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LYSOZYME TRANSMISSION THROUGH POLYMERIC BASED ULTRAFILTRATION MEMBRANE: EFFECT OF PH AND IONIC STRENGTH

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ABSTRACT

Fractionation and purification of complex protein mixture has becomes a great interest and has attracted a considerable amount of attention in recent years. This study aimed is to demonstrate the factors influencing the lysozyme separation through polyethersulfone ultrafiltration (UF) membrane. Asymmetric UF membrane with 15% polymer composition (UF15) was developed via phase inversion technique. Membrane was characterized in term of morphology, membrane surface charge and molecular weight cut-off to ensure its suitability for lysozyme separation. Effect of pH on the transmission of lysozyme through the UF15 membrane has been examined at different pH (5, 7, 9, 11 and 13) and ionic strength (0.1M, 0.2M and 0.3M). Results displayed that at optimum pressure 3 bars, permeation with pH 9 and 0.1M ionic strength of feed solution obtained the optimum flux and lysozyme transmission for about 36.6 x 10^{-5} $m^3/m^2$and 92.9%, respectively. This study has proved that pH and ionic strength were found to be greatly affected the lysozyme transmission and promoted the lysozyme separation to a significant degree.

Keywords: lysozyme, membranes, ultrafiltration, protein, pH, ionic strength.

INTRODUCTION

Ultrafiltration (UF) is a pressure-driven separation process which walk in the path of development and yet attempting to search for the new quality product through the worldwide. It has a wide variety of application, ranging from the processing of biological macromolecules, electrocoat paint recovery, enzyme and pharmaceutical preparations to wastewater treatment (Ghosh, 2002). Some of the major applications are the fractionation of nucleic acids, concentration of macromolecules, dialfiltration, removal of cells and debris from fermentations broth, virus removal from therapeutic products, harvesting of biomass and effluent treatment (Cheryan, 1986) and also in many process engineering with significant technical and commercial impact. On top of that, total usage of UF membrane in food and biotechnology applications are also currently increase. In the case of high value therapeutic protein based product, separation and purification cost can be high. Thus, it makes good economic sense to develop cost-effective and scaleable purification processes for such product by using ultrafiltration membrane in separation process. When it comes to proteins processing, UF is mainly used for removal of high molecular weight compounds and particles from protein solution (Ghosh, 2003).

Ultrafiltration lies between nanofiltration (NF) and microfiltration (MF) membranes and capables of retaining species in the range of 300-500,000 Daltons of molecular weight (MW). This type of membrane is typically used to retain macromolecules and colloid from a solution that has a mixture of some desirable components and some that are not. UF processes operate at low pressure, between 1 to 10 bars (Mulder, 1996) and is based on variety of synthetic polymers, have high thermal stability, chemical resistivity, and restricted the use of fairly harsh cleaning chemicals (Reis and Zydney, 2007; Zydney and Kuriyel, 2000). Most of polymeric membranes used for protein ultrafiltration are asymmetric structure which demonstrates a heterogeneous morphology and generally consists of a very thin dense top layer, supported by a porous sub layer with thickness ranging from 50 to 150μ (Mulder, 1996). This type of membrane also promoted a better permeation rate than symmetric membrane of comparable thickness of the actual barrier layer (Idris *et al*., 2002). On top of that, asymmetric structure of UF membrane gives the membranes its required mechanical strength (which is provided by the support layer) along with its desired separation properties (which are governed by the skin layer). It is recognised that the separation properties of porous membranes also depends on their physical properties including porosity and pore size distributions (Field *et al*., 2009).

Currently, the total usage of ultrafiltration (UF) membrane in biotechnology applications has increased up to 40% (Ghosh and Cui, 2000). One of the high demand proteins currently is lysozyme, which is a significant enzyme for different applications including as a food additive in milk products, a cell-disrupting agent for extraction of bacterial intracellular products and as a drug for treatment of ulcers and cancers. Lysozyme is normally found in chicken egg white (CEW) together with the other proteins such as ovalbumin, conalbumin and ovomucin.

