CHAPTER VI

Single-step Cross-flow Ultrafiltration for Recovery and Purification of Surfactin Produced by *Bacillus subtilis* ATCC 21332 and *Bacillus subtilis* MSH1

6.1. Introduction

Bacillus subtilis is a sporulating rod bacterium that thrives in the soil and is nonpathogenic to human beings (Zweers *et al.,* 2008), making it one of the most studied Gram-positive bacteria (Driks, 2002). The ability of *B. subtilis* strains to produce a series of lipopeptides (surfactin, iturin and fengycin) has been documented over 60 years (Xiao *et al.,* 2008) and it is a high-value bioproduct that offers advantageous application in various fields as an alternative to replace chemical surfactant. Surfactin is a heptapeptide linked to a β-hydroxy fatty acid chain of 13–16 carbon chains and consisting of series of isoforms. However, surfactin is an expensive lipopeptide, which makes it unable to compete effectively with chemical surfactants due to downstream processing contributes up to 60% of its production cost (Oka *et al.,* 1993) caused by the complexity of fermentation broth, which contains impurities such as proteins, sugar, lipid compounds and different types of amino acids (Keller *et al.,* 2001; Mulligan *et al.,* 1990).

In recent years, a lot of effort has been expended to reduce the downstream processing costs, including the use of foam fractionation (Davis *et al.,* 2001), acid precipitation (Chen *et al.,* 2007; Reis *et al.,* 2007) extraction using organic solvent, adsorption chromatography or a combination of these techniques. Unfortunately, all of these techniques give low surfactin purity (<65%), which makes them not good enough and leaves room for improvement in order to achieve higher performance of downstream processing. In addition, some of the approaches involving two-step treatment of fermentation broth (Lin *et al.,* 1997; Isa, *et al.,* 2007; Isa *et al.,* 2008) make it impractical and less attractive for industry purposes. Furthermore, most of the conventional methods the use of toxic organic solvents such as chloroform and dichloromethane and these causes the final product suffer from the loss of biosurfactant activity. Hence, there is a demand to develop more economic and environmentally friendly method to improve the current downstream processing technique.

Surfactin separation efficiency from fermentation broth is the most essential requirement in developing commercial-scale processes. One of the alternative techniques for downstream processing is membrane filtration. Membrane filtration system was considered by researcher (Lin *et al.,* 1997; Isa, *et al.,* 2007; Isa *et al.,* 2008) for the purpose of recovery and purification of biosurfactants because it is environmentally friendly and economical for the purpose of the downstream processing. Membrane filtration, which is widely applied in various chemical and biochemical processes, uses pressure-driven force applied to a membrane to suspend species based on the size and molecular shape (Oka *et al.,* 1993). More importantly, the membrane filtration process involves no phase change (Mulligan *et al.,* 1990) and thus enables the molecular structure to be preserved. In much of the literature, membrane filtration meets downstream separation needs because the concentration and purification of the final product surpasses the limitations of traditional methods (Sen *et al.,* 2005; Chen *et al.,* 2008a).

The excellent characteristics of UF include the minimal physical damage of biomolecules from shear effects, minimal denaturation, high recovery yield, and the avoidance of resolubilization. In this study, cross-flow UF system equipped with hydrosart membrane (HT) and polyethersulfone membrane (PES) with a molecular weight cut-off (MWCO) of 10 kDa and 30 kDa was used for the filtration of crude fermentation broth of *B. subtilis* MSH1 and *B. subtilis* ATCC 21332. The final surfactin and protein concentration both in permeates and retentates were analysed to evaluate the performance of UF. The aim of this work is to evaluate the type of membrane which can offer better recovery and purifity of surfactin from crude fermentation broth operated under different transmembrane membrane pressure (TMP) ranging from 0.5 bar- 2.0 bar.

6.2. Materials and Methods

6.2.1. Preparation of Culture Media

A defined mineral salts medium (MSM) were prepared as described in section 5.3.1.

