0273-2289/95/5503--0249$06.20

β-Transelimination of Citrus Pectin Catalyzed by *Penicillium italicum* Pectin Lyase in a Membrane Reactor

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Received November 14, 1994; Accepted May 1, 1995

ABSTRACT

Continuous β-transelimination of citrus pectin was achieved using pectin lyase [PNL, poly(methoxygalacturonide)lyase, EC 4.2.2.10] purified from *Penicillium italicum* and confined in a membrane reactor. Various operational parameters, such as enzyme and substrate concentrations, filtrate flowrate, and reaction volume were optimized. Kinetic studies indicated that β-transelimination of pectin in the continuous reactor occurred following a first-order reaction with respect to substrate concentration for up to 90 min at 1 mg/mL pectin. This time period could be extended up to 120 min when the reactor was fed with 3–4 mg/mL pectin. During the first 50 h of operation, the system was capable of maintaining a viscosity reduction of 55% below the initial value when it was continuously fed with 4 mg/mL pectin.

Index Entries: Pectin lyase; EC 4.2.2.10; β-transelimination; citrus pectin; membrane reactor; *Penicillium italicum.*

INTRODUCTION

Traditionally, the industrial utilization of pectic enzymes in fruit juice processing has been conducted in conventional batch reactors using soluble enzymes. Unfortunately, after each cycle of opeation the enzymes

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could not be recovered for further use, and inevitably they were present in the final product. In this context, the immobilization of pectolytic enzymes has proven to be very advantageous for continuous industrial use (*1*). However, the activity yields of these immobilized enzymes appeared to be negatively influenced by the colloidal characteristics of pectin solutions and by the high molecular weight of pectic substances (*2*). These diffusional limitations can be overcome, at least partly thanks to the development of membrane reactors (*3*).

In fact, membrane reactors are becoming increasingly attractive because of their potential use in continuous fermentation operations (*4*). They combine efficiently the advantages of conventional bioreactors and membrane technology, by bringing together a continuous reaction and the simultaneous separation of products from the reaction mixture. In addition, apart from permitting the continuous removal of the resulting small-sized products from the reaction mixture containing the enzyme and the remaining large substrate, membrane reactors show, in principle, higher degrees of conversion than other types of bioreactors (*4*).

In this work we have designed and optimized a membrane reactor for the continuous β-transelimination of citrus pectin using the extracellular pectin lyase (PNL) purified from *P. italicum*. Factors affecting the activity and stability of the reactor such as filtrate flowrate, reaction volume, and concentrations of substrate and enzyme, among others, were determined in order to maximize the productivity and yield of the process.

MATERIALS AND METHODS

Materials

Citris pectin (degree of esterification 70%) was purchased from Sigma (St. Louis, MO). All other chemicals were reagents of analytical grade supplied by Merck (Darmstadt, Germany).

Enzyme Source

Pectin lyase activity [PNL, poly(methoxygalacturonide)lyase; E.C. 4.2.2.10] produced by *P. italicum* (obtained from Colección Espanola de Cultivos Tipo, CECT 2294, also now available as ATCC 66636) in surface bran cultures (*5*) was used. The extracellular enzyme was purified to the phenyl-sepharose step as previously reported (*6*).

Determination of Pectin

The galacturonic acid content of pectin was estimated as anhydrogalacturonic acid by the carbazole colorimetric method (*7*) as modified by Dietz and Rouse (*8*).

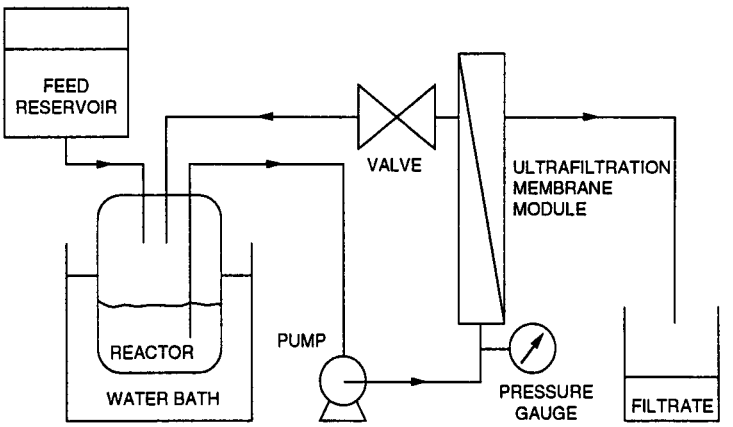

Fig. 1. Scheme of the membrane reactor system.

