Concentration of Proteins and Decrease of Salt Content in a Hydrolyzed Fermentation Broth Using Membrane Filtration

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Preface and Acknowledgements

I have worked with this thesis during the spring of 2023, and it concludes my years of studying to become an engineer. The work was done at the Department for Chemical Engineering at Lund University in cooperation with the company Bioextrax AB.

I want to thank Basel, for taking on the role as main supervisor of this project, and for your help throughout the last months. Thank you to everyone at the Department of Chemical Engineering, for always helping out when I had questions, and for being excellent fika-company. Thank you Lina and Gabi, for being my opponents and giving me valuable feedback on this report, but especially for all the help in the lab. Thank you also to previous students that have contributed to the Overleaf-template that was used to write this report.

Thank you to my co-supervisor Klas, and everyone else at Bioextrax, for giving me the opportunity to work with you and this project. It's been inspiring as a new engineer to work with Bioextrax, with research and development at its core.

Thank you to my family and friends, for cheering me on the tough days. And lastly, I want to thank my sambo Joel. Thank you for always supporting and believing in me. Seeing you work and grow into an excellent engineer is my biggest inspiration. I am excited to see where the future takes us.

Linnea, 2023-06-11

Abstract

Membrane filtration was used to concentrate proteins and decrease the salt concentration in a hydrolyzed fermentation broth. The broth was a by-product from the bacterial production and extraction of polyhydroxyalkanoates (PHA), that can be used to produce biobased plastics.

3 ultrafiltration-membranes, RC70PP, GR70PP and ETNA10PP from Alfa Laval Corporate AB, were evaluated at varying cross-flow velocities (CFV) of 0.3-0.5 m/s and transmembrane pressures (TMP) of 1-9 bar. High flux rates were measured, >100 L/m^2h at CFV = 0.5 m/s and TMP 3-9, and the protein retention was >80% for RC70PP and >90% for GR70PP and ETNA10PP. Due to a limited amount of broth being available, it was necessary to dilute it before using it as feed in the study. This dilution appears to have significantly affected the filtration process, as there was a notable disparity between the results from this study and the results in the following concentration studies.

Concentration studies were conducted with RC70PP and GR70PP, where the original, non-diluted broth was concentrated until a volume reduction of 90% was reached. Much lower flux rates, generally <20 L/m^2h, were measured for both membranes. A high protein retention was achieved, of 97.1 % for RC70PP and 98.3 % for GR70PP. Both RC70PP and GR70PP measured fluxes around 5 L/m^2h after the 90 % volume reduction, but due to unintentionally using a more concentrated feed in the study with GR70PP, the final protein concentration in the retentate was approximately doubled compared to the retentate of RC70PP. From this, the conclusion was drawn that GR70PP performed the best, as the flux was similar as when using RC70PP, but at a higher concentration of solutes.

Diafiltration was performed with RC70PP with a diafiltration factor of 5 and this successfully decreased the conductivity in the retentate with approximately 50%, but it was at the cost of a small protein loss.

Populärvetenskaplig Sammanfattning

Biprodukt från bioplatstillverkning kan bli framtidens kosttillskott

Det är möjligt att tillverka plast från socker med hjälp av bakterier, och även om detta bara görs på en relativt liten skala idag så kommer tillverkningen av biobaserad plast troligtvis öka väsentligt de kommande decennierna. Denna typen av plast tillverkas på förnybara råvaror, och är därför ett bra alternativ till plast som är baserad på fossila råvaror, som petroleum. För att öka hållbarheten av processen, både eknomiskt och miljömässigt, är det bra att utnyttja så mycket av råmaterialet som möjligt, och ta tillvara på både huvudprodukten och biprodukter som produceras.

Biobaserad plast går att tillverka med många olika metoder, och en metod är att bakterier omvandlar socker till polymerer som heter polyhydroxialkanoater (PHA), som sedan extraheras ur bakterierna, renas upp och bearbetas till olika produkter. Ett företag i Lund, Bioextrax AB, tillverkar PHA och använder inga farliga kemikalier i processen, vilket gör att lösningen som är kvar efter PHA har blivit upprenad är en näringsrik blandning av bl.a. vatten, protein, fetter och överblivna celldelar.

I detta examensarbete har membranfiltrering används för att koncentrera lösningen som ursprungligen var väldigt utspädd. Slutmålet med projektet var att den koncentrerande lösningen ska torkas för att användas som t.ex. kosttillskott eller djurfoder. Tre membran av olika material testades och jämfördes med varandra. I slutändan gav alla membranen ganska lika resultat, och det var möjligt att koncetrera lösningen 10 gånger, och behålla majoriteten av proteinet.

Abbreviations and Symbols

Abbreviations

CFV	Cross Flow Velocity	m/s
DF	Diafiltration	
MWCO	Molecular Weight Cut Off	
PHA	Polyhydroxyalkanoates	
PWF	Pure Water Flux	L/m^2h
TMP	Transmembrane Pressure	bar
TS	Total solids	%
VR	Volume reduction	%

Symbols

c	Concentration	mg/mL
J	Flux	L/m^2h
μ	Dynamic viscosity	Pa s
ω	Rotational speed	s^{-1}
P	Pressure	bar
r	Radius (of magnetic stirrer)	m
R_{true}	Retention, true	g/g or %
R_{obs}	Retention, observable	g/g or %
R_m	Membrane resistance	m^{-1}
V	Volume	m^3

Contents

Introduction, aim and background

1.1 Introduction

In bioprocesses, microorganisms are utilized to produce various products, such as food, pharmaceuticals and polymers. A well-designed production process is often needed to achieve a high productivity, and in the end, make the process profitable. Once the process design for the main product has been optimized, one strategy to further increase profitability is to take advantage of the excess biomass and by-products that will inevitably be produced. This is also preferable when considering the sustainability of the process, to utilize the raw material as much as possible and minimize waste.

This thesis was done in cooperation with the company Bioextrax AB. Bioextrax has developed a method to produce and extract polyhydroxyalkanoates (PHA), which is a group of polymers that can be used in the production of biobased plastics [1]. PHA can be made from renewable carbon sources and serves as a more sustainable alternative to plastics based on fossil fuels, such as petroleum. It is possible to produce PHA using carbon from industrial waste streams, for example from wastewater treatment plants or from food production, which could both decrease the cost and environmental impact of the production [2].

1.2 Aim

After the PHA has been produced, extracted and separated using Bioextrax's method, there is a nutrient rich broth remaining. The aim with this thesis was to develop and optimize a method to concentrate the proteins and decrease the salt concentration in the broth. This will be the first steps in a larger project of developing a method to utilize the broth and refine it into a product, for example animal feed or a dietary supplement.

The main separation method used was membrane filtration, and the sub-goals with this thesis were to test and evaluate different membrane materials and pore sizes, and investigate how an increasing concentration will affect the filtration process. Fouling and cleaning of the membranes were also evaluated.

1.3 PHA production and extraction

PHA is a group of polymers, polyesters, produced by microorganisms that is both biobased and biodegradable. PHA can have similar properties to petroleum-based plastics such as polypropylene and can be processed in different ways and be used for packaging, films, paper coatings and many other applications [3].

