



Assessment on Flux Reduction and Protein Rejection Behavior in Fractionating Tilapia By-Product Protein Hydrolysate by Ultrafiltration Membrane

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ABSTRACT

Modification of tilapia by-products (TB) into fish protein hydrolysate (FPH) using enzymatic treatment is a favorable approach for enhancing their values and applications in the food industry. The TB protein hydrolysate has a wide range of sizes, which is possible to be fractionated using ultrafiltration (UF) membrane for obtaining small sized peptides. Thus, the present study aims to assess the flux reduction behavior of ultrafiltration membrane by varying transmembrane pressure, stirring speed and solution pH. Regenerated cellulose membrane of 10 kDa molecular weight cut-off (MWCO) was used throughout of the study. It was found that the trends for flux behavior of all parameters were reaching steady state within 60 – 70 minutes. At completion of filtration (at 70 minutes), the highest permeate flux for each operating and physicochemical parameters were at 3 bar (13.6 L/m²h), 600 rpm (42.8 L/m²h) and pH 8 (53.4 L/m²h). In term of protein rejections, significant effects were attained for stirring speeds with reduction of 57% from 0 to 600 rpm and lowest protein

rejection (9.6%) was obtained at pH 8. Thus, controlling the operating parameters of the UF process could reduce membrane fouling.

Keywords: Fish protein hydrolysate, flux reduction, protein hydrolysate, protein rejection, tilapia by-product, ultrafiltration

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INTRODUCTION

Processing of tilapia fish for the production of tilapia fillet would eventually generates a huge amount of by-products such as bones, frames, heads, and skin. Disposal of these by-products to the land fill may result in serious environmental problem (Arvanitoyannis & Kassaveti, 2008). Alternatively, such by-products can be converted into valuable products such as bioactive peptides for applications in food, healthcare and pharmaceutical products. Enzymatic hydrolysis is the most preferred method used for converting fish by-products into fish protein hydrolysate which composed of peptide mixtures with different sizes and biological functions (Kristinsson & Rasco, 2000). Several studies have reported that small-sized peptides have great biological activities such as antioxidant (Suwal et al., 2018; Sabeena Farvin et al., 2016; Zhong et al., 2011; Picot et al., 2010), antihypertensive (Roslan et al., 2017; Raghavan & Kristinsson, 2009; Bougatef et al., 2008; Theodore & Kristinsson, 2007) and antimicrobial (Najafian & Babji, 2012). Production of peptides with small-sized (<10 kDa) through enzymatic hydrolysis is a challenging process which cannot be achieved by merely controlling the degree of hydrolysis (DH). Therefore, it requires downstream processes such as fractionation and purification approaches to ensure that the production of peptides on a large scale and the desired molecular sizes is successful. Purification of bioactive peptides from protein hydrolysates can be achieved through chromatographic methods due to its high selectivity, but the process required high cost especially for scale-up applications (Agyei et al., 2016).

In this regard, ultrafiltration (UF) process is the most promising technology that offers several advantages including high throughput, ability to separate protein hydrolysate effectively, easily controlled and scale-up and could maintain the peptide with high biological activity (Abejon et al., 2018; Raghavan & Kristinsson, 2009; Je et al., 2005). Ultrafiltration process has been widely used as a tool for obtaining peptides with sizes less than 10 kDa (Raghavan & Kristinsson, 2009; Kim et al., 2007; Ranathunga et al., 2006; Jeon et al., 1999). Production of these specific peptide sizes in greater amounts using ultrafiltration membranes still continues as a major constraint. In UF processes, the occurrence of membrane fouling cannot be avoided, eventually decreasing the separation of the FPH and consequently reducing the yield of peptide (Prata-Vidal et al., 2001). This is because the effectiveness of peptides fractionation is not merely influenced by membrane properties (pore size), but also affected by the hydrodynamic conditions and feed solution characteristics (She et al., 2009). Protein aggregation may also occur as a result of protein-protein and protein-membrane interactions in the feed solution. Such interactions could lead to extensive accumulation of protein near the membrane surface, thus causing detrimental effect to the final product throughput. An efficient UF process for improving the yields is highly desirable. Several studies have demonstrated that proteins