Enzyme Assay

PNL activity was routinely determined at 40°C with citrus pectin as a substrate in 0.2*M* citrate-phosphate buffer, pH 6.0, by recording the increase in aborbance at 235 nm as reported by Albersheim and Killias (*9*). One unit (U) of enzyme catalyzed the increase of one unit of $A_{235\ nm}$/min. PNL was also assayed at 40°C by measuring, at the indicated times, the reaction mixture viscosity using a Cannon-Fenske 100 viscometer (5). In this case, samples were momentarily withdrawn from the reactor and their viscosity determined. The enzyme activity was corrected for any endogenous decrease in viscosity found in the blank assays conducted in parallel in the absence of added PNL.

Membrane Reactor

Figure 1 shows a scheme of the membrane reactor system used in this work that was similar to that described by Nakajima et al. (*10*) for the hydrolysis of soluble fish proteins. The ultrafiltration system consisted of a stirred tank coupled to a Minitan system (Millipore Intertech, Bedford, MA) with a plate and frame module. Ultrafiltration membranes (Millipore PTGC, 10 kDa cutoff) with a membrane area of 60 cm^2 were used. The membranes were assembled, each separated by silicon retentate separators. Packets of two membranes were utilized in all experiments.

Pectin solutions were preadjusted to the required pH and temperature (in the feed reservoir) and pumped into the reaction vessel. The reaction mixture was continuously recycled with a pump inserted in-line between the reaction vessel and the membrane module inlet. The enzymatic reaction occurred in both the reactor tank and the recirculation tube. The system was initially operated at a filtrate flowrate of 1–1.5 mL/min and samples for product determination (as $A_{235\ nm}$) were collected from the outlet product. As the reaction tank was pressure tight, the inlet flowrate

was equal to the outlet flowrate, thus keeping a constant reaction volume. The reactor temperature was kept constant at 40°C by immersion in a water bath. The filtration unit was operated at a pressure of 10 *psi* (0.69×10^5 N/m^2). The total reaction volume was 100 mL, including both the membrane module and recirculation tubes.

RESULTS AND DISCUSSION

The optimum reactor operational conditions for continuous β-transelimination at 40°C of a citric pectin solution were investigated. Thus, the effect of other performance variables, i.e., filtrate flowrate, reaction volume, and initial enzyme and substrate concentrations, on the conversion of pectin was studied. The reactor was initially operated at 1.5 mL/min flowrate using a reaction volume of 100 mL, 100 mU/mL PNL, and 5 mg/mL pectin. During these studies, the effect of each performance variable was evaluated independently, keeping the other three conditions constant.

Effect of PNL Concentration

As shown in Fig. 2A, the amount of product in the filtrate increased progressively with increasing enzyme concentrations until a maximum value corresponding to a PNL concentration of 100 mU/mL was reached. A higher PNL concentration (200 mU/mL) did not significantly increase the amount of product in the filtrate. Similar patterns indicating enzyme saturation were reported by Mannheim and Cheryan (*11*) and Lozano et al. (*1*) in their studies on continuous hydrolysis of milk protein in a membrane reactor and the operational behavior of a cross-flow reactor with immobilized pectolytic enzymes, respectively.

In addition, it was found that the time necessary to obtain the highest conversion value was reduced when increasing the enzyme concentration (Fig. 2B). This could be because of the fact that the time necessary to obtain the maximum conversion is only related to the catalytic capability of the system, which is controlled by both the amount of enzyme and the recycling flowrate (*1*). The time required for the product to appear in the filtrate decreased with increasing enzyme concentrations (Fig. 2B). PNL from *P. italicum* is, in fact, an endo-PNL, implying that its catalytic action takes place randomly within the pectin molecule (*6*). Consequently, it seems plausible that low concentrations of PNL are initially unable to quickly catalyze the β-transelimination of the substrate into fragments small enough to pass through the ultrafiltration membrane.

Appearance of product in the filtrate at the steady-state reached saturation at PNL concentrations of about 100 mU/mL, and enzyme concentrations higher than this value were ineffective in improving the calculated reaction yield (Fig. 2C).