A simplified overview of Bioextrax's PHA production process can be seen in Figure 1.1 and it consist of three main steps: production of intracellular PHA, extraction of PHA through lysis of the cell walls and a solid-liquid separation.
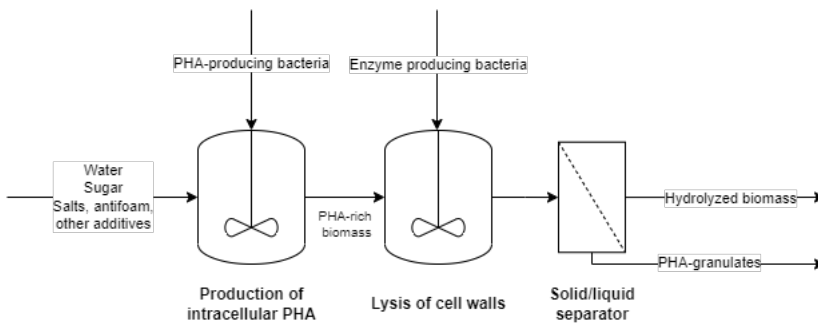

Figure 1.1: Simplified process diagram over Bioextrax's PHA production and extraction process

The PHA is produced by bacteria that metabolize sugars, such as sucrose, into biomass. Most of the biomass, 50-80 % of the dry weight, will be in the form of intracellular PHA-granulates, but other compounds will also be produced, such as proteins [4].

The main techniques used to extract PHA from cells are solvent extraction, chemical digestion and biological extraction methods ("bioextraction") [5]. One of the main advantages using bioextraction methods is the avoidance of chemicals that can be harmful to humans and/or the environment. There are several methods that could be classed as "bioextraction", for example using bacteriophages or mealworms [5], and Bioextrax has developed their own, patented extraction method [6].

Bioextrax's extraction method works through adding enzyme-producing bacteria to the PHA-rich biomass. The enzyme will lysis the cell wall of the PHA-containing cells, releasing the granulates into the liquid [6]. The PHA-granulates are separated from the broth, for example through centrifugation, and then washed and processed into an appropriate form, such as pellets. The broth that is remaining is rich in proteins and other components from the cells.

1.4 Membrane filtration

Membrane filtration will be used to concentrate the hydrolyzed broth. Cross flow membrane filtration is different from traditional, dead-end filtration, and the difference is illustrated in Figure 1.2.
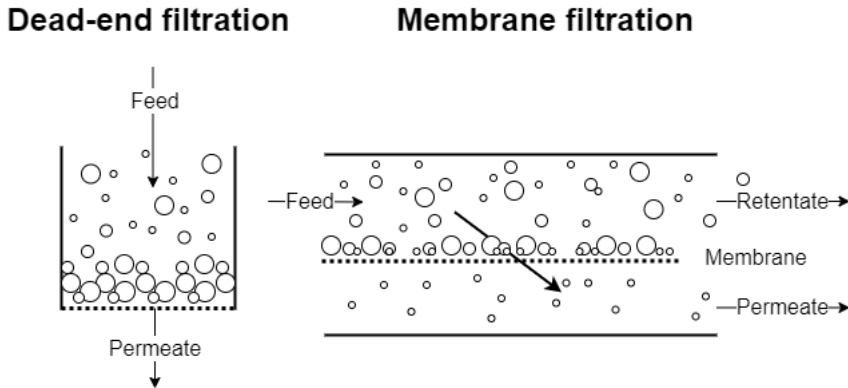

Figure 1.2: Illustration showing the difference between dead-end filtration and cross flow membrane filtration

In dead-end filtration, all of the liquid that is going to be filtrated, the feed, flow perpendicularly through the filter or membrane, which will retain larger particles while letting through smaller particles and liquid. In cross-flow membrane filtration, the feed flows parallel to a semi-permeable membrane, which also separates the components based by particle size. The liquid that goes through the membrane is called the permeate and the liquid that is retained by the membrane is called the retentate. The linear velocity of the feed is called the cross-flow velocity (CFV) and is often in the unit m/s. The volumetric flow of permeate through the membrane per area is called the flux, J, commonly with the unit L/m^2h.

The main driving force for the separation will be the difference in pressure across the membrane, called the transmembrane pressure (TMP). There are membrane filtration processes that have other driving forces, for example membrane distillation and dialysis, but these are not relevant for this thesis. The TMP will vary throughout the membrane and the average TMP is often used, defined by equation 1.1, using the pressure, P, on the sides of the feed, retentate and permeate [7].

$$\text{TMP} = \frac{P_{feed} + P_{retentate}}{2} - P_{permeate} \qquad (1.1)$$

The flux of a pure solvent can be described by Equation 1.2 where R_m is the membrane resistance and μ is the dynamic viscosity of the liquid [8].

$$J = \frac{\text{TMP}}{R_m \mu} \qquad (1.2)$$

The flux found when filtrating pure water is often called the Pure Water Flux (PWF) and can be used to characterise a membrane, for example, through calculating the membrane resistance R_m or to evaluate fouling. As can be seen in Equation 1.2, the PWF will be linear when plotting the flux against TMP, since the membrane resistance and viscosity will remain constant.

If there is more than one component in the liquid, the concentration will increase closer to the membrane surface, c_m, compared to in the bulk, c_b. This phenomenon is called concentration polarization and is illustrated in Figure 1.3 [7]. There are three main models to describe concentration polarization: The first, the osmotic pressure model, is mostly relevant for reverse osmosis and nanofiltration, is dependent on the concentration difference across the membrane, which creates an osmotic pressure. The cake model, which is mostly relevant for microfiltration, is based on that larger particles gather on the membrane surface to create a cake layer, which increases the hydraulic resistance. The last model is the gel model, which is similar to the cake model, but it based on that the saturation concentration is reached on the membrane surface, making substances aggregate and gather on the membrane surface [8].

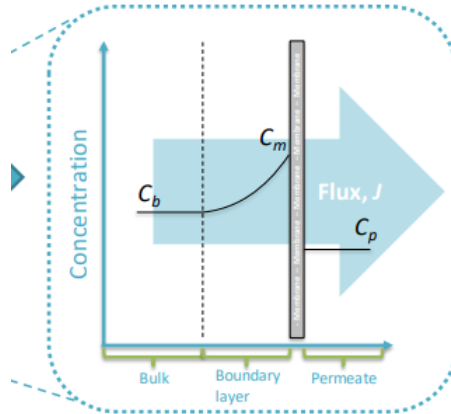**Figure 1.3:** Illustration of concentration polarization in membrane filtration [7]

The particle retention, R_{true}, is described by Equation 1.3 and can be used as a measurement of the separation of the membrane. In practice, the observable retention, R_{obs} is often used instead, see Equation 1.4, since it is easier to measure the concentration in the bulk solution than on the membrane surface [7].

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$$R_{true} = 1 - \frac{c_{\text{permeate}}}{c_{\text{membrane}}} \tag{1.3}$$

$$R_{obs} = 1 - \frac{c_{\text{permeate}}}{c_{\text{bulk}}} \tag{1.4}$$

Membrane filtration can be used to concentrate a liquid, as the volume of the retentate will decrease when liquid goes through the membrane. The volume reduction (VR) can be used to describe how much the retentate has been concentrated and is defined as the fraction of the volume of the permeate, $V_{permeate}$, to the original volume of the feed, V_{feed}, shown in Equation 1.5.

$$\text{VR (\%)} = 100 * V_{permeate}/V_{feed} \tag{1.5}$$

The main pressure-driven membrane filtration methods are microfiltration, ultrafiltration, nanofiltration and reverse osmosis. The fundamental difference between these methods is how large particles that will permeate through the membrane. To give some examples on applications, microfiltration can be used to separate cells and yeast, or for clarification of beverages, ultrafiltration can be used to separate proteins, nanofiltration can be used to separate micropollutants and reverse osmosis can be for desalination [8]. The operational ranges for the methods differ: the methods that separate larger particles will operate at a higher flux and lower TMP while the methods that separate smaller particles operates at a lower flux but higher TMP. To separate smaller particles, the membranes are denser, increasing the inertia through the membrane, therefore a higher TMP is needed. An overview over the different methods and their operational ranges can be seen in Table 1.1 [8]. It is possible for a membrane to compress at high pressures, which makes the porous structure denser, decreasing overall flux [8].