6.3.2. Culture Conditions and Fermentation

Culture conditions and fermentation of *Bacillus subtilis* MSH1 and *Bacillus subtilis* ATCC 21332 were conducted as described in section 5.3.2.

6.2.3. Analytical Methods

6.2.3.1. Measurement of Bacterial Growth

Bacterial growth was determined by method proposed in section 5.2.3.1.

6.2.3.2. Measurement of Surfactin Concentration

Culture samples was determined by method proposed in section 5.2.3.2.

6.2.3.3. Measurements of Surface Tension

Each sample, including surfactin standard was prepared in 5 mM of Tris buffer using deionized water. The surface tension of each sample was measured by ring method using a digital tensiometer (KRÜSS, Germany). A platinum ring was automatically submerged into each solution and then slowly pulled through the air/water interface. The ring was washed, flamed and cooled between each measurement. Each measurement was taken at room temperature.

6.2.3.4. Measurement of Surfactin Purity

Here, surfactin concentration was measured with HPLC according method proposed in section 6.2.3.2. The purity of surfactin in the dried sample was calculated by Equation 7 (Chen *et al.*, 2008b).

$$\text{Purity (\%)} = \frac{\text{Concentration of surfactin determined by HPLC}}{\text{Weight of dried sample powder}} \times 98\% \qquad \text{(Eq. 9)}$$

The purities of surfactin in the recovered product and in the treated broth were used to calculate the recovery of surfactin.

6.3.5. Measurement of Protein Concentration

The total amount of protein present at each stage of the purification procedures was determined by using Equation 10 (Eq. 10) (Chen *et al.*, 2007). As crude fermentation broth was centrifuged at 10 000 rpm to remove biomass. Later the supernatant was called raw broth was further treated by acid precipitation (Chen *et al.*, 2007) by addition of 1 M HCl to a pH 4 then was centrifuged at 10 000 rpm for 15 minutes. Later, the crude powder obtained was oven-drying at 37 °C and weight until constant reading achieved (Chen *et al.*, 2007).

Protein concentration (mg/L) = Weight of dried sample - Weight of surfactin (Eq.10)

6.2.4. Recovery and Purification of Surfactin by UF

Figure 11 shows a schematic diagram of UF process conducted in this study. Microfiltration was applied to completely remove biomass and small particle present in fermentation broth and thus to prevent them from blocking the membrane pore at the UF stage. Small-scale crossflow UF procedures were carried out using a benchtop crossflow filtration device (Sartorius Stedim, Germany) equipped with two sets of membranes which are polyethersulfone (PES) membrane and hydrosart (HT) membrane each with molecular weight cut-off (MWCO) of 10 kDa and 30 kDa with an effective area of 0.02 m^2. The driving force of the permeate flow was the pressure supplied by the external pump towards the system. In general, a feed volume of 250 mL was added to the reservoir and the volume was reduced to 25 mL. Later, retentates and permeates were recovered and analyzed to determine surfactin concentration, protein concentration, the rejection coefficient of surfactin by membrane (R) and the total recovery of surfactin in the final fraction.

Recovery is defined as:

$$\text{Recovery}(\%) = \left(1 - \left(\frac{C_p}{C_f}\right)\right) \times 100 \qquad \text{(Eq. 11)}$$

Where C_p and C_f are the concentration of surfactin in permeates and feed respectively. Th recovery were calculated according to feed and permeate concentrations obtained at tend of the experiments for all components cause the filtration system was operated in concentration mode.

