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ABSTRACT: The objective of this paper is to assess if a membrane microbioreactor system could potentially be used to diagnose consequences of different process design and reactor operation options relevant for larger-scale enzymatic degradation of pectin reactions. The membrane microbioreactor prototype was fabricated from poly(methylmethacrylate) (PMMA) and poly(dimethylsiloxane) (PDMS) with a working volume of ∼190 μL. The prototype also contained the necessary sensors and actuators, i.e., pressure transducer, mixing via magnetic stirrer bar and a temperature controller. The functionality of the prototype was demonstrated by performing a continuous enzymatic degradation of pectin experiment for a range of reactor conditions: different membrane molecular weight cutoff (MWCO) values, enzyme-to-substrate ratios (E/S), and substrate feeding rates (F) were assessed. Based on the experimental data, it was found that the apparent reaction rate increased from 0.11 μmol/h to 0.13 μmol/h when the E/S ratio was doubled from 0.2% (g/g) to 0.4% (g/g). In contrast, when the substrate feeding rate was reduced from 200 μL/h to 100 μL/h (i.e., longer residence time), a higher yield was achieved (producing a pectin fragment concentration of 0.82 mM in the permeate) and the apparent reaction rate increased by ∼50% (i.e., from 0.11 μmol/h to 0.17 μmol/h). Clearly, this signifies that the substrate feeding rate is a critical variable that influences the conversion rate and the process yield. The data also showed that the process design affected the membrane rejection profile. The results obtained thus underlined the suitability of a miniature membrane reactor system for evaluating different process design options that are relevant for larger-scale reactions of enzymatic pectin degradation.

INTRODUCTION

Pectic substances can be found in fruits and vegetables, and they are abundant in many different agro-industrial waste streams. Agro-industries are highly interested in converting these low-value waste streams into useful high-value products (for example, into biofunctional oligosaccharides (carbohydrates containing between 2 and ∼20 monomers1,2)). Such a conversion of pectin is possible through enzymatic reactions catalyzed by pectinolytic enzymes (typically of fungal origin), such as the pectin lyase (PL) (EC 4.2.1.10), pectin methylesterase (PME) (EC 3.2.1.11), endopol-galacturonase (PG) (EC 3.2.1.15), etc.3,4

A number of studies have convincingly shown that enzymatic modification of pectin can benefit from the application of membrane bioreactors.5–10 With the use of membrane bioreactors, enzymes are retained in the reaction system while, at the same time, allowing for (continuous) product removal and substrate feeding. It is indeed a rational processing option, as the high viscosity of pectin in solution prevents batch processing of high initial substrate levels, which, in turn, is a barrier to obtaining high enzyme efficiencies. Moreover, potentially inhibitory low-molecular-weight compounds—produced from the continuous degradation of the pectin polymer—can be continuously removed from the reaction mixture and, thus, the reaction rates increase. An increase by 40% in volumetric productivity (ksp red × m−1 reactor volume h−1)—compared to the productivity in a traditional batch reactor—has been reported when low-molecular-weight substances (e.g., D-galacturonic acid) produced from the depolymerization of pectin were continuously removed from the system.6 It has also been demonstrated that a membrane reactor may run stably for up to 15 days of continuous operation with the possibility of achieving a pectin substrate conversion as high as 90%.7 In addition, proper selection of the membrane molecular weight cutoff (MWCO), coupled with a feasible operation strategy (i.e., batch, fed-batch or continuous operation) and appropriate reactor residence time, offer the possibility of controlling the molecular weight size distribution of the end-product mixture, which is an outcome that cannot be achieved with conventional batch reactors.5,9,11

To date, academia relies mostly on bench-scale membrane bioreactors to conduct studies on continuous enzyme-catalyzed pectin hydrolysis reactions.5–10 Considering the huge number of experiments usually needed to elucidate novel enzyme-catalyzed production processes and the large amount of enzymes and substrates consumed per experiment (i.e., bench-scale reactors normally operate with a volume from a few hundred milliliters and upward), performing experiments in bench-scale membrane bioreactors can be very costly, especially when multiple parallel experiments are needed.