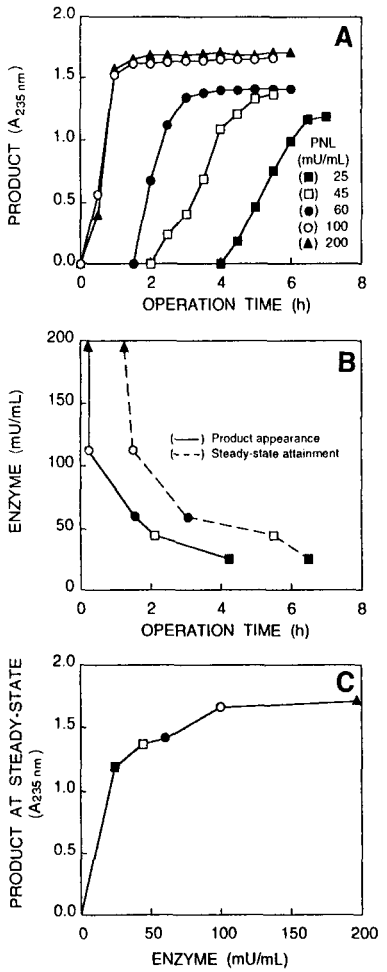

Fig. 2. Effect of PNL concentration on: (**A**) time-course of product appearance in the filtrate; (**B**) time required to detect product in the filtrate or to reach steady-state performance conditions; and (**C**) attained steady-state concentration of product in the filtrate. Operational conditions were: 1.5 mL/min filtrate flowrate and 300 mL/min recycling flowrate using a reaction volume of 100 mL and 5 mg/mL pectin.

Effect of Filtrate Flowrate

Increased filtrate flowrates led to lower amounts of product in the filtrate (Fig. 3A). The corresponding shorter residence times for the substrate (accompanying increased filtrate flowrates) imply that the catalytic reaction is less likely to occur because of time limitations. Thus, when steady-state condition was achieved, the shorter the residence time, the lower the amount of product in the filtrate (Fig. 3B). Analogous results have been reported by several authors (*1,12–15*). Furthermore, from Fig. 3A it can also be inferred that the time required to reach maximum conversion was not affected by filtrate flowrate. Lozano et al. (*1*) drew the

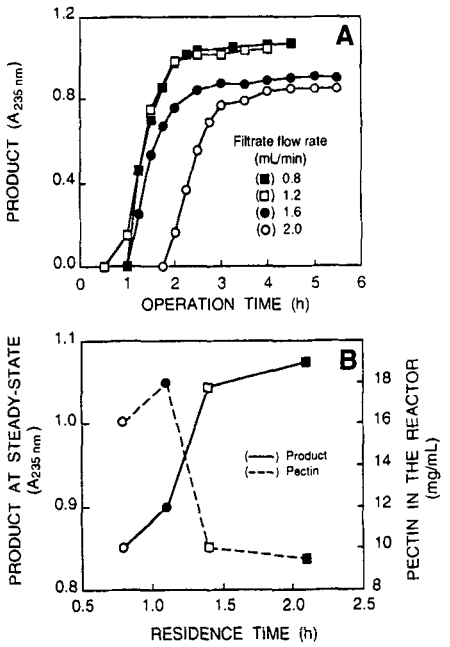

Fig. 3. Effect of filtrate flowrate on the time-course of product appearance in the filtrate (**A**); and effect of the residence time on the steady-state concentrations of pectin in the reactor and product in the filtrate (**B**). In all cases operational conditions were: 300 mL/min recycling flowrate and the indicated filtrate flowrates using a reaction volume of 100 mL, 100 mU/mL PNL, and 5 mg/mL pectin.

same conclusion from their studies on the pectolytic enzymes immobilized on Nylon membranes in a crossflow reactor. Finally, Fig. 3B shows the relationship between the residence time and the amount of substrate accumulated inside the reactor. In this graph, again, it can be seen that the longer the residenctime, the less substrate accumulated in the reactor, probably because of the higher degrees of pectin β-transelimination obtained when the enzymatic reaction had more time to occur.

The concentration of the intact substrate depended not only on the residence time but on the activity (concentration) of the enzyme as well (*16*). Nakajima et al. (*12*) also found that a short mean residence time resulted in the accumulation of substrate in their studies of the continuous hydrolysis of soluble starch by free β-amylase and pullulanase using an ultrafiltration membrane reactor.

