SEPARATION AND PURIFICATION OF RECOMBINANT PROTEINS BY USING ULTRAFILTRATION MEMBRANES

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURE AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

BEGÜM AKCAN

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
CHEMICAL ENGINEERING

SEPTEMBER 2017

Approval of the thesis:

SEPARATION AND PURIFICATION OF RECOMBINANT PROTEINS BY USING ULTRAFILTRATION MEMRANES

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ABSTRACT

SEPARATION AND PURIFICATION OF RECOMBINANT PROTEINS BY USING ULTRAFILTRATION MEMRANES

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September 2017, 85 pages

Membrane based processes have an increasing role in separation processes and used in many industrial areas like water treatment, food industry and biotechnology. Ultrafiltration can be an alternative to conventional techniques such as crystallization and chromatography due to superior advantages such as having low energy and chemical consumption and easy scale up in the field of bioseparation.

Within the scope of the separation of the recombinant proteins from fermentation environment by using ultrafiltration membranes, two separate production media were considered. First production media contained recombinant human growth hormone (rhGH). In size based separation with cellulose membranes, it was observed that desired product, hGH, was specifically retained by the membrane. High retention of the hGH could arise from the agglomeration of the hGH molecule. Furthermore, larger molecular weight proteins passed to the permeate side whereas hGH was highly retained. In this environment, hGH was produced extracellularly so, all metabolites including salts were present in the feed solution. Presence of the salts may have made the permeation of the proteins easier since they decreased the electrical double layer thickness of the proteins. To improve separation performance, pH change and modification on membrane surface were performed. As a result of these experiments,

changing pH of the feed solution did not affect the separation significantly but the positively modified membrane increased the retention of all proteins.

For this production medium, diafiltration was also conducted to investigate the effect on separation behavior. Although separation performance increased, high rejection of hGH was observed from first set of the filtration. In addition to that, PES membranes were tried for filtrations due to suspected damage of cellulose membranes at the end of the diafiltration. However, there was still high retention of hGH and unexpected permeation of the larger molecular weight proteins.

Second production medium containing recombinant granulocyte-colony stimulating factor (rGCSF) which was produced intracellularly, was used as feed solutions in ultrafiltration experiments after centrifugation and washing step with urea solution. In both 100 kDa and 30 kDa MWCO membrane filtration case, size based separation was achieved to some extent. For further separation of the desired protein, GCSF, pH of the feed solution was adjusted to different pH values and it was observed that the best result was achieved when the pH was 5.2 and permeate samples of the 100 kDa membrane filtration were used as feed solution for 30 kDa membrane filtration.

Consequently, in hGH environment, hGH was selectively rejected by the membrane in any case. On the other hand, in GCSF environment, GCSF had selectivity over the other proteins in the pH range 4-6.5 especially in the case of pH was equal to 5.2.

Keywords: Ultrafiltration Membranes, Recombinant Proteins

ÖZ

ULTRAFİLTRASYON MEMBRANLARI İLE REKOMBİNANT PROTEİNLERİN AYIRIMI VE SAFLAŞTIRILMASI

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Eylül 2017, 85 sayfa

Membrana dayalı yöntemler ayırma süreçlerinde gittikçe daha önemli bir yere sahip olmakta ve su arıtımı, gıda endüstrisi ve biyoteknoloji gibi bir çok alanda kullanılmaktadır. Ultrafiltrasyon, düşük enerji ve kimyasal kullanımı ve kolay ölçeklendirilebilmesi gibi üstün özellikleri sayesinde kristallendirme ve kromatografi gibi geleneksel yöntemlere kıyasla biyo-ayırma alanında alternatif bir yöntem olabilir.

Rekombinant proteinlerin fermentasyon ortamından ultrafiltrasyon membranları ile ayrılması kapsamında iki ayrı üretim ortamı seçilmiştir. İlk üretim ortamı insan büyüme hormone (rhGH) içermektedir. Boyuta bağlı, selüloz mebranlarla yapılan ayırımda, istenilen ürün olan hGH'nin membran tarafından özellikle tutulduğu görülmüştür. hGH'nin bu denli tutulması hGH molekülünün kümeleşmesinden kaynaklı olabilir. Buna ek olarak hGH yüksek oranda tutulurken, yüksek molekül ağırlığındaki proteinler süzüntü kısmına geçmiştir. Bu üretim ortamında hGH hücre dışında üretilmektedir, bu yüzden tuzlar dahil bütün metabolitler besleme çözeltisinde bulunmaktadır. Ortamda tuzların bulunması, elektriksel çift katmanını azalttığı için proteinlerin geçişini arttırabilmektedir. Ayırma performansını arttırmak amacıyla pH değişikliği ve membran yüzeyi modifikasyonu gerçekleştirilmiştir. Bu deneylerin

sonucunda, besleme çözeltisinin pH değişiminin ayırmayı önemli ölçüde değiştirmediği ancak pozitif modifiye edilmiş membranın bütün proteinlerin tutulmasını arttırdığı görülmüştür. Bu üretim ortamı için ayırma davranışı üzerindeki etkisini anlayabilmek için diafiltrasyon deneyleri de yürütülmüştür. Ayırma performansının artmasına rağmen, ilk filtrasyon setinde hGH'nin yüksek oranda tutulduğu gözlemlenmiştir. Buna ek olarak selüloz membranlarında diafiltrasyon sonucunda hasar meydana geldiğinden kuşkulanılmış bu nedenle de PES membranları ile filtrasyon da denenmiştir. Ancak yine de hGH yüksek oranda tutulmuş ve beklenmedik şekilde yüksek melekül ağırlıklı proteinler geçmiştir.

Hücre içi yolla üretilen rekombinant Granulocyte-Colony Stimulating Factor (rGCSF) içeren ikinci üretim ortamı, santrifüj ve üre ile yıkama gibi birkaç ön işlemden sonra besleme çözeltisi olarak ultrafiltrasyon deneylerinde kullanılmıştır. Hem 100 kDa hem de 30 kDa MWCO membran filtrasyonlarında boyuta bağlı ayırıma bir noktaya kadar ulaşılmıştır. İstenilen proteinin daha ileri ayırımı için, besleme çözeltisinin pH'ı değiştirilmiş ve pH 5.2'ye eşit olduğunda ve 100 kDa'luk membranla yapılan filtasyon sonucunda alınan süzüntü örneklerinin 30 kDa'luk membrane filtrasyonu için besleme çözeltisi olarak kullanıldığı durumda en iyi sonuca ulaşıldığı gözlenmiştir.

Sonuç olarak, hGH ortamında, hGH her durumda seçici olarak tutulmuştur. Öte yandan GCSF ortamında, GCSF özellikle pH 5.2'ye eşit olduğunda diğer proteinlere göre seçiciliğe sahiptir.

Anahtar Kelimeler: Ultrafiltrasyon membranları, Rekombinant Proteinler

To My Family and Friends

ACKNOWLEDGEMENTS

I would like to express my great appreciation to my advisor Assoc. Prof. Dr. Zeynep Çulfaz Emecen her endless support, patience and guidance. I would also like to thank to my co-advisor Prof. Dr. Pınar Çalık for her interest and support throughout my thesis. I am very grateful to them for giving me such opportunity to work with them.

I would like to thank to Çulfaz-Emecen research group where I felt like by the side of my family and shared such a great time with them as a bio-person. I am very grateful to Kübra, Elif, Zeynep, Kaan, Canan, Faqih, Hazal and Fatma individually for accepting me as a part of the membrane girls and their amazing friendship. Also, I would like to thank to all my labmates in Industrial Biotechnology and Metabolic Engineering Laboratory for their contributions to my work in the laboratory and friendship.

I would like to express my appreciation to my dear friends Handes, Zühra, Cansu and Serap for being more than friends for me. I owe them so much. I am very grateful to İlker for being in my life with his endless support, encouragement and love.

My deepest gratitude is definitely for my family who always support me and believe me in any time. I am very fortunate to have such a great family in my life. Without them, I would not be here.

I would like also to acknowledge the financial for provided by METU Scientific Research Projects: BAP-03-04-2016-007.

Begüm Akcan

6.9.2019

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NOMENCLATURE

BSA	: Bovine Serum Albumin
CP	: Concentration Polarization
FTIR	: Fourier Transform Infrared Spectroscopy
rGCSF	: Recombinant Granulocyte Colony Stimulating Factor
rhGH	: Recombinant Human Growth Hormone
MWt	: Molecular Weight
MWCO	: Molecular Weight Cut-Off
NMP	: N-methyl-2-pyrrolidinone
KDa	: Kilodalton
PBS	: Phosphate Buffered Saline
PES	: Polyethersulfone
pI	: Isoelectric Point
PWP	: Pure Water Permeance
RC	: Regenerated Cellulose
SEM	: Scanning Electron Microscope
SDS-PAGE	: Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis
TMP	: Transmembrane Pressure
UF	: Ultrafiltration
XPS	: X-Ray Photoelectrom Spectroscopy

CHAPTER 1

INTRODUCTION

Separation comprises a large portion of the processes in the biotechnology since it is necessary to separate desired product from production environment with high purity and yield at the end of the process. Therefore, the separation must be feasible technically and economically. To achieve this objective, various types of techniques have been investigated for concentration, fractionation and purification of the product like crystallization, affinity chromatography. Among these processes, membrane technology has become an important alternative recently due to its benefits like consuming low energy and chemicals, having easy scale-up, installation and being a continuous process.

Membrane can be basically defined as a selective barrier that separates species in a mixture. In this process, one or more species are able to pass through the membrane in preference to others as a result of a driving force which leads to the permeate stream becoming enriched in these species rather than feed stream. On the other hand, some species are not allowed to pass to the other side and remain in the retentate stream [1].

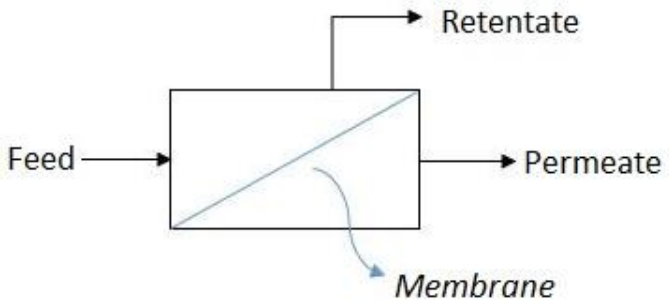


Figure 1-1.Schematic of the membrane system

Membrane applications can be applied to many industrial areas from waste water treatment to biotechnology. Especially, with the rapid growth in the field of

biotechnology, separation of biotechnological products has become tremendously important. Most of the protein based products need to be purified prior to use. Since, bioseparation cost can achieve up to 80% of the total cost, searching for cost-effective, more environmentally friendly processes gained importance in this field [2].

In protein bioseparation, achieving the product with high purity and recovery is very important [3]. The bioproducts are usually fragile hence depending on their application require specific separation conditions [1]. There are many processes for the separation and purification these products. Mostly used techniques including precipitation and crystallization suffer from poor selectivity, on the other hand, chromatographic methods like affinity chromatography have low yield of product with higher cost [4]. Since these conventional techniques have some drawbacks like having complicated steps and higher cost, alternative ways have been gaining importance at recent years. Separation with membranes do not require additives, salt and buffers in contrast to chromatographic methods. Moreover, they can be integrated into other separation processes [5]. Furthermore, membrane based processes are energy efficient, easy to scale up and require lower cost and less chemicals [4].

1.1. Ultrafiltration

There are many membrane based processes, based on different separation principles and mechanisms such as reverse osmosis, microfiltration and ultrafiltration. These processes have been already used for industrial applications. Reverse osmosis (RO) membranes are dense membranes that can reject ions and hence main application of RO membranes is the desalination of the water. Microfiltration (MF) and ultrafiltration (UF) are basically similar in terms of separation mechanism, however; microfiltration membranes have larger average pore size (0.1-10 μm) and mostly used to removal of suspended solids and bacteria. On the other hand, the average pore diameter of an ultrafiltration membrane is in the range of 1-100 nm.

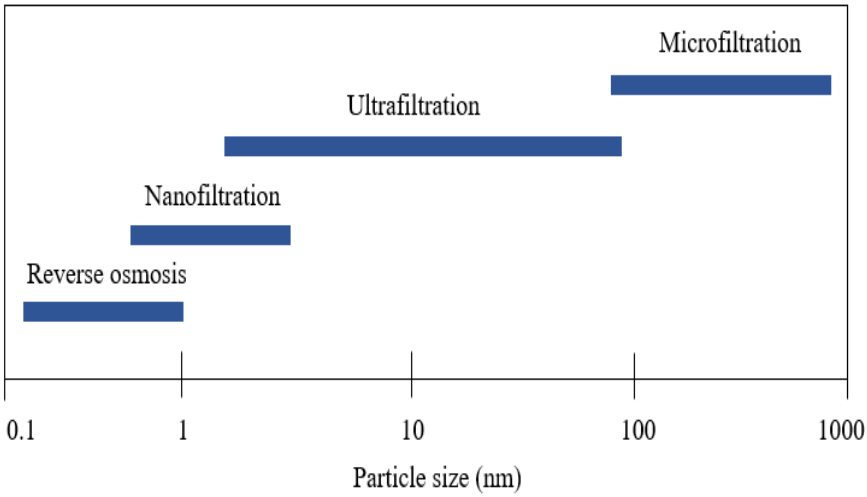


Figure 1-2. Average pore sizes of the membranes.

Ultrafiltration membranes consist of a finely porous skin layer where the separation takes place and the macroporous support layer which gives mechanical strength. Ultrafiltration is pressure-driven process which means separation occurs as a result of pressure as the driving force [6].

In ultrafiltration, basically two process modes of operation exist, dead-end and cross-flow filtration. In dead-end filtration the feed flow is perpendicular to the membrane surface so that feed solution is pressurized and forced through the membrane. Therefore, accumulation of retained particles on the membrane surface or inside the pores is easier. On the other hand, in cross-flow operation, the feed flows tangentially along the membrane surface. This way, the retained solutes accumulate less compared to the dead-end filtration that minimize fouling. For industrial applications especially, a cross-flow operation is preferable because of having less tendency to foul, however; it is a more complex and expensive operation relative to the dead-end mode [1,7]. A representative image of dead-end and cross-flow system can be seen in Figure 1-3.

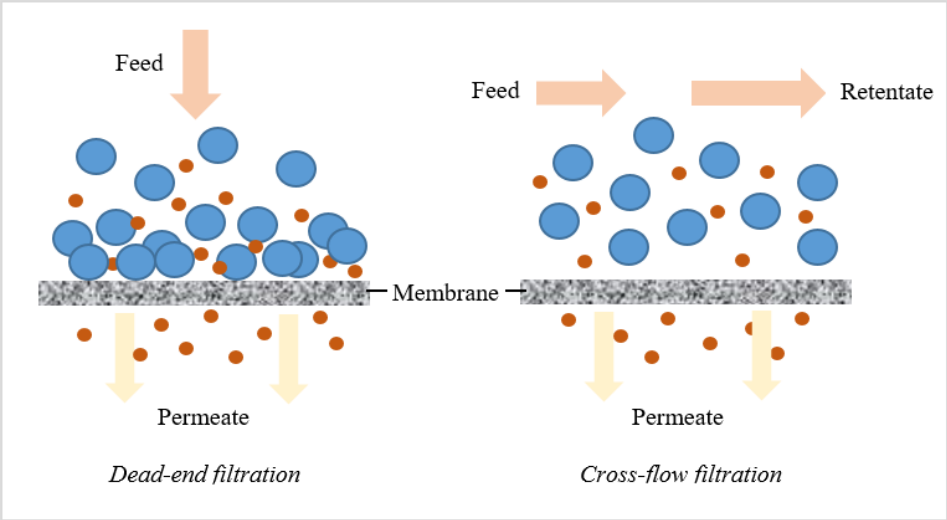


Figure 1-3. Schematic representation of dead-end and cross-flow systems.

Ultrafiltration is used especially for separation of dissolved macromolecules (like proteins) from the mixtures and also applicable for concentration and desalting of the proteins [7]. For desalting of the solution and buffer exchange, diafiltration mode is used most of the time. It is an effective method that washes out non-retained solids, in order to increase the protein content in the retentate part. In diafiltration, the initial volume in the feed tank remains constant because water or buffer is added at the same rate at which permeate is collected. With the addition of the solvent, the retentate is diluted so that the low molecular weight solutes are washed out [8]. For example, in one study, concentration of the whey proteins was performed by Baldasso and coworkers via ultrafiltration membranes instead of thermal evaporation method. In this study, diafiltration mode was used to obtain high protein content with continuous operation. It was stated that whey proteins could be separated from lactose, minerals and compounds with low molar mass. Moreover, protein concentrate having greater than 70% protein by weight was achieved [9].

Apart from protein concentration, ultrafiltration is applied for protein purification/separation. Most of the studies only deal with model protein mixtures. Since the real biological solutions usually contains low and high molecular weight materials and many of them having similar properties, separation of the proteins from

bioreactor environment is complicated process [1]. To achieve effective separation of the proteins, the system needs to be identified in terms of operating and physicochemical conditions [10]. In ultrafiltration, protein separation and purification can be accomplished basically according to the size difference as well as charge difference [4].

1.1.1. Sized Based Separation

In principle, separation by ultrafiltration is based on size exclusion. Difference between the pore size of the membrane and the molecular size of the proteins in a mixture plays role in the size based separation.

Detecting the average pore diameter in a membrane is difficult, so *molecular weight cutoff (MWCO)* concept is used most of the time. The mean molecular weight of the globular protein molecule that is 90% rejected by the membrane is defined as MWCO of an ultrafiltration membrane [6]. Therefore, by looking at the MWCO of a membrane, one can select a proper membrane for sized based separation.

In literature, separation based on size was performed with model protein mixtures; however, some studies were conducted with chicken egg white (CEW) that may be more close to real biological mixtures. Ghosh et al reported that the purification of lysozyme from CEW with 25 kDa polysulfone membrane was achieved by using size difference. CEW is a protein mixture that mainly contains ovalbumin (Mwt 45 kDa, pI=4.5), conalbumin (Mwt 80 kDa, pI=6.1), ovomucin (Mwt 5.5-8.3 x 10^3 kDa, pI=4.7) and lysozyme (Mwt 14.3 kDa, pI=11). Since the proteins except lysozyme had high molecular weights, it was largely retained by the 25 kDa MWCO membrane. Moreover, ultrafiltration was also carried out with 50 kDa MWCO membrane. However, in this case, permeation of ovalbumin as well as other proteins was expected to be higher than 25 kDa MWCO membrane since its molecular weight was too close to membrane MWCO value. For this reason, it was more difficult to obtain pure lysozyme by using only size difference. Therefore, the process parameters were altered and optimized for efficient purification of the lysozyme with this membrane filtration [4].

In another study, α-lactalbumin (α-LA) and β-lactoglobulin (β-LG) were tried to purified from whey protein isolate by using 100 kDa and 30 kDa composite regenerated cellulose membranes. In this study, two-staged ultrafiltration process was applied by using 100 kDa membrane filtration followed by 30 kDa and one with reverse order. In both strategies, α-LA was obtained at above 85 % yield. β-LG yield was achieved as about 70% [11].

In all of these studies, separation is purely based on size exclusion that was limited even in model protein mixtures. Some interactions such as protein-protein or membrane-protein interactions are involved during the separation of proteins. Therefore, further improvement is needed for the separation of proteins having similar size by adjusting some process parameters.

1.1.2. Charged Based Separation

In the separation of similarly sized proteins, size based separation may not be sufficient. Therefore, changing some process parameters such as changing pH of the solution to alter the charge of the proteins and the membrane, or membrane modification becomes an alternative in order to obtain better separation. In this case, separation is dominated by electrostatic attractive/repulsive interactions that take place between protein-protein or protein-membrane [12].

Within this context, pH of the feed solution is the most commonly used method. In one study, ultrafiltration was performed again with chicken egg white. Wan et al, tried to separate lysozyme from natural chicken egg white by using 30 kDa polyethersulfone (PES) membranes by manipulating some parameters like pH. As a result of this study, transmission of lysozyme through PES membrane was found to be pH dependent. At pH 6.0 and 10.0, higher lysozyme transmission was observed contrary to expectations because generally highest transmission was expected at pI of the proteins. Although pI of the lysozyme is 11, transmission decreased when pH was adjusted to 11. Several reasons were reported for this behavior such as pH change leading alteration on the electrical double layer thickness. Other reason could be protein-membrane repulsion. Moreover, pH change could result in conformational changes and dimerization of lysozyme that effect the transmission through the membrane [10].

Apart from pH change of the feed solution with the aim of using electrostatic attraction/repulsion effects in ultrafiltration, Van Reis and coworkers invented a method for the modification of the membrane to enhance the separation behavior. In this method, membrane was modified in order to create charged groups that can cause electrostatic interactions between membrane and proteins. By this way, it is expected that desired protein can be separated from other proteins in the medium selectively since like-charged proteins are highly rejected due to repulsion by same charged membrane whereas uncharged or having oppositely charged proteins tend to pass to the permeate side [13]. In this invention, cellulose membranes were modified either negatively or positively based on the procedure given in patent [14]. Moreover, modified membranes were tried for the separation of the binary protein mixtures. For example, using negatively charged membrane, the separation of BSA (pI 4.8) and Fab (Antibody-binding fragments) (pI 8.1) was achieved. The filtration was conducted using pH 8 buffer at which Fab was uncharged and BSA was negatively charged. Therefore, BSA was highly rejected while uncharged Fab was allowed to pass through the negatively modified membrane [14].

Arunkumar and co-workers also investigated the membrane modification effect. In this work, α-LA (Mwt 14.4 kDa, pI=4.4) and β-LG (Mwt 18.4 kDa, pI=5.2) were separated from bovine milk serum with the help of a 300 kDa positively charged regenerated cellulose ultrafiltration membrane. As it was seen, membrane MWCO was 15-20 times higher than molecular weight of the proteins in the mixture. However, making modification to the membrane led to repulsion between β-LG and the membrane since they both were positively charged. On the other hand, α-LA had no net charge at pH 4.3, so it had no influence from charge interactions and easily passed through the membrane. At the end of the experiments, it was reported that it was possible to have a selectivity 71% greater by replacing this positively modified membranes with unmodified ones [15].

A similar study has been made on the fractionation of the binary mixture containing bovine serum albumin (BSA) and lactoferrin (LF) by using 100 kDa charged composite regenerated cellulose ultrafiltration membranes. In this study, pH optimization and

membrane surface modification were combined to enhance the separation of these similarly sized proteins where BSA is a 66.5 kDa protein with pI approaching 5 and molecular weight of LF is 78 kDa and its pI is 9.0. When the pH was set to 5.0, it was seen that LF was positively charged and BSA had negative charge. At this pH, almost complete separation was achieved with positively, negatively charged or uncharged membranes but positively charged membrane had highest separation factor in this case. It was reported that it was probably because of the repulsion between membrane and LF since both were positively charged. When pH was equal to 9, both BSA and LF was found to be negatively charged. This was unexpected in terms of LF because of having pI point of 9 that made LF was uncharged at his pH. Therefore, in this case, with the use of negatively charged membrane, BSA was highly rejected since there was protein-membrane charge repulsion [16].