In this respect, miniature-sized bioreactors (i.e., microfabricated reactors with working volumes of <1 mL12,13) with integrated...
sensors and actuators are better-suited for research and process development studies. This is because, at such a small scale of operation, enzyme and substrate consumption are significantly reduced (normally in the range of micrograms or microliters per experiment) while heat- and mass-transfer rates are notably increased.\(^{12,13}\) Muller et al.\(^ {14}\) compared the running cost of similar experiments performed in a different scale of operation and reported that the running cost for continuous reactions in a microreactor—relative to the bench-scale reactor—decreases by a factor of 50. Delattre et al.\(^ {15}\) proposed the use of a convective interactive media (CIM) disk as a support for immobilization of pectin lyase (PL) in a microreactor and demonstrated the usefulness of such a microsystem in investigating the optimum temperature, optimum pH, and thermal stability of the enzyme. In a previous study,\(^ {16}\) we developed a disposable membrane microbioreactor system and showed that the kinetic profiles achieved in microscale were congruent with those obtained in a bench-scale setup (i.e., a reproducible result is obtained in a miniature-scale membrane reactor while only requiring a fraction of the resources typically needed to operate a bench-scale membrane bioreactor).

In this paper, we aim to further examine the functionality of the membrane microbioreactor prototype and assess if such a miniature membrane reactor can be applied to evaluate consequences of process operation choices relevant for larger-scale enzymatic degradation of pectin reactions. Different reactor design options—namely, membrane molecular weight cutoff (MWCO), enzyme-to-substrate ratio (E/S), and substrate feeding rate (\(F\))—were tested to assess their influence on the reaction yield and productivity. The MWCO, E/S ratio, and feeding rate (i.e., reactor residence time) all are important reactor variables that have direct influence on the enzymatic degradation of pectin reaction rates/yield and selectivity toward the production of desired oligomers.\(^ {2,8,9}\)

**MATERIALS AND METHODS**

**Microbioreactor Design and Fabrication.** The scheme of the experimental setup for the membrane microbioreactor is illustrated in Figure 1. Prototyping of the microbioreactor was done with three-dimensional (3D) computer-aided design (CAD) software, SolidWorks 2006, and micromachining in which poly(methylmethacrylate) (PMMA) (Plexiglas GS, Klar 233, Nordisk Plast, Auning, Denmark) and poly(dimethylsiloxane) (PDMS) were used as the materials for fabrication. Development of the prototype also encompassed setting up the necessary measurement apparatus and machinery to drive the microbioreactor systems. Mixing of the reactor content was achieved by means of a magnetic stirrer bar and the recirculation pump. Both the magnetic stirrer and the recirculation pump were set to operate at a rotational speed (\(N\)) of 500 rpm and at a recirculation rate (\(R_p\)) of 2700 \(\mu\)L/h, respectively. These operating conditions remained unchanged in each experimental run to provide a consistent mixing performance.

Programs for measurement and control routines were written in LabVIEW v8.5 software (National Instruments, Austin, TX, USA), and were implemented by interfacing LabVIEW with a data acquisition (DAQ) card (NI USB-6229, National Instruments) for data logging and sending signals to actuators. Further analysis of online measurement data (temperature and pressure) was done using Matlab v7.0 software (The Mathworks, Natick, MA, USA). Details for the design and fabrication of the membrane microbioreactor prototype have been described elsewhere.\(^ {16}\)

**Startup and Reactor Operation.** The startup of the reaction in the membrane microbioreactor first involved filling the reactor with enzyme solution. The enzyme solution was loaded into the reactor by disconnecting one of the inflow/outflow interconnects at the side of the bottom PMMA layer. While the enzyme solution filled the reactor, air that was trapped inside the PFA tubes, the microchannels, and the reaction chamber was pushed out of the reactor simultaneously through one of the already-opened side ports. Since the PFA tubes and the microbioreactor were both transparent, bubble-free conditions were assessed visually before starting the reaction. Once the reactor was completely filled, the loop was reconnected. The reactor temperature then was set and the reaction was initiated by starting

![Figure 1. Two-dimensional (2-D) schematic of the membrane microbioreactor experimental setup.](image-url)
the feeding of the substrate solution. Substrate solution was continuously fed into the reactor using a syringe pump (Model MA1 70-2211, Harvard Apparatus, Holliston, MA, USA) and circulated by means of a micro gear pump (Model mzs 2905, HNP Mikrosysteme GmbH, Parchim, Germany).