The low content of lysozyme in chicken egg white (~3.4% of total protein) makes its purification process becomes a challenge since a large quantity of raw material has to be processed in order to get a reasonable

amount of pure lysozyme. Thus, an efficient, large scale-protein purification process is required to apply for lysozyme purification. At present, conventional techniques are most of the current process widely used in lysozyme purification which included chromatography, electrophoresis and centrifugation (Wan *et al*., 2006). Those techniques however only suited for producing a small quantity of proteins and difficult to scale-up. To overcome this obstacle, UF membranes are seen as an alternative separation technique which is cost effective and can be fine tuned to achieve high productivity in protein separation process. Selective transmission of a protein through membrane proves to be a major drawback for UF process. This is due to the transportation of solute through the membrane does not depend only on size, but also several factors such as solute-solute and solute-membrane interactions (Ghosh and Cui, 1998). Thus, physicochemical parameters such as pH value and salt concentration need to be optimized in order to get a high throughput of product.

In the present study, 15% PES UF membranes were fabricated to be used for lysozyme separation. The fabricated membranes were characterized by means of pure water permeability, membrane morphology and membrane surface charged and molecular weight cut-off. The effects of different pH and ionic strength on lysozyme transmission have been studied, aiming at optimization of operating conditions for the efficient transmission of lysozyme through UF membrane.

METHOD

Materials

For dope preparation, Polyethersulfone (purchased from merck) was used as polymer, N-methyl-2-pyrrolidone (NMP) (supplied by Merck) was used as solvent and pure water (H_2O) as non-solvent in PES/NMP solution. All chemicals and reagents used are of analytical grade. Distilled water (H_2O) was used as a coagulation bath medium and methanol as post treatment medium.

Membrane preparation

A homogenous dope solution consists of PES 15 % (w/w), NMP 77% (w/w) and water 8% (w/w). Membranes were fabricated via simple dry/wet phase inversion technique using an electrically casting machine at shear rate $200s^{-1}$ and then immersed directly into a coagulation bath for 24 hours. Post-treatment was carried out using methanol for about 8 hours to remove any residual solvent left.

Permeation with pure water and lysozyme single solution

All permeation experiments were carried out using dead end cell, supplied by Sterlitech HP4750 with 300 ml processing volume and effective permeation membrane area of 14.6 cm^2. Distilled water was used for pure water permeation to obtain pure water permeability and ensure the membrane stability. The operating pressures of eachlysozyme permeation test, 500 ppm lysozyme single solution was prepared fresh via used by dissolving the lysozyme powder (supplied by sigma aldrich) in NaCl solution at room temperature. Lysozyme solution was adjusted to three different pH values which are pH 4.5, 8.5, and 10 by adding 0.01M hydrochloric acid (HCl) or 0.01M sodium hydroxide (NaOH). NaCl solutions were also prepared in three different concentrations which are 0.1M, 0.2M and 0.3M. Concentration of lysozyme present in feed and permeate were analyzed using a UV-Visible Spectrophotometer at a wavelength of λ_{max}=280 nm. Membrane performance is expressed by the percentage of transmission (T), which is defined by the concentration of solute in the permeate phase, C_p, relative to the concentration of the solute in feed, C_f.

$$T (\%) = (Cp/C_f) \times 100 \quad (1)$$

Membrane characterization

Characterization in term of membrane morphology was employed using Scanning Electron Microscopy (SEM) (JSM P/N HP475 model). Automatic coater JFC 100 model has used to coat the membrane specimen and the cross section of membrane was obtained by liquid nitrogen freeze fracturing followed by auto-coating step with a thin gold layer using the automatic coater.

To determination of membrane molecular weight cut-0ff, aseries of protein (myoglobin [17kD], ovalbumin [40kD], Pepsin [35kD] and bovine serum albumin [66 kD]) with different molecular weights were used for rejection studied to determine MWCO of the fabricated membrane. From the feed and permeate concentrations, the percentage of solute rejection (SR) was calculated using equation 2.

$$\%SR = [1-(C_p/C_f)] \times 100 \quad (2)$$

whereC_p/ and $C_f$are the concentration of permeate and feed, respectively.

The prepared membrane was also characterized in term of membrane zeta potential using Electro Kinetic Analyzer (EKA) (Anton PaarGmbh Graz, Austria). The conductivity Dip-in-cell was calibrated before used and the membrane sheets were cut into a rectangular size (12.8cm x 5.1cm) that mounts on the measuring cell. EKA was rinsed with deionized water before measurement of zeta potential to remove the bubbles from the sample. The results were analyzed using Visiolab software after measurement.