Lebreton et al. were studied with the feed stream containing recombinant proteins as well as host cell proteins. In this study, purification of DNA derived antibody fragment (Fab'2) by using high performance tangential flow filtration (HPTFF) were tried to be performed. Positively charged 100 kDa MWCO composite regenerated cellulose membranes were used in filtrations under the optimized conditions. It was stated that, Fab'2 has molecular weight of 99 kDa and pI point of 9.1. Optimum operating condition was selected as pH=5.6 at which Fab'2 is positively charged. Apparently, positively charged Fab'2 was highly retained by positively charged membrane. At the end of the process, overall yield was obtained as 98% which was 86% when the conventional chromatography method was applied [17].

Consequently, the fractionation of the proteins benefits from charge interactions by selecting proper pH values, or modifying the membrane surface can enhance the protein separation performance.

1.2. Concentration Polarization and Fouling Concept

Membrane-based systems have been subject of considerable interest in the field of protein separation and purification. However, the major factors determining the membrane performance which are concentration polarization and membrane fouling can create some drawbacks for these systems. All membrane processes are affected by these problems that can cause decrease in membrane life and accordingly increase the cost of the separation process [6].

During filtration, some components not permeating thorough the membrane are enriched in the feed side of the membrane that increase the concentration of these retained components near the membrane surface. This phenomenon is named as concentration polarization which is a reversible process. At low pressures, concentration polarization is small since flux is low. As applied pressure is increased, flux and the concentration of the retained molecules increases. Further increase in the concentration of the retained components leads to deposition of these molecules on the membrane wall. After some point which is known as limiting flux, a cake or gel layer starts to form on the membrane surface resulting in membrane fouling. In addition to the gel/cake layer formation, solid particles can penetrate into the membrane pores that leads to blockage of the pores known as internal fouling. Most of the time fouling create irreversible alteration on membrane that can affect the membrane performance by lowering its flux and reducing the transportation of the solute thorough the membrane [7,19,20].

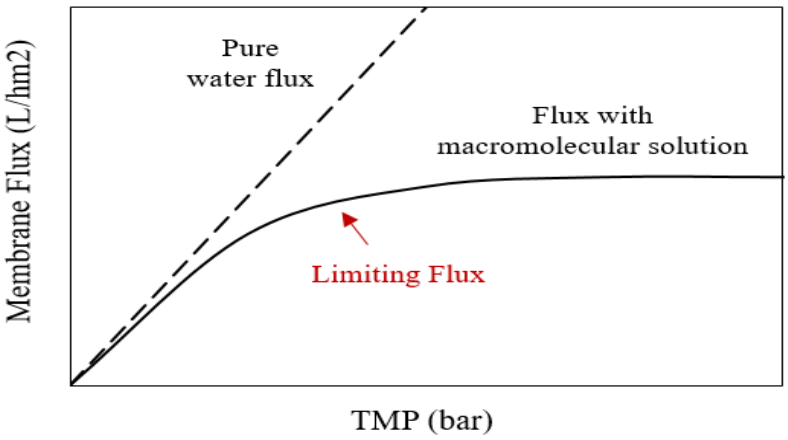


Figure 1-4. Membrane flux vs TMP graph.

Since the adsorption of the proteins onto the membrane surfaces or pore walls is easy, they are difficult foulants to deal with. In the case of fouling, cake/gel layer can behave as a secondary barrier that can cause a decrease in permeate flux and change the solute transmission [20].

1.3. Recombinant Proteins

Current research and developments in the area of protein bioseparation focus on therapeutic proteins which can be defined as proteins that are used in the treatment of some diseases [2]. Since proteins have become an integral part of the pharmaceutical industry and biotechnology for diagnostic and therapeutic purposes, these proteins can be produced by using genetic engineering techniques including recombinant DNA technology [21].

In recombinant DNA technology, desired DNA sequences that do not occur in nature can be created artificially. With the help of this method, DNA fragments containing specific genes of interest are taken from different species, combined and transferred into the host organisms like yeast or bacteria. By this way, organism can be able to produce a desired product which can be purified from production medium in order to use in related area [22].

There are various types of recombinant DNA derived proteins available for pharmaceutical industry such as monoclonal antibodies, growth factors, therapeutic enzymes [23]. In our study, granulocyte-colony stimulating factor (GCSF) and human growth hormone (hGH) were selected as recombinant proteins that were produced in bioreactor environments and needed to be purified for further applications.

1.3.1.Human Growth Hormone (hGH)

Human growth hormone (hGH) is a single chain polypeptide consisting of 191 amino acid residues with a molecular mass of approximately 22 kDa [24]. hGH is synthesized naturally in human body under normal conditions and controls growth so it is necessary to be taken in the case of its deficiency. It has been used for therapeutic applications in the treatment of dwarfism, bone fractures and skin burns. Furthermore, recent studies

show that hGH plays critical role in the aging process and regulation of the immune functions [31, 32].

Various separation techniques have been used to purify recombinant hGH from fermentation broths that requires high purity and low pyrogen (bacterial endotoxin) level due to its therapeutic use [24]. For the isolation of high purity proteins chromatographic methods have been performed typically using adsorption chromatography including ion exchange, hydrophobic interaction and metal-chelate affinity columns [23]. A widely employed purification method involves the use of peptide affinity tags like polyhistidine residues. Polyhistidine affinity tags are protein-specific moities which are fused to the protein of interest and able to bind a ligand that are used in recombinant protein purification via affinity chromatography. A polyhistidine tag is inserted within the recombinant sequence to purify the desired protein on an affinity column. However, in this method, polyhistidine tags can bind unspecific parts of the proteins due to natural abundance of histidine in protein structure [33,34]. In addition to that, resin-based chromatographic systems are expensive and require large quantities of salts and buffers during process.

As an alternative to the chromatography, Catzel and coworkers worked with gradiflow preparative electrophoresis technology in order to purify recombinant hGH which was produced extracellularly. This technology is based on electrophoretic movement of the proteins through polyacrylamide membranes with defined pore size. By this way, separation is affected by both size and charge. In this study, pH values were controlled with buffer solutions. As a result, 50 mM Tris/Hepes buffer at pH 7.5 with 50 kDa MWCO membrane gave the best result for the separation. Consequently, recombinant hGH purity was obtained as around 98% [23].

1.3.2. Granulocyte-Colony Stimulating Factor (GCSF)

Granulocyte-colony Stimulating Factor (GCSF) is a glycoprotein and naturally occurring hormone that stimulates the bone marrow to produce white blood cells. GCSF is hydrophobic molecule and has a molar mass of about 19 kDa [29]. GCSF can be produced via recombinant DNA technology in order to be used in therapeutic applications [30]. GCSF plays critical role in immune functions and host defense

against bacterial and fungal infections since it stimulates the bone marrow to produce white blood cells [26,27,28]. Therefore, GCSF has been widely used in the treatment of the cancer patients suffering from chemotherapy-induced side effects [34].

1.4. Aim of the Study

In this study, separation and purification of desired recombinant proteins from production medium by ultrafiltration membranes was investigated. The recent studies about protein separation by using membranes mainly focus on the separation of model binary protein mixtures rather than complex bioreactor environment. In this study, two separate production media containing human growth hormone (hGH) and granulocyte colony stimulating factor (GCSF) were selected as recombinant proteins. For this purpose, ultrafiltration experiments were performed to separate these proteins from complex media based on size and charge difference. The effect of some process parameters such as MWCO of the membrane, pH of the feed solution, membrane modification and membrane type were observed and tried to optimized to obtain better separation.

CHAPTER 2

EXPERIMENTAL METHODS

2.1. Materials

Commercial regenerated cellulose flat membranes of MWCO 100, 30, 10 and 5 kDa and polyethersulfone (PES) membranes of MWCO 100 kDa were purchased from Millipore. Model proteins, casein, from bovine milk (Mwt=22-25, pI= 4.2-5.1) and albumin, from bovine serum (BSA, Mwt= 66 kDa, pI=4.7) were supplied by Sigma Aldrich and Fluka respectively. 1-bromopropanesulfonic acid sodium salt (≥97%) and (3-bromopropyl) trimethyl-ammonium bromide (97%) were used for membrane modifications and both provided from Sigma Aldrich. For dye sorption tests, Brilliant Blue R (826 Da) were purchased from Sigma Aldrich, Crystal Violet (408 Da) from Merck.

Phosphate buffered saline (PBS), Pluronic F-127, N-Methyl-2-pyrrolidone (NMP) (99%), hydrochloric acid (HCl), sodium hydroxide (NaOH), pure ethanol and urea were purchased from Sigma Aldrich. Poly(ether sulfone) (PES,Ultrason E6020P) sodium hypochloride (NaOCl) was provided from BASF and Merck respectively.

For silver staining SDS-PAGE washing step procedure, acetic acid, potassium carbonate and sodium dodecyl sulfate were obtained from Sigma Aldrich, methanol and silver nitrate from Merck, sodium thiosulfate pentahydrate from Fluka. For gel preparation step, TGX Stain-Free FastCast^{TM} Acrylamide kit, ammonium persulfate (APS) and N,N,N',N'tetramethylethylenediamine (TEMED) were used supplied from Bio-Rad.

Ultra-pure (UP) water was used in solutions and permeance measurements and reverse-osmosis water (RO) was used as nonsolvent for coagulation step.

2.2.Solution Preparation

2.2.1. Model Proteins

Model protein solutions were prepared at the concentration of 0.1 g/L by dissolving, BSA and casein in buffered solution (PBS, Phosphate Buffered Saline) at pH=7.4. Casein solution was also heated up to 38°C to achieve complete dissolution.

2.2.2. Polymer Solutions for Membrane Preparation

PES was used as polymer in the membrane production and dried at 80°C. In order to make the surface of the membrane hydrophilic, Pluronic F-127 was used and put into vacuum overnight prior to use. After addition of the NMP as solvent, solutions were allowed to stir with magnetic stirrer. Final polymer solutions were filtered with 300 mesh metal filter to get rid of any suspended solids. Different polymer solutions were prepared having different polymer concentrations in order to be used in membrane preparation (Table 2-1).

Table 2-1. Polymer solutions for membrane preparation.

Solution Code	**Composition**
A solution	10 wt% PES, 5 wt% Pluronic F-127, 85 wt% NMP
B solution	15 wt% PES, 7.5 wt% Pluronic F-127, 77.5 wt% NMP

2.3.Membrane Preparation

In order to obtain flat sheet PES membranes, prepared polymer solution was spread on a glass plate by using 250 μm casting bar at room temperature. Cast polymer film was directly put into coagulation bath about for 10 minutes. After coagulation, membranes were washed in RO water by refreshing the water a few times for 24 hours. After that, membranes were kept in 20% ethanol-80% water solution to prevent any microbial growth.

2.4. Production Medium Pretreatment

2.4.1. rhGH

The production medium containing rhGH was produced by *Pichia pastoris* extracellularly [35] in bioreactor (Sartorius Stedim, Frigomix 1000). Composition of produced medium and concentration of the components are listed in Appendix E.

After collection of the samples, which coming from bioreactor, centrifugation was applied (Hermle Z 323 K) at 4°C, 1500 rcf, 10 minutes to remove cells from production medium. Final solution can be used for ultrafiltration experiments as feed or stored at refrigerator at -80°C.

2.4.2. rGCSF

GCSF was produced by *Escherichia coli* intracellularly. As GCSF is an intracellular product, all proteins and metabolites comes from organism are needed to be removed to the outside of the cell. For this reason, high pressure homogenizer was used for cell disruption in METU Central Lab- Molecular Biology and Biotechnology R&D Department. Solution was pretreated prior to use with urea solutions to get rid of the refolding of the inclusion bodies. To achieve final solution, 2.5 M urea solution was prepared and mixed with production media in proportion of 4:1. After complete dissolution of the mixture, solution was centrifuged at 25°C, 10000 rcf, 10 minutes. Solid part was mixed with another 2.5 M urea solution and same procedure was followed. At the end, again solid part was taken but in this case, mixed with 7.5 M urea solution and left to shake for 6 hours in ice bath [36]. After all steps were completed, final centrifugation was applied at the same conditions and liquid part of the solution (supernatant) was collected in order to use in ultrafiltration experiments or stored at refrigerator at 4°C. in Appendix E.

2.5.Ultrafiltration Experiments

Ultrafiltration experiments were conducted in dead-end mode using stirred ultrafiltration cell (Amicon, Millipore) of 10 ml or 50 ml volume. Schematic drawing for ultrafiltration system can be seen in Figure 2-1.

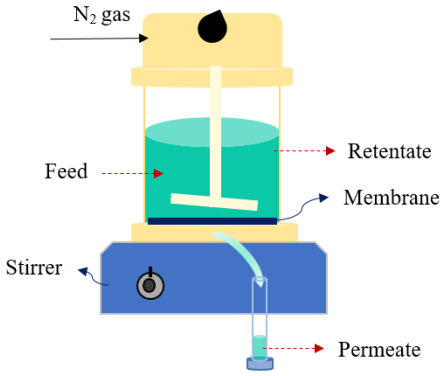


Figure 2-1. Schematic Drawing of Filtration System

Before and after filtration experiments, pure water permeance (PWP) values were measured to examine the fouling behavior of the membrane. PWP was calculated according to the formula given below in L/hm^{2}bar.

$$PWP = \frac{J}{TMP} \qquad 2.1$$

where J refers to permeate flux (L/hm^2) and TMP is transmembrane pressure difference (bar).

Different pressures depending on membrane MWCO was applied. The slope of the J vs TMP graph gave the pure water permeance value.

For filtration, solutions were fed to the system at certain volume while stirring at 250 rpm in order to minimize concentration polarization. Under certain TMP, every 2 or 10 ml of sample was collected as permeate and time was recorded for each permeate sample. After filtration, the remaining solution in the stirred cell was taken as retentate. Then, physical cleaning was applied by filling empty cell with water and stirring the water for 15 minutes. In case physical cleaning was not sufficient to remove fouling,

chemical cleaning was performed with 0.1 M NaOH or 500 ppm NaOCl solution where the membrane was allowed to stay for 10 minutes. After cleaning, PWP was expected to reach its initial value if fouling were totally cleaned.

In cases where the pH of the medium was changed before filtration, pH of the feed solution was adjusted to desired value by using 0.1 M HCl or 0.1 M NaOH and was controlled with pH meter (Sartorius).

2.6. Silver Staining SDS-PAGE

2.6.1. Gel Preparation and Electrophoresis

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was used to visualize separation performance of the ultrafiltration experiments and identify the retention values of proteins.

For this analysis, feed, permeate and retentate samples from ultrafiltration experiments were prepared based on the procedure. In this procedure, 20 μL of each sample was mixed with 10 μL of loading buffer, and then 15 μL of the mixture were loaded to the wells of SDS-PAGE gels. To make comparison, prestained protein molecular weight marker was loaded as 2 μL and standard (commercially available, pure protein) was loaded as 15 μL like other samples. Under constant electrical current (200 V), samples were run through the gel about 50 minutes.

SDS polyacrylamide gel preparation and electrophoresis procedures in detail are as follows:

1. Gel solutions were prepared based on procedure in 12% TGX Stain-Free ™ FastCast™ acrylamide kit (Bio-Rad).

2. 1 mm Bio-Rad glass plates were placed for dropping of the gel solution between them.

3. After pouring of the solutions, comb was inserted and sufficient time (at least 45 minutes) was allowed for polymerization reaction.

4. Before sample loading, wells were dried completely with filter paper.

5. The glass plates were put into container and then, the space between glass plates and gel wells were completely covered with running buffer solution.

6. Samples were loaded and electrical current was applied as it is described above.

Solutions used during SDS-PAGE were as follows:

- 10% (w/v) APS (Ammonium PerSulfate): 0.1 g APS is added to 1 mL of distilled H_2O.
- Resolving gel: 6 ml BioRad FastCast© resolving gel solution, 30 µl ammonium persulfate and 3 µl TEMED are mixed.
- Stacking gel: 2 ml BioRad FastCast© stacking gel solution, 10 µl ammonium persulfate and 2 µl TEMED are mixed.
- 4X SDS-PAGE Sample loading buffer: 200 mM Tris-HCl at pH 6.8, 40% glycerol; 6% SDS and 0.013% ml Bromophenol blue were mixed and stored at -20°C.
- 5X SDS-PAGE Running buffer: 15 g Tris Base, 72 g glycine, 5 g SDS was put into bottle and completed with distilled H_2O to 1 L and stored at +4°C. Before it is used for electrophoresis, the solution was diluted at the ratio of 1:4.

2.6.2. Silver Staining Procedure

After running was finished, gels were separated from glass plates and washing steps were applied for each gel as described in Laemmli et al [37]. At the end of the process, proteins were visualized with the help of the silver impregnation. Solutions and detailed procedure was given as follows:

- Fixer: 100 ml methanol, 24 ml acetic acid, 100 µL 37% formaldehyde were mixed and ultra-pure water was added up to 200 mL. The gels in the mixture were left to shake at least for 1 hour.
- Ethanol: %50 ethanol solution was prepared and gels were washed with this solution three times for 20 minutes.

- Pretreatment: 0.05 g $Na_2S_2O_3.5H_2O$ was dissolved in 200 ml distilled water. 2 ml of the solution was set aside for further use in developing solution preparation.
- Silver nitrate: 0.2 g silver nitrate was put into bottle and 100 ml ultra-pure water and 75 µl 37% formaldehyde were added. Gels were treated for 20 minutes with this mixture.
- Developing: 2.25 g potassium carbonate was dissolved in 100 mL ultra-pure water. 2 ml from pretreatment solution and 75 µl 37% formaldehyde were added. Gels was washed this solution until protein bands starts to appear.
- Stop: 50 ml methanol, 12 ml acetic acid were mixed and ultra-pure water was added up to 100 mL. This mixture was used to store gels.

2.7. Retention Measurement

For model proteins, % retention was calculated according to the formula given in Equation 2.2.

$$\%\ Retention = \left(1 - \frac{< C_P >}{(C_R + C_F)/2}\right) X\ 100\ \% \qquad 2.2$$

where $<C_P>$ is average concentration value for permeate, C_R and C_F represent retentate and feed concentrations respectively. Concentrations were determined by using UV/VIS spectrometer (Schimadzu UV-1601). Calibrations for BSA and casein solutions can be seen in Appendix A.

Retention values for production environment were determined by a using computer program (LabWorks) based on optical density difference after SDS-PAGE analysis. Optical densities were measured for feed, permeate and retentate protein bands. Concentration of standard was known. Therefore, the other values were compared with this known concentration to achieve unknown concentration. %Retention was calculated based on Equation 2.2. This method gives a rough estimate of optical densities. Therefore, the concentrations as well as the retentions were approximate values. Sample calculation can be found in Appendix B.

2.8. Bradford Assay

Bradford assay is performed to determine the total protein concentration in a sample. In this assay, the absorbance is measured for each sample at 595 nm by UV/VIS spectroscopy. The unknown concentration of the proteins in a sample were found by making comparison to calibration curve which was prepared using BSA with known concentrations as it was stated in the procedure (Appendix C).

2.9. 2D-Electrophoresis

In order to get information about molecular weight and isoelectric points of the proteins, two dimensional electrophoresis was performed. One dimension represents the molecular size distribution of the proteins like SDS-PAGE while the other dimension shows isoelectric points of the proteins. 2D-electrophoresis analysis was conducted at METU Central Lab- Molecular Biology and Biotechnology R&D Department.

2.10. Surface Modification

Modification of the membrane surface were performed according to the procedure of van Reis [14]. Briefly, for positive modification, 0.5 M (3-bromopropyl) trimethyl-ammonium bromide (97%) was dissolved in 0.1 M NaOH and the membranes were waited in this mixture for 24 hours. On the other hand, to obtain negatively charged membrane similar procedure was followed with different chemical. The mixture of 2 M 1-bromopropanesulfonic acid sodium salt (≥97%) in 0.1 M NaOH was prepared and again membranes were waited for 24 hours.

2.11. Dye Sorption Test

Dye sorption test was performed to understand whether the charge modification was achieved or not. In order to do this test, negatively charged dye was selected as Brilliant Blue R. Positively modified and unmodified membranes put into separate containers which contains same amount of Brilliant Blue R-water solution. Color change on the membrane surface and the solution was observed over time. Same procedure was applied with positive dye which is crystal violet to check negatively modified membranes.

2.12. XPS Analysis

X-ray Photoelectron Spectroscopy (XPS) analysis was conducted to observe the extent of modification. After modification, it was expected that some groups were connected to the membrane surface. For positive modification, substitution of the hydroxyl groups of cellulose membrane with propyl-trimethyl-ammonium groups expected to take place. For negative modification, on the other hand, propyl sulfonate groups have to be connected if the modification is achieved successfully. As a result of this, sulphur (S) and nitrogen (N) elements in the added groups which are not present in the base membrane can be detected by XPS analysis that was conducted in METU Central Lab.

2.13. FTIR Analysis

Fourier Transform Infrared Spectroscopy (FTIR) measurement was also performed for modified membranes. For this purpose, membranes were allowed to stay in the modification solution for 2,6,23 and 30 hours to observe degree of modification and then, put into vacuum before FTIR analysis. The spectra of membranes were taken between 500 to 4000 cm^{-1} wavenumber. The peaks are expected at 1000-1350 cm^{-1} for C-N bond for positively modified membrane and at around 1350 cm^{-1} for S=O bond for negatively modified membrane. Moreover, OH bond gives broad peak around 3200-3600 cm^{-1} that must be disappear as a result of the modification. FTIR analysis results can be found in Appendix D.

2.14.Membrane Morphology

Membrane morphologies were examined by using scanning electron microscope (SEM, QUANTA 400F Field Emission SEM) in METU Central Lab. Before analysis, membranes were frozen in liquid nitrogen, and then broken immediately. After drying in vacuum overnight and the surface and cross-section of the fractured membrane were sputter coated with Pd/Au. SEM micrographs generally indicate the surface of the membranes as well as the cross-sections of them. Cross-section can be either support side or non-solvent side. Nonsolvent side can be defined as the side of the membrane that is facing the nonsolvent during coagulation, on the other hand, support side represents the part contacts with the glass plate during coagulation.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Model Proteins

Since production medium is a complex mixture of proteins, salts and other dissolved molecules, first of all, model proteins were used in ultrafiltration experiments to understand the separation behavior of the membranes selected. As model proteins, bovine serum albumin (BSA, Mwt= 66 kDa, pI=4.7) and casein (Mwt=22-25 kDa, pI= 4.2-5.1) were selected and ultrafiltration experiments were conducted by using different MWCO commercial regenerated cellulose membranes at certain transmembrane pressure at ambient temperature. For BSA, 30 kDa MWCO membrane was chosen and as a result of the ultrafiltration experiment, it was seen that retention value of the BSA was very high (99.5%), as expected. On the other hand, casein was tested with 10 kDa and 30 kDa MWCO membranes. Retention of the casein was determined as 98% when 10 kDa MWCO membrane was used. This was expected due to the size difference between membrane pore size and the size of the protein. In the case of 30 kDa MWCO membrane filtration, retention value was detected as 97%. High retention in this filtration was thought to result from fouling during the filtration. Since casein is smaller in size, it was easier to go into the membrane pores. Therefore, it can create pore blockage which cause fouling of the membrane. Moreover, fouling was understood from permeance values in Figure 3-1. As it was seen in the figure, for this filtration, permeance values during filtration were lower than PWP values that was the indication of fouling. However, in the case of 10 kDa MWCO membrane filtration, there was less fouling compared to 30 kDa MWCO membrane filtration since decrease in permeance values during filtration were less. As the pure water permeance values before and after ultrafiltration experiments were in the same level for all filtrations, it was understood that physical cleaning (mixing with water) of the membranes was possible and the membranes were reusable.