Then, the reconcentration factors (R_F) as shown in Equation 10 was used to asses the reconcentration increase of surfactin in the feed according to their initial feed concentration (C_o):

$$R_F = \frac{C_f}{C_o} \qquad \text{(Eq. 12)}$$

Throughout the UF procedure, the flow rate across the membrane was estimated by collecting permeates volumes during a precisely controlled period of time. Permeate flux was calculated by using the following equation:

$$\text{Flux}\left(\text{LMH or}\frac{L}{m^2h}\right) = \frac{\text{flow rate (L/h)}}{\text{membrane area } (m^2)} \qquad \text{(Eq.13)}$$

The relative flux (RF) was defined as

$$RF(\%) = \frac{J_s}{J_w} \times 100 \quad \text{(Eq.14)}$$

The Flux recovery (FR) was defined as

$$FR(\%) = \frac{J_f}{J_w} \times 100 \quad \text{(Eq.15)}$$

Where J_w, J_s and J_f are the pure water flux of clean membrane, fermentation broth flux and pure water flux of fouled membrane, respectively.

The irreversible and reversible flux decline caused by fouling were calculated according the Equation 14 while the reversible flux decline caused by either concentration polarization or reversible adsorption phenomena were calculated according Equation 15 respectively (Doulia *et al.*, 2007).

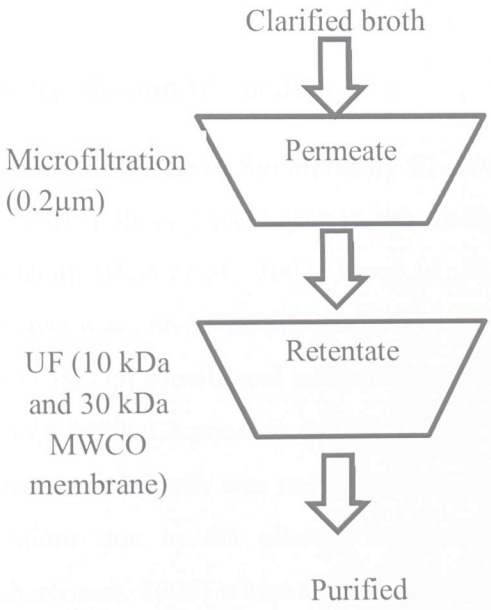
$$\text{Fouling} = 100 - FR \quad \text{(Eq.16)}$$

$$\text{Reversible flux decline} = FR - RF \quad \text{(Eq.17)}$$

The four different TMPs for this procedure ranged from 0.5 bar to 2.0 bar was conducted manually by adjusting the valve and pump controller. TMPs of filtration process were calculated following Eq. 15. The highest TMP applied was up to 2.0 bar.

$$TMP = \left(\frac{P_{in} - P_{out}}{2}\right) - P_{permeate} \quad \text{(Eq.18)}$$

Figure 11: Schematic representation of filtration process carried out.



6.3.5. Membrane Cleaning Experiments

The membrane cleaning experiments of the used membrane after each filtration process were directly carried out with *in situ* cleaning according to Chen *et al.,* (2008) with slight modifications. Cleaning solutions used were deionized water, NaOH solution (pH 10, 12 and 14). The cleaning time was set up 5–10 min, 10-15, 15–20 min, 20-25 min or more than 25 minutes to restore the membrane performance. The experiments were performed at 25 °C with a continuos feed of the cleaning solution from reservoir tanks. As comparison, the cleaning solutions were either flowed in the same direction as the filtration experiments (flushing) or in the reverse direction (back-flushing). When the filtration and membrane cleaning experiments were completed, the used membranes were immediately flushed with deionized water for 30 min each in order to restore the hydraulic permeability. The cleaned membrane were finally stored in 30% ethanol (EtOH) as recommend by the manufacturer. The cleaned membranes were repeatedly employed in every filtration step with differences of pure water flux between the cleaned and fresh membranes were smaller than 5%.

6.2.6. Statistical Analysis

Data analysis consisted of calculating mean, standard deviation of the mean value and determination of the level of the significance was employed using Student's t-test and

Tukey-Kramer multiple comparison test. The differences between measurements were considered significant at the level of $P < 0.05$.