RESULTS

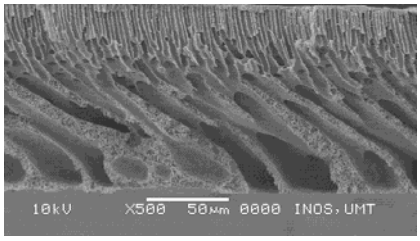

Figure-1. Cross section of UF15 membrane.

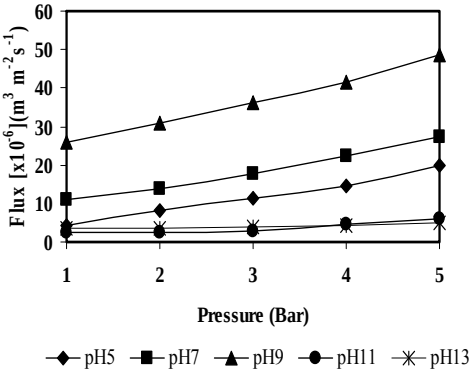

Figure-2. Filtrate flux of lysozyme transmission with UF15-M at different pH value.

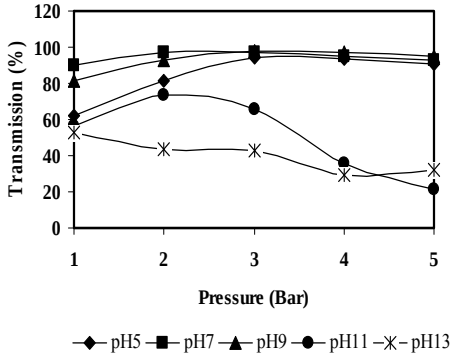

Figure-3. Lysozyme transmission through UF15-M with at different pH.

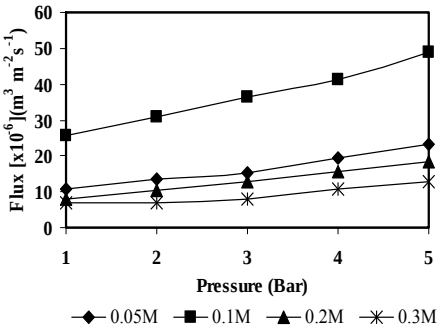

Figure-4. Filtrate flux of lysozyme transmission at different ionic strength.

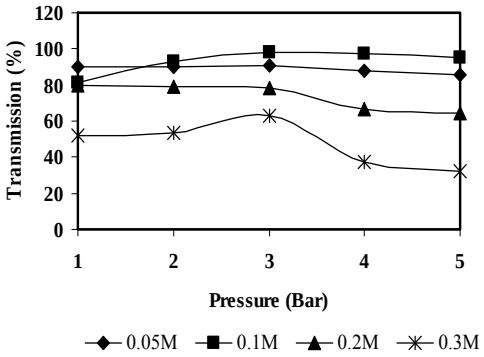

Figure-5. Lysozyme transmission through UF15-M at different ionic strength.

DISCUSSIONS

Membrane characteristics and properties

Permeability coefficient which indicated the membrane stability for UF15 was determined by pure water permeation. According to Mulder (1996), pure water permeability for ultrafiltration membrane was in the range of 10-50 L/m^2.h.bar (2.8x 10^{-6} to 1.4 x 10^{-5} m^3/m^2.s.bar) for operating pressure 1-5 bars. The result postulated that UF15 is a loose UF membrane when its permeability coefficient iswas slightly higher the range of ultrafiltration membrane, for about 2.3 x 10^{-5} m^3/m^2.s.bar. This was due to a lower polymer concentration used in the dope solution has led to reduce the membrane thickness and thus improved the membrane permeability.

Figure-1 illustrates the morphology of UF15 membrane which comprises of well developed skin layer and supported by a porous support layer with large finger like, sponge like and macrovoid structures. Different structure is formed was due to the solvent-non-solvent exchange, leading to the different starting conditions for phase separation at layers far from the surface. Macrovoids formation giving rises in UF15 membrane due to its lower polymer concentration (Koros and Mackelvey, 1996). On top of that, large finger like structure in UF15 membrane was resulted from the rapid solvent precipitation during phase inversion process (Young and Chen, 1995). Separation behavior occurs at the skin (active) layer of the membrane and the bottom layer which is the support layer acts as a mechanical strength of the membrane.