During the course of reaction (which occurred in both the reactor chamber and the recirculation line), enzyme and unreacted substrate solutions were retained in the system by the membrane while low-molecular-weight products (less than the membrane MWCO) were continuously separated from the system. Regenerated cellulose membranes with MWCO = 1 and 10 kDa, respectively (Model PLGC06210, Millipore, Billerica, MA, USA) were used for separation.

**Enzymatic Depolymerization of Sugar Beet Pectin by Pectin Lyase.** Sugar beet pectin with a degree of methoxylolation of ∼60% and a degree of acetylation of ∼19% (Danisco A/S, Brabrand, Denmark) was used as a substrate. The pectin lyase used in this study was supplied by Novozymes A/S (Bagsværd, Denmark). It was a cloned monocomponent enzyme from *Aspergillus aculeatus* with an enzyme activity on citrus pectin of 0.0085 U/mg. (One unit of activity (U) is defined as the production of 1 μmol of double bonds per minute at pH 4.5 and 50 °C.) A 0.1 M sodium acetate buffer solution (pH 4.5) was used to prepare both the substrate and the enzyme solutions.

Continuous enzymatic pectin degradation experiments were performed for a limited range of different reactor variables, namely, membrane MWCO (1 kDa and 10 kDa), E/S (0.2%, 1%, and 5%), and F (100 and 200 μL/h). For every experimental condition, the reaction was performed with an initial substrate concentration ([S]₀) of 10 g/L at pH 4.5, and the temperature was controlled at 50 °C. Duplicates were made for every experiment to check data reproducibility, and the reaction was carried out until a steady state was reached.

**Ultraviolet (UV) Absorption at 235 nm.** Similarly to the analytical method applied in our previous work, the UV absorption analysis was performed in order to monitor the progress of the depolymerization reaction. This was done by measuring sample absorbance via a microtiter plate reader at 235 nm. Beer—Lambert law (A = εcL) was used to estimate the concentration of product fragments (c) generated from the depolymerization reaction. A molar absorption coefficient (ε) of 5500 M⁻¹ cm⁻¹ was used.

**Monogalacturonic and Oligogalacturonic Acid Electrochemical Measurement via HPAEC-PAD.** Identification and quantification of monogalacturonic and oligogalacturonic acids in hydrolysates were performed using an ICS-3000 system coupled to an AS50 autosampler (Dionex Corp., Sunnyvale, CA). Separations were performed using a CarboPac PA20 (3 mm × 150 mm) analytical column (Dionex Corp.), according to the method described previously. Two-eluent system comprised of deionized water (18.2 mS/cm at 25 °C) and 500 mM NaOH aqueous solution was used. Monosaccharides and oligosaccharides were eluted isocratically with 2.5 mM NaOH for 20 min, followed by a second isocratic elution at high NaOH concentration (500 mM) for 10 min to elute any present acidic species. The eluent flow rate was always kept at 0.5 mL/min. All eluents were prepared from high-performance liquid chromatography (HPLC)-grade chemicals. The quantification of monogalacturonic and digalacturonic acids was carried out using external standards. Trigalacturonic acids were quantified using the response factor of galacturonic acid. Data were collected and analyzed on computers equipped with Chromeleon 6.80 Sp8 software (Dionex Corp.).

**RESULTS AND DISCUSSION**

The kinetic profiles of the enzymatic pectin degradation experiments followed a general trend where the concentration of the pectin fragments in the permeate increased progressively with time until a steady state was reached. In each case, a steady-state production level was reached when a balance between the feed flow rate (F, in units of μL/h), the kinetic conversion rate (i.e., the overall rate of the enzymatic reaction, as measured from the product formation rate), and the membrane flux of the system was reached. Based on the experimental data with two different membranes, i.e., MWCO = 1 and 10 kDa (Figure 2), it was found that the membrane with MWCO = 1 kDa showed a higher membrane rejection at the beginning of the reaction, compared to the membrane with MWCO = 10 kDa, resulting in an apparently lower product formation rate of pectin fragments (Figure 2). Theoretically, the membrane rejection coefficient (R) is defined as a function of the ratio between the solute concentration in the permeate (C_p) and the solute concentration on the surface of the membrane (C_m) (i.e., R = 1 - C_p/C_m). Thus, C_m is a function of the solute concentration in the reactor. From this relation, a lower permeate concentration (C_p) indicates a higher membrane rejection characteristic when C_m is constant. Hence, the results obtained are as expected for a membrane with smaller pore size (i.e., 1 kDa vs 10 kDa) as the membrane with the lower MWCO will reject pectin fragments with molecular weights of >1 kDa.