6.3. Results and Discussions

6.3.1. Production of Surfactin by *B. subtilis* Strains

Various authors have reported the abilty of *B. subtilis* ATCC 21332 to produce surfactin (Reis *et al.,* 2007; Isa *et al.,* 2007) and Cooper's media with 4% (w/v) of glucose was chosen as the media because it has been designed to supply nutrients for bacterial cell growth and surfactin synthesis by *Bacillus* strains (Reis *et al.,* 2007; Isa *et al.,* 2007; Charcosset, 2006). During the course of fermentation, the pH of the fermentation broth was maintained at pH 7 to avoid the acidification of the culture medium due to the change from aerobic to anaerobic respiration by the cell (Charcosset, 2006) where the cell grows in the absence of oxygen. Surfactin will lose its ability to solubilize if the pH drops to pH 5, causing surfactin to precipitate (Chen *et al.,* 2007).

6.3.1.1. Production of Surfactin by *B. subtilis* ATCC 21332

Figure 12: Production of surfactin by *B. subtilis* ATCC 21332.

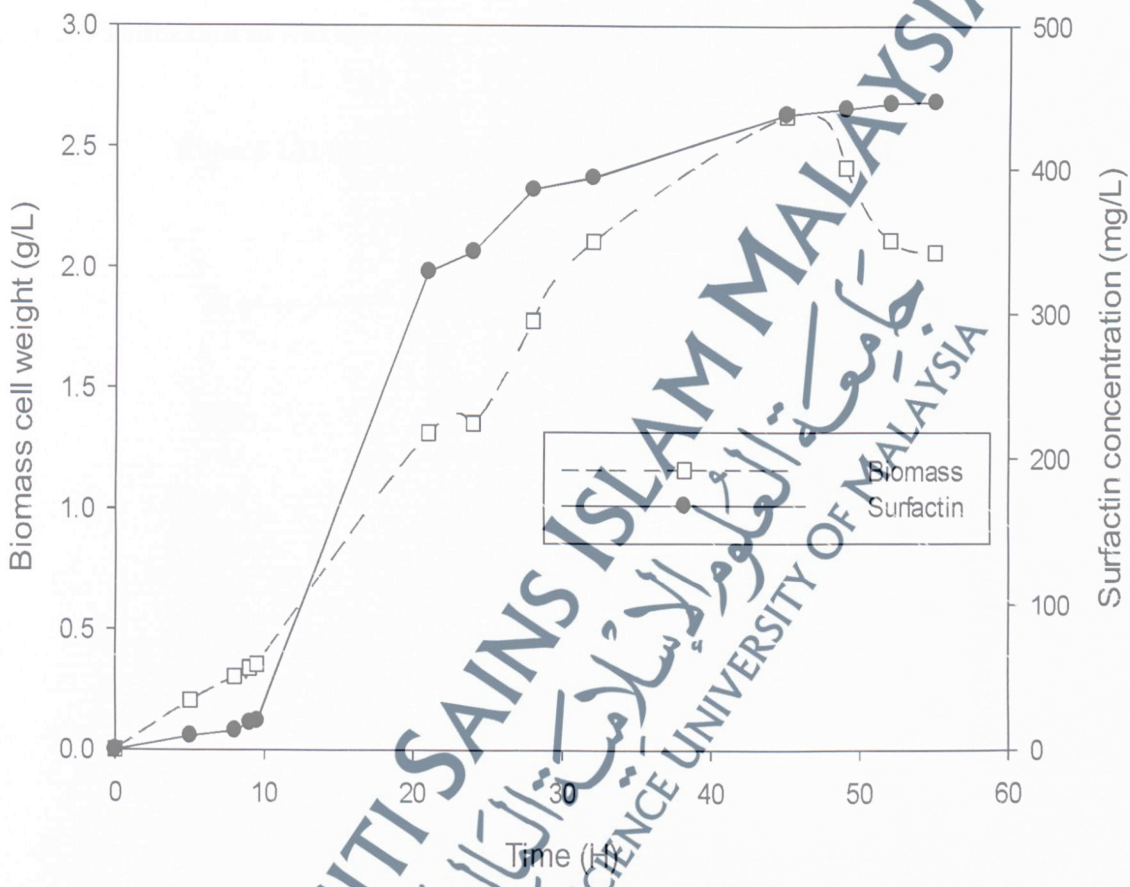


Figure 12 shows bacterial cell growth and surfactin production during the fermentation of *B. subtilis* ATCC 21332. The beginning of cell growth of *B. subtilis* ATCC 21332 lasted for about 10 h, implying that the cells take time to adapt to Cooper's media. This lag phase shows almost no apparent cell growth due to the adaptation of microorganism to the new environment in which the rate of cell growth is very low. Later the cells grow exponentially between 