Table 1.1: Pressure driven membrane filtration methods, their cut-offs and approximate operational ranges [8]

Type	Pore size	Pressure range [bar]	Flux range [L/m² h]
Microfiltration	0.05-10 micrometer	< 2	> 50
Ultrafiltration	10-100 nm	1-10	10-50
Nanofiltration	1-10 nm	10-25	1 - 12
Reverse osmosis	<2 nm	> 15	0.05 - 1.4

With pressure driven filtration methods, especially microfiltration and ultrafiltration, it is common to see a decrease in flux over time. There are two main reasons for this: concentration polarization and fouling [8]. Fouling is when components from the bulk solution, such as proteins or salts, adheres to membrane surface or in the

membrane pores [8]. Fouling can be reversible or irreversible, where reversible fouling can be removed by switching from feed to water, and irreversible fouling can either be removed through cleaning the membrane or is not removable [7]. The fouling degree of a membrane can be found through comparing the original PWF before experiments with the PWF after experiments and cleaning, according to Equation 1.6 [9].

$$\text{Fouling grade (\%)} = 100 * \frac{\text{PWF}_{Before} - \text{PWF}_{After}}{\text{PWF}_{Before}} \tag{1.6}$$

For membrane filtration to be a feasible separation method from an economic standpoint, it is important to maintain a high flux. There are some important factors to maintaining a high flux, such as doing the appropriate pretreatment, choosing the correct membrane material and pore size, and operating at appropriate conditions [8]. When filtrating a liquid, the flux plotted against TMP will often be linear in the beginning and then level of as fouling increases until it becomes constant, where the flux does not change with a change in TMP [10]. The point when the flux starts to become non-linear is called the critical flux. The final flux, where an increase in TMP does not change the flux, is called the limiting flux. It is often beneficial to run industrial filtration processes at or close to the critical flux, to minimize fouling [10].

Cleaning the membrane is an important part of the process, since it can remove irreversible fouling and increase the flux again, making it possible to reuse membranes. There are a couple of strategies for cleaning membranes, and the main ones are mechanical, chemical, and hydraulic [8]. Mechanical cleaning could for example be through scraping the membrane. Chemical cleaning is done through running the filtration with a detergent solution and there are different types of agents, depending on what membrane is used and what solution is being filtrated. The main cleaning solutions are acidic, alkaline, enzymatic, or fluorinated, and these can be used both individually or successively in different combinations and concentrations. Hydraulic cleaning methods are for example backflushing and backpulsing, where the direction of the flow of the retentate is reversed [8].

Diafiltration is a special way of running membrane filtration to decrease the concentration of small components, for example salts, in the retentate. A buffer, often water, is added to dilute the retentate and then filtrated out. This will retain the larger components in the retentate and wash out small components with the permeate [8]. The diafiltration factor is defined as the fraction of the volume of the diluent (water) added to the volume of the retentate, according to Equation 1.7.

$$\text{Diafiltration factor} = V_{diluent}/V_{retentate} \tag{1.7}$$

Method and Materials

2.1 Raw material

The hydrolyzed fermentation broth remaining after the PHA extraction, described in Section 1.3, was used as the feed in all experiments. The broth contains various components from the cells, such as protein, oligopeptides, free amino acids, lipids and nucleic acids. The broth will also contain the salts $NaCl$, NH_4Cl, NH_4SO_4, $MgSO_4 \cdot 7H_2O$, K_2PO_4 and $Na_2PO_4 \cdot 7H_2O$. There can also be small amounts of carbohydrates present and PHA remaining after the separation.

The broth that was used in the experiments came from the same batch of fermentation, but it had been split into several containers, and there were small differences in the composition in the different containers as the broth was not homogenised before. The feed had to be frozen before the experiments due to a short longevity. The broth was estimated to be stable in room temperature for only one day, then it had to be discarded. A limited amount of feed was available, only 15 L for all experiments, and it had to diluted to be able to perform the planned studies. Samples that were collected during experiments were kept on ice and frozen as quickly as possible after being sampled.

Prefiltration was done before all experiments to remove any larger solids, through pouring the feed through a series of metal meshes of increasing fineness of 150, 60, and 40 µm.

2.2 Membranes

The membranes used in the studies were GR70PP, ETNA10PP and RC70PP, and they are made from polysulphone, composite fluoro polymer and regenerated cellulose acetate. GR70PP and ETNA10PP have hydrophobic surfaces while RC70PP have a hydrophilic surface. All membranes are manufactured by Alfa Laval Corporate AB, have a nominal MWCO of 10 kDa [1] and use polypropylene as a support material [11, 12]. The recommended operational ranges given from the manufacturer can be seen

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[1]for GR70PP, the 10 kDa MWCO was measured on typical dairy products [11]

in Table 2.1. All experiments were done at room temperature and at pressures below 10 bar, which is within the recommended operational ranges from the manufacturer.

Table 2.1: Membranes used in experiments, their material and recommended operational ranges [11, 12]

Membrane	Material	Temperature (°C)	pH	Pressure (bar)
GR70PP	Polysulphone	5 - 75	1 - 13	1 - 10
ETNA10PP	Composite fluoro polymer	5 - 60	1 - 11	1 - 10
RC70PP	Regenerated cellulose acetate	5 - 60	1 - 10	1 - 10

The table has a spanning header "Operational ranges" over Temperature, pH, and Pressure columns.

2.3 Method

The experimental set up for the parameter study (a) and concentration study (b) can be seen in Figure 2.1, illustration from [9].
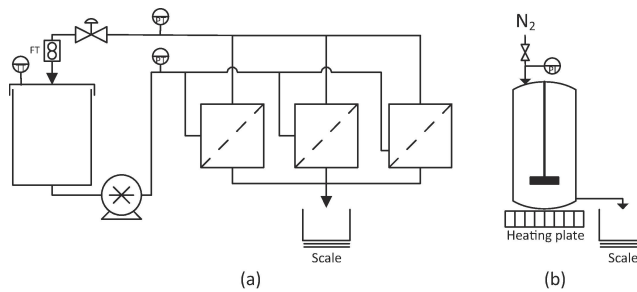

Figure 2.1: Experimental set up for parameter study (a) and concentration study (b) [9]

2.3.1 Parameter study

A parameter study, varying the CFV and TMP, was performed to evaluate the performance of the three membranes compared to each other and to find an operating point for the oncoming concentration study. The original plan was to do two parameter studies, to evaluate both different membrane materials and different MWCOs. In the first study, both hydrophobic and hydrophilic membranes with the same MWCO, 10 kDa, were studied and evaluated based on their performance. Then, in a second parameter study, 3 membranes with different MWCO made of the material that performed best would be evaluated against each other. After the first parameter study was finished, it was decided to not do the second parameter study, as the results with the 10 kDa membranes was satisfactory in regards to flux (generally over 100 L/m^{2}h at CFV of 0.5 m/s) and protein retention (over 80% for RC70PP and 90% for GR70PP and ETNA10PP).