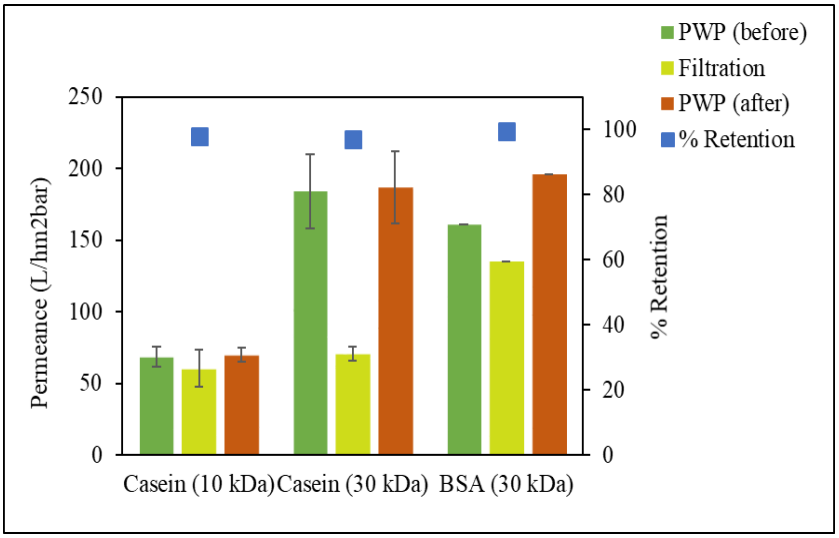


Figure 3-1. Permeance and retention values for model proteins

3.2. Recombinant hGH

Separation behavior was investigated with different production media. The first medium contained rhGH which was produced extracellularly in bioreactor environment.

3.2.1. Characterization of the Production Medium

After collection and centrifugation of the bioreactor sample, size and charge distribution of the proteins in the final solution was analyzed by using SDS-PAGE and 2D-electrophoresis. Each lane in the SDS-PAGE in Figure 3-2 represents the bioreactor sample with a certain dilution factor. Marker shows the certain molecular weights (Appendix F). Therefore, by observing the level of the marker, size of the proteins in a sample can be determined. Moreover, standard (pure protein) is used to ensure that the presence of desired protein in the production medium.

Molecular weight of rhGH can be easily seen as 22 kDa from SDS-PAGE image. Other proteins in the production environment line up from 10 to 170 kDa range. Additionally, isoelectric point of hGH was detected as around 5.5 from the 2D-electrophoresis results.

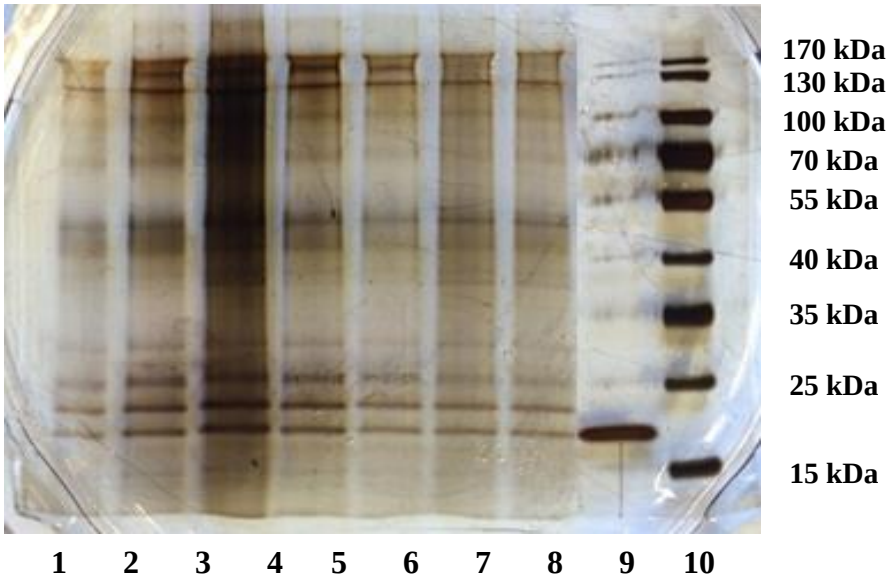


Figure 3-2. SDS-PAGE image of the hGH production medium (BR).
1) Reactor sample (2X); 2) Reactor sample (4X); 3) Reactor sample (7X);
4) Reactor sample (10X); 5) Reactor sample (20X); 6) Reactor sample (30X);
7) Reactor sample (40X); 9) hGH standard (50 mg/L); 10) Marker.

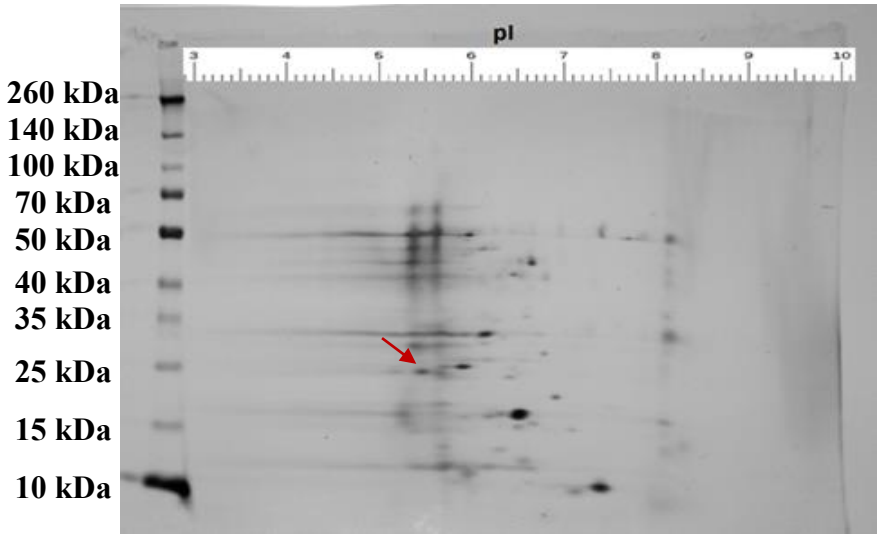


Figure 3-3. 2D-electrophoresis image of the hGH production medium (BR).

3.2.2. Separation Behavior for rhGH Environment

Ultrafiltration experiments were conducted based on size and charge difference of the proteins and the membrane to separate rhGH from production medium. Information about filtrations for this medium was listed in Table 3-1.

Table 3-1. Filtration codes and conditions for rhGH environment.

Filtration Code	Bioreactor Sample No	Membrane MWCO	Pressure (bar)	pH
H-BR15-100	BR15	100	0.5	5.5
H-BR15-30	BR15	30	2	5.5
H-BR15-10	BR15	10	3	5.5
H-BR15-5	BR15	5	3	5.5
H-BR14-100	BR14	100	0.5	5.5
H-BR14-30-7.7	BR14	30	2	7.7
H-BR14-30-6	BR14	30	2	6
H-BR14-30-4.5	BR14	30	2	4.5
H-B6-100	B6	100	0.5	5.5
H-B6-30	B6	30	2	5.5
H-BD-100	BD	100	0.5	5.5
H-BD-30P-2b	BD	30	2	5.5
H-BD-30D-0.5b	BD	30	0.5	5.5
H-BD-30D-0.1b	BD	30	0.1	5.5
H-BD-30P-PM	BD	30	2	5.5
H-BD-30P-NM	BD	30	2	5.5
H-BD-100-PM	BD	100	0.5	5.5
H-BD-30-DIA	BD	30	0.1	5.5
H-BD-30-DIA-4.5	BD	30	0.1	4.5
H-BD-30-DIA-7	BD	30	0.1	7
H-BD-100-PES	BD	100	0.1	5.5

In Table 3-1, D represented that direct production medium was used as feed solution for filtrations whereas P represented that permeate samples of 100 kDa membrane filtration were used as feed solution for filtrations. PM and NM indicated that filtrations were conducted by using positively modified and negatively modified membranes respectively. DIA term was used for diafiltration experiments.

3.2.2.1. Size Based Separation

Initially, ultrafiltration experiment was performed with 100 kDa MWCO membrane under the transmembrane pressure of 0.5 bar by using centrifuged production medium (BR15) as feed solution. Before and after each filtration, pure water permeance (PWP) were calculated. As a result of the experiment of H-BR15-100 (hGH-100 kDa), the proteins having molecular weight higher than 55 kDa were rejected by the membrane while the other proteins including hGH passed to the permeate side. Although, hGH was seen in the permeate side, retention was calculated as about 86% which was a high retention value that can arise from cake formation during filtration. From Figure 3-6, it was clearly seen that there was sharp decrease in permeance which implies presence of fouling. However, physical cleaning can restore the PWP.

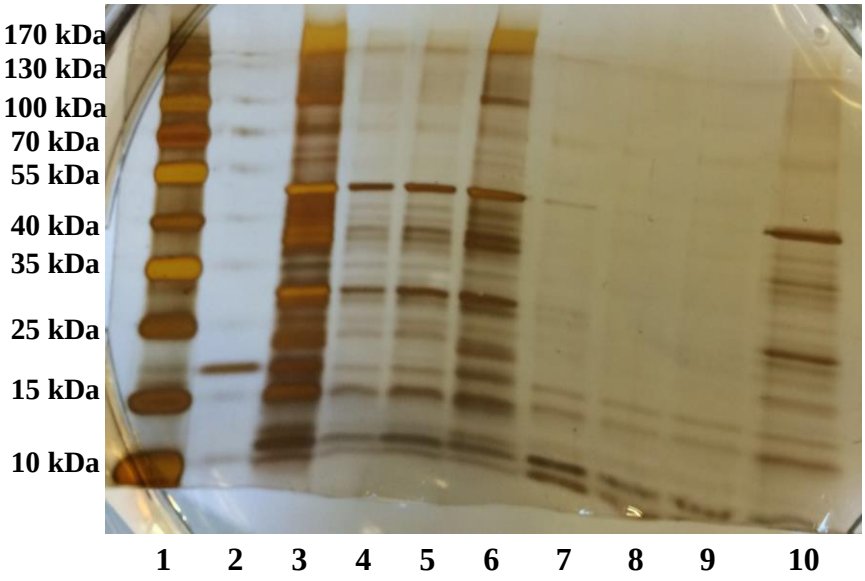


Figure 3-4. SDS-PAGE images of H-BR15-100 and H-BR15-30 filtrations. 1) Marker; 2) hGH standard (50 mg/L); 3) Feed solution of H-BR15-100 filtration (10X); 4-5) Permeates of H-BR15-100 filtration (10X); 6) Retentate of H-BR15-100 filtration (20X); 7-8-9) Permeates of H-BR15-30 filtration (no dilution); 10) Retentate of H-BR15-30 filtration (10X).

After that, permeate sample of H-BR15-100 filtration were used as feed solution for H-BR15-30 (hGH-30 kDa) filtration. In this case, hGH and the proteins below the hGH passed to the permeate side while the other proteins were rejected over time. At the end of the filtration, presence of hGH and smaller molecular sized proteins in the permeate

side can be seen from Figure 3-4. In this time, retention of the hGH was measured as 93%. While losing much of hGH, separation from larger proteins was achieved to some extent with these filtrations.

H-BR15-30 filtration permeate samples were collected to be used as feed solutions for H-BR15-10 and H-BR15-5 filtrations. H-BR15-10 was the filtration in which 10 kDa MWCO membrane was used. In this filtration, it was observed that, there was no change between the feed and the permeates. On the other hand, in H-BR15-5 filtration was performed with 5 kDa MWCO membrane, there was no protein transmission thorough the membrane (Figure 3-5). In Figure 3-6, there was sharp decrease in permeance values for H-BR15-100 and H-BR15-30 filtrations that indicated that the formation of fouling which was removed by physical cleaning. In H-BR15-10 and H-BR15-5 filtrations, on the other hand, PWP and permeance values during filtration were almost same level.

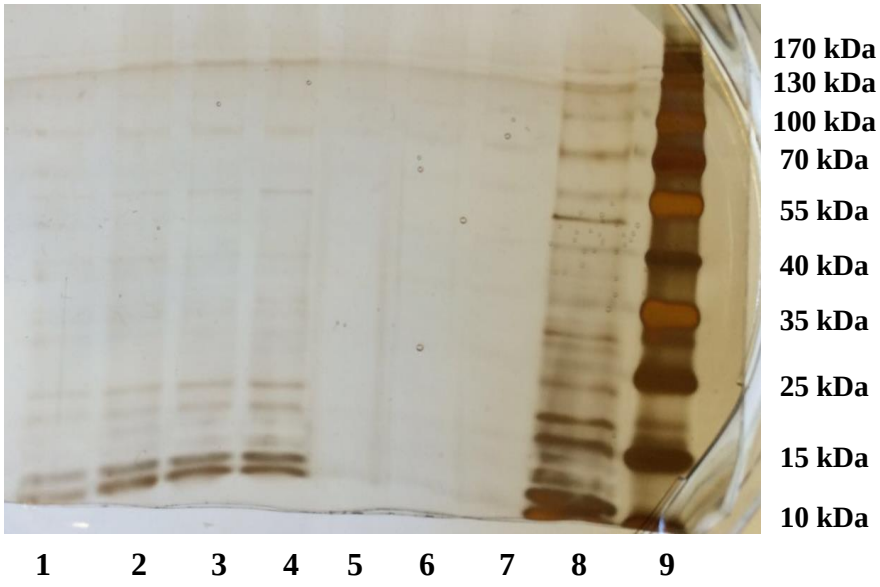


Figure 3-5. SDS-PAGE images of H-BR15-10 and H-BR15-5 filtrations.
1-2-3) Permeates of H-BR15-10 filtration (no dilution); 4) Retentate of H-BR15-10 filtration (no dilution); 5-6-7) Permeates of H-BR15-5 filtration (no dilution); 8) Retentate of H-BR15-5 filtration (no dilution); 9) Marker.

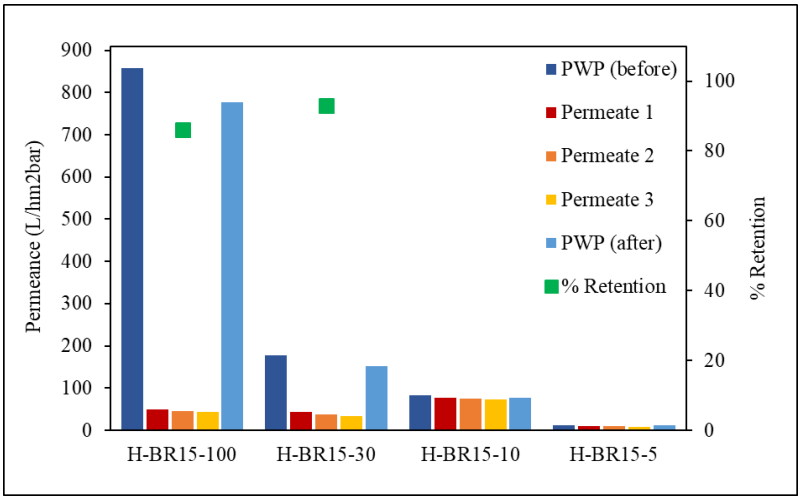


Figure 3-6. Permeance and Retention Values for H-BR15-100, H-BR15-30, H-BR15-10 and H-BR15-5 filtrations.

After all these filtrations, separation of similarly sized proteins based on size difference was found limited. For this reason, it was continued with the charge based separation.

3.2.2.2 . Charge Based Separation

3.2.2.2.1. pH change

However, in order to obtain better results in terms of separation of the similarly sized protein mixtures, it is possible to alter charge of the proteins or membrane. Within this scope, changing charge of the proteins in the solution may be applied by adjusting pH of the solution to desired value. As it is known, at isoelectric point, proteins have no net charge. However, if pH is under the isoelectric point, proteins become positively charged, if pH is above the isoelectric point, proteins become negatively charged. Isoelectric points of the proteins in the production medium were determined from 2D-electrophoresis analysis. Isoelectric point of the hGH was around 5.5, while the other similarly sized proteins that were not separable with based on size, have isoelectric points as around 6.5 and 7.5.

In this production medium, altering pH of the feed solution strategy was applied first. Different pH values were selected before ultrafiltration experiments. Before continuing with the pH change, H-BR14-100 filtration were conducted with 100 kDa MWCO

membrane based on size. Permeate samples of H-BR14-100 filtration were used as feed solution for H-BR14 -30-7.7 (hGH-30 kDa, pH=7.7) filtration after pH was adjusted to 7.7. Initial pH of the feed solution was measured approximately as 5.5. At pH 7.7, most of the proteins in the production medium were negatively charged including hGH. SDS-PAGE image from Figure 3-7 shows that feed and the permeate samples were almost same.

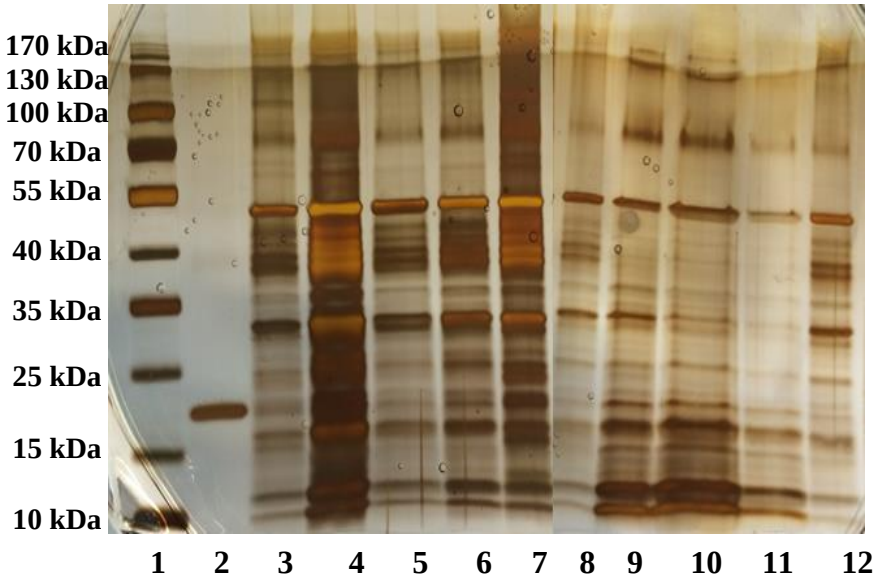


Figure 3-7. SDS-PAGE images of H-BR14-100 and H-BR14-30-7.7 filtrations. 1) Marker; 2) hGH standard (50 mg/L); 3) Feed solution of H-BR14-100 filtration (20X); 4-5-6) Permeates of H-BR15-10 filtration (10X); 7) Retentate of H-BR14-100 filtration (20X); 8) Feed solution of H-BR14-30-7.7 filtration (10X); 9-10-11) Permeates of H-BR14-30-7.7 filtration (no dilution); 12) Retentate of H-BR14-30-7.7 filtration (10X).

When the pH was decreased from 7.7 to 4.5 before H-BR14-30-4.5 (hGH-30 kDa, pH=4.5) filtration, transmission of the proteins to permeate side decreased. At this pH value, all proteins were positively charged. When the pH value was adjusted to 6 at which some proteins were positively charged and some were negatively charged. In H-BR14-30-6 filtration, permeation of some proteins like 45 kDa line increased but passing of the larger molecular weight proteins to permeate side continued (Figure 3-8).

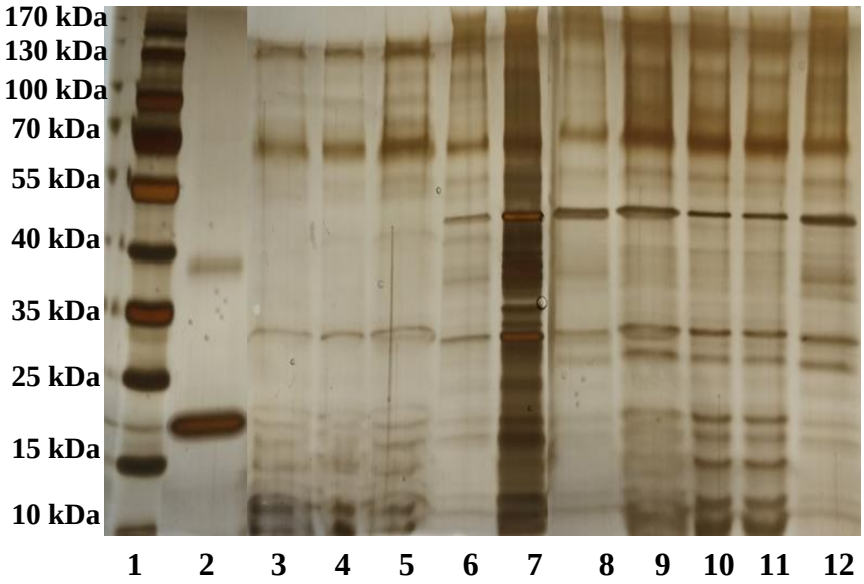


Figure 3-8. SDS-PAGE images of H-BR14-30-4.5 and H-BR14-30-6 filtrations. 1) Marker; 2) hGH standard (50 mg/L); 3-4-5) Permeates of H-BR15-10 filtration (no dilution); 6) Feed solution of H-BR14-30-4.5 filtration (10X); 7) Retentate of H-BR14-30-4.5 filtration (10X); 8) Feed solution of H-BR14-30-6 filtration (10X); 9-10-11) Permeates of H-BR14-30-6 filtration (no dilution); 12) Retentate of H-BR14-30-6 filtration (10X).

At all pH values it was seen that, although some proteins were rejected by the membrane, there was still transmission to the permeate side. Contrary to expectations, larger sized proteins like 70 kDa could pass through the 30 kDa MWCO membrane. In all filtrations, there was severe decrease in permeance values during filtration that can show cake formation during filtration on the membrane surface. Fouling was not able to be cleaned physically in H-BR14-100 filtration contrary to H-BR14-30-7.7, H-BR14-30-6 and H-BR14-30-4.5 filtrations which was probably because of significant pore blockage. Permeance values for filtrations could be seen in Figure 3-9.