10 h and 45 h in which the cell growth increased in a logarithmic pattern. The stationary growth phase for *B. subtilis* ATCC 21332 began after 45 h of incubation time. The final concentration of surfactin in the fermentation broth was 470 mg/l, which was higher than that obtained by Davis *et al.* (2001) of 439 mg/l under the same experimental conditions. Cooper *et al.* (1981) suggested that biosurfactant production by *B. subtilis* strain was closely

related to microbial cell growth, while Shepard and Mulligan (1987) stated that biosurfactant production mainly occurs at the end of the exponential phase or at the stationary phase of microbial growth. This study shows that the production of surfactin is very closely related to the growth of the strains where the maximum production is found at the end of the exponential growth phase.

6.3.1.2. Production of Surfactin by *B. subtilis* MSH1

Figure 13: Production of surfactin by *B. subtilis* MSH1.

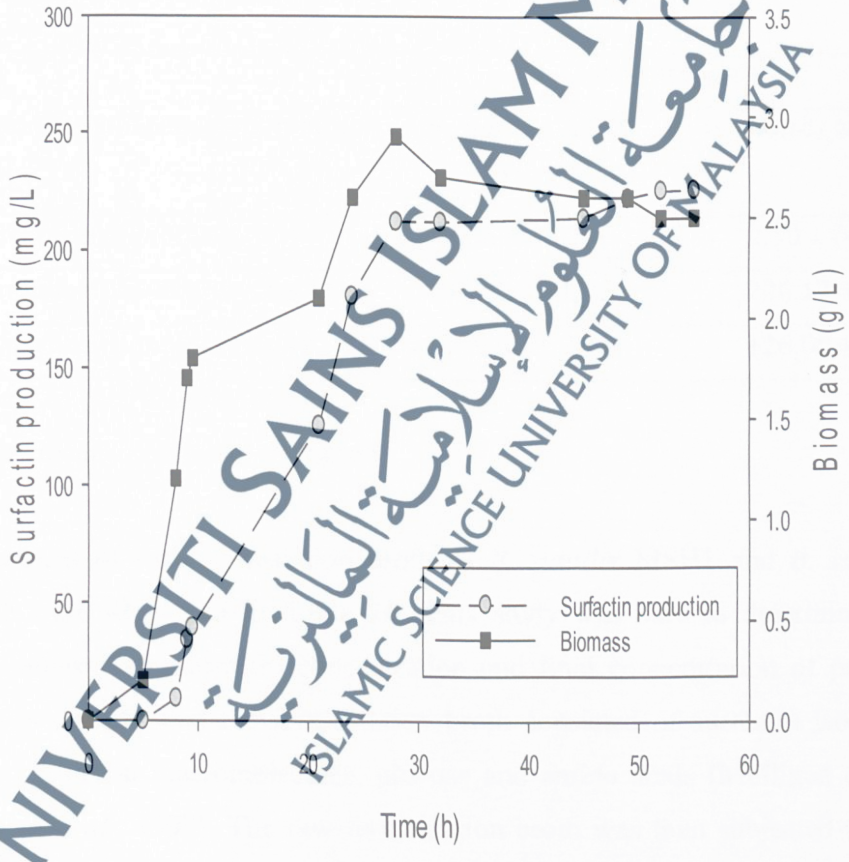


Figure 13 shows bacterial cell growth and surfactin production during the fermentative of *B. subtilis* MSH1. As can be seen, the beginning of cell growth (lag phase) for *B. subtilis* MSH1 was short (about 5 h) implying the fact that *B. subtilis* MSH1 took less time to adapt compared to other strains of *Bacillus*. This lag phase shows almost no apparent cell growth due to adaptation of microorganism to the new environment in which cell growth is very low. Later the cell growth showed