The experimental set up, seen to the left in Figure 2.1, had a feed tank of 15L and the CFV was controlled with a pump. The permeate and retentate were recirculated

to ensure a constant concentration in the bulk solution. There were pressure gauges on both the feed and retentate side, and the average of these two pressures gave the TMP. The TMP was controlled by adjusting a needle valve on the retentate side. Three membranes were set up in parallel and the permeate from each membrane was collected on scales to measure the flux.

Before the study, the membranes were cleaned with Ultrasil 110 (Ecolab AB) and the PWF was measured. Three different cross-flow velocities were tested, 0.5, 0.4 and 0.3 m/s, starting with the highest to avoid fouling. The TMP was increased from 1 to 9 bar at each CFV. The system was left running for a couple of minutes to stabilize before the logging of the flux was started. The pressure was increased in increments of 1 bar, and the flux was logged for around 10 minutes at each pressure interval. After a TMP of 9 bar was reached, the TMP and CFV was decreased, and the procedure was redone at a CFV of 0.4 m/s and lastly 0.3 m/s. Samples of the permeate were collected at each pressure. When the study was finished, the feed was emptied out, the system was rinsed with water and the PWF was measured. Then the membranes were cleaned with Ultrasil 110, and the PWF was measured a final time.

The feed had to be diluted, 3 L broth with 10 L water (corresponding to a dilution factor of 4.33) due to the limited amount and longevity of the feed. The parameter study was done over two days, and broth from two different containers was used. Samples from the feed was taken at the beginning of each day.

2.3.2 Concentration study

Concentration studies were performed to study how an increasing concentration of the retentate would influence the performance of the membranes. Diafiltration was also tested during the concentration study, to see if would be possible to decrease the salt concentration in the retentate.

Concentration studies was done with one hydrophilic and one hydrophobic membrane, RC70PP and GR70PP. GR70PP was prioritized over ETNA10PP because it measured a slightly higher critical flux in the parameter study. Due to a lack of time and availability of equipment, diafiltration was only done with one membrane, the RC70PP.

The experimental setup, seen to the right in Figure 2.1, consisted of a 400 ml chamber with the membrane fixed at the bottom. The permeate was collected in a beaker on top of a scale to measure the flux. The CFV was controlled by a magnetic stirrer and the TMP was increased through adding N_2 gas into the chamber. The stirring of magnetic stirrer was translated into CFV using Equation 2.1 [9] where ω is the rotational speed (s^{-1}) and r is the radius of the stirrer (2.5 cm).

$$CFV = \omega r \pi \qquad\qquad (2.1)$$

The membranes were cut to the correct size, assembled, washed with Ultrasil 110 and the PWF was measured. The concentration studies were run with a high CFV of 0.5 m/s to avoid fouling and at the TMP at the critical flux found in the parameter study, which was at a TMP of 5 bar for RC70PP and 3 bar for GR70PP. The concentration was stopped when a volume reduction of 90 % was achieved. Samples from retentate and permeate were collected, the membrane was rinsed with water and the PWF was measured again. For the GR70PP membrane, it was directly washed with Ultrasil 110 and the PWF was measured again.

For RC70PP, diafiltration was performed before cleaning by adding deionized water to the retentate, with a diafiltration factor of 5, corresponding to 150 mL. The filtration was run with the same conditions as before, CFV of 0.5 m/s and TMP of 5 bar. Samples were taken from permeate and retentate again. Cleaning was performed and the PWF was measured.

2.3.3 Pure Water Flux

The pure water flux in the parameter study was measured at 30 °C and at a CFV of 0.3 m/s. A 4-point measurement was done, at permeate pressures of 0.5, 1, 1.5 and 2 bar and the flux was logged at each pressure increment for 5-10 minutes. The flux was plotted as a function of the transmembrane pressure, and the pure water flux (with a unit $L/(m2 * h * bar)$) was found as the gradient of a linear regression of the points.

In the concentration studies, the PWF was measured at one point, with a TMP of 0.5 bar, at 25 °C and with a CFV of approximately 0.3 m/s. The flux was logged for 5-10 minutes and the average flux was calculated.

2.3.4 Cleaning membranes

The membranes were cleaned before and after the experiments with an alkaline mixture of water and Ultrasil 110 (Ecolab AB). All cleaning was done at 50 °C for 1 hour. For the parameter study, a 0.04 wt.% mixture was used, with an approximate pH of 11.7.

After the parameter study, it was noted that RC70PP was not recommended to be cleaned with a pH over 11.5 [12], so for the concentration study the RC70PP membrane was cleaned with Ultrasil 110 and water that was mixed until the measured pH was 11.3 (the exact wt.% was not noted).

2.3.5 Analysis methods

Protein was quantified using the Bradford method [13], which works by utilizing a colour agent, Coomassie Brilliant Blue G-250, that binds to the proteins in the sample. This method was chosen since it is easy and fast to do, relatively cheap and can quantify small quantities of protein. The Bradford reagent, 1.5 ml, was mixed with the samples, 0.05 ml, and the absorbance of light at 595 nm was measured using a spectrophotometer. A 5-point calibration curve with known concentration of protein (Bovine Serum Albumin) ranging from 0 - 1.5 mg/mL was made and the linear regression of these points was later used to convert the measured absorbance of samples to protein concentration. A modified microanalysis method was used for a majority of the permeate samples, as these had protein concentrations below 0.1 mg/mL. Another calibration curve, with a protein concentration ranging from 0 - 15 µg / ml, was made and a higher ratio between the Bradford reagent and sample was used (1 ml of sample was mixed with 1 ml of reagent).

Total solids (TS) were found through drying a 3 ml sample in an oven at 105 °for 24 hours, and comparing the weight before and after.

Ash content was found through ashing 3 mL of samples in a ceramic crucible in a muffle furnace at 575 °for 4 hours. The samples were let to cool in a desiccator and then weighed and the ash content was found through comparing the weight before and after ashing.

Conductivity was measured to monitor the salt concentration in the fractions, as the conductivity of a liquid is proportional to the ion concentration. It was measured with a conductivity probe (HI 76306, HI 99301, Hanna Instruments Inc.).

Results and Discussion

3.1 Parameter study

In the parameter study, filtration with the membranes RC70PP, GR70PP and ETNA10PP were run at the CFVs of 0.5 m/s, 0.4 m/s and 0.3 m/s and at TMPs of 1-9 bar. The study was performed over two days, with the CFV of 0.5 m/s performed on one day and 0.4 and 0.3 m/s performed the second day. The feed had to be replaced on the second day, due to its short longevity, and there were small differences in the feed across the different days. The broth was also diluted, 3 L broth with 10 L water, due to the limited amount broth available.

3.1.1 Flux

The averages of the measured flux at each TMP for each membrane can be seen in in Figure 3.1.
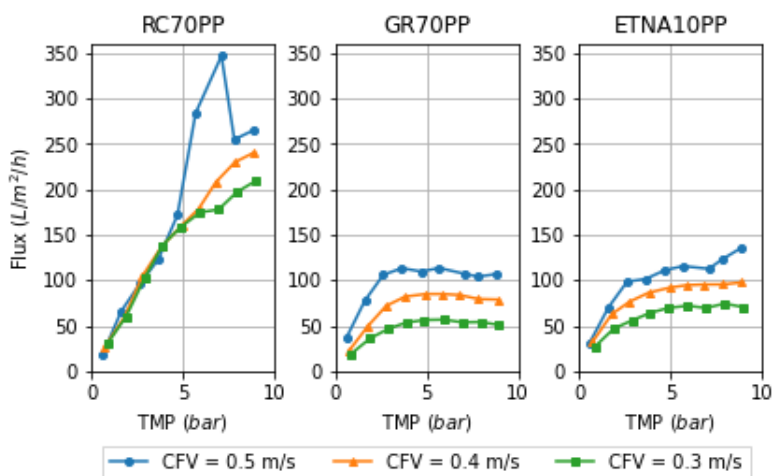

Figure 3.1: Average fluxes at different TMP for membranes RC70PP, GR70PP and ETNA10PP for CFV of 0.5 m/s (blue), 0.4 m/s (orange) and 0.3 m/s (green).

