig. 7. Catalytic efficiency of the FEMR for pectin hydrolysis assessed as viscosity in both reaction mixture and permeate. (Operation conditions: 23.3 substrate/enzyme ratio; 48 °C; pH 4.8; 36 l h^{-1} recycling flow rate; 34.5 kPa transmembrane pressure).

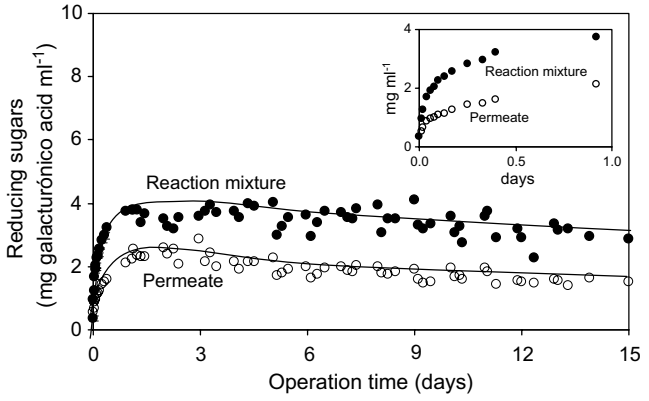

Fig. 8. Catalytic efficiency of the FEMR for pectin hydrolysis assessed as release of reducing sugars in both reaction mixture and permeate. (Operation conditions: 23.3 substrate/enzyme ratio; 48 °C; pH 4.8; 36 l h^{-1} recycling flow rate; 34.5 kPa transmembrane pressure).

caused by local increases in temperature and by the presence of air bubbles in the system. It seems likely that severe polarization and fouling of membranes can cause loss of bioreactor efficiency (Mountzouris, Gilmour, Grandison, & Rastall, 1999). Finally, an inhibition of enzyme activity by one or several products arising from the pectin degradation was observed in a kinetic study on pectin hydrolysis in an FEMR with endopectinases (Rodriguez-Nogales et al., 2005).

4. Conclusion

From the results obtained in this study, *A. niger* CECT 2088 can be successfully cultured in a cheap raw material (apple pomace) producing a significant level of pectinolytic activity. Thus, an industrial process such as apple juice production, could be completed with a waste utilization step: manufacturing a valuable product, pectinases. Additionally, this study could be further widened by adapting the strain to this by-product for obtaining a higher enzyme activity.

The use of the crude enzyme preparations allowed a drastic reduction of the pectin viscosity in the FEMR in comparison with the membrane reactor configuration without enzyme. Moreover, the FEMR was a successful technique for continuous pectin hydrolysis over a long time of operation. These results show that the use of pectinases in a membrane reactor appears to be of potential interest for the clarification of fruit juices and wine. However, further studies are necessary for a future commercial application.

Acknowledgement

We thank the financial support from the "Ministerio de Ciencia y Tecnología", Grant No. AGL2000-1688.

References

Alaña, A., Alkorta, I., Domínguez, J. B., Llama, M. J., & Serra, J. L. (1990). Pectin lyase activity in a *Penicillium italicum* strain. *Applied and Environmental Microbiology, 56*, 3755–3759.

Albersheim, P., & Killias, U. (1962). Studies relating to the purification and properties of pectin transeliminase. *Archives of Biochemistry and Biophysics, 97*, 107–115.

Alkorta, I., Garbisu, C., Llama, M. J., & Serra, J. L. (1995a). Beta-transelimination of citrus pectin catalyzed by *Penicillium italicum* pectin lyase in a membrane reactor. *Applied Biochemistry and Biotechnology, 55*, 249–259.

Alkorta, I., Garbisu, C., Llama, M. J., & Serra, J. L. (1995b). Viscosity decrease of pectin and fruit juices catalyzed by pectin lyase from *Penicillium italicum* in batch and continuous-flow membrane reactors. *Biotechnology Techniques, 9*, 95–100.

Bailey, M. J., & Pessa, E. (1990). Strain and process for production of polygalacturonase. *Enzyme and Microbial Technology, 12*, 266–271.

Belafi-Bako, K., Eszterle, M., Kiss, K., Nemestothy, N., & Gubicza, L. (2007). Hydrolysis of pectin by *Aspergillus niger* polygalacturonase in a membrane bioreactor. *Journal of Food Engineering, 78*, 438–442.

Berovic, M., & Ostroversnik, H. (1997). Production of *Aspergillus niger* pectolytic enzymes by solid state bioprocessing of apple pomace. *Journal of Biotechnology, 53*, 47–53.

Burns, J. K. (1991). The polygalacturonase and lyases. In R. H. Walter (Ed.), *The chemistry and technology of pectin* (pp. 165–174). New York: Academic Press.

Fane, A. G., & Radovich, J. M. (1990). Membrane systems. In J. A. Asenjo (Ed.), *Separations process in biotechnology* (pp. 1–52). New York: Marcel Dekker.