and peptides can be effectively separated by manipulating the operating conditions such as solution pH and salt concentration which greatly influence the electrostatic interaction between the proteins and membrane (Saidi et al., 2013; Das et al., 2009; Ghosh & Cui, 1998). The effectiveness of proteins and peptides separation using ultrafiltration membrane could be monitored through the flux reduction (decline) and protein rejection behaviour which are the most important indicator of fouling and selectivity (Almecija et al., 2007; Das et al., 2009; Datta et al., 2009; Saidi et al., 2013). In order to minimize membrane fouling during fractionation, controlling the operating parameters, particularly the transmembrane pressure, system hydrodynamics, and pH of feed solution are necessary in order to achieve high throughput of the desired product. Fractionation of tilapia protein hydrolysate using UF membranes has been conducted by several researchers (Raghavan & Kristinsson, 2009; Dekkers et al. 2011). However, in their studies, the UF membrane was merely used as a tool for separation of small-sized peptides and lacked discussion on the effect of operating parameters on the fractionation process. Hence, the objective of this study is to investigate the effectiveness of UF membrane in fractionating tilapia by-product (TB) protein hydrolysate by manipulating the operating parameters such as pressure, stirring speed, and pH to obtain high yield of peptide.

MATERIALS AND METHODS

Tilapia By-product Mince Preparation

Tilapia (*Oreochromis niloticus*) (500-600 g) was bought from a local fish farm in Rawang, Selangor, Malaysia. The sample was kept cool using ice and afterward cleaned, washed and filleted to get the tilapia by-products (TB) (frames, head, and tail). A high-speed grinder was used to mince the TB, kept in polyethylene plastic bags and frozen it at -20°C . Prior to enzymatic hydrolysis, the frozen minced TB was defrosted in a refrigerator ($4 \pm 1^{\circ}\text{C}$).

Tilapia By-product Protein Hydrolysate Preparation

Hydrolysis of the TB was conducted by mixing 7.5 g of the minced TB with 50 mL (15% w/v, 0.05 M) phosphate buffer solution (pH 7.5) and 60 AU/kg of alcalase enzyme (a bacterial endoproteinase from a strain of *Bacillus licheniformis*, Novo Nordisk, Denmark) followed by 60 min incubation at 60°C . After that, the mixture was heated in a water bath (temperature at 90°C) for 15 min with intermittent agitation to terminate the hydrolysis reaction. Ice was used to cool the mixture, then a refrigerated high-speed centrifuge (Eppendorf, Model 5804 R) at 10,000 rpm ($14,136 \times g$) for 20 min was used to separate the particle (sedimentation) and solution, which the supernatant (top solution) was saved (Roslan et al., 2015) for further processes. Analytical grade with $> 95\%$ purity of chemical reagents were used in this experiment.

Membrane Module and Type

Dead-end ultrafiltration membrane (Amicon Model 8200 stirred ultrafiltration cell, Amicon Corp., Danvers, MA) with 10 kDa MWCO flat sheet regenerated cellulose (RC) membrane (Ultracell PLGC; 28.7 cm² filtration area) was used to fractionate the TB protein hydrolysate. Setup of the UF process is shown in Figure 1. The UF stirring system consisted of suspended bar impeller that magnetically driven stirring hot plate (Favorit). A digital photo tachometer (HYELEC Model MS6208B) was used to monitor the stirring speed.

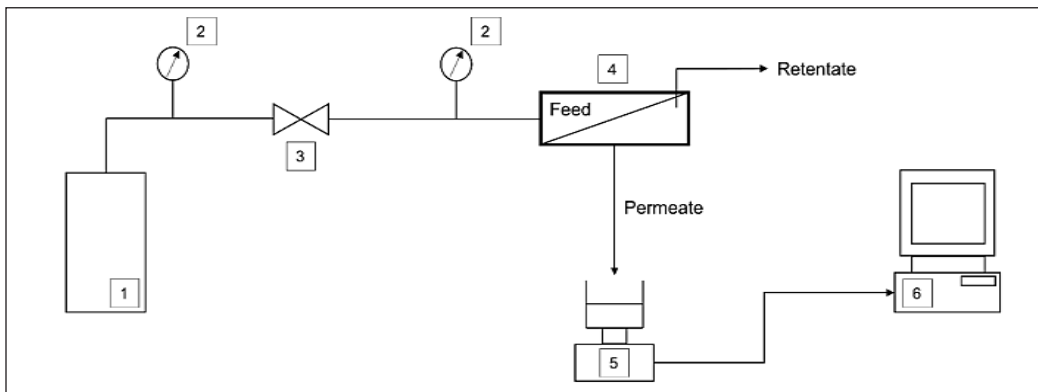


Figure 1. Schematic of experimental set-up (1. Oxygen gas tank; 2. Pressure gauge; 3. Valve; 4. Stirred cell module; 5. Balance; 6. Computer).