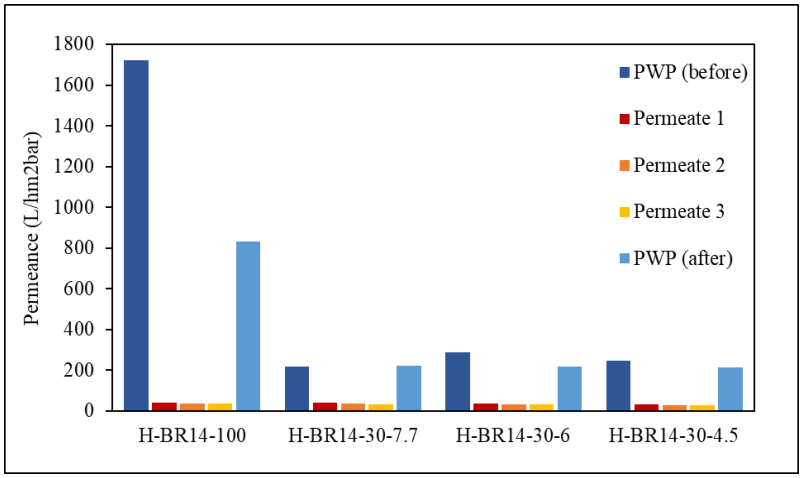


Figure 3-9. Permeance values for H-BR14-100, H-BR14-30-7.7, H-BR14-30-6 and H-BR14-30-4.5 filtrations.

After this point, it was considered to change pH of the feed solution before 10 kDa MWCO membrane filtration. For this purpose, another reactor sample was used named as B6. First of all, filtrations based on size were done with 100 kDa and 30 kDa membrane filtrations. However, in both H-B6-100 and H-B6-30-P filtrations, results were not compatible with before (Figure 3-10). In these filtrations, some proteins were rejected, while some of them passed to the permeate side. In fact, even proteins larger than 70 kDa passed through the membrane. When looking at the production medium, presence of glycerol in the medium was detected. In literature, it is reported that glycerol can have an effect on the permeation of the proteins by leading to compression of the double layer of the proteins [38]. This may be the reason of the unexpected transmission of the proteins in this case.

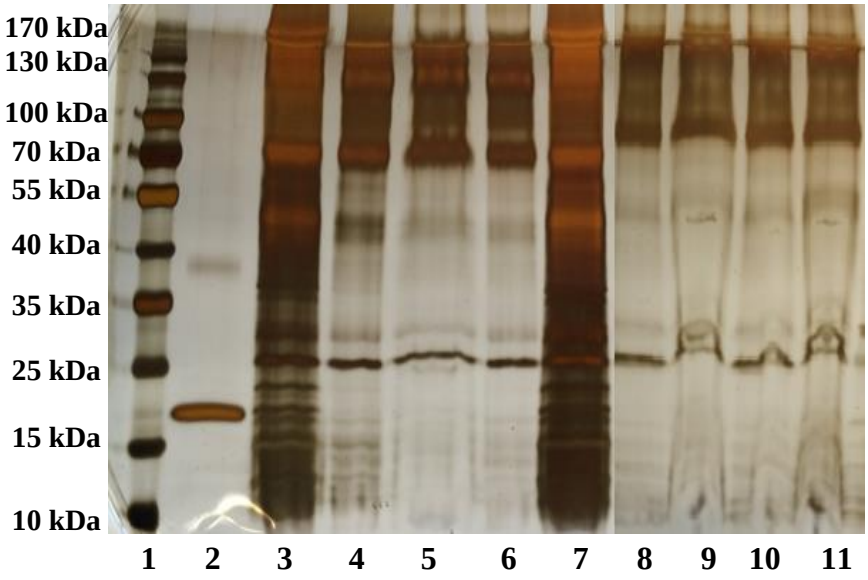


Figure 3-10. SDS-PAGE images of H-B6-100 and H-B6-30-P filtrations. 1) Marker; 2) hGH standard (50 mg/L); 3) Feed solution of H-B6-100 filtration (no dilution); 4-5-6) Permeates of H-B6-100 filtration (no dilution); 7) Retentate H-B6-100 filtration (no dilution); 8-9-10) Permeates of H-B6-30-P filtration (no dilution); 11) Retentate of H-B6-30-P filtration (no dilution).

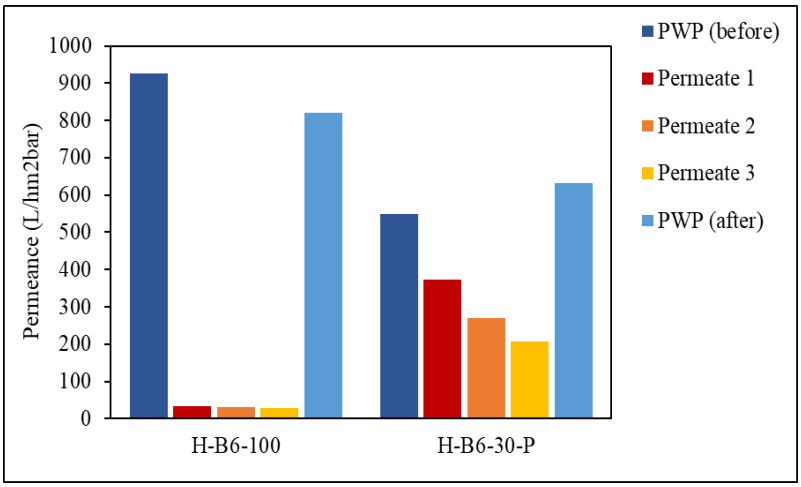


Figure 3-11. Permeate values for H-B6-100 and H-B6-30-P filtrations.

As it was understood from Figure 3-11, there was significant decrease in permeance values for H-B6-100 filtration which could probably create fouling. On the other hand, in H-B6-30-P filtration, permeance decreased less than H-B6-100 filtration. At the end, for both filtrations, PWP values could be restored by physical cleaning.

Because of getting different results from same filtrations, production medium was produced once more with different strategy [39] and then, molecular weight of the hGH was specified as 22 kDa and isoelectric point was detected as around 5.5 that were same as before. However, in this production medium (BD), it was seen that isoelectric points of the other proteins in the medium were resulted in different distribution leading to different charge interactions.

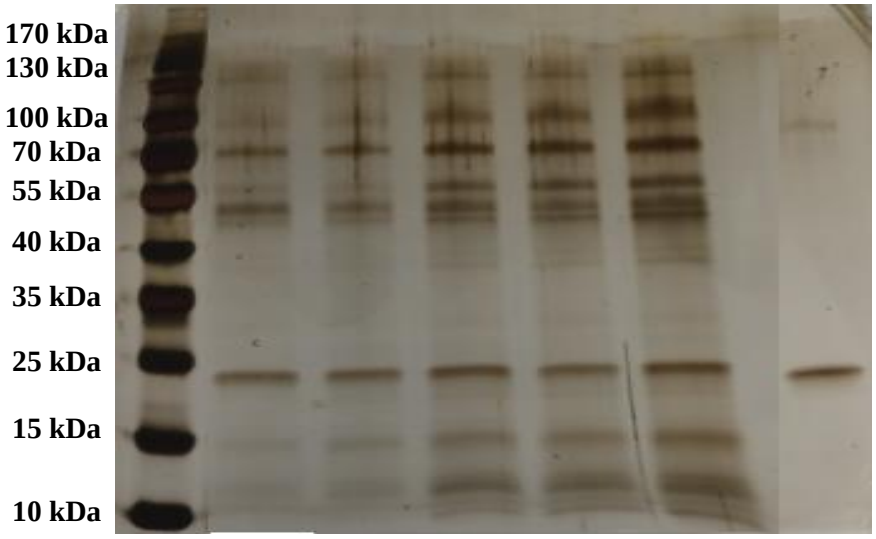


Figure 3-12. SDS-PAGE image of the hGH production medium (BD).

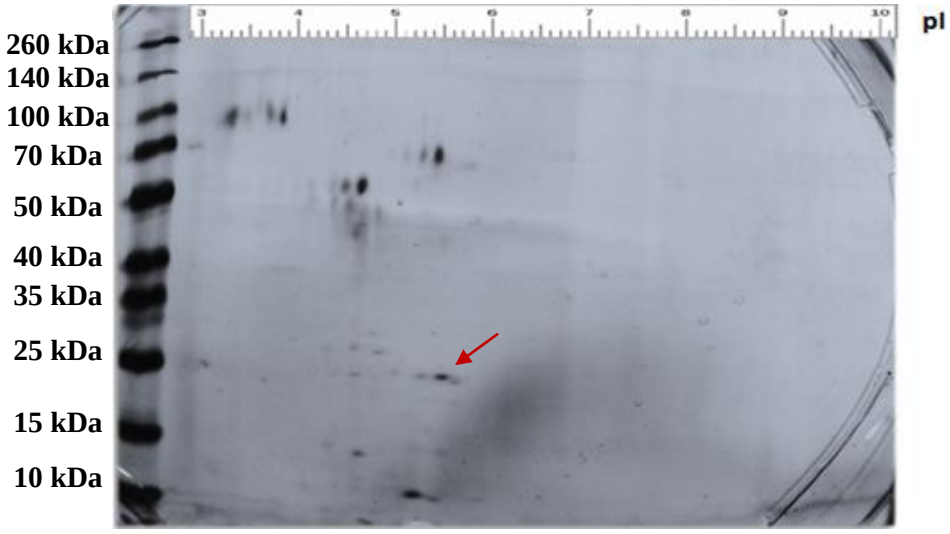


Figure 3-13. 2D-electrophoresis image of the hGH production medium (BD).

In the same way, sized based separation was applied first by using 100 kDa (H-BD-100) and 30 kDa MWCO membranes (H-BD-30P-2b). It was observed that all proteins passed to the permeate side in H-BD-100 filtration. However, retention of the hGH calculated for this filtration around 70% which was still high. Permeate samples of H-BD-100 filtration used for H-BD-30P-2b (hGH-30 kDa-2 bar) filtration where hGH was retained significantly (98%). Contrary to this high retention of hGH, larger molecular weight proteins like the proteins at 100 kDa line could also permeate through membrane pores (Figure 3-14).

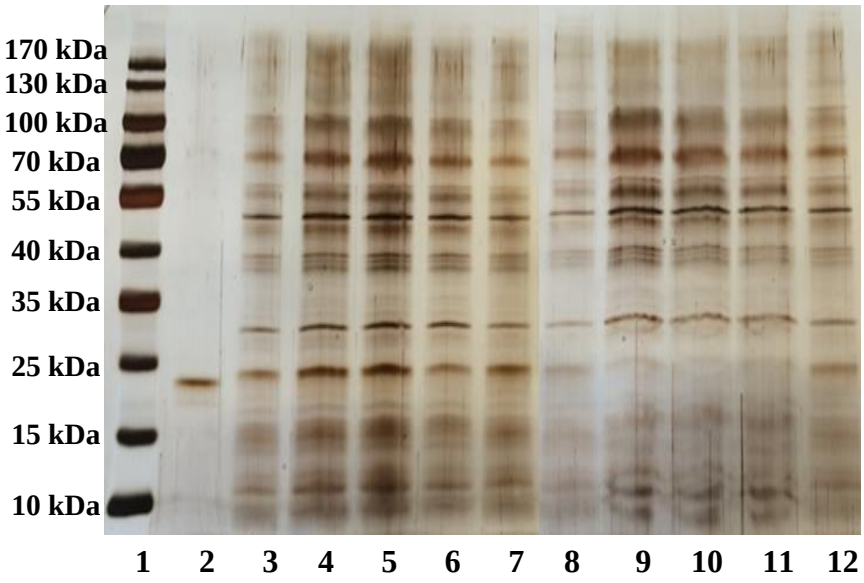


Figure 3-14. SDS-PAGE image of H-BD-100 and H-BD-30P-2b filtrations. 1) Marker; 2) hGH standard (10 mg/L); 3) Feed solution of H-BD-100 filtration (10X); 4-5-6) Permeates of H-B6-100 filtration (4X); 7) Retentate H-BD-100 filtration (10X); 8) Feed solution of H-BD-30P-2b filtration (7X); 9-10-11) Permeates of H-BD-30P-2b filtration (no dilution); 12) Retentate of H-BD-30P-2b filtration (20X).

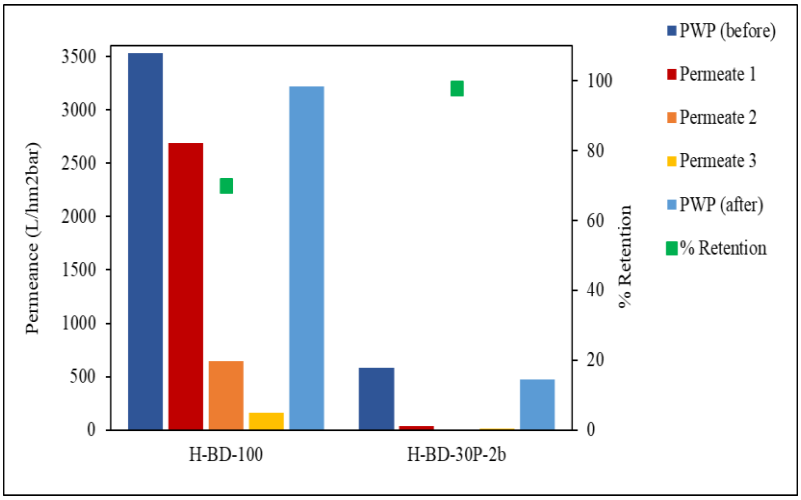


Figure 3-15. Permeance and retention values for H-BD-100 and H-BD-30P-2b filtrations.

In membrane filtrations, concentration polarization is the most problematic issue that can cause fouling. Fouling can decrease the membrane life and bring extra cost to the process as well as effect the separation performance. Because of the possibility of the fouling, it would be better if the filtration could be done below the limiting flux where the fouling begins [19]. For this reason, filtrations were conducted by using 30 kDa MWCO membrane at three different pressure values (2-0.5-0.1 bar) and average flux values during filtration were compared.

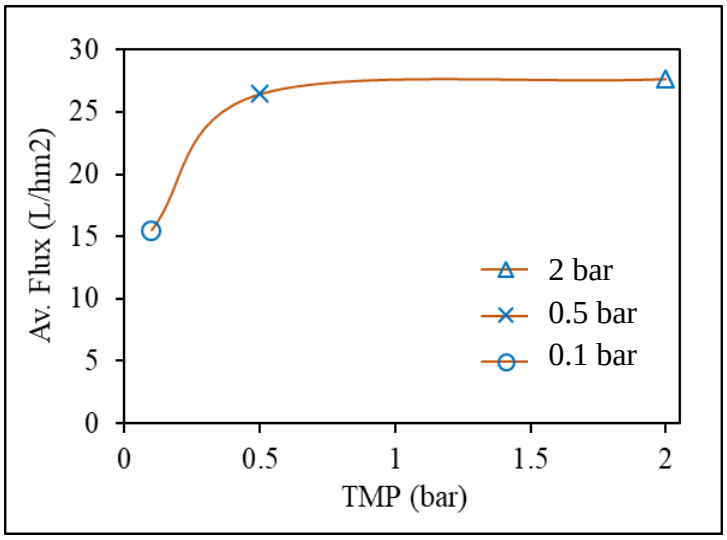


Figure 3-16. Limiting Flux for 30 kDa MWCO membrane (hGH)

As it was seen in the Figure 3-16, limiting flux of 26 L/hm^2 was achieved around at 0.5 bar, in fact, it was below the 0.5 bar. From the separation point of view, there was no significant change between H-BD-30D-0.5b (hGH-30 kDa-0.5 bar) and H-BD-30D-0.1b (hGH-30 kDa-0.1 bar) filtrations can be seen in Figure 3-17. Therefore, in order to prevent the possibility of fouling, further filtrations were conducted at 0.1 bar.

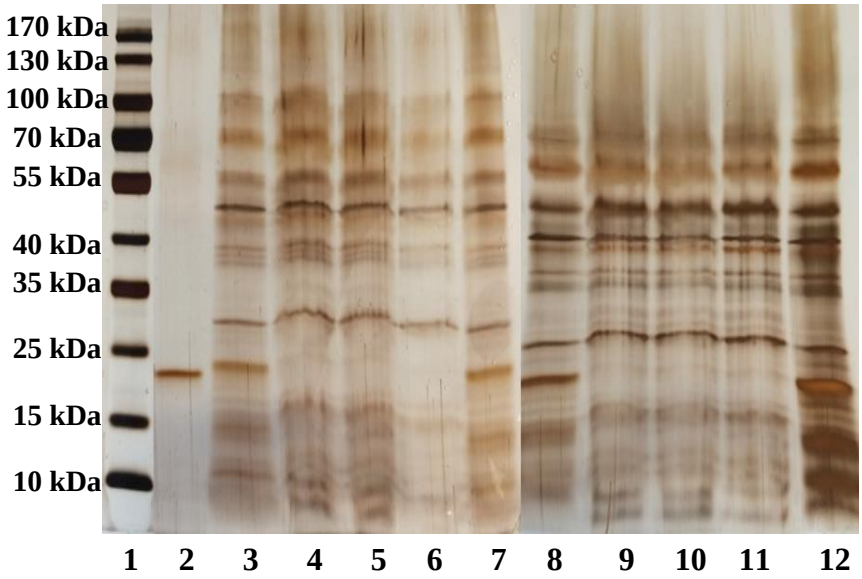


Figure 3-17. SDS-PAGE image of H-BD-30D-0.5b and H-BD-30D-0.1b filtrations. 1) Marker; 2) hGH standard (10 mg/L); 3) Feed solution of H-BD-30D-0.5b filtration (10X); 4-5-6) Permeates of H-BD-30D-0.5b filtration (no dilution); 7) Retentate H-BD-30D-0.5b filtration (10X); 8) Feed solution of H-BD-30D-0.1b filtration (10X); 9-10-11) Permeates of H-BD-30D-0.1b filtration (no dilution); 12) Retentate of H-BD-30D-0.1b filtration (10X).

3.2.2.2.2. Modification of Membranes

Another strategy in the separation of the similarly sized proteins is the modification of the membrane surface. In this case, membrane gains a positive or negative charge after the chemical modification. Therefore, attractive or repulsive forces play critical role in the membrane-protein interaction.

Membrane surface modifications, either positive or negative, were performed according to the procedure given in literature [14]. During modification, chemical reaction takes place between OH bonds in the cellulose structure and alkyl halogens

and form modified membranes. At the end of the reaction, acid evolves which leads pH decrease in the environment. Reactions can be seen in Figure 3-18 and 3-19. For this reason, in order to understand whether the modification was performed or not, pH values were measured for each samples. Due to absence of sufficient solution, pH control was conducted by using pH strips instead of pH meter. In the figure, it was clearly seen that there was pH change over time for positive modification. On the other hand, no change was observed for negative modification (Figure 3-20).

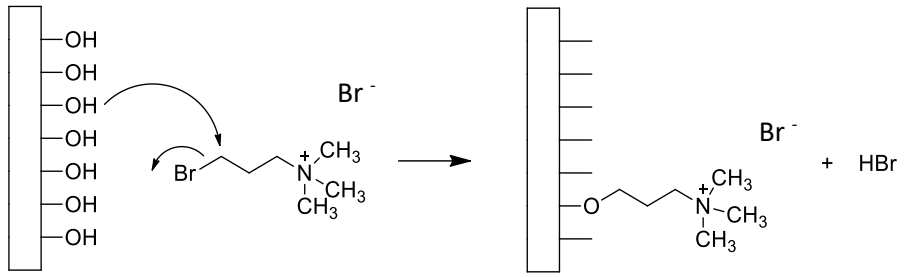


Figure 3-18. Positive modification reaction

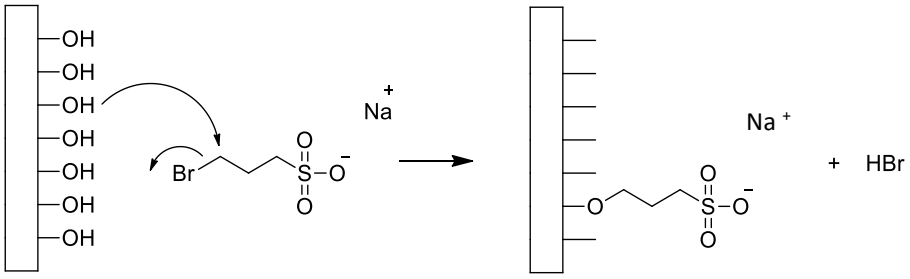
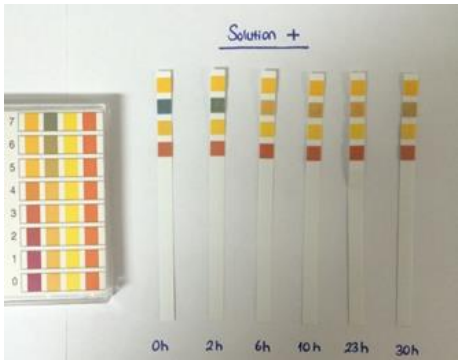
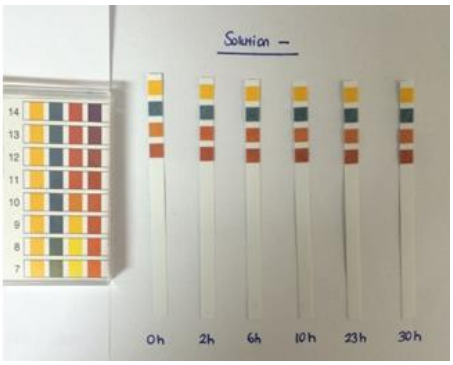


Figure 3-19. Negative modification reaction



Time (hours)	pH
0 h	9
2 h	7
6 h	5
10 h	5
23 h	5
30 h	5



Time (hours)	pH
0 h	10
2 h	10
6 h	10
10 h	10
23 h	10
30 h	10

Figure 3-20. pH change vs time for positive and negative modification solutions.

In order to ensure that whether modification was performed or not, X-ray Photoelectron Spectroscopy (XPS) was carried out. With the help of this analysis, it was able to be achieved the information about surface of a material in atomic and molecular degree. For positive modification, it was expected that nitrogen must be found while for negative modification, sulphur was the expected element besides C, H, O coming from cellulose structure must be observed. In positive modification case, nitrogen was observed so it was thought that positive modification was achieved successfully (Figure 3-21). In the negative modification case, sulphur was observed in the analysis (Figure 3-22). However, there was same amount sulphur in positive modification case. Therefore, it was not clear whether the negative modification was actually successful.

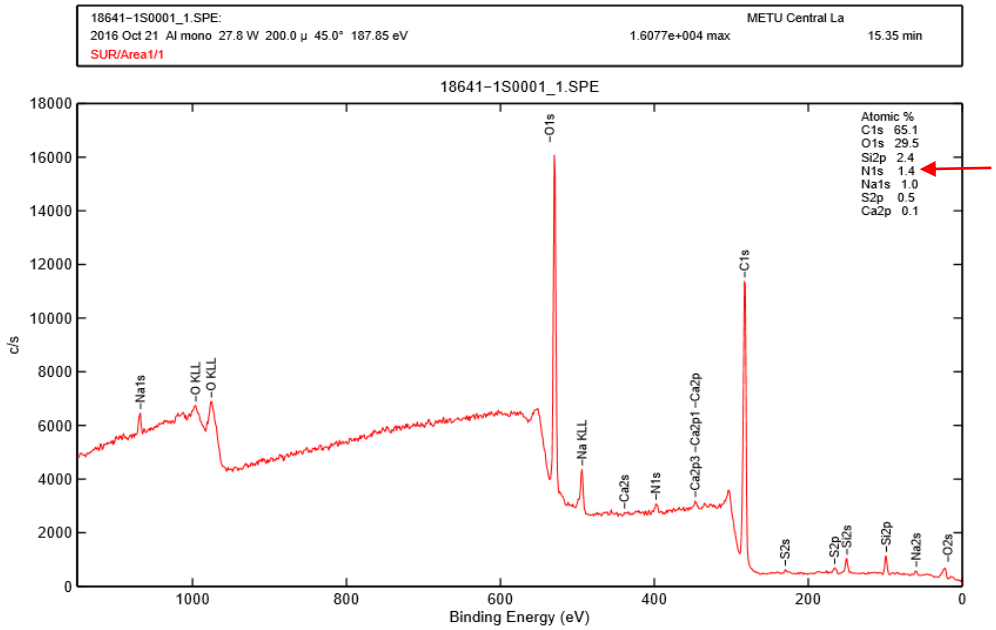


Figure 3-21. XPS results for positive modification.