Fogarty, W. M., & Kelly, C. T. (1983). Pectic enzymes. In W. M. Fogarty (Ed.), *Microbial enzymes and biotechnology* (pp. 131–182). London: Applied Science Publishers.

Grzeskowiak-Przywecka, A., & Slominska, L. (2007). Saccharification of potato starch in an ultrafiltration reactor. *Journal of Food Engineering, 79*, 539–545.

Guadix, A., Camacho, F., & Guadix, E. M. (2006). Production of whey protein hydrolysates with reduced allergenicity in a stable membrane reactor. *Journal of Food Engineering, 72*, 398–405.

Kashyap, D. R., Vohra, P. K., Chopra, S., & Tewari, R. (2001). Applications of pectinases in the commercial sector: a review. *Bioresource Technology, 77*, 215–227.

Lang, C., & Dornenburg, H. (2000). Perspectives in the biological function and the technological application of polygalacturonases. *Applied Microbiology and Biotechnology, 53*, 366–375.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry, 193*, 265–275.

Lozano, P., Manjon, A., Iborra, J. L., Canovas, M., & Romojaro, F. (1990). Kinetic and operational study of a cross-flow reactor with immobilized pectolytic enzymes. *Enzyme and Microbial Technology, 12*, 499–505.

Mannheim, A., & Cheryan, M. (1990). Continuous hydrolysis of milk protein in a membrane reactor. *Journal of Food Science, 55*, 381–385.

Mountzouris, K. C., Gilmour, S. G., Grandison, A. S., & Rastall, R. A. (1999). Modeling of oligodextran production in an ultrafiltration stirred-cell membrane reactor. *Enzyme and Microbial Technology, 24*, 75–85.

Mulder, M. H. V. (1996). *Basic principles of membrane technology*. Boston: Kluwer Academic.

Mutlu, M., Sarioglu, K., Demir, N., Ercan, M. T., & Acar, J. (1999). The use of commercial pectinase in fruit juice industry. Part I: viscosimetric determination of enzyme activity. *Journal of Food Engineering, 41*, 147–150.

Naidu, G. S. N., & Panda, T. (1998). Production of pectolytic enzymes – a review. *Bioprocess and Biosystems Engineering, 19*, 355–361.

Nair, S. R., Rakshit, S. K., & Panda, T. (1995). Effect of carbon sources on the synthesis of pectinase by Aspergilli. *Bioprocess and Biosystems Engineering, 13*, 37–40.

Nelson, N. (1944). A photometric adaptation of the Somogyi method for the determination of glucose. *Journal of Biological Chemistry, 153*, 375–380.

Panda, T., Nair, S. R., & Prem Kumar, M. (2004). Regulation of synthesis of the pectolytic enzymes of *Aspergillus niger*. *Enzyme and Microbial Technology, 34*, 466–473.

Pariza, M. W., & Foster, E. M. (1983). Determining the safety of enzymes used in food processing. *Journal of Food Protection, 43*, 453–468.

Prazeres, D. M. F., & Cabral, J. M. S. (1994). Enzymatic membrane bioreactors and their applications. *Enzyme and Microbial Technology, 16*, 738–750.

Rodriguez-Nogales, J. M., Ortega, N., Perez-Mateos, M., & Busto, M. D. (2005). Operational stability and kinetic study of a membrane reactor with pectinases from *Aspergillus niger*. *Journal of Food Science, 70*, 104–108.

Rodriguez-Nogales, J. M., Ortega, N., Perez-Mateos, M., & Busto, M. D. (2007). Experimental design and response surface modelling applied for the optimisation of pectin hydrolysis by enzymes from *A. niger* CECT 2088. *Food Chemistry, 101*, 634–642.

Rombouts, F. M., & Pilnik, W. (1980). Pectic enzymes. In A. H. Rose (Ed.), *Microbial enzymes and bioconversions* (pp. 227–282). London: Academic Press.

Solis-Pereira, S., Favela-Torres, E., Viniegra-Gonzalez, G., & Gutierrez-Rojas, M. (1993). Effects of different carbon sources on the synthesis of pectinase by *Aspergillus niger* in submerged and solid state fermentations. *Applied Microbiology and Biotechnology, 39*, 36–41.

Somogyi, M. (1952). Notes on sugar determination. *Journal of Biological Chemistry, 195*, 19–23.

Szaniawski, A. R., & Spencer, H. G. (1997). Effects of immobilized pectinase on the microfiltration of pectin solutions by macroporous titania membranes: resistance model interpretation. *Journal of Membrane Science, 127*, 69–76.

Van Rijssel, M., Gerwig, G. J., & Hansen, T. A. (1993). Isolation and characterization of an extracellular glycosylated protein complex from *Clostridium thermosaccharolyticum* with pectin methylesterase and polygalacturonate hydrolase activity. *Applied and Environmental Microbiology, 59*, 828–836.