Membranes Preparation

The 10 kDa MWCO flat sheet regenerated cellulose (RC) membrane was soaked in deionized water (DI) overnight. To prevent any alterations in membrane hydraulic resistance throughout the process, the membrane had undergone compaction at 3.5 bar until the water flux value became steady (Das et al., 2009). The water flux was determined at different pressure from 0.5, 1, 1.5, 2, to 2.5 bar, that was used as a reference for cleaning process.

Cleaning Procedure

The RC membrane was first rinsed with deionized water, followed by soaking in 0.1% of NaOH solution and sonicated for 15 minutes. The membrane was then moved into a beaker containing deionized water and once more sonicated for 15 minutes. Finally, rinsed the membrane using deionized water up to neutral condition and then measured the water flux. If water flux obtained did not reach the initial value, the cleaning procedure needed to be repeated. The cleaned membrane was stored in a chiller at 4°C until further use.

Evaluation of the Membrane Performance

The membrane performance was assessed based on permeate flux and protein rejection factor.

Permeate Flux. In a membrane separation process, the permeate flux is used as indicator of productivity of the process. The flux value is influenced by several factors such as properties of the membrane, the system hydrodynamics, the transmembrane pressure, the concentration of protein in the feed, and the protein and solvent properties. the permeate flux was measure using equation (1) (Ghosh, 2003):

$$\text{Flux} = \frac{\text{Total quantity passed through membrane}}{\text{Membrane area} \times \text{time}} \text{ L}/(\text{m}^2\text{h}) \quad (1)$$

Protein Rejection Factor. Another factor used to describe the membrane system performance is the rejection factor. Protein rejection factor is expressed by the following equation (Lin et al. 2008):

$$\text{Protein rejection factor, } R_f (\%) = 1 - \frac{C_p}{C_f} \times 100 \quad (2)$$

where C_f is the concentration of solute in the feed solution and C_p is the concentration of solute in the permeate.

Membrane Filtration Experiments at Different Trans-Membrane Pressure, Stirring Speed and Solution pH. *The Effect of Trans-Membrane Pressure (Tmp) on Permeate Flux Reduction and Protein Rejection.* TB protein hydrolysate was fractionated using RC membrane (pore size 10 kDa) at TMP of 1, 2 and 3 bars while the other parameters such as stirring speed and pH were fixed at 0 rpm, pH 6.8 (original pH solution), respectively. Permeate was collected at 5 minutes interval until 70 minutes and permeate flux was measured. The protein rejection was calculated based on the permeate at 70 minutes. All experiments were carried out in duplicate. The highest TMP value is used for the next experiment. The same procedure was repeated for the effect of stirring speed and pH.

Statistical Analysis

Statistical analysis was performed using the Statistical Analysis System (1989) with ANOVA and Duncan's multiple range test for multiple comparisons. The same software was used to calculate the standard deviation.

RESULTS AND DISCUSSION

Effects of Transmembrane Pressure (TMP) on Flux Reduction and Protein Rejection

The fractionation was conducted under different transmembrane pressure (TMP; 1 to 3 bar) with the feed solution was kept at original pH (pH 6.8) and without stirring. The flux reduction behaviour was shown in Figure 2. Generally, the percentages of flux reduction