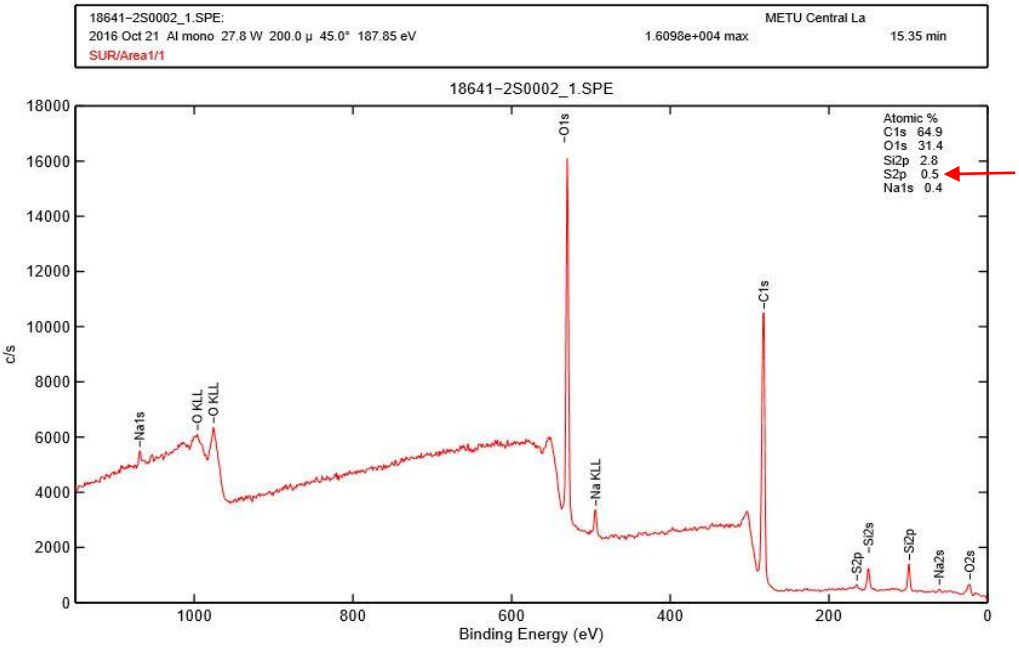


Figure 3-22. XPS results for negative modification.

In addition to the XPS analysis, dye sorption tests were conducted for modified membranes (M) and unmodified membranes (UM). For positive modification, negatively charged brilliant blue was selected as dye. As it was easily seen from Figure 3-23, positively modified membrane sorbed negatively charged brilliant blue more than unmodified membrane. In negative modification, positive crystal violet was chosen. However, in this case, there was almost no difference between modified and unmodified membrane in terms of dye sorption. As a result of these experiments, it was concluded that positive modification was achieved successfully while negative modification was not achieved at least to large extent.

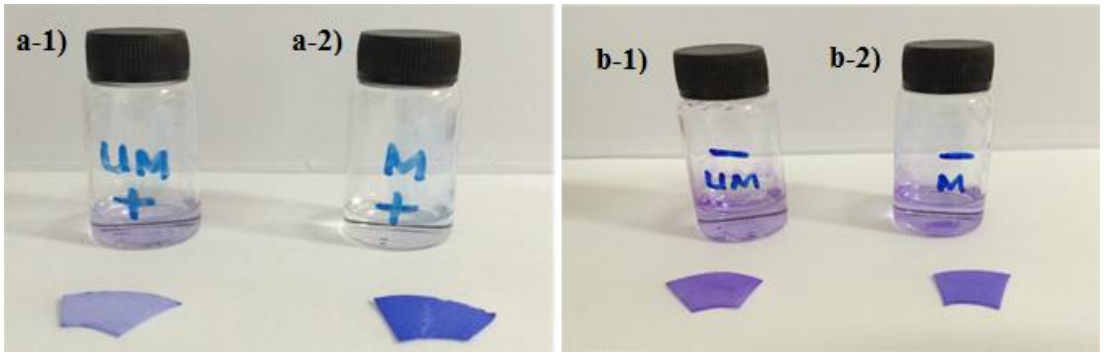


Figure 3-23. Dye sorption tests images for modified and unmodified membranes. a-1) Brilliant blue test with un-modified membrane; a-2) Brilliant blue test with positively modified membrane; b-1) Crystal violet test with un-modified membrane; b-2) Crystal violet test with negatively modified membrane.

30 kDa MWCO membranes were modified and used in the ultrafiltration experiments. Performances of them are in agreement with the observation of dye sorption test, XPS and pH change. H-BD-30P-PM filtration was conducted with positively modified membrane which rejected almost all proteins in the production medium including hGH. Retention of hGH was measured as 99% from SDS-PAGE approximately. In fact, since the pH of the production medium as close to 5.5, all proteins in the medium were expected to be negatively charged. High rejection of negatively charged proteins by positively charged membrane can possibly be explained in terms of size exclusion rather than electrostatic interactions. Van Reis et al. reported that in the case of chemical modification, pore size of the membrane decreases because of the connected groups which are larger than OH groups in the cellulose structure [14].

On the other hand, H-BD-30P-NM filtration negatively modified membrane gave the same result as unmodified membrane filtration (H-BD-30P-2b). Moreover, hGH rejection was found as 99.8%. Consequently, it was concluded that negative modification was performed probably less than positive one. SDS-PAGE results for H-BD-30P-PM and H-BD-30P-NM filtrations can be seen in Figure 3-24.

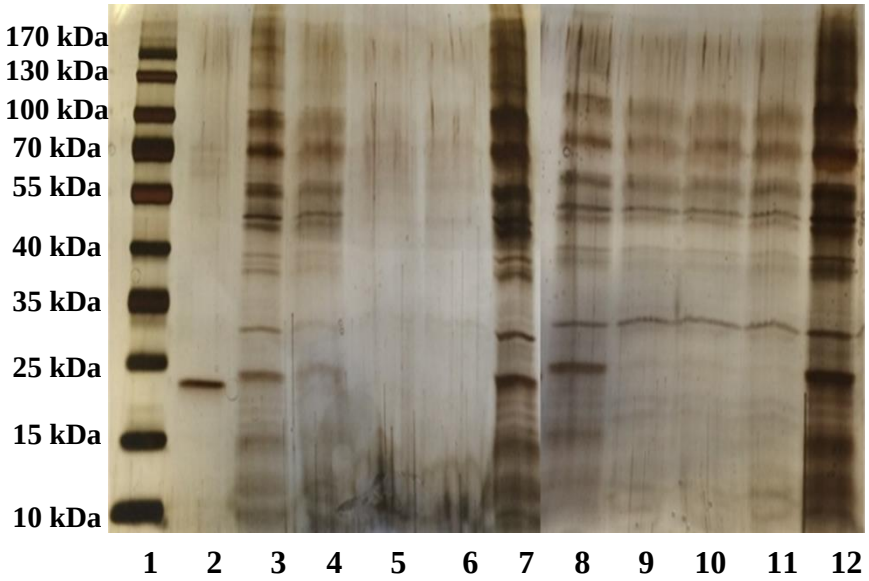


Figure 3-24. SDS-PAGE image of H-BD-30P-PM and H-BD-30P-NM filtrations. 1) Marker; 2) hGH standard (10 mg/L); 3) Feed solution of H-BD-30P-PM filtration (7X); 4-5-6) Permeates of H-BD-30P-PM filtration (no dilution); 7) Retentate H-BD-30P-PM filtration (20X); 8) Feed solution of H-BD-30P-NM filtration (10X); 9-10-11) Permeates of H-BD-30P-NM filtration (2X); Retentate of H-BD-30P-NM filtration (20X).

Positive modification was also applied for 100 kDa MWCO membrane (H-BD-100-PM). Before modification almost all proteins could pass to the permeate side and retention of hGH was measured as 70% (H-BD-100 filtration). However, when the modification was performed, it was seen that there was still permeation but hGH was highly retained by the membrane which was calculated as 90%. Furthermore, some proteins which were passed to the permeate side in the case of H-BD-100 filtration were rejected by the modified membrane (Figure 3-25). For all three filtrations where the modification was performed, permeance values was lower compared to PWP values especially in H-BD-100-PM filtration case (Figure 3-26). Therefore, most probably fouling formed during filtration that was removed after physical cleaning.

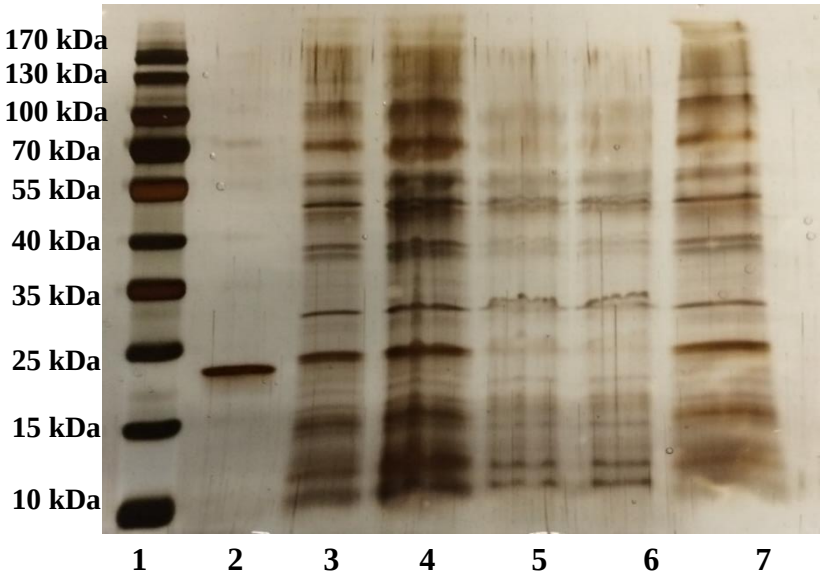


Figure 3-25. SDS-PAGE image of H-BD-100-PM filtration.
1) Marker; 2) hGH standard (10 mg/L); 3) Feed solution of H-BD-100-PM filtration (10X); 4-5-6) Permeates of H-BD-100-PM filtration (4X); 7) Retentate H-BD-100-PM filtration (20X).

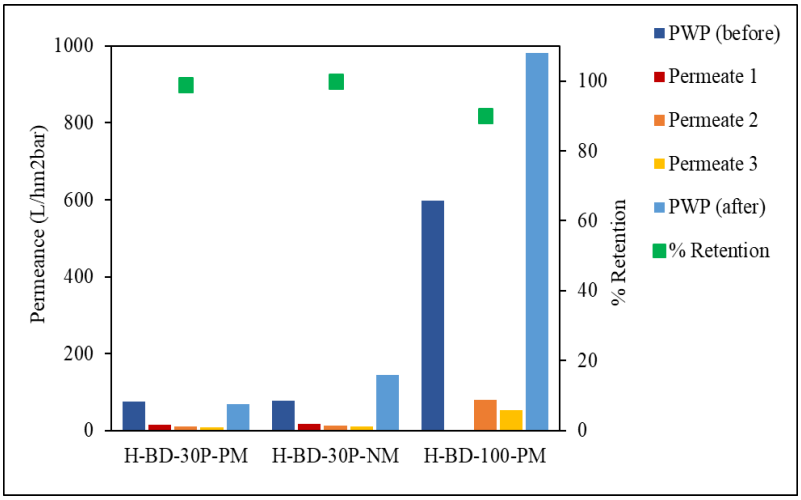


Figure 3-26. Permeance and retention values for H-BD-30P-PM, H-BD-30P-NM and H-BD-100-PM.

Transmission of the larger molecular weight proteins to the permeate side and specific retention of the hGH in any case supported the idea of the possible agglomeration of

the hGH molecule. It is known in the literature that even small amount of protein aggregates affects the solute transport during filtration, thereby the separation behavior [40]. Moreover, transmission of larger sized proteins to the permeate side may have arisen from the presence of salt in the extracellular hGH medium since salt content also affects the separation behavior by decreasing the electrical double layer of the proteins [11]. For this reason, ionic strength was calculated as 2.1386 for hGH environment (Appendix E) and diafiltration was performed to explore the effect of salt content on separation performance.

3.2.2.3. Diafiltration

In order to observe if there is an effect of the high protein concentration and salt content in the medium on the separation behavior, diafiltration was conducted. For this purpose, 30 kDa MWCO membrane was used. In here, initial feed solution volume was kept constant by adding solvent at the end of the filtration. Other filtration was started after volume adjustment. This way, the medium is desalted and diluted.

At the end the H-BD-30-DIA filtration, it was observed that permeation of the other proteins decreased and no permeation was observed after some point (Figure 3-27 and Figure 3-28). Therefore, it was implied that, diafiltration had an influence on the separation behavior. In the meantime, desired protein, hGH, was highly retained by the membrane from first filtration, so it was not a good option for the moment.

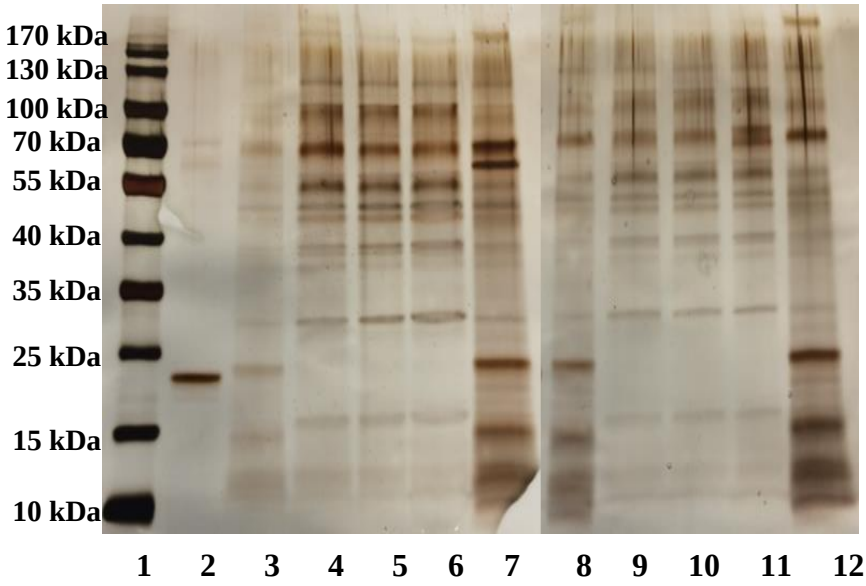


Figure 3-27. SDS-PAGE image of H-BD-30-DIA filtration.
1) Marker; 2) hGH standard (10 mg/L); 3) Feed solution of 1st filtration (5X); 4-5-6) Permeates of 1st filtration (no dilution); 7) Retentate of 1st filtration (10X); 8) Feed solution of 2nd filtration (2X); 9-10-11) Permeates of 2nd filtration (no dilution);12) Retentate of 2nd filtration (5X).

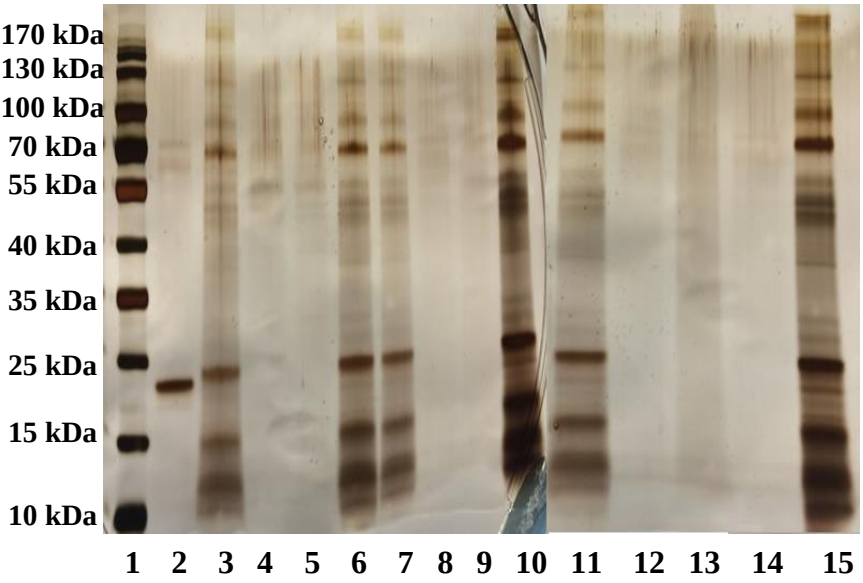


Figure 3-28. SDS-PAGE image of H-BD-30-DIA filtration.
1) Marker; 2) hGH standard (10 mg/L); 3) Feed solution of 3rd filtration (no dilution); 4-5) Permeates of 3rd filtration (no dilution); 6) Retentate of 3rd filtration (3X); 7) Feed solution of 4th filtration (no dilution); 8-9) Permeates of 4th filtration (no dilution); 10) Retentate of 4th filtration (no dilution); 11) Feed solution of 5th filtration (no dilution); 12-13-14) Permeates of 5th filtration (no dilution); 15) Retentate of 5th filtration (no dilution).

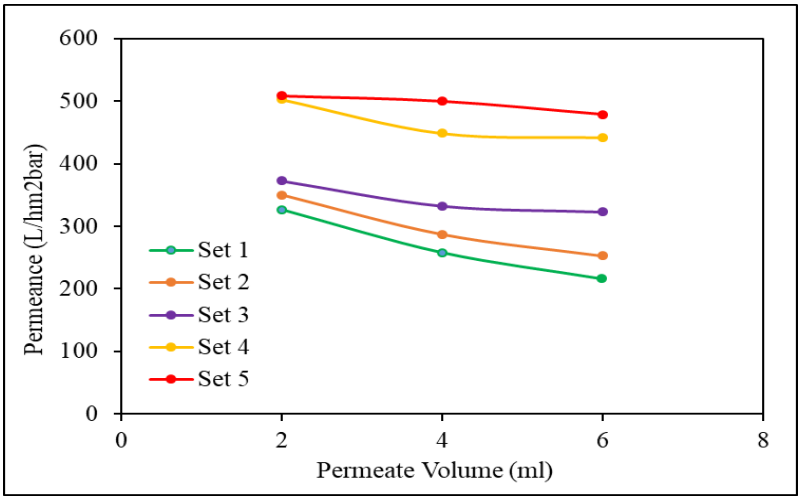


Figure 3-29. Permeance values for each set of H-BD-30-DIA filtration.

Permeance values for each set can be observed from Figure 3-29. As it was seen, permeance during filtration increased which makes sense because there was dilution of the proteins.

Same procedure was followed but this time pH of the feed solution was changed and adjusted before each filtration. At both pH 4.5 (H-BD-30-DIA-4.5) and 7.0 (H-BD-30-DIA-4.5), there was still high retention of hGH and permeation of the other proteins even larger molecular sized ones (Figure 3-32 and Figure 3-33).

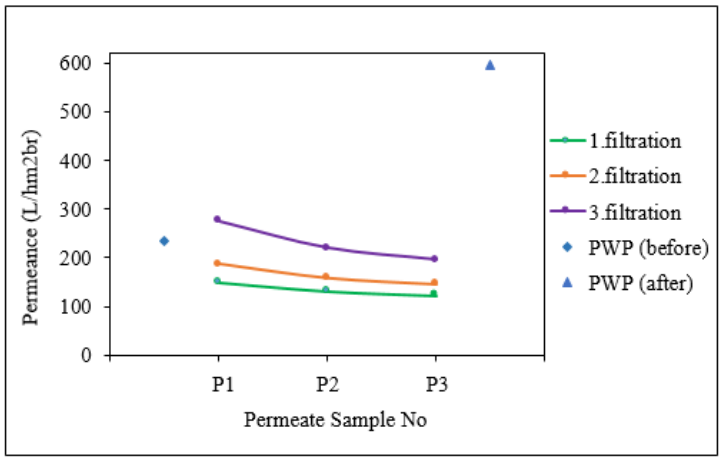


Figure 3-30. Permeance values for each set of H-BD-30-DIA-4.5 filtration.

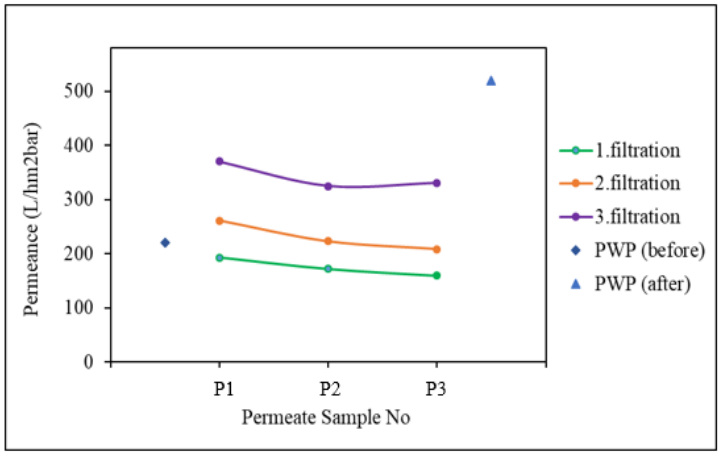


Figure 3-31. Permeance values for each set of H-BD-30-DIA-7 filtration.

PWP measurements were carried out before 1st filtration and after all filtrations were completed. There was extreme increase in PWP values at the end of the filtrations. In fact, it was clearly seen that from Figure 3.30 and 3.31, permeance values were gradually increasing during filtration. For this reason, it was considered that there was some kind of degradation of the cellulose membrane during filtrations.

Figure 3-32. SDS-PAGE image of H-BD-30-DIA -4.5 filtration.
1) Marker; 2) hGH standard (10 mg/L); 3) Feed solution of 1st filtration (10X); 4-5-6) Permeates of 1st filtration (no dilution); 7) Retentate of 1st filtration (20X); 8) Feed solution of 2nd filtration (5X); 9-10-11) Permeates of 2nd filtration (no dilution); 12) Retentate of 2nd filtration (12X); 13) Feed solution of 3rd filtration (4X); 14-15) Permeates of 3rd filtration (no dilution); 16) Retentate of 3rd filtration (no dilution).

Figure 3-33. SDS-PAGE image of H-BD-30-DIA-7 filtration.
1) Marker; 2) hGH standard (10 mg/L); 3) Feed solution of 1st filtration (10X); 4-5-6) Permeates of 1st filtration (no dilution); 7) Retentate of 1st filtration (20X); 8) Feed solution of 2nd filtration (6X); 9-10-11) Permeates of 2nd filtration (no dilution); 12) Retentate of 2nd filtration (12X); 13) Feed solution of 3rd filtration (4X); 14-15-16) Permeates of 3rd filtration (no dilution); 17) Retentate of 3rd filtration (7X).