Molecular weight cut off (MWCO) is customarily used to indicate the pore size of ultrafiltration membrane, and its value can be determined from the solute rejection of membranes against the stable molecules with various weights, which can be measured with ultrafiltration process (Becht *et al.*, 2008). In this study, molecular weight cut off of UF15 has been estimated to be 43 kD and this large MWCO would possible to allow the lysozme molecule to pass through the membrane at maximum amount.

Zeta potential is an important indicator of the membrane surface charge and it was observed that zeta potential of UF15 was about -62mV. Negatively charge membranes are widely used since it can selectively partition the ions in the salt mixture through the electrostatic interaction between ions and membrane (Wang and Chung, 2005). In this study, negatively charge UF15 would attract the positively charge lysozyme onto the membrane surface and membrane pores. Operating pressure applied would desorbed the lysozyme molecule to pass through the membrane pores which tend to improve the filtrate flux and lysozyme transmission.

Effect of pH on lysozyme transmission

Transmission of lysozyme through UF membrane was found to be very dependent on pH as the electrical charge carried by protein was greatly influenced by solution pH. A change in solution pH can alter the electrical charge on protein as well as the membrane due to the ionization or deionization of various acidic/basic groups on the protein and the membrane surface which can cause either repulsive or attractive interactions (Aravind *et al*., 2007). Furthermore, this factor also can alter the conformation of proteins, which can affect the protein diffusion coefficient (Pujar and Zedney, 1998; Burns and Zedney, 1999).

The isoelectric point (p*I*) of lysozyme is 11 (Narsaiah and Agarwal, 2007) and the lysozyme molecules would be cationic at pH values below than 11. Previous section has mentioned that UF15 is negatively charged membrane with zeta potential for about -62 mV. Thus, UF15 and lysozyme carry opposite charge at three pH examined in this study (pH 5, 7 and 9). The attraction between the membrane and lysozyme molecule were occurred and led it to be easily passed through the membrane. At isoelectric point, the net charge of lysozyme would be zero. At pH 13, lysozyme molecule would be anionic and the repulsion between the lysozyme molecule and membrane surface charge tends to give high resistance for lysozyme to pass through the membrane. Figures 2 and 3 represent the flux and transmission of lysozyme versus pressure at different pH using UF15 membrane.

At all pH value, the flux increased with the increasing of applied pressure. In the beginning of filtration period, protein interaction is low and lysozyme mainly adsorbed onto the membrane surface to form a dense cake layer. Thus, lowest filtrate flux at lowest pressure was due to the strong electrostatic interaction (Tung *et al*., 2007). However, the result obtained was not reliable with the findings from previous researchers since they found that the flux declined will be occurred after a certain point during the filtration process due to the fouling phenomena (Muller *et al*., 2003). High porosity of UF15 might provide this promising result which tends to avoid a crucial fouling phenomenon. Besides, the membrane has flushed with distilled water after permeation in order to remove the bounded protein on the membrane pores.

pH 9 was determined as an optimum pH for lysozyme separation since it was presented the highest of average filtrate flux (36.6 x 10^{-6} m^3/m^2.s) along with high average lysozyme transmission; around 92.9%. This high flux and transmission obtained since this pH 9 was closer to the isoelectric point of of lysozyme and thus the net positive charge of lysozme has decreased. Lysozyme was best transmitted through the membrane with a low positive charge since the electrostatic interaction of lysozyme decreases while that of the membrane electrostatic interaction increases. At the beginning of the filtration process, the interaction between the protein was low. Thus, lysozyme was mainly absorbed onto the membrane to form a dense cake layer which lead to the reduction in the filtrate flux since the membrane electrostatic interaction here was very strong. The highest lysozyme transmission at high solution pH is also proven since the applied pressure and vertical drag force during filtration process desorbed the lysozyme from the membrane surface and pore wall, allow it to be easily passed through the membrane (Tung *et al.,* 2007). Reduced the pH of lysozyme solution from 9 to 7 has also reduced the filtrate flux but slightly increased the lysozyme transmission. The average flux and lysozyme transmission for pH 7 was around 18.5 x 10^{-6} m^3/m^2.s and 94.5%, respectively. These values were considered as moderate flux and high transmission since the pH was shifted quite far from the isoelectric point of lysozyme. Thus the positive charge of lysozyme and interaction between the lysozyme molecule become increased and promoted high potential of lysozyme adsorbtion onto the membrane surface. Accumulation of lysozyme molecule onto the membrane surface has reduced the filtrate flux. However the highest average transmission was obtained at this pH and the reason for this finding was not clear yet since the highest transmission was expected to be occured at pH 9 due to the low electrostatic interaction between lysozyme molecule and membrane surface.