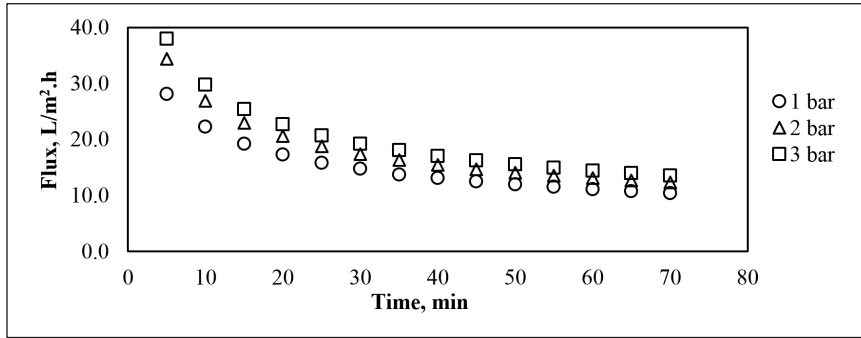


Figure 2. Flux reduction with different transmembrane pressure

up to 70 minutes of operating time were different for each pressure, which 64.3% for 1 bar, 64.7% for 2 bar and 63.2% for 3 bar. As the TMP increased from 1 to 3 bar, the permeate flux also increased significantly ($p < 0.05$) and the highest flux value was found at 3 bar.

As expected, the highest initial flux was achieved at 3 bar with 38 L/m²h followed by 2 bar (34.4 L/m²h) and 1 bar (28.1 L/m²h). The rate of flux reduction is higher in the first 20 min of the fractionation for all pressures studied. This result is consistent with the previous studies by Wang & Tang (2011) who found that higher initial flux tended to stimulate severe fouling due to increase in permeate drag force and concentration polarization. A moderate flux reduction was observed for subsequent period (30 to 55 min), and stable permeate flux was achieved within 60 to 70 min. All pressures studied have shown a different stable flux (at 70 minutes) with the value of 13.6, 12.3 and 10.4 L/m² h at 3, 2 and 1 bar, respectively.

As for the protein rejection, it was observed (Figure 3) that with the increase of TMP from 1 to 3 bar would reduce the protein rejection significantly ($p < 0.05$) with the values of 81.73%, 78.88% and 77.93%, respectively. The lower protein rejection at the higher TMP could be explained by the sufficient driving force provided under high pressure, thus allowing more solute to be transported pass through the membrane (Sarkar et al. 2009; Das et al., 2009; Datta et al., 2009). In addition, the tendency for the formation of layer cake on membrane surface is high at lower TMP making a resistance for solute to pass through the

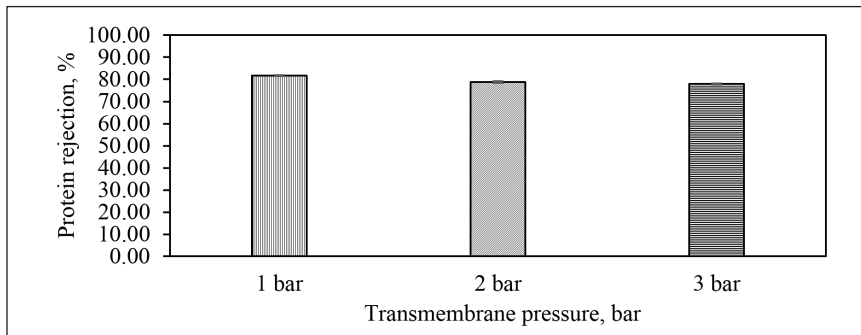


Figure 3. Protein rejection with different transmembrane pressure

membrane, thus resulting in higher protein rejection (Datta et al. 2009). This observation is in agreement with study reported by Das et al. (2009), who found the fall in protein rejection with increase in TMP. The TMP of 3 bars was selected for further experiments as it provided highest permeate flux and reasonably protein rejection.

Effects of Stirring Speed on Flux Reduction and Protein Rejection

Stirring speed is one of the major operating parameters in membrane separation process particularly for dead-end UF membrane. Figure 4 shows the effect of stirring speeds (0, 300 and 600 rpm) on flux reduction over time with other conditions were fixed at TMP of 3 bar and pH 6.8. The flux reduction percentages up to 70 minutes of operating time for each stirring speed were 63.2% for 0 rpm, 51.6% for 300 rpm and 43.4% for 600 rpm which indicated that the flux reduction decreased as the stirring speed increased. The cumulative values of permeate flux increases significantly ($p < 0.05$) with the increase in stirring speed due to the high shear field at the membrane surface. At the initial stage, the permeate flux was observed to be decreasing rapidly and eventually as the fractionation progress, the flux value became stable within 60 to 70 min. Changing the stirring speed to 300 and 600 rpm, the steady state permeate fluxes increased by 114% and 207%, respectively as compared to 13.6 L/m²h without stirring. This is possibly due to the high shear field near the membrane that could remove the solutes accumulated on the membrane surface, which reduces the effect of concentration polarization and ultimately resulting in high permeation rate (Datta et al., 2009; Sarkar et al., 2010).