All three membranes show a linear increase in flux at low TMP, and at higher TMP the curve flattens out or slightly decreases, which can be expected. When the overall

resistance for filtration is dominated by the membrane resistance, which can occur with low to no fouling at low TMPs, the curve is expected to be linear just as the curve for pure water is linear, according to Equation 1.2.

At TMPs below 3 bar, the flux of RC70PP is close to flux of GR70PP and ETNA10PP at CFV 0.5 m/s, while at higher TMPs RC70PP show a significantly higher flux than the other membranes. The limiting flux for ETNA10PP at all CFVs is slightly higher than for GR70PP and the limiting flux for RC70PP was not reached, but it would end up significantly higher than for the other membranes. The large difference in flux of the membranes is believed to be caused by the different characteristics, especially the hydrophilicity, of the membrane materials. The two hydrophobic membranes, GR70PP and ETNA10PP, consistently have relatively similar results to each other, which is contrasted with the results from the hydrophilic RC70PP that often display other trends, both in regard to flux and retention.

In theory, the gel formation should be equal for all membranes, considering that the gel concentration is dependent on the composition of the feed and this should remain identical for all membranes since they were attached in parallel, sharing feed. But the different material properties of the membrane could potentially make gel and solutes more or less prone to stick to the membrane surface, and therefore affect the overall gel and cake formation, consequently affecting both the flux and retention of particles.

For GR70PP and ETNA10PP, the critical flux at CFV of 0.5 m/s was determined to be at a TMP of 3 bar. For RC70PP, it is harder to determine the critical flux, as the flux for CFV of 0.5 m/s starts increasing instead of decreasing. There is a peak in the flux for RC70PP at 6-7 bars, and then the flux decreases. During the measurements at 6-7 bars, it was observed that the flux increased instantly after the TMP had been increased, and then declined for a couple of minutes before stabilising. The reason for this is not clear, as for the other measurements, the flux stabilized close to the average flux quickly after an increase in TMP. If the flux would have been recorded for a longer time period, the average flux would decrease and the points would probably follow a more similar trend to those at CFVs of 0.4 and 0.3 m/s. But for all CFVs, the linearity is broken at around a TMP of 5 bar, and this was used at the operating point for the concentration study later.

3.1.2   Protein

The protein concentration was measured in all samples. The diluted feed used for CFV 0.5 m/s had a protein concentration of 0.131 mg/mL and the feed used for CFV 0.4 and 0.3 m/s had a concentration of 0.148 mg/mL. The protein concentration in the permeate samples ranged from 0.008 to 0.029 mg/mL. The calculated protein retention plotted against TMP can be seen in Figure 3.2.
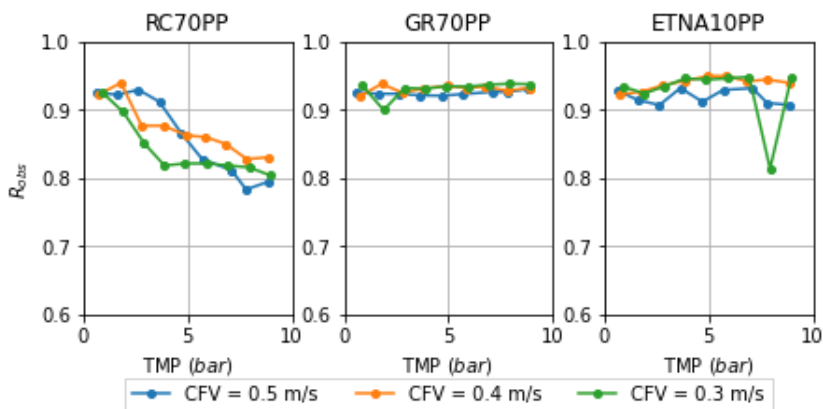
Figure 3.2: Observable retention of protein plotted against TMP for RC70PP, GR70PP and ETNA10PP at CFV of 0.5 m/s (blue), 0.4 m/s (orange) and 0.3 m/s (green)

Both GR70PP and ETNA10PP had a high retention of protein, consistently 90% or higher. The RC70PP membrane had a similar retention at low TMP of 1-2 bar, but as the TMP increased, the protein retention decreased with around 10%. It is not clear why the protein retention for RC70PP decreases, but it could happen if retention is dependent on flux, as the flux for RC70PP kept increasing when it stagnated for the other membranes. The retention was considered satisfactory for all membranes as it was expected to increase even further in the following studies where a feed with a higher concentration would be used.

The sample from ETNA10PP at TMP 8 bar and CFV of 0.3 m/s deviated from the trend of the rest of the samples, both for protein concentration, and also later for the conductivity measurements. Both measurements lied much lower than the surrounding data points. The reason for this most likely that there has been a mix-up when filling the test tubes with permeate, so that the RC70PP permeate have been poured in both the RC70PP and ETNA10PP sample tubes. These measurements have been disregarded in the overall analysis of the results.

A number of samples, mostly RC70PP-samples, measured absorbance that lied above the calibration curve that was used to calculate the protein concentration. This was noted first after the analyses was finished, and therefore, the curve was extrapolated for these samples and the concentration was calculated with the same linear relationship. This extrapolation increases the uncertainty in the measurements of the protein concentration. If the analysis were to be redone, the samples should be diluted to fit in the calibration curve to increase accuracy.

3.1.3 Ash and Conductivity

Ash was measured for 8 samples of 3 ml each, both the original feed and the diluted feed, and samples from each membrane at the lowest and highest TMP at the CFV of 0.5 m/s. The ash weight, content and retention can be seen in Table 3.1.

Table 3.1: Ash weight (of a 3 mL sample), ash content in sample and observable retention for samples from the parameter study at CFV = 0.5 m/s

TMP [bar]	Sample	Weight ash [g]	Ash content in sample [%]	R_{obs}
—	Feed$_{original}$	0.01221	0.415	
—	Feed$_{diluted}$	0.00297	0.102	
0.6	RC70PP	0.00197	0.068	0.337
0.6	GR70PP	0.00170	0.058	0.428
0.6	ETNA10PP	0.00160	0.055	0.461
8.8	RC70PP	0.00223	0.076	0.249
8.8	GR70PP	0.00100	0.034	0.663
8.8	ETNA10PP	0.00114	0.039	0.616

The ultrafiltration membranes were not expected to separate any significant amount of salt, and it was therefore surprising when the retention of ash was relatively high, of 0.25 - 0.66. But this is most likely because around 1% of the total cell weight is inorganic ions [14], and if most of the cell matter was retained by the membrane, this would lead to an overall higher ash retention. Ashing did therefore not work as a good measurement of dissolved salts, and it was decided to instead measure the conductivity.