Effect of Reaction Volume

As shown in Fig. 4A, by decreasing the reaction volume, the maximum degree of conversion was also increased, whereas the time necessary to obtain this maximum value (V_{max}) was reduced. According to Lozano et al. (*1*), this effect is a consequence of the high dependence of V_{max} on

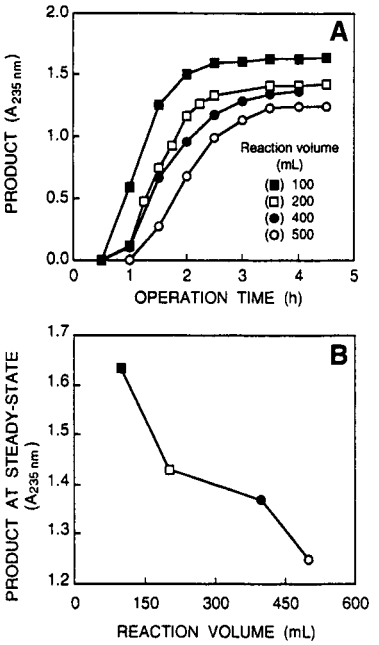

Fig. 4. Effect of reaction volume on: (**A**) time-course of product appearance of the filtrate; and (**B**) attained steady-state concentration of product in the filtrate. Operational conditions were: 1.5 mL/min filtrate flowrate and 300 mL/min recycling flowrate using the indicated reaction volumes, 100 mU/mL PNL and 5 mg/mL pectin.

Q_r/v, where Q_r is the recycling flowrate and v is the reaction volume. This effect could again be observed in Fig. 4B, where the reaction volume vs product concentration in the filtrate at steady-state was analyzed.

Effect of Pectin Concentration

The highest concentration of product in the filtrate was obtained when the reactor was continuously fed with pectin solutions between 3 and 4 mg/mL (Fig. 5A). Higher pectin concentrations (i.e., 6 and 8 mg/mL) led to a decrease in the amount of product found in the filtrate.

The effect of the initial pectin concentration on the amount of product found in the filtrate at steady-state is shown in Fig. 5B. At low pectin concentrations, the higher the substrate concentration the higher the amount of product in the filtrate, until a maximum value of 4 mg/mL was reached. Nonetheless, above this value, an increase of substrate concentration resulted in a concomitant decrease in the amount of product found in the filtrate. This type of behavior suggested a substrate inhibition effect already found for the β-transelimination of citrus pectin by the soluble PNL from *P. italicum* (data not shown). This substrate inhibition has also been observed by Nakajima et al. (*17*) and Schmidt-Steffen and Staude

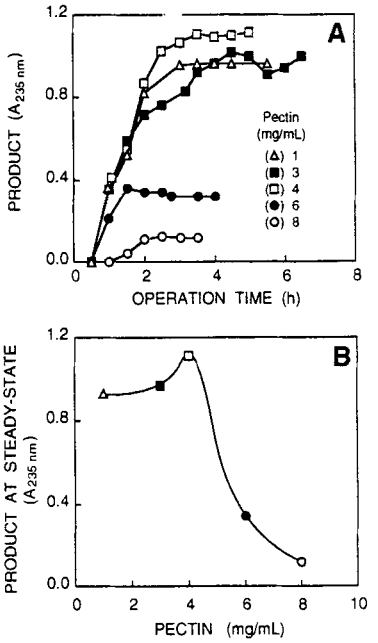

Fig. 5. Effect of pectin concentration fed to the reactor on: (**A**) time-course of product appearance in the filtrate; and (**B**) attained steady-state concentration of product in the filtrate. Operational conditions were: 1.5 mL/min filtrate flowrate and 300 mL/min recycling flowrate using a reaction volume of 100 mL, 100 mU/mL PNL, and the indicated pectin concentration.

(*15*) in their studies on the conversion of sucrose by immobilized invertase in an asymmetric membrane reactor and the use of ultrafiltration membranes for chemical bonding of urease, respectively.