Figure 5 shows the effect of stirring speed on protein rejection at three different stirring speeds (0, 300 and 600 rpm). Similar trend of peptide rejection were observed for stirring speeds and TMP, where protein rejection decreases significantly ($p < 0.05$) with the increase in stirring speed. The lowest protein rejection was found at 33.7% (600 rpm) followed by 51.3% (300 rpm) and 77.9% (without stirring). Low protein rejection observed at a high stirring speed is mainly due to the reduction of membrane fouling effect on the membrane surface, as discussed in the previous section (Datta et al., 2009). The results obtained

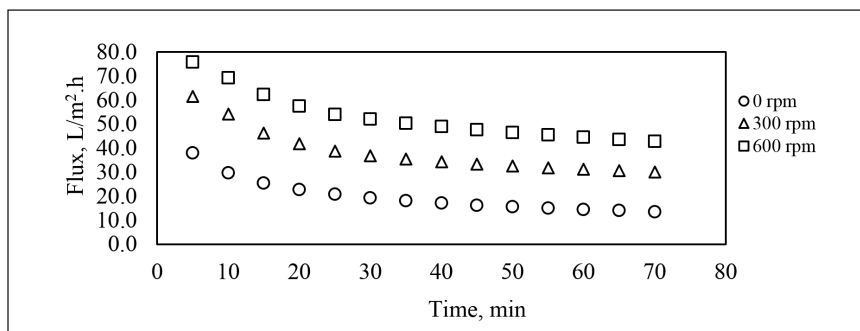


Figure 4. Flux reduction with different stirring speed

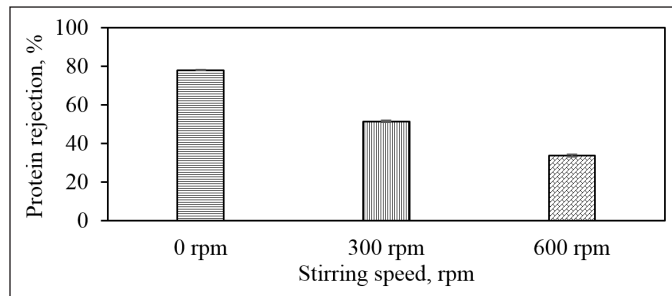


Figure 5. Protein rejection with different stirring speed

here suggested the strong correlation between stirring speed and flux value as well as the protein rejection. Therefore, for the next section, stirring speed of 600 rpm was selected with respect to the highest permeate flux and protein rejection obtained.

Effects of Solution pH on Flux Reduction and Protein Rejection

The effect of solution pH (pH 3, 5, 7, 8 and 9) over time on permeate flux is shown in Figure 6 with other conditions were fixed at 3 bar (TMP) and 600 rpm (stirring speed). It was found that the flux reduction for all pH range were 31.9% (pH 3), 48.7% (pH 5), 27.8% (pH 7), 29.3% (pH 8) and 49.4% (pH 9). The lowest flux was achieved at pH 5 with the initial flux of 78 L/m²h. After this point, permeate flux decreased dramatically between 10 to 45 min of fractionation and then reached a steady value (40 L/m²h) within 50 to 70 min. It seems that the effect of fouling is more pronounced at this pH, which is most likely to be at the isoelectric point. Generally, fouling is more apparent when the protein in the feed solution is uncharged, which is known as the isoelectric point (IEP). At this point, protein adsorption and aggregation are more likely to occur due to the less repulsive forces between the protein molecules (McDonogh et al., 1990, Kelly & Zydney, 1995). Accumulation of the aggregated protein on the membrane surface causes a low permeate flux (Das et al., 2009).