3.2.2.4. Membrane Type

In ultrafiltration, variety of polymers such as polysulfone (PS), polyethersulfone (PES) and regenerated cellulose (RC) are available as membrane material. RC membranes are preferable especially in bio-based applications due to leading less fouling, being more easily cleaned and having mechanical strength [41]. On the other side, for example PES membranes have significant thermal and chemical stability [5]. Due to having significant increase in permeance values and possibility of degradation in cellulose membrane structure, filtrations were also performed with PES membranes.

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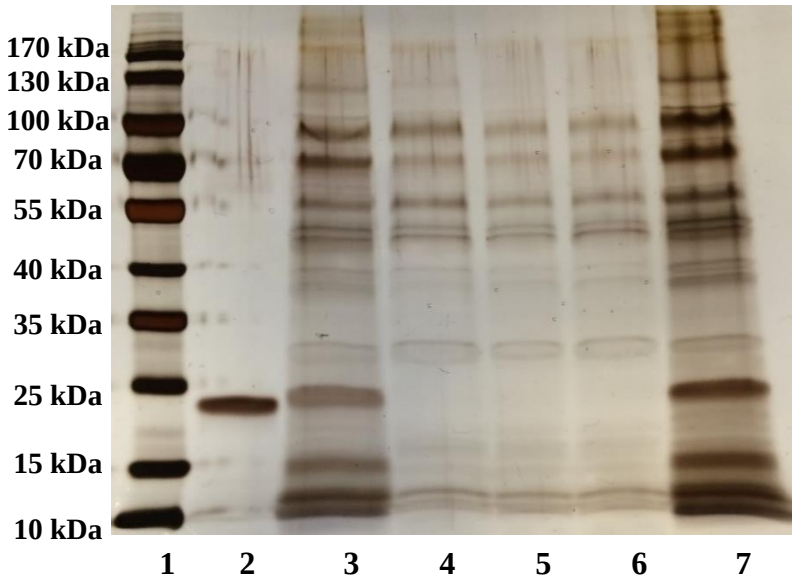

Figure 3-34. SDS-PAGE image of H-BD-100-PES filtration.
1) Marker; 2) hGH standard (10 mg/L); 3) Feed solution (10X); 4-5-6) Permeates (4X); 7) Retentate (20X).

As it was seen from SDS images and filtration values in Figure 3-34 and 3-35 respectively, there was no change in terms of separation because hGH was highly rejected, however; significant amount of fouling was observed at the end of the filtration. Fouling could not be removed even with the chemical cleaning which was conducted using 0.1 M NaOH and 500 ppm NaOCl. It was known that PES membranes tend to foul more than cellulose membranes that information was compatible with the results.

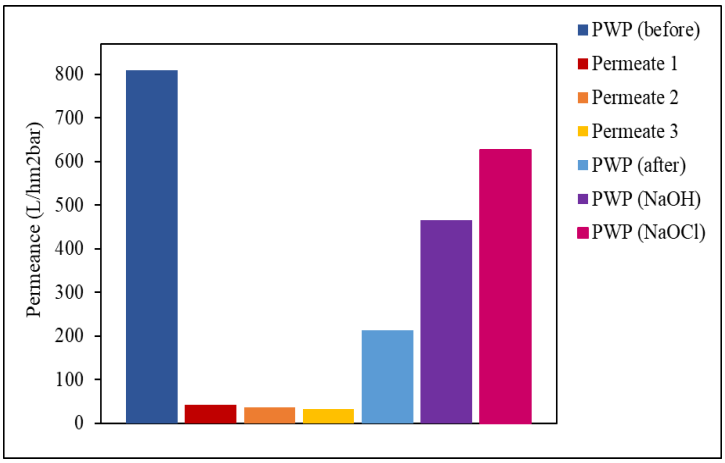

Figure 3-35. Permeance values for H-BD-100-PES filtration.

As a consequence of the results, in hGH environment, it was observed that hGH was specifically rejected by the membrane in any case (Table 3-2) while the larger molecular weight proteins passed to the permeate side. In this environment, there was extracellular production, so all metabolites including salts present in the feed solution. It was known that the presence of the salt can decrease the electrical double layer thickness of the proteins thereby increase the transmission of them [11]. Furthermore, it was considered that agglomeration of the hGH can lead to high retention. For this reason, filtrations were conducted with other production medium containing GCSF that have extra washing steps which can prevent the salt effect.

Table 3-2. Summary of the retentions for rhGH environment.

Filtration Code	**Retention of rhGH**
H-BR15-100	86%
H-BR15-30	93%
H-BD-100	70%
H-BD-30P-2b	98%
H-BD-30P-PM	99 %
H-BD-30P-NM	99.8 %
H-BD-100-PM	90%

3.3.Recombinant GCSF

3.3.1. Characterization of the Production Medium

Size and charge distribution of the proteins for rGCSF production medium was analyzed by using SDS-PAGE and 2D-electrophoresis. It is known that molecular weight of the GCSF as 18.8 kDa, however in the SDS-PAGE image in Figure 3.36, molecular weight of this protein can be seen as about 20 kDa. This difference can be caused from the polyhistidine-tag which is amino acid sequence that consist of at least six histidine used for easy purification or detection of the recombinant protein [28]. Since there are extra amino acids comes from polyhistidine-tag, molecular weight of the GCSF in the production medium is higher than normal case. Molecular size of the other proteins in the production environment line up from 10 to 170 kDa range. Additionally, isoelectric point of the GCSF can be detected as around 7 from the 2D-electrophoresis image (Figure 3.37). Almost all other proteins have isoelectric point lower than 7.

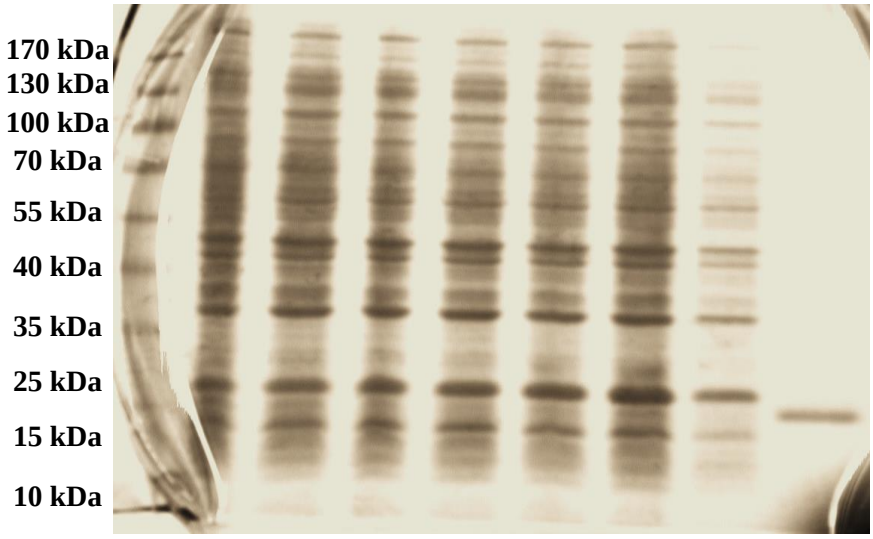

Figure 3-36. SDS-PAGE image of the GCSF production medium.

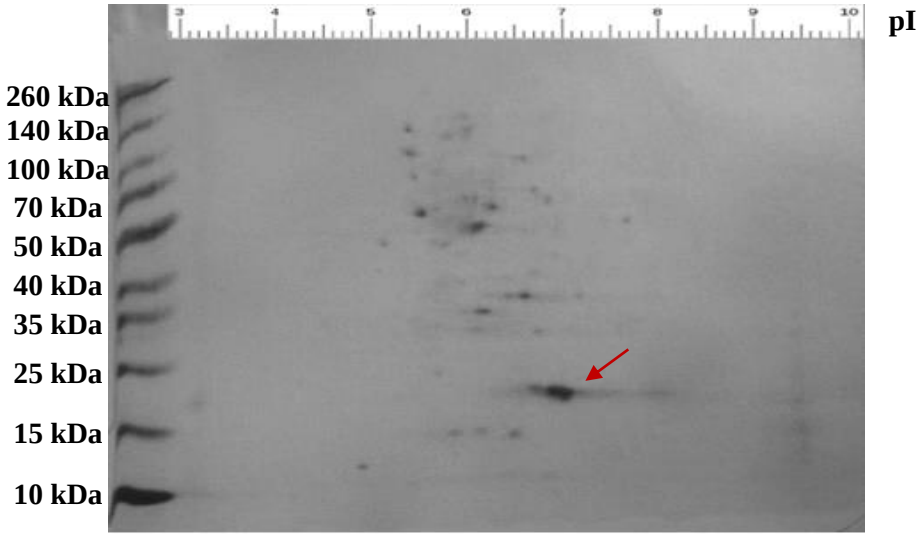

Figure 3-37. 2D-electrophoresis image of GCSF production medium.

3.3.2.Separation Behavior for rGCSF Environment

In order to obtain pure rGCSF, ultrafiltration experiment results which were performed based on size and charge difference of the proteins and the membrane were summarized in Table 3-3. Separation behavior was observed by SDS-PAGE analysis.

Table 3-3. Filtration codes and conditions for rGCSF environment.

Filtration Code	Membrane MWCO	Pressure (bar)	pH
G-100-0.5b	100	0.5	7
G-30-D	30	0.1	7
G-30-P	30	0.1	7
G-30-D-6.5	30	0.1	6.5
G-30-D-5.2	30	0.1	5.2
G-30-D-4	30	0.1	4
G-30-P-5.2	30	0.1	5.2
G-30-P-4	30	0.1	4
G-100-0.03b	100	0.03	7
G-30-P-0.03b-5.2	30	0.03	5.2

3.3.2.1. Size Based Separation

Initially, ultrafiltration was performed with 100 kDa MWCO membrane under the transmembrane pressure of 0.5 bar by using pretreated production medium as feed solution. Before and after filtration, pure water permeance (PWP) were measured. Analysis of the separation behavior was observed by using SDS-PAGE method. As a result of the experiment of G-100-0.5b (GCSF-100 kDa-0.5 bar) can be seen in Figure 3-38, the proteins having molecular weight higher than 55 kDa were rejected by the membrane while the other proteins including GCSF passed to the permeate side.

Retention of the GCSF was calculated from SDS-PAGE approximately as 74 % for G-100-0.5b filtration. However, the permeate samples appear to contain of larger proteins compared to GCSF. As a result, it can be said that size based separation was achieved partially with this membrane filtration.

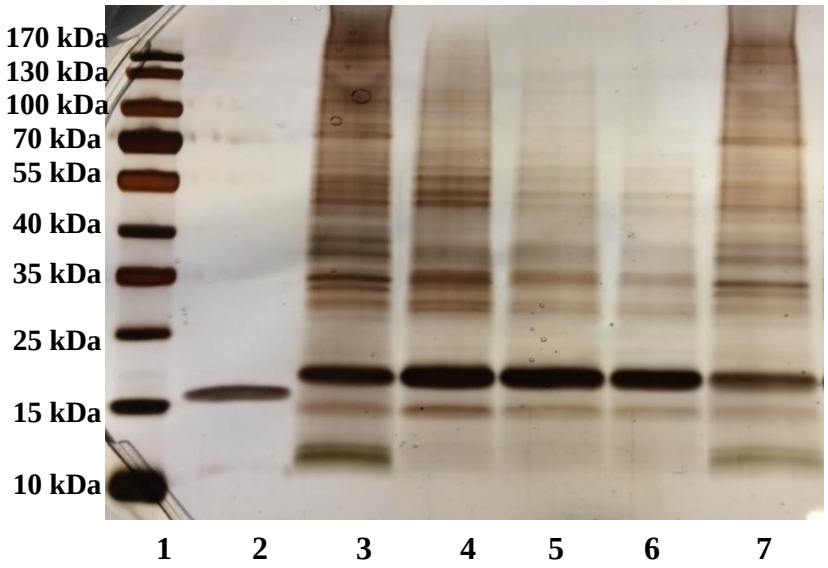

Figure 3-38. SDS-PAGE images of G-100-0.5b filtration.
1) Marker; 2) GCSF standard (10 mg/L); 3) Feed solution (20X); 4-5-6) Permeates (5X); 7) Retentate (60X).

From figure 3-38, it was understood that proteins were rejected over time during filtration. This brings us to the idea of cake formation on the membrane surface or clogging of the membrane pores. This is in accordance with the observation that there was sudden decrease in the permeance value and it was significantly lower than PWP before filtration. Moreover, PWP after filtration did not approach to its initial value that means physical cleaning with water was not sufficient for getting rid of the fouling which implies that the presence of the internal fouling. Therefore, chemical cleaning was carried out with 0.1 M NaOH (Figure 3-39).

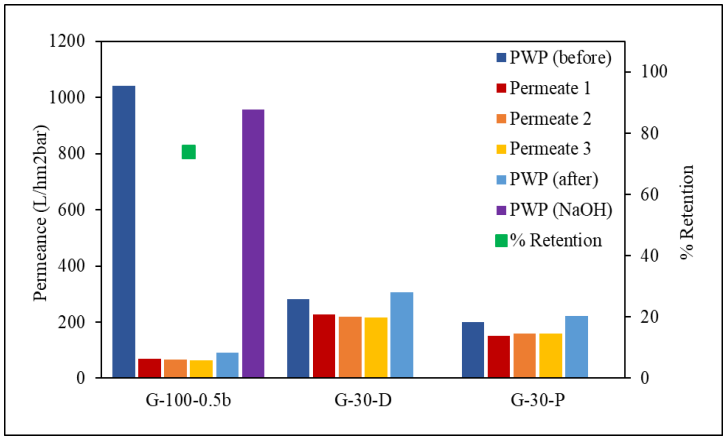

Figure 3-39. Permeance values for G-100-0.5b, G-30-D and G-30-P filtrations.

After these observation, G-30-D filtration was tried and pretreated (centrifuged and washed with urea solutions) production medium was directly used as feed solution at ultrafiltration experiment which was performed at 0.1 bar. In this case, after first permeate, there is no measurable protein transmission thorough the membrane. Therefore, collected permeate samples of the G-100-0.5b filtration were used as feed solution in G-30-P filtration. It was seen that all proteins were again retained by the membrane (Figure 3-40). In fact, in G-100-0.5b filtration, GCSF was highly retained.

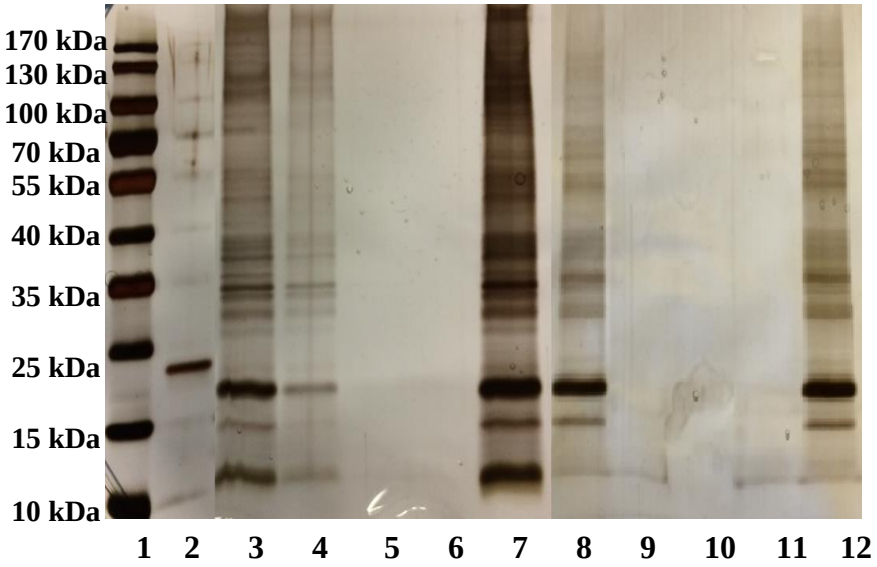

Figure 3-40. SDS-PAGE images of G-30-D and G-30-P filtrations.
1) Marker; 2) GCSF standard (10 mg/L); 3) Feed solution of G-30-D filtration (20X); 4-5-6) Permeates of G-30-D filtration (5X); 7) Retentate G-30-D filtration (50X); 8) Feed solution of G-30-P filtration (20X); 9-10-11) Permeates of G-30-P filtration (3X); 12) Retentate of G-30-P filtration (60X)

3.3.2.2. Charge Based Separation

Experiments conducted based on size difference were successful to some extent for the separation of the GCSF from production medium. However, to enhance the separation performance, charge based separation could be applied. For this purpose, different pH values were determined by looking at the charge distributions of the proteins in the 2D-electrophoresis image. Difference between pI points of these proteins were significant, so it was thought that separation can be achieved by using electrostatic interactions like repulsion/attraction.

At the beginning, pH value of the production medium was measured as 7. This solution was treated with 0.1 M HCl to achieve desired value of pH. G-30-D-6.5 filtration was the filtration that the pH of the feed solution was adjusted to 6.5 and pretreated production medium is directly used without 100 kDa pre-filtration for this experiment. It was observed that permeation of GCSF is higher in this filtration while the other proteins having higher molecular weight than GCSF have been rejected by the membrane. At pH 6.5, GCSF has slightly positive charge due to having isoelectric point

at pH=7. Almost all others proteins except GCSF were negatively charged in the production medium at this pH value. SDS-PAGE image for G-30-D-6.5 can be seen in Figure 3-41 shows that there was slight transmission of the GCSF while the other proteins were rejected. This result is probably because membrane tends to permeate positively charged GCSF more than other negatively charged proteins. It is reported that cellulose membranes are negatively charged due to deprotonated OH groups in a wide pH range [42]. This observation also appears to be in agreement with the literature.

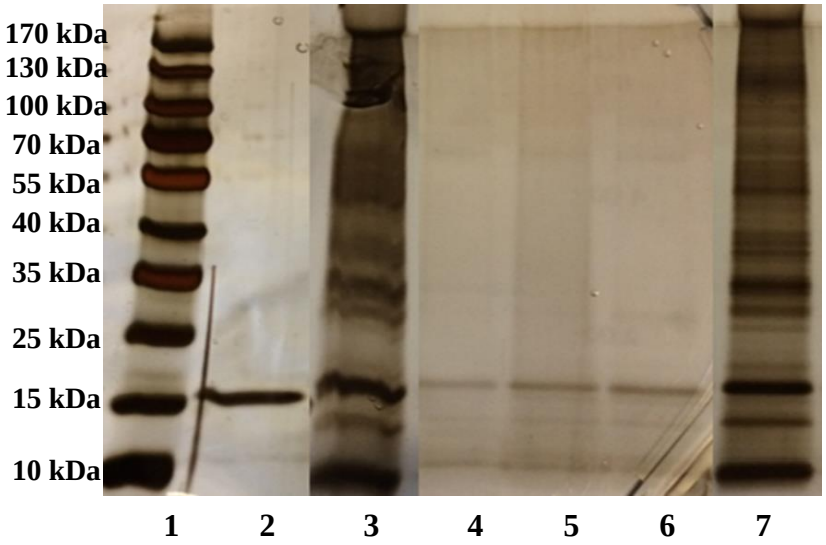

Figure 3-41. SDS-PAGE images of G-30-D-6.5 filtration.
1) Marker; 2) GCSF standard (10 mg/L); 3) Feed solution (20X); 4-5-6) Permeates (no dilution); 7) Retentate (60X).

When the pH value was decreased to 5.2, it was seen that permeation of the GCSF as well as the other proteins having larger size (25 kDa to 50 kDa range) increases in the G-30-D-5.2 filtration. In this case, all proteins including GCSF were positively charged. Therefore, transmission of them through negatively charged membrane became easier due to attraction of the proteins and membrane. If the pH was adjusted to 4, transmission of the larger molecular weight proteins increased more in G-30-D-4 filtration where all proteins in the production medium had also positive charge. Moreover, they have higher charge compared to pH=5.2 (Figure 3-42). Higher protein transmission at this pH value could be due the fact that, the charge on the proteins were

higher at pH=4 compared to pH=5.2. This may create two effects on the transmission during filtration. First is, attraction forces may act more to the proteins and the membrane can aid the protein transmission. Second is, more charge on the proteins may prevent their agglomeration.

From these results, it was implied that changing pH of the feed solution could affect the separation behavior significantly for rGCSF production medium.

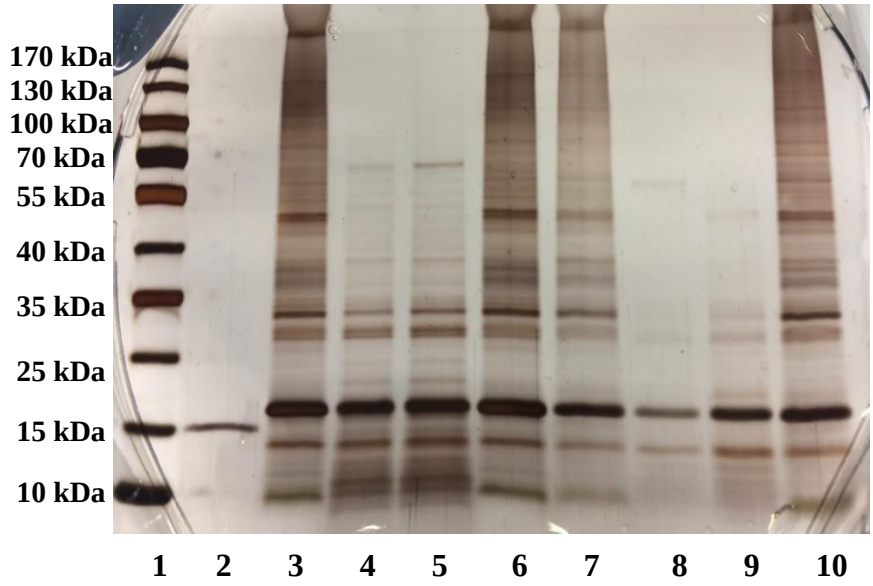

Figure 3-42. SDS-PAGE images of G-30-D-4 and G-30-D-5.2 filtrations.
1) Marker; 2) GCSF standard (10 mg/L); 3) Feed solution of G-30-D-4 filtration (20X); 4-5) Permeates of G-30-D-4 filtration (no dilution); 6) Retentate G-30-D-4 filtration (60X); 7) Feed solution of G-30-D-5.2 filtration (20X); 8-9) Permeates of G-30-D-5.2 filtration (no dilution); 12) Retentate of G-30-D-5.2 filtration (60X).

In filtrations at all three pH values, GCSF retention was significantly high, however; there was some selectivity for GCSF over the other proteins. However, retention values were calculated as about 99% and 98% for the filtrations of G-30-D-5.2 and G-30-D-4 respectively which show that the yield was quite low. On the other hand, PWP values before and after filtration were almost same. Therefore, membranes were able to be cleaned physically (Figure 3-43).