The conductivity was measured in all samples. The feed for CFV = 0.5 m/s had a conductivity of 1.12 mS/cm and the feed for CFV = 0.4 and 0.3 m/s had a conductivity 1.15 mS/cm. The conductivity of the permeate samples ranged from 0.69 to 1.23 mS/cm. The retention of conductivity can be seen in Figure 3.3.

The conductivity measurements results in a low retention, <10%, for RC70PP and a bit higher retention for GR70PP and ETNA10PP. GR70PP and ETNA10PP also show a trend of an increasing retention with increasing TMP, while the retention for RC70PP remains more stable across all TMP. An increased gel and/or cake formation on the membrane surface will increase the resistance for salts to go through the membrane, and therefore increase the retention. There could also be other components present in the broth that was detected by the conductivity meter, for example small, charged proteins, affecting the measurements.

Across all membranes, samples at CFV of 0.4 m/s resulted in the higher retention than those at 0.3 m/s and 0.5 m/s. This was unexpected, as would be more logical if the trend was in chronological order. This could be because the retention is calculated from the ratio between the concentration in permeate and the feed (Equation 1.4), so
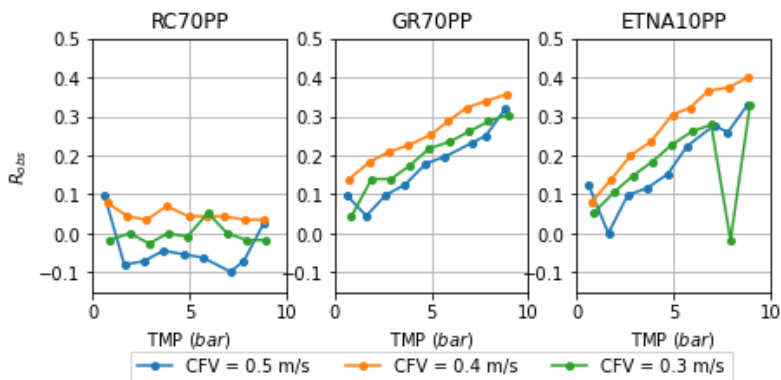
Figure 3.3: Observable retention for conductivity measurements plotted against TMP for RC70PP, GR70PP and ETNA10PP at CFV of 0.5 m/s (blue), 0.4 m/s (orange) and 0.3 m/s (green)

all samples are compared to the feed measurement. If the feed sample was measured incorrectly, this would cause all the retention values to shift (decrease or increase). Two different feeds were used for the experiments at CFV 0.5 m/s and 0.4-0.3 m/s since the experiments were conducted over two days, so the retention was calculated using two different feed concentration measurements (one for 0.5 and one for 0.4 and 0.3 m/s). Likely, one of these measurements were not accurate, causing a shift of all points compared to that.

The samples from the lowest and highest TMP, and also the feed, at CFV of 0.5 m/s for all membranes was measured on a different day than the rest of the samples. This can be seen in Figure 3.3, as these values lie a bit higher than the rest. This is most likely due to a small temperature difference when measuring the samples on different days.

3.1.4 PWF

Washing of the membranes was performed before and after experiments with an alkaline wash of Ultrasil 110, 0.04 wt.% in water. The measured PWF with linear regressions plotted against the TMP can be seen in Figure 3.4. The fouling degrees, based on the linear regression, were for RC70PP 12.8 %, GR70PP 14.2 % and ETNA10PP 17.5 %.

The RC70PP membrane had a higher flux directly after the experiments compared to the other membranes, but it did not change considerably after cleaning. Both GR70PP and ETNA10PP measured lower fluxes directly after the experiments, indicating more fouling, but the flux did also increase significantly after cleaning. These results line up with the flux-measurements in the study when broth was used, Fig-
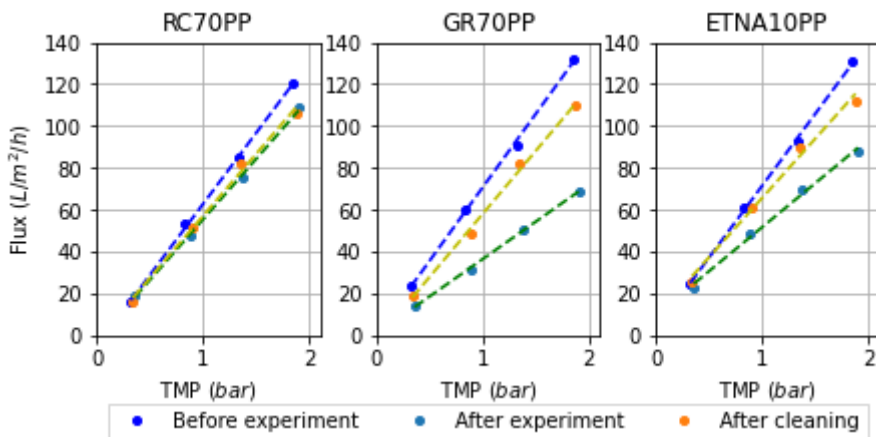
Figure 3.4: Pure water flux plotted against TMP for RC70PP, GR70PP and ETNA10PP before experiments, after experiments and after cleaning.

ure 3.1, as GR70PP and ETNA10PP reached its limiting flux quickly, which could indicate more fouling compared to RC70PP.

After cleaning, all membranes end up with a similar PWF. The decrease, or a part of it, in the PWF before experiment compared to after experiments could have been caused by compression of the membrane. During the parameter study, the membranes were operated at TMPs close to their maximum operational pressure, see Table 2.1, and if the porous structure of the membrane was compressed, this could lead to an irreversible decrease in flux. To evaluate this in future experiments, it is recommended to do the parameter study two times consecutively or to pre-compress the membranes before the study. This would make it possible to evaluate how much of the decrease in PWF is caused by compression and how much is caused by irreversible fouling.

In general, it would be beneficial to do several consecutive studies to evaluate if the cleaning regiment is sufficient in removing all fouling, or if the flux decreases over time, even with cleaning. Removing as much of the fouling as possible is advantageous from an economic standpoint when scaling up, as it would make it possible reuse the same membrane for a longer time. The cleaning regiment could also be altered and optimized, for example by adding an enzymatic wash to remove fouling proteins.

After the parameter study was finished, it was noticed that the RC70PP membrane was not recommended to be washed with a pH over 11.5. There is a risk that this can have affected the membrane and deteriorated the surface, but this seems to have been negligible. If the cleaning solution would have damaged the membrane, the PWF would most likely increased after the second cleaning session, but the PWF was the same or lower across all TMP, so the higher pH has most likely not made a significant

damage to the membrane.

3.1.5 Conclusion of Parameter Study

The conclusion from the parameter study was that RC70PP had similar or higher fluxes than GR70PP and ETNA10PP at all TMPs, indicating less fouling overall. This was also seen in the PWF-measurements, where the PWF after the experiments was lower for GR70PP and ETNA10PP compared to RC70PP. RC70PP did also have a slightly lower protein retention and a lower salt retention. Overall, GR70PP and ETNA10PP had very similar results to each other, and this is believed to be because of their hydrophobic surface, compared to the hydrophilic RC70PP. It was difficult to determine which membrane performed the best, as it depends on if the flux or protein retention is prioritised, and these objectives had not been pre-defined.