Interestingly, when the enzymatic reaction came to steady state (Figure 2), the final concentrations of pectin fragments in the permeate were comparable (yield ∼ 0.55 mM) for both membranes (i.e., 1 and 10 kDa). This result indicated that, as the reaction proceeded to steady state, the membrane pore size was no longer influencing the membrane rejection, despite the fact that the pore size of the membranes differed by a factor of 10. This observation may be due to increased viscosity and/or fouling resulting from the buildup of unreacted substrate in the reactor as well as at the membrane surface (i.e., concentration polarization effects). Such effects lead to an increase of C_m and, hence, the rejection R of the 10 kDa membrane. It was
anticipated that the membrane with the lowest MWCO value would have the highest membrane selectivity while removing pectin fragments from the reactor system, i.e., shorter fragments were to be expected in the permeate mixture when using a membrane with smaller pore size (e.g., 1 kDa). Size-exclusion chromatography (SEC) profiles confirmed the presence of different sizes of pectin oligomers in the reactor permeate (data not shown), but, as shown in Figure 3, it was also found that similar types and relatively similar levels of the very-low-molecular-weight pectin oligomers, i.e., galacturonic acid (GalA) monomers, dimers, and trimers, were recovered in the permeate for both membranes (the molecular weights of the dehydrated, methylated forms of the monogalacturonic, digalacturonic, and trigalacturonic acids are ∼190, 380, and 570 Da, respectively). The oligomer concentration profiles for both membrane systems indicated a sharp increase of the oligomer concentration during the first 2–4 h of reaction, before reaching a steady state after a reaction time of ∼4 h (Figure 3). Although the concentrations of the recovered low-molecular-weight pectin oligomers in the permeate were slightly higher for the 10 kDa membrane system, compared to the 1 kDa system, suggesting that the enzymatic rates could be slightly higher on pectin oligomers than on the native polysaccharides, it can be concluded that the concentrations of the low-molecular-weight pectin oligomers were within the same order of magnitude for both membranes. In our previous work, it was observed that the system pressure gradually increased after the initiation of the enzymatic reaction (i.e., a relative pressure increase as high as 1.5 bar was registered). The increase of the system pressure was believed to be caused by the accumulation of the unreacted substrate molecules in the system. This was confirmed, since the system pressure no longer increased when the substrate feeding was stopped and the system was flushed with buffer solution (i.e., solution without the presence of any pectin substrates). Under a pressurized state—due to continuous feeding—the unreacted substrates will tend to deposit on the surface of the membrane, which leads to the formation of a gel layer on the membrane surface. Hence, it creates a concentration gradient between the substrate concentration in the bulk solution and the concentration on the membrane surface (i.e., concentration polarization effects). Considering the typical diffusivity ($D$) values of pectin molecules ($\sim 10^{-11} \text{ m}^2/\text{s}$) and a standard membrane boundary layer thickness, $\delta$ ($\sim 15 \times 10^{-6} \text{ m}$ for 1% (w/v) of pectin\cite{22}); it is possible to deduce that, under a constant feeding rate of 200 μL/h, the concentration of pectin close to the membrane surface, $C_M$, could be $\sim 7–10$ times higher ($\sim 70–100 \text{ g pectin/L}$) than the concentration of pectin in the bulk solution ($C_b$, i.e., in accordance to $C_M/C_b = \exp(J_v/k)$). The rejection capacity of the system can be greatly influenced by this additional gel layer on the membrane surface.\cite{9}

Since the substrate feeding rate ($F$) is equivalent to the permeate flux ($J_v$), because the microbioreactor volume does not change with time (i.e., $dV/dt=0$ and no headspace), the pectin oligomer concentration in the bulk solution ($C_b$) can be assumed to be the same as the concentration of the pectin oligomers in the permeate ($C_p$). The results from the HPLC analysis also supported the hypothesis of identical concentrations of small pectin fractions in the bulk solution and in the permeate (see Table 1). In the steady state, comparable concentrations of GalA monomers, dimers, and trimers were detected at both sides of the membrane (i.e., retentate and permeate) for both 1 and 10 kDa membranes. Hence, based on this analysis, the apparent higher production rate of pectin fragments observed for the 10 kDa membrane as assessed by the amount of dehydrated