The filtrate flux and lysozyme transmission were seemed to be declined as the pH solution was reduced to pH 5. The average filtrate flux and transmission were 11.5 x 10^{-6} m^3/m^2.s and 84.5%, respectively. This result was in good agreement with the previous research which mentioned that the protein transmission would be lower at pH values which is away from its isoelectric point (Muller *et al*., 2003). The membrane carries a moderately negative charge and at pH 5 lysozyme molecule is expected to carry a large net positive charge since the pH was shifted away from the protein's p*I*. Hence, some of lysozyme molecule would be adsorbed onto the membrane surface which could possibly lead to "self-rejection" of positively charged lysozyme in the solution bulk by positively charged lysozyme adsorbed onto the membrane surface. This result is in good agreement with the previous finding which mentioned that greater self-rejection of protein would occur at pH far away from the isoelectric point of protein (Ghosh and Cui, 1998). Protein-membrane interaction is very important when it comes to membrane fouling. Accumulation of protein lysozyme on the

membrane surface leading to form a dense cake layer and consequently lowered the permeate flux when the solution pH was adjusted to pH 5.

In addition, at low pH value which far apart from the isoelectric point of lysozyme, the protein charge become increased, led to increase the effective volume of lysozyme molecule due to the presence of a diffuse ion cloud around the protein (Aravind *et al.*, 2007). This condition would give high resistance to the protein lysozyme to pass through the membrane. Some of lysozyme molecule would retain onto the membrane surface which consequently reduced the flux and transmission due to the fouling phenomena. Besides, high charge density of lysozyme was increased the lysozyme adsorption onto the negatively charged membrane. However, the low pressure applied during the filtration process would not desorb some of lysozyme molecule in the membrane pore, leading to pore blocking phenomena which has reduced the lysozyme transmission.

Separation of lysozyme at its isoelectric point (pH 11) was presented a great decay in flux and lysozyme transmissionwhich averages around 3.7 x 10^{-6} m^3/m^2.s and 50.6%, respectively. This result was not reliable with the expectations based on the findings of some earlier researchers who generally assumed that the highest transmission of a protein through a membrane would be at isoelectric point (p*I*) of the protein (Ghosh *et al*., 1998). The highest transmission obtained only 73.5%, at pressure 2 bars. The transmission was further decreased even a higher operating pressure was applied. The low filtrate flux and protein transmission at pH 11 might be caused by cake layer on the membrane surface and membrane fouling phenomenon which occurred at the initial stage of filtration. At isoelectric point of lysozyme, this proteins tend to be comapacted, which result in higher density of the protein layer. Therefore, this dense layer has reduced the flux and lysozyme transmission through the membrane (Muller *et al*., 2003).

The lowest lysozyme transmission was observed at pH 13, which averages only 40.1%. Above the isoelectric point, lysozyme would be negatively charge creating a strong electrostatic repulsion between protein and membrane (Tung *et al*., 2007). Thus, a smaller concentration polarization occurred and reduced the lysozyme transmission but slightly increased the filtrate flux with average 4.08 x 10^{-6} m^3/m^2.s. Repulsion effect between the protein and negatively charge membrane has also led to the accumulation of lysozyme onto the membrane surface which finally resulted in cake formation, consequently reduced the flux and lysozyme transmission.

Overall results clearly determined that the percentage of lysozyme transmission at five different pH started to decrease after the highest transmissions obtained. This situation is mainly contributed from the formation of highly resistant filter cake, resulting from accumulation of the protein solutes drawn toward the filtering surface by tangential flow of filtrate through the membrane (Iritani *et al*., 1995). Therefore even the applied pressure was further increased, lysozyme transmission was continued to decrease.

The effect of ionic strength on lysozyme transmission

Optimization of ionic strength forms an integral part of many strategies of protein separation process by virtue of its effectiveness and ease of control. Protein separation using ultrafiltration is drastically influenced by the nature of solute-solute interaction and also depends on salt concentration (Wan *et al*., 2006). Value of pH was fixed at 9 since the previous result showed the optimum condition for lysozyme separation was found to be at pH 9.