The highest and stable permeate flux was achieved at pH 8 with an initial permeate flux of 75.1 L/m²h. A slight flux reduction occurred in the first 10 min with the value of 72.6 L/m²h and then gradually decreased from 15 to 70 min (71.1 to 53.4 L/m²h) which seemed to have reached the steady-state permeation flux. This might be explained by a reduced tendency to aggregate at higher pH value (basic condition) as the membrane and the peptides are repulsed by their negative charge (Groleau et al., 2003). When the pH was changed to pH 9, the initial permeate flux was obtained at the value of 87.0 L/m²h. A rapid permeate flux reduction occurred from the 87.0 L/m²h (initial flux) to 55.1 L/m²h in 30 min of fractionation. Then, a steady permeate flux (46.4-44.4 L/m²h) was obtained within 60 to 70 min. However, this result is inconsistent with the previous studies where the permeate flux is expected to be higher at pH 9 as a result of the electrostatic repulsion between

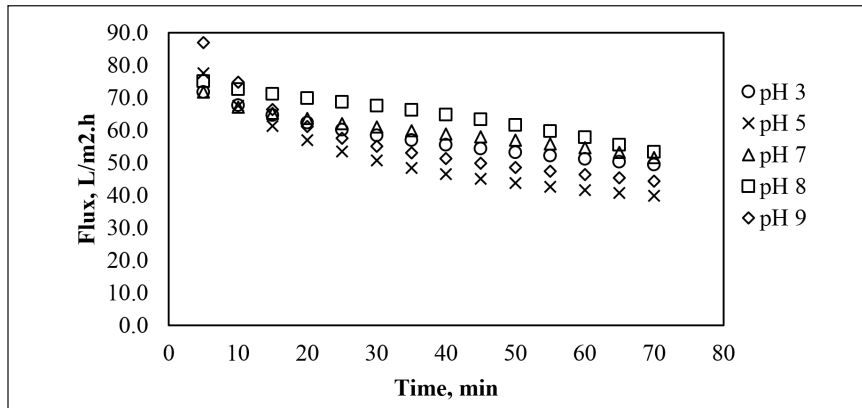


Figure 6. Flux reduction with pH effects

similar charge (negatively charged) owing by the membrane and the peptides (Lapointe et al., 2005; Groleau et al., 2004; Pouliout et al., 1999). This contradicted result possibly is due to the interaction between hydrophobic peptides that leads to peptide aggregation and eventually resulted in a significant flux reduction ($p < 0.05$) (Groleau et al., 2004).

When the fractionation was conducted at pH 7 (neutral condition), a slightly lower permeate flux was obtained as compared to pH 8 with an initial flux of 71.8 L/m²h. Permeate flux decreased gradually from 67.2 to 59.9 L/m²h from 10 to 35 min, and then flux declined slowly from 58.9-51.7 L/m²h within 40 to 70 min. At pH 3 (acidic condition), the permeate flux reduction behaviour was exactly similar with pH 7 where the initial permeate flux was obtained at 71.8 L/m²h and then decreased steadily from 67.7 to 57.0 L/m²h in 10 to 35 min. Further flux reduction was much milder and attaining a steady permeate flux value within 60 to 70 min with values ranging from 51.3-49.5 L/m²h. There was a significant difference ($p < 0.05$) in the stable permeate flux values for all pH studied, which indicated that pH of the solutions significantly affected the permeate flux performance.

The minimum permeate flux was found at the isoelectric point (IEP) (pH 5). Apparently, the permeate flux increased as the pH is shifted away from the isoelectric point. The membrane permeability becomes more pronounced when the fractionation was performed in alkaline condition (above the isoelectric point), where the permeate flux shows the maximum value. This is possibly due to the strong electrostatic repulsive force between the membrane and peptide that promotes permeate flux increment. Several studies have reported a similar behavior where the better flux performance was observed under alkaline condition (Saidi et al., 2013; Wang & Tang, 2011; She et al., 2009). Low permeate flux observed at pH 3 might be the result of strong interaction between the oppositely charged peptides (positive) and membrane surface (negative) which caused the peptide to accumulate on the surface of the membrane thus lowering the permeate flux (Saidi et al., 2013). The difference in flux values obtained for fractionation conducted in acidic and basic conditions probably

due to the greater electrostatic repulsion at the alkaline condition as stated by Lapointe et al. (2005) and Pouliot et al. (1999).