### Table 1. Concentration of Oligomers (Monomers, Dimers, and Trimers) in the Retentate and in the Permeate at Steady State for the Membranes Used in the Experimental Work: (a) MWCO = 1 kDa and (b) MWCO = 10 kDa

<table>
<thead>
<tr>
<th>MWCO (kDa)</th>
<th>Retentate</th>
<th>Permeate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$C_b$ (mg/L)</td>
<td>$C_p$ (mg/L)</td>
</tr>
<tr>
<td>(a) 1 kDa</td>
<td>8.03</td>
<td>4.5</td>
</tr>
<tr>
<td>(b) 10 kDa</td>
<td>8.5</td>
<td>4.6</td>
</tr>
</tbody>
</table>
After a residence time of 1 h, the steady-state rate, which was calculated as the apparent steady-state reaction rate, increased; with the 1 kDa MWCO membrane at a feeding rate of 200 μL/h. The data points are averages of two experimental runs, shown ± standard deviation (σ < 0.04 mM).

Similarly, when the enzymatic reaction was performed under the same conditions as those reported in Figure 2, but with a lower feeding rate (i.e., system flux ≈ 100 μL/h and residence time of τ = 2 h), a significantly higher product yield and recovery rate were observed (see Figure 6). The apparent steady-state product formation level increased with longer residence time (i.e., up to ~0.82 mM and 0.65 mM for the 10 kDa and 1 kDa membranes, respectively (Figure 6)). Hence, the reactor residence time of 1 h limited the apparent steady-state product formation level indicated that the apparent steady-state product level could be controlled (at least within certain boundaries) by adjusting the feed rate, which influenced both the substrate supply and the residence time. There was apparently plenty of substrate, even at the low feed rate, which, in turn, must signify that the enzymatic rate in the reactor (i.e., at the apparent steady state) was running near maximum.

In the present work, we demonstrated that the impact of important process operation design choices—namely, the substrate

Figure 4. Concentration profiles of pectin fragments in the permeate (given in units of mM) for a membrane with a molecular weight cutoff (MWCO) of 1 kDa, operated under different enzyme-to-substrate (E/S) ratio (i.e., 0.2% or 0.4% (g/g)) at a feeding rate (F) of 200 μL/h. The data points are averages of two experimental runs, shown ± standard deviation (σ < 0.04 mM).

Figure 5. Concentration profile of pectin fragments in the permeate (given in units of mM) for a membrane with MWCO = 10 kDa, operated at different feeding rates (i.e., 100 or 200 μL/h) at an E/S ratio of 0.2% (g/g). The data points are averages of two experimental runs, shown ± standard deviation (σ < 0.04 mM).