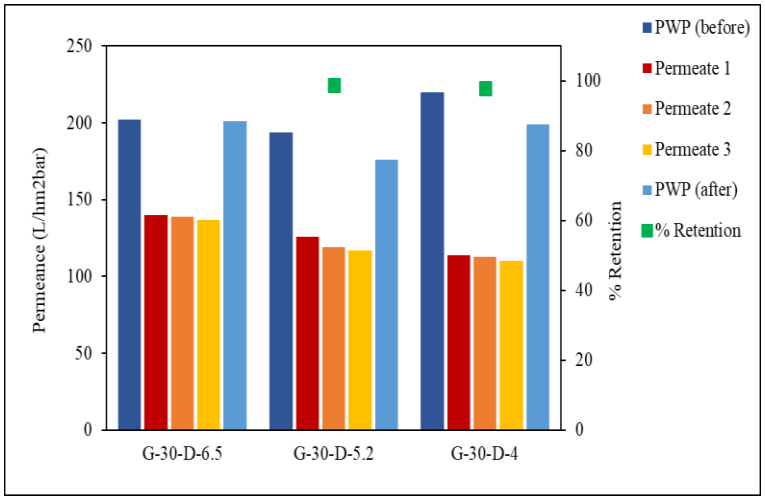

Figure 3-43. Permeance and retention values for G-30-D-6.5, G-30-D-5.2 and G-30-D-4 filtrations.

Changing pH of the feed solution strategy was applied for another case as well. For this time, 100 kDa membrane filtration was conducted and permeate samples of this filtration were collected and then pH change was carried out. Final solution was used as feed solution for 30 kDa membrane filtration. If the pH was adjusted as 5.2, GCSF and the proteins having smaller size could pass through the membrane whereas the other proteins having larger molecular weight than GCSF were rejected by the membrane G-30-P-5.2 filtration. In G-30-P-4 filtration, pH was equal to 4, other proteins having larger size can also pass to the permeate side contrary to pH=5.2 case.

Actually, similar behavior was observed at this strategy compared to the filtrations of G-30-D-5.2 and G-30-D-4. However, transmission of larger sized proteins was observed as lower in G-30-P-5.2 and G-30-P-4 filtrations. From these results it was implied that other proteins having larger size were highly rejected in 100 kDa membrane filtration.

Consequently, it was implied that the best result was achieved when pH was equal to 5.2 when the 100 kDa membrane filtration permeate samples used as feed solution for 30 kDa membrane filtration (G-30-P-5.2). Comparison of the filtrations were shown in Figure 3-44.

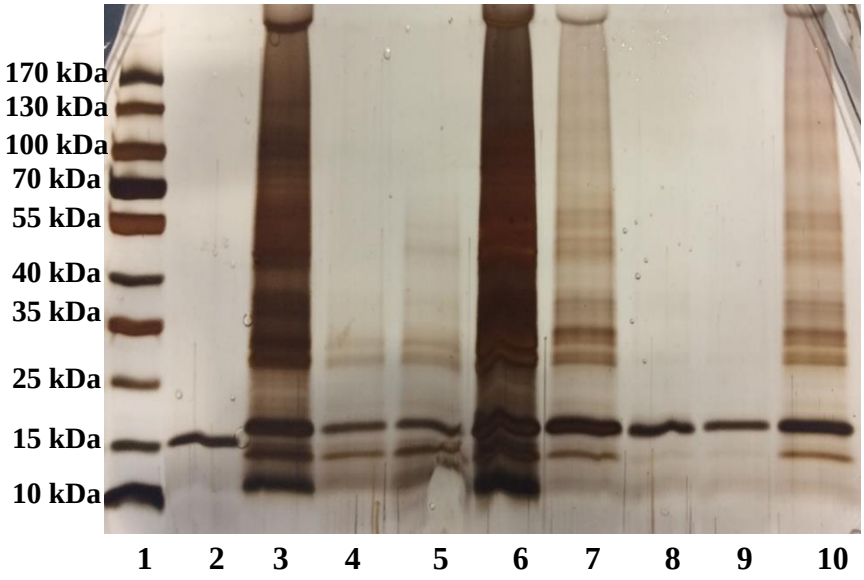

Figure 3-44. SDS-PAGE images of G-30-P-4 and G-30-P-5.2 filtrations. 1) Marker; 2) GCSF standard; 3) Feed solution of G-30-P-4 filtration (3X); 4-5) Permeates of G-30-P-4 filtration (no dilution); 6) Retentate of G-30-P-4 filtration (7X); 7) Feed solution of G-30-P-5.2 filtration (3X); 8-9) Permeates of G-30-P-5.2 filtration (no dilution); 12) Retentate of G-30-P-5.2 filtration (7X).

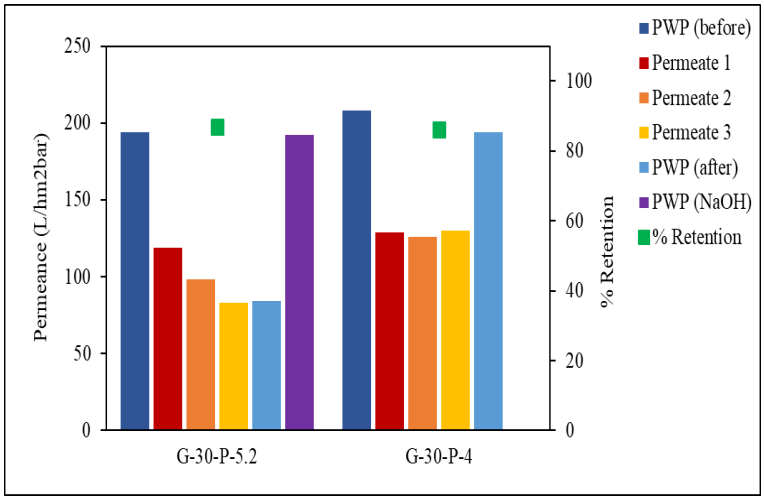

Figure 3-45. Permeance and retention values for G-30-P-5.2 and G-30-P-4 filtrations.

Figure 3-45 indicates the permeance and retention values in G-30-P-5.2 and G-30-P-4 filtrations. Chemical cleaning was performed with 0.1 M NaOH to regenerate the membrane since fouling was not cleaned physically. On the other hand, only by

physical cleaning, fouling could be removed in G-30-P-4 filtration. GCSF rejections were measured as 86% and 87% for G-30-P-5.2 and G-30-P-4 respectively.

In hGH production medium, limiting flux was observed to minimize possibility of fouling. In GCSF environment, pressure was reduced as much as possible (0.03 bar) to minimize the concentration polarization during filtration. G-100-0.03b (GCSF-100 kDa-0.03 bar) and G-30-P-0.03b-5.2 (GCSF-30 kDa-permeate-0.03 bar, pH=5.2) filtrations were performed at this pressure and it was observed in Figure 3-47 that the permeance values during filtration increased as expected and became more close to the PWP values. Therefore, it was considered that concentration polarization was lower at 0.03 bar due to lower pressure value.

Average flux values for this filtration was also compared with the filtrations which were conducted at 0.1 and 0.5 bar to get information about limiting flux. In the Figure 3-46, It was observed that limiting flux of 30 L/hm^2 was achieved around at 0.1 bar for 100 kDa MWCO membrane. Therefore, the flux values at 0.03 bar were below the limiting flux for this membrane. For 30 kDa MWCO membrane, filtrations were performed only at 0.1 bar and 0.03 bar. However, it was clearly seen that flux at 0.1 bar was higher than the flux at 0.03 bar. Therefore, it could be said that the flux at 0.03 bar was below the limiting flux, however; to learn whether it was reached to the limiting flux at 0.1 bar or not one more filtration must be conducted at higher pressure.

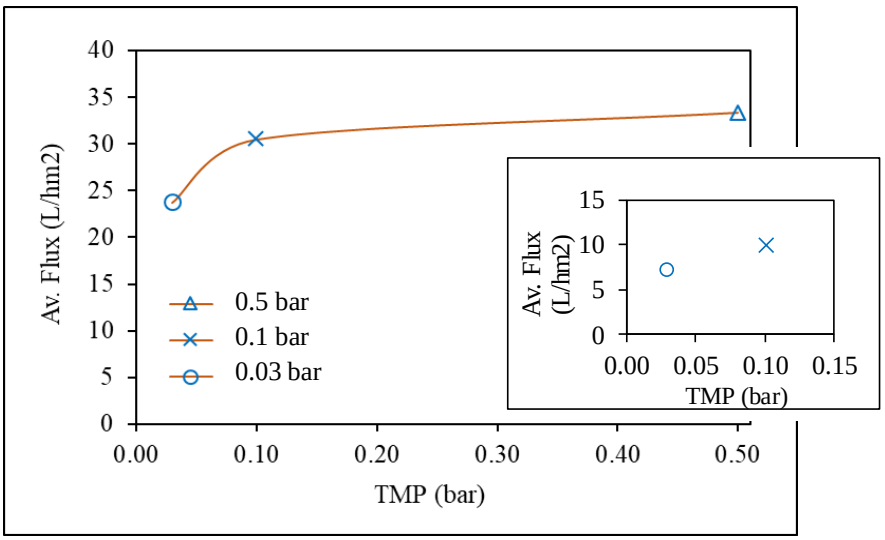

Figure 3-46. Limiting Flux for 100 kDa and 30 kDa MWCO membranes (GCSF).

In addition, retention values were detected as 96% G-100-0.03b filtration and 91% for G-30-P-0.03b-5.2 filtration which were a little higher than the filtrations at 0.1 bar (Figure 3-47). On the other hand, SDS-PAGE images in Figure 3-48 show that there was no significant change in terms of separation. For this reason, since 0.03 bar is very low pressure that is hard to keep it stable so it was not used for further experiments.

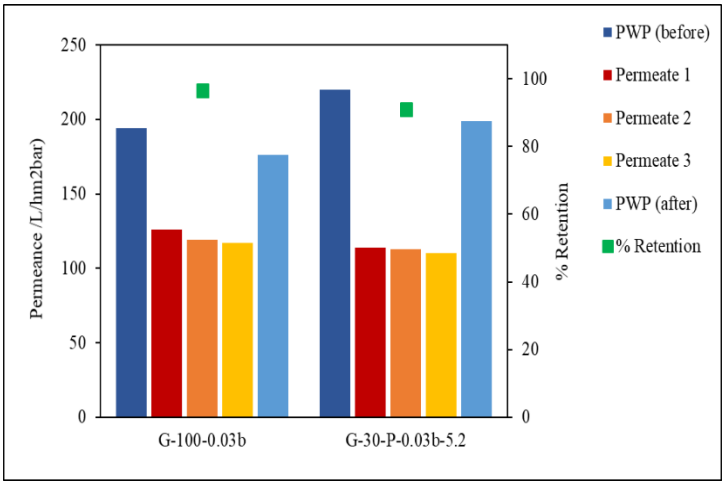

Figure 3-47. Permeance and retention values for G-100-0.03b and G-30-P-0.03b-5.2 filtrations.

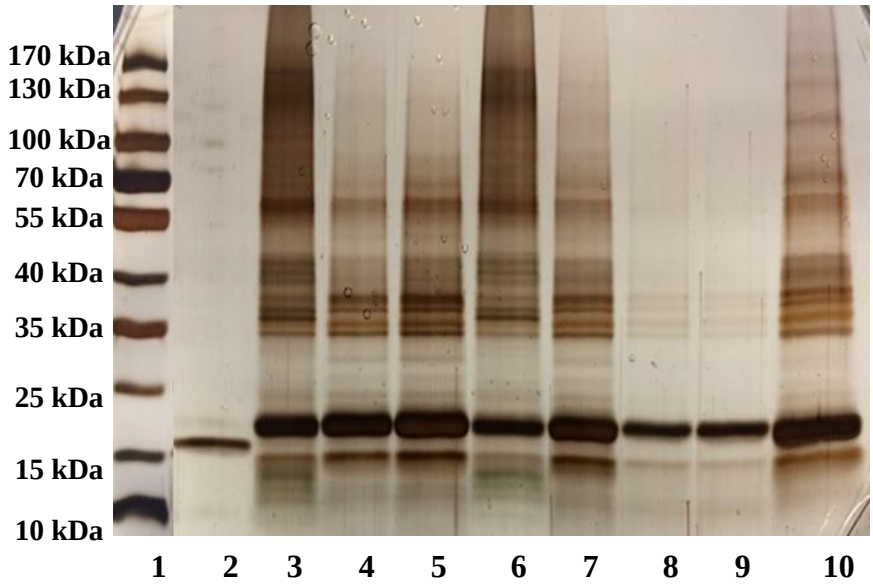

Figure 3-48. SDS-PAGE images of G-100-0.03b and G-30-P-0.03b filtrations. 1) Marker; 2) GCSF standard; 3) Feed solution of G-100-0.03b filtration (20X); 4-5) Permeates of G-100-0.03b filtration (no dilution); 6) Retentate of G-100-0.03b filtration (60X); 7) Feed solution of G-30-P-5.2 filtration (3X); 8-9) Permeates of G-30-P-5.2 filtration (no dilution); 12) Retentate of G-30-P-5.2 filtration (7X).

3.3.3. Retentions and Enrichment Factor for the rGCSF Environment

As a result of the experiments conducted with GCSF production medium, high GCSF retentions were measured. Retention of desired recombinant protein for each filtration were summarized in Table 3-4.

Table 3-4. Summary of the retentions for rGCSF environment.

Filtration Code	Retention of rGCSF
G-100-0.5b	74%
G-30-D-5.2	99%
G-30-D-4	98%
G-30-P-5.2	86%
G-30-P-4	87%
G-100-0.03b	96%
G-30-P-0.03b-5.2	91%

To explore the extent of enrichment for GCSF in the filtrations, enrichment factor was calculated as follows;

$$Enrichment\ factor = \frac{\left(C_{GCSF}/C_{Total}\right)_{permeate}}{\left(C_{GCSF}/C_{Total}\right)_{feed}} \qquad 2.3$$

Protein concentrations (C_{Total}) in each sample were calculated by Bradford assay. GCSF concentration was determined from LabWorks program. As it was observed from Table 3-5, GCSF enriched more in permeate for G-30-P-4 filtration case compared to other filtrations.

Table 3-5. Enrichment factor for rGCSF production medium.

		Absorbance	C_{Total} (mg/L)	C_{GCSF} (mg/L)	C_{GCSF}/C_{Total}	Enrichment Factor
G-30-D-5.2	Feed	0.753	1051.037	573.957	0.546	
	Permeate 1	0.074	103.754	5.848	0.056	**0.103**
	Permeate 3	0.110	153.538	20.547	0.134	**0.245**
	Retentate	1.299	1812.679	1859.137	1.026	
G-30-D-4	Feed	0.768	1072.440	661.900	0.617	
	Permeate 1	0.040	55.832	30.131	0.540	**0.874**
	Permeate 3	0.084	117.247	37.223	0.317	**0.514**
	Retentate	1.294	1806.165	2441.270	1.352	
G-30-P-5.2	Feed	0.298	415.948	55.372	0.133	
	Permeate 1	0.046	64.207	11.955	0.186	**1.399**
	Permeate 3	0.064	89.331	6.452	0.072	**0.543**
	Retentate	0.492	686.268	84.516	0.123	
G-30-P-4	Feed	0.615	858.882	28.379	0.033	
	Permeate 1	0.056	77.700	5.249	0.068	**2.045**
	Permeate 3	0.103	143.767	6.945	0.048	**1.462**
	Retentate	1.197	1670.773	56.097	0.034	

3.4. Membrane Production

PES-Pluronic 127 blend membranes with different preparation recipe were also tested for the filtrations of GCSF production environment. PES was used as the membrane polymer. Since PES membranes were hydrophobic, protein adsorption thereby the membrane fouling is easier compared to cellulosic membranes. Therefore, Pluronic was used as additive to minimize fouling by making the surface hydrophilic for these membranes [40,44]. It was aimed to tune the pore size more finely compared to different commercial membranes.

In Figure 3-49, SEM images of produced PES membranes are given for cross-section at total, non-solvent side of the cross section and surface of the membranes. Microporous skin layer and macroporous support layer can easily be seen from figures for both membranes. Moreover, as it was expected, PES-B membranes were denser so that having smaller pore size than PES-A membranes since the polymer concentration in B solution was higher (15%).

Furthermore, pore size measurement was performed by using ImageJ software. As a result, pore sizes was measured for PES-A membrane approximately between 14-70 nm whereas PES-B membrane had pores having size between 10-20 nm.

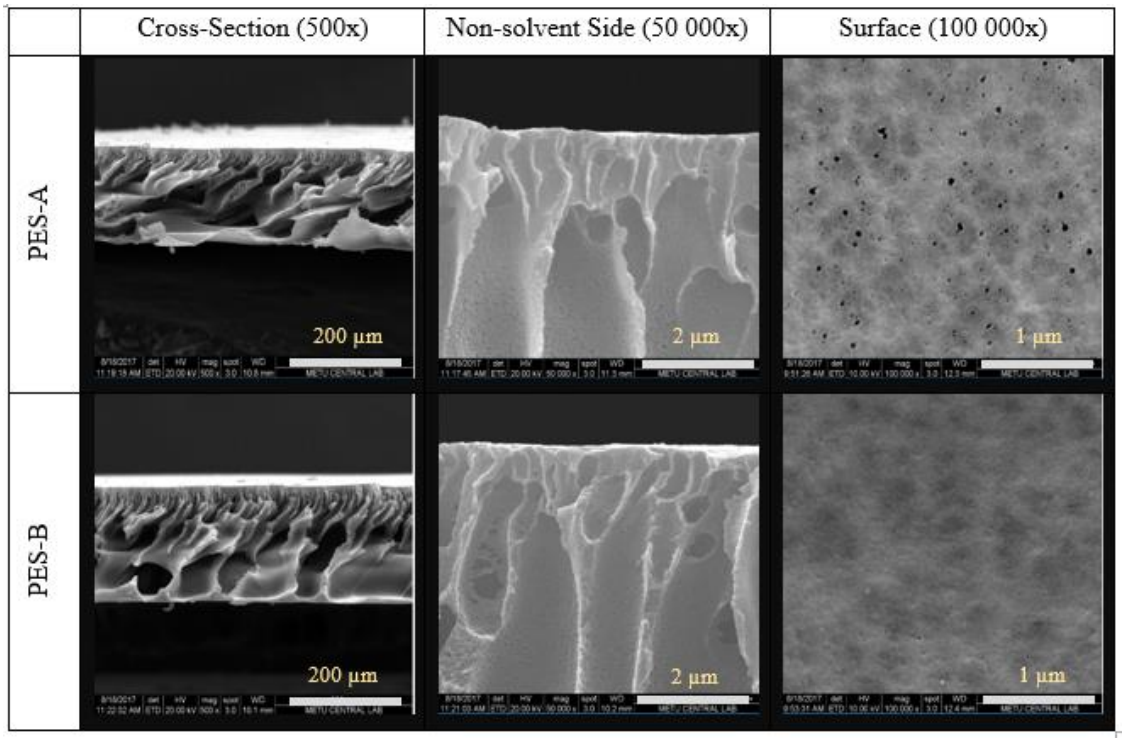

Figure 3-49. SEM images of produced PES membranes.
Scale bars are 200 µm (500x) for cross-section, 2 µm (50 000x) for side and 1 µm (100 000x) for surface images.

GCSF production medium was used as feed solution in these experiments, however, pH of the solution was adjusted before filtration started. Initially, the membranes were casted by using A solution (PES-A), and then used for ultrafiltration experiments. After measuring of the PWP of the membrane, filtration was performed at pH 4. There was

sharp decrease in the permeance value according to the PWP. Moreover, it was seen that from SDS-PAGE image, Figure 3-50, the first permeate sample was the same as feed solution whereas after first permeate sample, all proteins were retained by the membrane. This implies that proteins blocked the pores of the membranes that caused fouling. Therefore, there was no protein transmission thorough the membrane after some time. PWP measurement after filtration showed that permeance did not reach first value which also supports the presence of fouling (Figure 3-51).

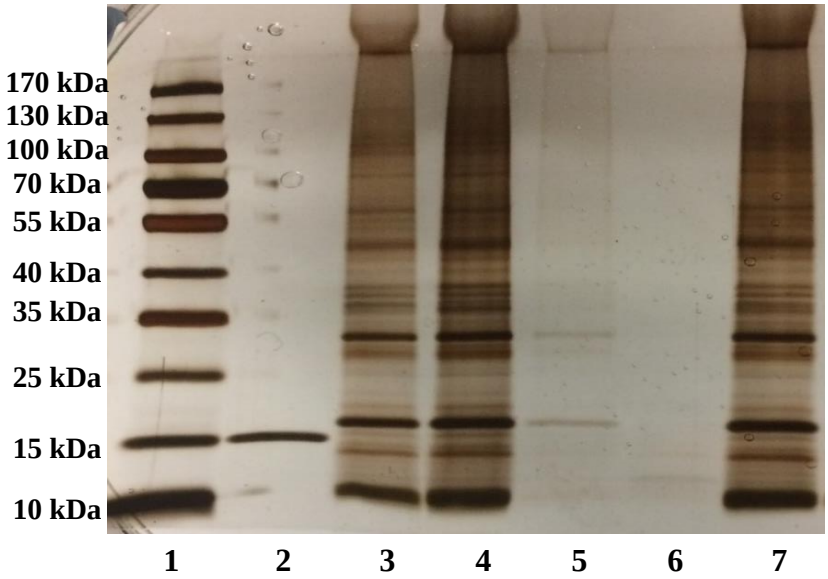

Figure 3-50. SDS-PAGE images of PES-A membrane filtration.
1) Marker; 2) GCSF standard; 3) Feed solution of PES-A membrane filtration (20X); 4-5-6) Permeates of PES-A membrane filtration (no dilution); 7) Retentate of PES-A membrane filtration (60X).

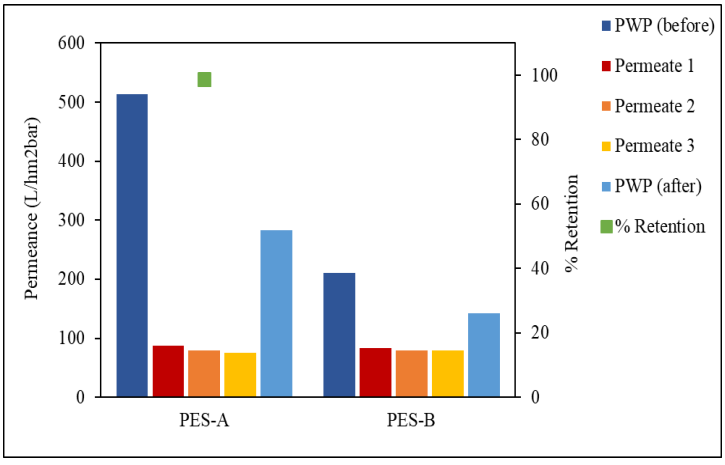

Figure 3-51. Permeance and retention values for PES-A and PES-B filtrations.