Figure 7 shows the effect of various pH on protein rejection of TB protein hydrolysate. The protein rejection results have shown a significant difference ($p < 0.05$) at all pH studied. It can be seen that the highest protein rejection was achieved at pH 5 (36.2%), followed by pH 9 (26.7%), pH 3 (20.7%), pH 7 (15.5%) and the lowest rejection was at pH 8 (9.6%). These results suggest that effect of pH on protein rejection is not certain as other pH dependent phenomena might take place during the process such as pH-electrostatic double layer interaction, pH-protein-membrane repulsion, and the pH-dependent conformation of protein as reported elsewhere (Ghosh & Cui, 1998; Das et al., 2009; Wan et al., 2006; Sarkar et al., 2010; Groleau et al., 2003).

Based on the lowest permeate flux and highest protein rejection obtained previously, pH 5 is considered as the IEP of the TB protein hydrolysate. This finding was also observed by Sarkar et al. (2009) and Das et al. (2009) who found that the protein rejection was highest at the isoelectric point. The possible explanation for this result is the high tendency of protein aggregation and adsorption to occur on dead-end ultrafiltration membrane (Das et al., 2009). Furthermore, at or closer to the isoelectric point, the protein molecules are more likely entangled/elongated which resulted in higher rejection or lower transmission (Sarkar et al. 2009).

When the solution's pH changed to pH 3 (acidic condition), the protein rejection was significantly decreased which could be due to the enhanced electrostatic repulsion and low tendency of peptide to accumulate on the membrane surface, allowing more peptides to pass through the membrane (Ghosh & Cui, 1998; She et al., 2009). The convective transport of solute as a result of high permeate flux at pH 3 can also contribute to the lower protein rejection (Almecija et al., 2007). However, the protein rejection in pH 3 is considered high as compared to protein rejection obtained at pH 7 and 8. The strong electrostatic repulsion between the negatively charged peptides and membrane in neutral and basic condition may limit the peptide aggregation and accumulation, therefore further peptide can permit through

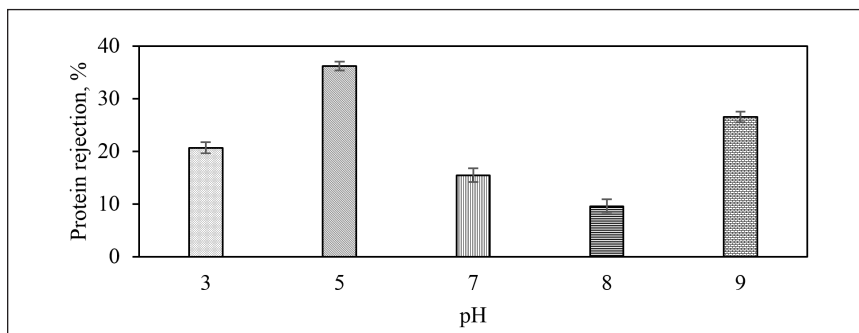


Figure 7. Protein rejection with the effect of pH

the membrane (Almecija et al., 2007). Less electrostatic repulsion may be responsible for the high protein rejection at pH 3 that can lead to the interaction between positively charged peptides on the oppositely charged surface of membrane. A slightly higher protein rejection was observed at pH 9 possibly due to the presence of hydrophobic peptides (positively charged) that had a tendency to adsorb or accumulates on the membrane surface (Groleau et al., 2004). This results clearly indicated that solution pH had a great influence on the fractionation of tilapia by-product protein hydrolysate. The best pH condition (pH 8) was obtained at the lowest protein rejection.

CONCLUSION

The flux reductions for all parameters reached steady state within 60 – 70 minutes. It seems that the trends of flux reduction behavior for TB protein hydrolysate were quite similar for each operating parameters, i.e transmembrane pressure (TMP), stirring speed and pH. The highest permeate flux at final filtration time (70 minutes) for each operating parameters were at 3 bar (13.6 L/m²h), 600 rpm (42.8 L/m²h) and pH 8 (53.4 L/m²h). In term of protein rejections, less significant effects were observed at various range of pressure, but significant effects were attained for both range of stirring speeds and pH. Controlling the stirring speed and pH could minimize the effect of concentration polarization and fouling, which is preferable due to higher permeate flux and less protein loss. The findings from this study provide an essential information on the effective separation conditions for obtaining a high yield of peptides from TB protein hydrolysate.

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