Figure 6. Concentration profile of pectin fragments in the permeate (given in units of mM) for membranes with MWCO = 1 kDa and 10 kDa, respectively, operated at a feeding rate of F = 100 μL/h with an E/S ratio of 0.2% (g/g). The data points are averages of two experimental runs, shown ± standard deviation (σ < 0.04 mM).
feeding rate \( F \) (i.e., reactor residence time or system flux), molecular weight cutoff (MWCO) of the membrane, and the enzyme-to-substrate ratio \( E/S \)—could be investigated with the use of a membrane microbioreactor prototype. The results highlight that \( F \) is a critical variable that can dramatically alter the rejection profile of a membrane bioreactor system. Obviously, there was a serious accumulation of unreacted substrates in our system. We deliberately operated our microbioreactor system under the chosen experimental conditions (i.e., at relatively low \( E/S \) ratios of 0.2% \((g/g)\)), because we wanted to closely monitor the progress and the dynamic changes of continuous degradation of pectin polymer kinetic profiles. We also set the reactor to work at a residence time of 1 – 2 h, because we need to generate sufficient volume of permeate for sampling and analysis. A low standard deviation, \( \sigma \) (i.e., \( \sigma_{\text{max}} < 0.04 \text{ mM} \) and also observable from the error bars in each plot), showed that data obtained in every experiment were highly reproducible. In this respect, we believe that the usage of a fresh membrane for every experiment is crucially important for achieving high data reproducibility. Every experiment requires only low amounts of enzymes and substrates, a small piece of membrane per experiment \( (A_m \approx 38.5 \text{ mm}^2) \), and also a relatively insensitive temperature control system (i.e., a few centimeters of wire as the heating element for temperature control\(^{23}\)); this allows for a large number of experiments to be tested against a relatively low cost. Finally, another aspect worth mentioning here is the potential for parallel reactor operation. This is indeed a challenging task as fluidic handling (e.g., feeding strategy) and local reactor monitoring and control become increasingly complex when the number of microbioreactors increases from one to many units. Nevertheless, parallel reactor operation is essential and it would definitely be useful for screening novel processes, in a process optimization step or in predicting effects of various operational parameters on the reaction rates/yield. The membrane microbioreactor presented here—in its current state—may still require some modifications before parallel operation becomes feasible. First, integration of on board micropumps and microvalves\(^{24}\) would be required as substitutes for the large and expensive pumps used in the current version of this setup. Second, the permeate stream should be interfaced with a microfluidic detection chip such that the progress of the experiment performed in the prototype can be monitored online, i.e., via a fully automated system, thus eliminating the need for sampling, which, in practice, can be very laborious and demanding for lengthy reactor operation.

CONCLUSIONS

A continuous \( \beta \)-elimination of sugar beet pectin catalyzed by pectin lyase has been successfully performed in a membrane microbioreactor prototype under various experimental conditions of membrane molecular weight cutoff (MWCO), substrate feeding rate \( F \) (given in units of \( \mu \text{L/h} \)), and enzyme-to-substrate (i.e., pectin) ratio \( E/S \). The experimental data indicated that the concentration polarization phenomenon—which is an effect that is typically encountered in a membrane separation system—has a strong influence on the product removal rate of the reactor system. While higher enzyme load increases reaction rates, the product recovery rate is highly governed by the system flux (i.e., feeding rate). Moreover, the membrane MWCO was also found to have little or no significant impact on membrane selectivity when concentration polarization became a limiting factor for product separation. From the practical point of view, we developed a disposable microbioreactor system that allows for inexpensive experimentation in a laboratory environment. Summarizing, the results demonstrate the potential use of the membrane microbioreactor prototype as a supporting tool for assessing novel “biorefining”-type processes (e.g., enzyme-catalyzed degradation of pectin) and especially allowing prediction of the consequences of reactor design choices, notably the membrane MWCO and the substrate feeding rate \( F \).

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LIST OF SYMBOLS

MWCO = membrane molecular weight cutoff (kDa) 
\( E/S = \) enzyme to substrate ratio \((g/g)\) 
\([S]_0 = \) initial substrate concentration \((g/L)\) 
\( F = \) substrate feeding rate \((\mu \text{L/h})\) 
\( A_{235} = \) absorbance at 235 nm 
\( c = \) concentration of product fragments (M or mM) 
\( \varepsilon = \) molar absorption coefficient \((M^{-1} \text{ cm}^{-1})\) 
\( R_p = \) recirculation rate \((\mu \text{L h}^{-1})\) 
\( N = \) rotational speed \((\text{rpm})\) 
\( C_b = \) concentration of oligomers in bulk solution/retentate \((g/L \text{ or mg/L})\) 
\( C_p = \) concentration of oligomers in permeate \((g/L \text{ or mg/L})\) 
\( C_M = \) concentration of oligomers in surface of membrane \((g/L \text{ or mg/L})\) 
\( D = \) molecule diffusivity \((\text{m}^2/\text{s})\) 
\( \delta = \) boundary layer (i.e., gel layer) thickness \((\text{m})\) 
\( k = \) mass-transfer coefficient; \( k = D/\delta \) \((\text{m/h})\) 
\( J_v = \) system flux \((\mu \text{L/h})\) 
\( J_p = \) permeate flux \((\mu \text{L/h})\) 
\( R = \) membrane rejection coefficient \((\text{dimensionless})\) 
\( A_{\text{mem}} = \) membrane surface area \((\text{mm}^2)\) 
\( \tau = \) residence time \((\text{h})\)

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