As it is known, PES membranes tend to foul more than cellulose membranes because of their hydrophobic characteristics [20]. Although Pluronic F-127 was used to make the surface of these membranes hydrophilic, it is possible that they are still more hydrophobic than cellulose. As the membranes could not be cleaned physically, chemical cleaning was applied with the use of 0.1 M NaOH. With this application, PWP reached to its original value that showed the fouling was removed. Retention of the GCSF in this filtration was calculated about as 99 %.

After that, the membranes which were expected to be denser membranes because of the higher polymer concentration of the B solution (PES-B) were used. As a result of the filtrations that were conducted with these membranes, there was also decrease in permeation value during filtration, however it was not as much as PES-A membrane. No separation was achieved with this membrane because it rejected all of the proteins in the medium including GCSF (Figure 3-52).

As a result, it was determined to produce another membrane with polymer concentration between A and B solutions for future studies.

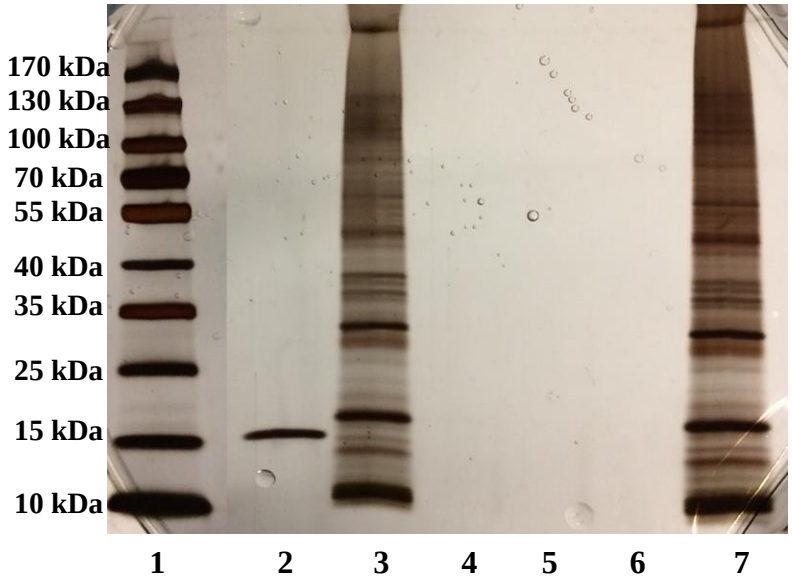

Figure 3-52. SDS-PAGE images of PES-B membrane filtration.
1) Marker; 2) GCSF standard; 3) Feed solution of PES-B membrane filtration (20X); 4-5-6) Permeates of B-B-C-1-1 membrane filtration (no dilution); 7) Retentate PES-B membrane filtration (60X).

CHAPTER 4

CONCLUSIONS

In this study, separation of the recombinant proteins from bioreactor environment by using ultrafiltration membranes was investigated. Ultrafiltration experiments generally were conducted in dead end mode at certain transmembrane pressure by using selected pore sized commercial regenerated cellulose membranes. To understand the separation mechanism, initially, BSA and casein were used as model proteins. High retention values was observed for BSA with 30 kDa MWCO membrane (99.5%). In the case of casein with 10 kDa and 30 kDa MWCO membranes, retentions were found as 98% and 97% respectively.

After conducting experiments with model proteins, to investigate complex medium, bioreactor samples containing recombinant proteins were collected. One of the production media contains rhGH produced in extracellularly. After removing the cells by centrifugation, solutions were used as feed solutions for ultrafiltration experiments. Sized based filtrations were performed and observed that hGH, was specifically retained by the membrane. It was considered that there was agglomeration of the hGH molecule in the medium. Furthermore, the transmission of the larger molecular weight of the proteins to the permeate side was observed. This unexpected transmission can arise from salt content of the hGH environment since production of hGH was extracellular and no washing step was applied before filtrations. It is known that presence of the salt can reduce the electrical double layer thickness of the proteins. Having compact double layer can increase the transmission of the proteins. To improve separation performance, pH change and modification on membrane surface were performed for hGH environment. As a result of the experiments, changing pH of the feed solution did not affect the separation significantly but positively modified membrane increased the retention.

For this production medium, diafiltration mode was also conducted to investigate its effect on separation behavior. Although separation performance increased, high rejection of hGH was observed from first set of the filtration. In addition to that, PES

membranes were tried for filtrations due to the possibility of degradation of RC membranes at the end of the diafiltration. However, there was still high retention of hGH and unexpected permeation of the larger molecular weight proteins.

Second production medium included recombinant Granulocyte-Colony Stimulating Factor (rGCSF) and was produced in intracellularly. Prior to use in ultrafiltration experiments, production medium was pretreated by washing with urea that can also reduce the salt content in the medium. Size based separation was achieved to some extent with the use of 100 kDa and 30 kDa MWCO membranes. To enhance the separation of GCSF, attraction/ repulsion forces between the proteins and the membrane were exploited. For this reason, pH of the feed solution was altered to desired value. The best result was achieved when the pH was equal to 5.2 and permeate samples of the 100 kDa membrane filtration were used as feed solution for 30 kDa membrane filtration.

GCSF production medium was also used in the filtrations with produced PES membranes instead of commercial ones. For the experiments, two different polymer solutions were prepared having different polymer (PES) concentration to tune the membrane pore size. After casting of the polymers, ultrafiltration experiments were performed at which no separation of the desired protein was achieved due to rejection of the all proteins and pore blockage. Therefore, as a recommendation, polymer concentration can be arranged to get transmission of GCSF and high rejection of the other proteins in the production medium.

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APPENDIX A

CALIBRATIONS OF MODEL PROTEINS

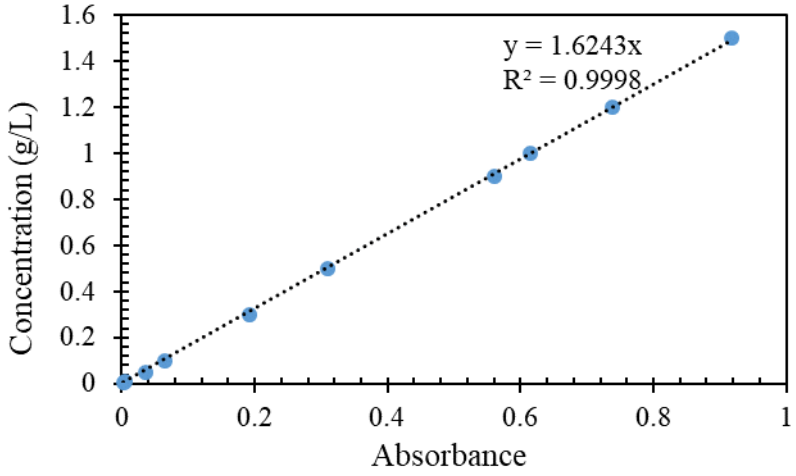


Figure A-1. Calibration Curve for BSA at 280 nm.

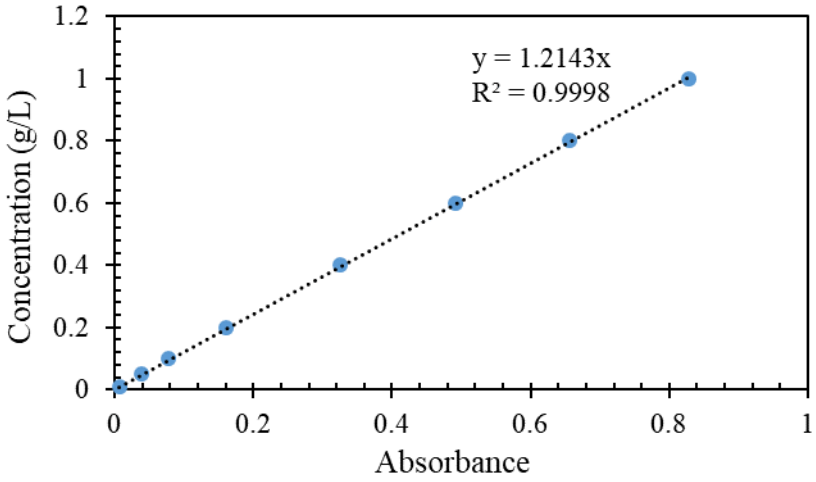
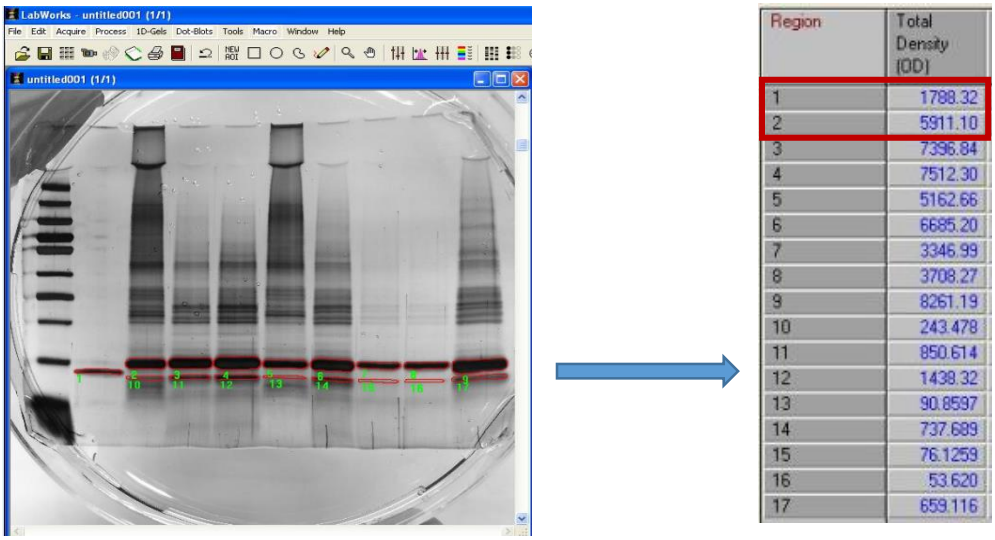


Figure A-2. Calibration Curve for Casein at 280 nm.

APPENDIX B

SAMPLE RETENTION CALCULATION



Region	Total Density (OD)
1	1788.32
2	5911.10
3	7396.84
4	7512.30
5	5162.66
6	6685.20
7	3346.99
8	3708.27
9	8261.19
10	243.478
11	850.614
12	1438.32
13	90.8597
14	737.689
15	76.1259
16	53.620
17	659.116

Figure B-1. LabWorks program output.

Optical densities are measured for each protein band by using computer program LabWorks. Optical density value of standard band is compare with optical densities of feed, permeates and retentate bands according to the formula. Since the concentration of the standard is known, unknown concentration of the protein bands is achieved approximately. By this way, % retention values can also be found from calculated concentrations by using formula given below.

$$\frac{Total\ density\ (OD)\ for\ sample * concentration.of\ standard}{Total\ density\ (OD)\ for\ standard} * dilution\ factor$$

$$= \frac{1788.32 * 10\ mg/L}{5911.1} * 20 = \mathbf{661.079\ mg/L}$$

Table B-1. Feed, average permeate, retentate concentrations and retention values for proteins calculated from SDS-PAGE.

	C_F (mg/L)	$\langle C_P \rangle$ (mg/L)	C_R (mg/L)	Retention (%)
GCSF	661.079	41.68	1732.126	**96.5**
13 kDa	27.230	6.40	30.484	**77.8**

$$\% \, Retention = \left(1 - \frac{< C_P >}{(C_R + C_F)/2}\right) X \; 100 \; \%$$

$$\% \, Retention = \left(1 - \frac{41.68}{(1732.126 + 661.079)/2}\right) X \; 100 \; \% = \mathbf{96.5} \; \%$$

APPENDIX C

CALIBRATION FOR BRADFORD ASSAY

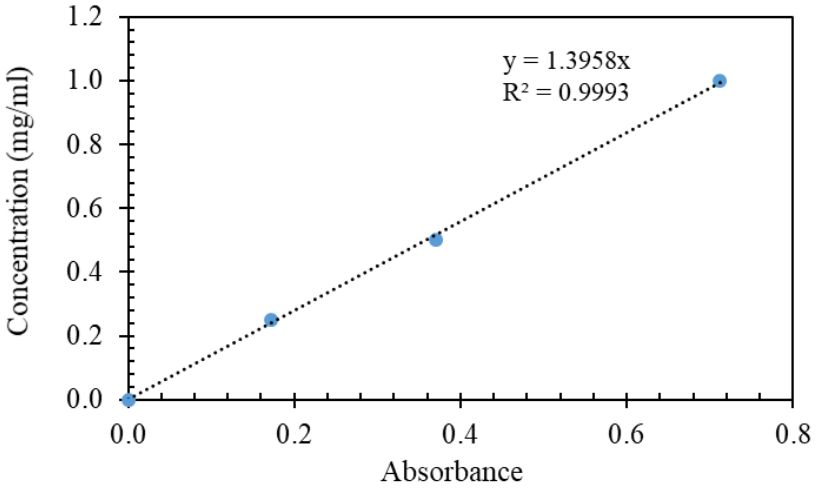


Figure C-1. Calibration Curve for Bradford Assay at 595 nm.

APPENDIX D

FTIR MEASUREMENTS

In FTIR analysis, it was expected to appear the peak between 1000-1350 cm^{-1} for C-N bond for positively modified membrane and around 1350 cm^{-1} for S=O bond for negatively modified membrane. Moreover, OH bond gives broad peak around 3200-3600 cm^{-1} that must be disappear as a result of the modification. Since C-N and S=O bonds overlap the bonds comes from cellulose structure, only change in the OH bond could be observed.

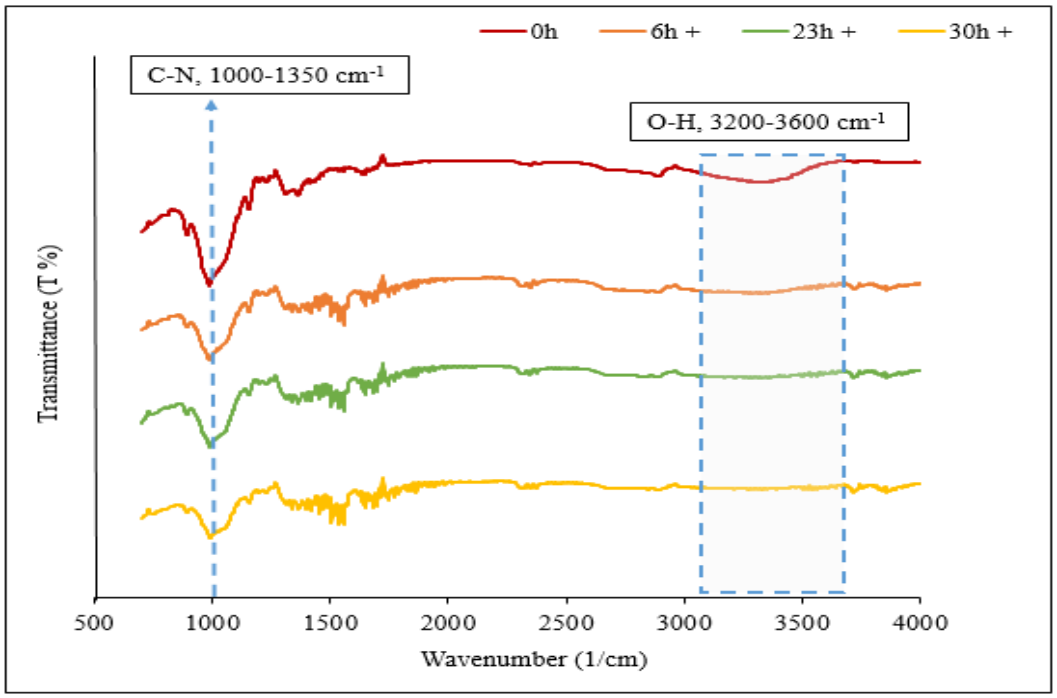


Figure D-1. FTIR result for positive modification.

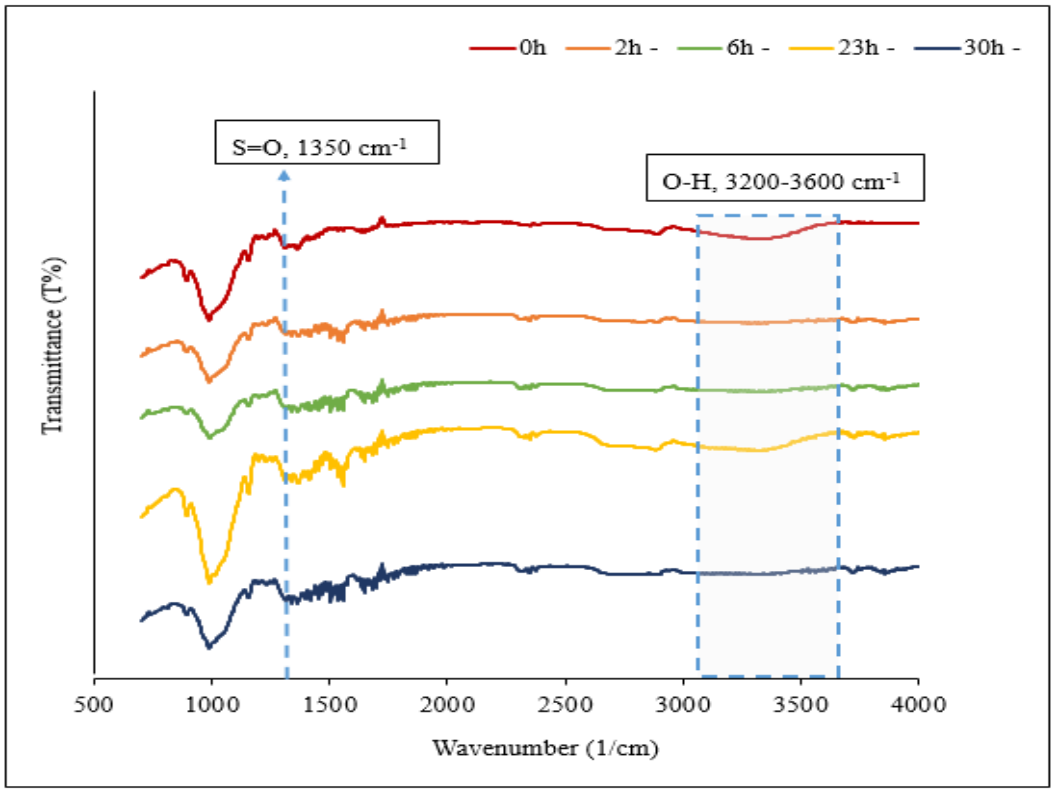


Figure D-2. FTIR result for positive modification.

APPENDIX E

COMPOSITON OF THE BIOREACTOR SAMPLES and IONIC STRENGTH CALCULATION

rhGH production medium was produced extracellularly and composed of different chemicals including salts were listed in Table E-1.

Table E-1. Composition of production medium of rhGH [35].

Component	**Concentration (g/L)**
Glycerol (86 %)	50 ml
85% H_3PO_4	26.7 ml
K_2SO_4	18.2
$MgSO_4.7H_2O$	14.9
KOH	4.13
$CaSO_4.2H_2O$	1.17
Chloromphenicol	1 mL
PTM	4.35 mL
10% antifoam	1 mL
Up water	to 1 L

PTM (Pichia trace minerals) is added to the production medium to maintain a healthy cell growth in production media [35]. As it was shown in Table E-2, PTM contains high amount of salt/mineral content which is involved in the production environment.

Table E-2. Composition of Pichia trace salts (PTM) in rhGH medium [35].

Component	**Concentration (g/L)**
$CuSO_4.5H2O$	6
H_3BO_3	0.02
NaI	0.08
$Na_2MoO_4.2H_2O$	0.2
$MnSO_4.H_2O$	3
$ZnCl_2$	20
$FeSO_4.7H_2O$	65
$CoCl.6H_2O$	0.916
H_2SO_4	5 mL
Biotin	0.2 mL
Up water	to 1 L

Since salt concentration was important factor in the transmission of the proteins, ionic strength of the medium was calculated according to the formula below and results were summarized in Table E-3.

$$I = \sum Z_i^2 C_i$$

where Z*i* and C*i* represent the charge of the ion *i* and molar concentration respectively.

Ionic strength was calculated as 2.1386 for rhGH production medium.

Table E-3. Ionic strength measurement for rhGH medium.

	Concentration (g/L)	**Concentration (mol/L)**	**Ionic Strength (mol/L)**
$CuSO_4.5H2O$	6	0.02403	0.09612
NaI	0.2	0.00083	0.00248
$Na_2MoO_4.2H_2O$	3	0.01775	0.07099
$MnSO_4.H_2O$	20	0.14675	0.44025
$ZnCl_2$	65	0.23380	0.93520
$FeSO_4.7H_2O$	0.916	0.00452	0.01357
K_2SO_4	18.2	0.10444	0.31332
$MgSO_4.7H_2O$	14.9	0.05973	0.23890
$CaSO_4.2H_2O$	1.17	0.00680	0.02718
			2.1386

On the other hand, intracellular rGCSF production medium also consisted of salts; however, due to washing steps with urea, salts in the production medium were removed before ultrafiltration experiments. Composition of rGCSF medium and trace minerals were listed in Table E-4 and Table E-5.

Table E-4. Composition of production medium of rGCSF [36].

Component	**Concentration (g/L) In Batch phase**	**Concentration (g/L) In Feeding phase**
Glycerol	30	750
KH_2PO_4	13.3	
$(NH_4)_2HPO_4$	4	
$MgSO_4.7H_2O$	1.2	14.7
Citric acid.H_2O	1.86	

Table E.5. Composition of trace minerals in rGCSF medium [36].

Component	**Concentration (g/L) In Batch phase**	**Concentration (g/L) In Feeding phase**
EDTA	14.1	9.56
$CoCl_2.6H_2O$	2.5	2.94
$MnCl_2.4H_2O$	15	17.3
$CuSO_4.5H_2O$	2.78	1.7
H_3BO_3	3	3.45
$Na_2MoO_4.2H_2O$	2.1	2.94
$ZnCl_2$	21	7.3
$FeCl_3.6H_2O$	111	32.4
Thiamine.HCl	4.5	3.3
Antifoam Y-30	500	
Ampicillin	50	50

APPENDIX F

MOLECULAR WEIGHT MARKER

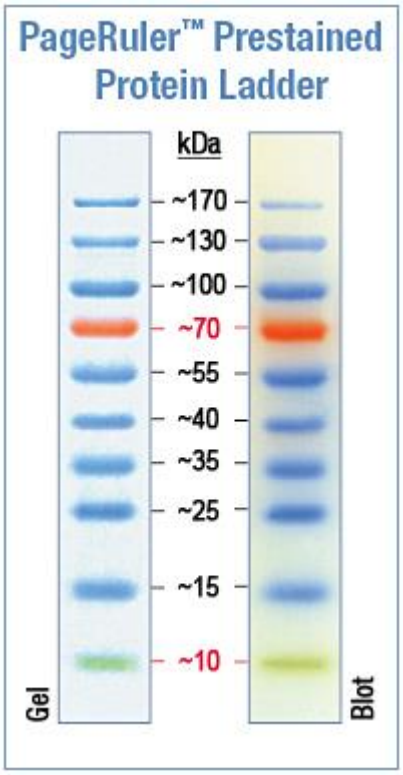


Figure F-1. PageRulerTM Prestained Protein Ladder.