To determine the reaction order, ln (1 − x) was plotted vs time at different initial pectin concentrations (where x stands for conversion, defined in this work as the increase in $A_{235\ nm}$ in the filtrate). A linear relationship would indicate that the reactor was operating under first-order kinetics (*16*). One such a plot at an enzyme concentration of 100 mU/mL and a residence time of approx 1 h was shown in Fig. 6. Based on the experimental results, first-order kinetics was only applicable during the initial stages of reactor start up. At a substrate concentration of 1 mg/mL, first-order kinetics was maintained for up to 90 min, whereas this period could be extended up to 120 min if a pectin concentration of 3–4 mg/mL was used. The nonlinearity of data at higher reaction time values illustrated a deviation from first-order kinetics. This type of substrate dependency was also reported by Sims and Cheryan (*16*) when studying the hydrolysis of liquefied corn starch in a membrane reactor using α-amylase from *Bacillus licheniformis*.

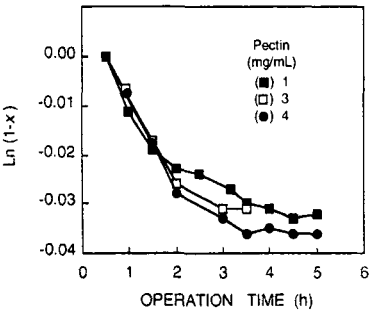

Fig. 6. Determination of the β-transelimination reaction order occurring in the membrane reactor at different pectin concentrations. Operational conditions were: 1.5 mL/min filtrate flowrate and 300 mL/min recycling flowrate using a reaction volume of 100 mL, 100 mU/mL PNL, and the indicated pectin concentrations. The substrate conversion (x) was estimated as increase in $A_{235\ nm}$ in the filtrate.

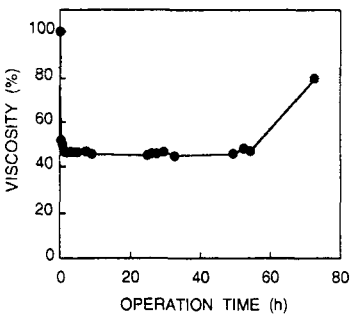

Fig. 7. Long-term operational stability of the membrane reactor for pectin degradation assessed as reduction in viscosity of the reaction mixture. One-hundred percent viscosity corresponded to 3.00 cSt at 40°C. Operational conditions were: 1.2 mL/min filtrate flowrate and 300 mL/min recycling flowrate, using a reaction volume of 100 mL, 100 mU/mL PNL, and 4 mg/mL pectin.

Operational Stability of the Membrane Reactor

In order to determine the operational stability of the system, the reactor was run for 4 d in a continuous mode at the optimum filtrate flowrate (1.2 mL/min) and fed with 4 mg/mL pectin, a substrate concentration that allowed the highest conversion value. The pectin solution was renewed daily to avoid any possible clogging caused by the presence of contaminating microorganisms. The reaction was followed by periodical measurements of decrease in viscosity of samples withdrawn from the reaction mixture.

As shown in Fig. 7, although the initial viscosity drop (55%) appeared to remain stable during the first 50 h of operation, but then the viscosity increased until it reached 80% of the initial viscosity value after 70 h. A

similar pattern was observed when determining the amount of product (as $A_{235\ nm}$) appearing in the filtrate (data not shown). From these results, it can be concluded that the system was stable during the first 50 h of operation, and after that time, the enzyme appeared to lose part of its activity, probably owing to sheer stress. Lozano et al. (*1*) also found that after reaching a maximum conversion value, the system became progressively less efficient. Furthermore, although Denis et al. (*18*) could not observe any loss of pectate lyase activity in their membrane reactor after 7 h of pumping, a longer performance (i.e., 6 d of operation) caused an activity drop of 36%.

Finally, it seems likely that the system stability may depend on the nature of the enzyme under study (*18*). Thus, invertase from *Candida utilis* immobilized in an ultrafiltration membrane appeared to lose activity after around 4 d of operation (*19*). In contrast, when aminoacylase from *Aspergillus* sp. was tested in a continuous stirred tank reactor attached to an ultrafiltration membrane, the system was stable for at least 54 d.

CONCLUSIONS

Because of the high viscosity of pectin solutions, the use of PNL from *P. italicum* in a membrane reactor provides a promising alternative for pectin degradation. In the present work, all four variables studied (i.e., enzyme and substrate concentrations, filtrate flowrate, and reaction volume) appeared to have an effect on pectin conversion. Although a gradual decay in the reactor activity was obseved over time, the system was capable of maintaining a viscosity decrease of 55% below the initial value for approx 50 h. Nonetheless, much further research is still needed to improve the productivity of the system for practical purposes.

ACKNOWLEDGMENTS

I. A. was the recipient of a scholarship from the Basque government.

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