3.2 Concentration study

Originally, the plan was to do one concentration study with diafiltration with the membrane with the best performance in the parameter study. But it was difficult to decide which membrane to use, as the RC70PP had higher fluxes, but also lower protein retention compared to the other membranes. It was therefore decided to do the concentration studies with one hydrophilic and one hydrophobic membrane, RC70PP and GR70PP. GR70PP was prioritized over ETNA10PP because it measured a higher critical flux in the parameter study, 106 L/m^{2}h compared to 98 L/m^{2}h. Considering that the GR-membranes are available in more MWCOs compared to ETNA-membranes [11, 12] it could be advantageous to prioritise the GR-series over ETNA if different MWCO would be investigated in the future. Due to a lack of time and availability of equipment, diafiltration was only done with one membrane, RC70PP.

In the concentration studies, an initial feed volume of 300 ml was used and a volume reduction of 90% was reached. For the RC70PP membrane, diafiltration was done through adding 150 ml, corresponding to a diafiltration factor of 5, to the retentate after 90% volume reduction had been reached. To avoid working alone in the lab after working hours, the experiment had to be stopped after 137 ml had permeated. The retentate after diafiltration was therefore diluted compared to the retentate before diafiltration, with a dilution factor of approximately 1.33.

The original, non-diluted, broth was used as the feed in the concentration study, compared to in the parameter study where diluted broth was used. The feed used for the experiments with RC70PP and GR70PP was from two different containers, and it can be seen later that they differ from each other, both in protein concentration, ion concentration and total solids. This should be taken into consideration when analysing the results.

3.2.1 Conductivity, Protein and TS

Conductivity, protein concentration and TS were measured of in the feed, permeate, retentate and in the fractions after diafiltration (DF in table) and are presented in Table 3.2.

Table 3.2: Conductivity, protein and TS in samples from before and after a 90 % VR with RC70PP and GR70PP, and after diafiltration (DF)

Membrane	Sample	Conductivity [mS/cm]	Protein [mg/mL]	Total solids [%]
RC70PP	Feed	5.80	0.401	1.91
RC70PP	Permeate	5.63	0.069	1.48
RC70PP	Retentate	7.25	4.384	—[a]
RC70PP	Permeate after DF	1.60	0.066	0.44
RC70PP	Retentate after DF	2.80	3.879	3.47
GR70PP	Feed	5.99	0.626	2.09
GR70PP	Permeate	5.89	0.067	1.58
GR70PP	Retentate	6.38	7.090	7.50

[a]not enough sample available

The average retention was calculated according to Equation 1.4 with the bulk concentration calculated as the average of the concentration in the feed in the beginning and concentration in the final retentate.

The average retentions for conductivity were 13.7 % for RC70PP and 4.8 % for GR70PP, which means that the membranes retain small amounts of salt. Ultrafiltration membranes are normally not expected to retain salts, but if there was gel formation and a filter cake on the membrane surface, this could work as a secondary filter, increasing retention. The retention was higher for RC70PP than GR70PP, which contradicted the results from the parameter study. It is not clear why, it could possibly be because of a larger gel or cake formation on the RC70PP. Diafiltration gave the desired result of significantly decreasing the salt concentration, as the conductivity decreased with around 50% (when taking dilution into account). But it was at the expense of a small protein loss, as some protein is still being filtrated out with the permeate.

The average retentions of protein were 97.1 % for RC70PP and 98.3 % for GR70PP. The proteins were successfully concentrated, which fulfils one of the main aims with the study. The retention is slightly higher for GR70PP than for RC70PP and this corresponds with the results from the parameter study. The retention is also noticeably higher than in the parameter study, which was expected with the increasing concentration of the bulk solution.

An analysis of the TS content was done, and a significant amount, around 2 %, was found in the feed. This indicates that the majority of the total solids are not free proteins, as the protein concentration in the feed for RC70PP of 0.4 mg/mL corresponds to a total protein content of 0.04 %. There have been indications from Bioextrax that there could be some whole cells left in the broth from their separation step, and this is believed to make up most of the TS. Adding a more thorough prefiltration step, for example through microfiltration, could decrease the amount of larger particles in the

feed, therefore possibly decreasing cake formation and increasing flux. Doing a more thorough analysis of the broth is advised, as it is still unclear what a large portion of the dry matter consists of. This would be valuable when interpreting results and make it possible to add analyses to evaluate how other components in the feed were separated by the filtration.

One improvement that could be made to the method is to take samples continuously throughout the concentration, to study how the retention changes with the VR.

3.2.2 Flux

The measured flux plotted against the volume reduction can be seen in Figures 3.5. The flux was recorded every 30th second, which created many data points since the concentration took several hours to complete, and therefore the rolling average of 10 consecutive measurements have been plotted.
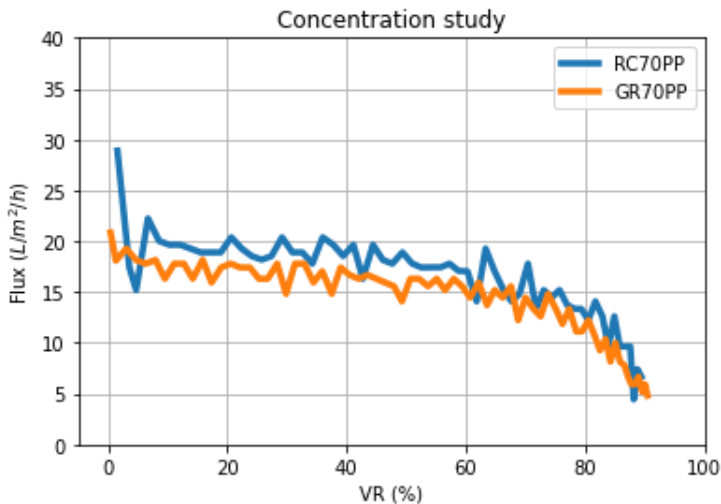

Figure 3.5: Flux plotted against VR (%) for RC70PP and GR70PP

Overall, the flux was a bit lower for GR70PP than RC70PP. For both membranes, the flux was higher in the beginning of the concentration and then decreases as the volume of the retentate decreases, which is to be expected. But overall, the fluxes are much lower than those measured in the parameter study and the reason for this was most likely the more concentrated feed. The gel concentration should be the same as in the parameter study, and since the feed concentration was higher, this gel concentration should be reached quicker, increasing overall gel formation and decreasing flux. There were also other solids than proteins in the broth, which can create a filter cake on the membrane surface, decreasing the flux.

It is difficult to compare the flux between the membranes directly, as the concentration studies were not conducted under identical conditions. There were a difference in protein concentration and TS in the feeds due to the heterogeneous broth, where RC70PP had a feed protein concentration of 0.40 mg/mL and GR70PP had 0.63 mg/mL and the final concentrations in the retentates were 4.38 mg/mL and 7.09 mg/mL. This difference in concentration could have affected the filtration process significantly, affecting factors such as concentration polarization, fouling and viscosity. In the end, the flux after 90 % VR were approximately 5 L/m^{2}h for both membranes, but the concentration of the retentate for GR70PP was much greater than it was for RC70PP. Based on this, the conclusion was that the GR70PP performed better than RC70PP, as the flux was close to that of GR70PP, but at a higher concentration of protein and other solutes.

The concentration studies were conducted at two different TMPs, which also should be taken into account. Generally, it is more expensive to run membrane filtration at a higher TMP, as the pump will need to work more. The study of RC70PP was done at 5 bar and GR70PP used 3 bar, and RC70PP had a slightly higher flux, but a techno-economic analysis would be needed to evaluate if that difference is large enough for it to be profitable.

The flux could most likely be increased with a few relatively easy measures. Adding a microfiltration step before ultrafiltration is believed to be able to decrease cake formation by removing larger solids from the broth. Increasing the filtration temperature could decrease the viscosity of the broth and therefore increase flux. The last separation step in Bioextrax's process is done at 50 °C and this is therefore the recommended temperature for future experiments.