The effects of ionic strength on filtrate flux and lysozyme transmission are shown in Figures 4 and 5, respectively. There was remarkable change in the filtrate flux through the change in ionic strength. A low ionic strength of protein solution (0.05M) presents a lower flux and protein transmission due to the lower electrostatic exclusion interaction between protein and membrane. At this point, the average flux and transmission was determined around 16.415 x 10^{-6} $m^3/m^2$and 88.9%, respectively. This result isacceptable since the lysozyme transmission was almost 90% according to moderate flux.

At a very low salt concentration (less than 0.05M), lysozyme is assumed to be retained onto the membrane surface since low shielding of positive charge of lysozyme has occurred. The strong interaction between the positively charged protein and negatively charged membrane led to lower the flux and transmission. The proteins were much more adsorbed on the membrane surface, resulting in an increase of fouling (Muller *et al*., 2003).

Increased the ionic strength to 0.1M was enhanced the flux and lysozyme transmission, with the average of 36.634 x 10^{-5} $m^3/m^2$and 92.9%, respectively. The highest transmission was obtained at pressure of 3 bars with 98% transmisssion. High transmission of lysozyme in 0.1M ionic strength can be explained in term of electrostatic interaction between the lysozyme molecule. At higher ionic strength, the shielding effect was enhanced so that the adsorption effect was weakened and higher flux and transmission was obtained (Muller et al., 2003). In other words, this high ionic strength; resulting from matching pore size and protein molecular weight would reduce the electrostatic interaction between the protein, therefore allow them to pass through the membrane easily, creating a higher flux and protein transmission.

Further increased of ionic strength above 0.1M would not improve the filtration process since the reduction of flux and transmission was observed when the ionic strength was shifted to 0.2M. Average transmission for 0.2M ionic strength was determined around 73.7% with a lower flux; 13.6 x 10^{-6} m^3/m^2. Shifting of ionic strength to 0.2M would more likely reduce the positive effects of the slight positive charge by causing additional shielding which could lead to lower the lysozyme transmission. Besides, with the increased of ionic strength,

the passage of lysozyme molecule through the membrane becomes more difficult. Although this effect could not be explained satisfactory, it can be said that higher NaCl concentrations facilitate protein-protein interaction (Scopes, 1994). This enables the formation of small agglomerates, making it more difficult for the protein to pass the membrane pores and therefore leading to a lower transmission (Muller, 2003).

The highest ionic strength used in this study (0.3 M) presented the lowest average flux (9.107×10^{-6} m^3/m^2) and lysozyme transmission (47.7%). This result is in good agreement from the findings of previous research which mentioned that the transmission of lysozyme was quite stable around 0.1M to 0.2M NaCl (Wan *et al*., 2006). Further increase in salt concentration has significantly reduced the lysozyme transmission. Besides, in high salt concentration range, protein solubility generally decreases (salt-out effect) due to the reduced activity of water and the neutralization of surface charge. This also favors the formation of protein aggregates, leading to the decrease in protein transmission. As the electrostatic interactions depend on the magnitude of protein surface charge and protein electrical double layer, both of which are ionic strength dependent (Wan *et al*., 2006). Therefore, the low transmission at 0.3M ionic strength might be caused by salt-out effect which occurs due to charge shielding effect of salt.

Figure-4 clearly shown that a similar trend of lysozyme transmission was found in four ionic strengths examined in this study, where the lysozyme transmission increased with pressures and started to decrease after the highest point was achieved even the applied pressure was higher. This phenomenon occurred since the most lysozyme molecule can pass through the membrane during the initial stage of filtration process when the pore size of membrane is larger than the pore radius of lysozyme. After it reached the saturating point, lysozyme starts to deposit onto the membrane surface and pore wall which leading to enhance fouling and reduced the lysozyme transmission.

CONCLUSIONS

This research proposed that fabricated UF15 membrane was profound as a good and efficient membrane for lysozyme separation and purification since it posses high selectivity and permeability behaviors. This study has also proved that ionic strength and pH were significantly affecting lysozyme transmission across the UF membrane. The optimum condition for lysozyme separation was found to be at pH 9 with 0.1M ionic strength.

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