3.2.3 PWF

The pure water flux was measured at 25 °C before the concentration, right after the concentration and after washing the membranes. The flux was measured for around 10 minutes and the average fluxes can be seen in Table 3.3 together with the calculated fouling degree.

Table 3.3: PWF for RC70PP and GR70PP before concentration, right after the concentration, after cleaning and the fouling degree

Membrane	PWF [L/m^2h] Before experiments	PWF [L/m^2h] After experiments	PWF [L/m^2h] After cleaning	Fouling degree [%]
RC70PP	57.2	119.4	124.9	-118.5
GR70PP	32.4	18.5	39.2	-20.8

The PWF for RC70PP increased significantly after the experiment, and even more after cleaning. But, a relatively large scratch was found on the membrane surface after

disassembling the experimental setup, which can be seen in Figure 4.1 in Appendix A. It is difficult to know exactly when the scratch happened, but it is believed to have happened right after the concentration study, as the flux behaved in the expected way during the concentration. The scratch is shaped like a half moon, and follows the edge on the membrane, and it is therefore most likely that it was the magnetic stirrer that caused it. It is difficult to evaluate how much this has affected the filtration and PWF without redoing the experiment.

For GR70PP, the PWF was lower after the concentration, which was expected, but it then increased after cleaning to higher values than the PWF before the experiments. The cause of this could be that the membrane was not cleaned enough before the experiments, so the second cleaning session removed decay from the membrane that should have been removed before the experiments. This could also have affected the RC70PP membrane. No scratches or other irregularities to the membrane was observed on GR70PP after the experiments. In the end it was difficult to draw any major conclusions regarding fouling from the PWF-results.

3.2.4   Conclusion of Concentration Study

In general, the results from the parameter study did not translate to the concentration study, and this is most likely due to the difference in the concentration of the feed. In the concentration study, a filter cake and/or gel formation has most likely decreased the flux and this was not as pronounced during the parameter study due to the lower concentrations.

GR70PP is concluded to have performed better than RC70PP. Even though the flux was slightly lower compared to RC70PP, the study with GR70PP was done at a lower TMP and the feed was more concentrated. Both the initial and final protein concentration for GR70PP was approximately twice as high as for RC70PP, indicating that GR70PP can concentrate the feed further than RC70PP while maintaining a higher flux.

Conclusion and Recommendations

4.1 Final Conclusion

The aim with this thesis was to concentrate a hydrolyzed fermentation broth, as well as decreasing the salt concentration, and in the end, these aims were fulfilled. At first, RC70PP, GR70PP and ETNA10PP was evaluated at varying CFVs and TMPs. The results were high flux-rates and protein retention, but due to a limited amount of broth, a highly diluted broth had been used which resulted in a higher flux than what was later measured in the studies without dilation. With RC70PP and GR70PP, the non-diluted broth was then concentrated to a volume reduction of 90% and the flux rates were noticeable lower, but the concentration also resulted in a very high protein retention. Diafiltration was tested with RC70PP to decrease the salt concentration in the retentate and this was successful, with a significant decrease in measured conductivity of the retentate. It is believed that membrane filtration is a good separation method for the desired end goal, but more work and optimization should be done to try and increase the flux further before scaling up.

4.2 Future Recommendations

The parameter study is recommended to be re-done with enough broth so that no dilution is needed, to better evaluate the appropriate operating point for the concentration. With the results from concentration study, GR70PP is the recommended membrane to use moving forward. It is advised to pre-compress the membrane, or do the parameter study twice consequently, to evaluate how compression affects the flux and PWF. Future experiments are recommended to be done at a higher temperature, for example the same temperature as the PHA-separation step, 50 °C, as this can increase flux. Adding a microfiltration step before ultrafiltration could decrease cake formation and therefore increase flux. Doing a more thorough analysis of the composition of the broth would helpful when analysing results and designing future experiments. A techno-economic analysis and a longer, scaled up study, to evaluate how flux depends on time, will be needed to determine if the process will be feasible to scale up to industrial scale. For this, it is also necessary to formulate more detailed objectives, for example what concentration of protein is satisfactory, what concentration of salts is acceptable or how much water is necessary to remove before drying.

References

[1] Bioextrax AB, "Technologies," 2023. https://bioextrax.com/technologies/, Accessed: 2023-03-10.

[2] G. Mannina, D. Presti, G. Montiel-Jarillo, J. Carrera, and M. E. Suárez-Ojeda, "Recovery of polyhydroxyalkanoates (PHAs) from wastewater: A review," *Bioresource Technology*, vol. 297, 2020. art. 122478.

[3] E. Bugnicourt, P. Cinelli, A. Lazzeri, and V. Alvarez, "Polyhydroxyalkanoate (PHA): Review of synthesis, characteristics, processing and potential applications in packaging.," *eXPRESS Polymer Letters*, vol. 8, no. 11, pp. 791 – 808, 2014.

[4] Bioextrax AB, 2023. Email correspondence, Received 2023-03-02.

[5] M. Haddadi, R. Asadolahi, and B. Negahdari, "The bioextraction of bioplastics with focus on polyhydroxybutyrate: A review," *International journal of environmental science and technology*, vol. 16, pp. 3935–3948, 2019.

[6] M. Takwa, M. H. A. Ibrahim, and R. Hatti-Kaul, "Process for extraction of bioplastic and production of monomers from the bioplastic." Patent WO2016085396A1, 2016.

[7] B. Al-Rudainy, *Galactoglucomannan recovery from softwood spent sulfite liquor: Challenges, process design and techno-economic evaluations.* PhD thesis, Lund University, 08 2020.

[8] M. Mulder, *Basic Principles of Membrane Technology*. Dordecht: Kluwer Academic Publishers, 2 ed., 1996.

[9] B. Al-Rudainy, M. Galbe, and O. Wallberg, "Influence of prefiltration on membrane performance during isolation of lignin-carbohydrate complexes from spent sulfite liquor," *Separation and Purification Technology*, vol. 187, pp. 380–388, 2017.

[10] P. Bacchin, P. Aimar, and R. Field, "Critical and sustainable fluxes: Theory, experiments and applications," *Journal of Membrane Science*, vol. 281, no. 1, pp. 42–69, 2006.

[11] Alfa Laval Corporate AB, "Alfa Laval UF flat sheet membranes — DAIRY. Flat sheet membranes for ultrafiltration (Dairy UF-pHt™)." https://www.alfalaval.com/globalassets/documents/products/separation/membranes/flat-sheet-membranes/dairy-flat-sheet-membranes_200000190-en-gb.pdf, Accessed: 2023-03-17.

[12] Alfa Laval Corporate AB, "Alfa Laval UF flat sheet membranes. Flat sheet membranes for ultrafiltration - FS, RC and ETNA types." https://www.alfalaval.com/globalassets/documents/products/separation/membranes/flat-sheet-membranes/uf-flat-sheet-membranes_200000311-4-en-gb.pdf, Accessed: 2023-03-17.

[13] M. M. Bradford, "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding," *Analytical Biochemistry*, vol. 72, no. 1, pp. 248–254, 1976.

[14] J. Berg, J. Tymoczko, and L. Stryer, *Biochemistry*. New York: W. H. Freeman and Company, 7 ed., 2010.

Appendix A - Damaged membrane

Figure 4.1: Picture of RC70PP membrane after experiments, with arrow pointing to damage