exponential phase where the cell growth increased in a logarithmic pattern which occur between 5 h to 28 h and indicates that *B. subtilis* MSH1 had achieved the maximum cell growth. The stationary growth phase began after 28 h of incubation time. *B. subitlis* MSH1 revealed the production of surfactin is very closely related to the growth of the strains where the maximum production is found at the end of the exponential growth phase and this was in agreement with other previous studies (Reis *et al.,* 2007; Cooper *et al.,* 1981).

6.3.2. Fermentation Broth Composition

Table 10: Major composition of fermentation broth of *B. subtilis* ATCC 21332 and *B. subtilis* MSH1 (means ± SD, n = 3).

Major composition of fermentation broth	Concentration[b]	
	B. subtilis ATCC 21332	*B. subtilis* MSH1
Final concentration of biomass (g/l)[a]	2.06 ± 0.02	2.50 ± 0.03
Final concentration of surfactin (mg/l)[a]	447.06 ± 1.25	226.17 ± 1.83
Final concentration of protein (mg/l)[a]	109.00 ± 2.11	126.00 ± 2.28

[a] Three reading of each sample (n=3)

[b] Calculated by means ± standard deviation

The major content of raw fermentation broth of *B. subtilis* MSH1 and *B. subtilis* ATCC 21332 were illustrated in Table 10. This study was able to determine the quantity of biomass cell, surfactin concentration and final concentration of protein however it was thought that the fermentation broth consisted of surfactin isomers, biomass cells, proteins, macromolecules, glucose and amino acids (Mulligan *et al.,* 1990; Yakimov *et al.,* 1997). The raw fermentation broth was then subjected to the single-step cross-flow ultrafiltration (UF) process for recovery and purification of surfactin.

6.3.3. Rejection Coefficient, Total Recovery and Total Purity of UF Retentate

Table 11: Total recovery and purity of fermentation broth of *B. subtilis* ATCC 21332 after UF treatment.

TMP	Type of membrane							
	PES10		HT10		PES30		HT30	
	Recovery (%)	Purity (%)	Recovery (%)	Purity (%)	Recovery (%)	Purity (%)	Recovery (%)	Purity (%)
0.5	94.5	83	96.0	83.9	93.0	92.6	95.3	95.5
1.0	94.9	83.6	96.1	86.6	92.5	91.3	94.6	95.2
1.5	94.2	85.9	95.1	87.0	92.2	90.5	93.3	93.1
2.0	94.4	87.2	96.3	88.9	91.4	89.4	92.8	92.0

Table 12: Rejection coefficient of raw fermentation broth of *B. subtilis* ATCC 21332 after UF treatment.

TMP	Type of membrane			
	PES10	HT10	PES30	HT30
	Rejection coefficient	Rejection coefficient	Rejection coefficient	Rejection coefficient
0.5	0.96	0.98	0.94	0.95
1.0	0.96	0.98	0.93	0.95
1.5	0.97	0.97	0.93	0.94
2.0	0.97	0.97	0.93	0.94

Table 13: Total recovery and purity of fermentation broth of *B. subtilis* MSH1 after UF treatment.

TMP	Type of membrane							
	PES10		HT10		PES30		HT30	
	Recovery (%)	Purity (%)	Recovery (%)	Purity (%)	Recovery (%)	Purity (%)	Recovery (%)	Purity (%)
0.5	92.9	78.0	95.8	84.0	92.8	92.2	94.1	94.5
1.0	93.1	80.4	96.2	85.4	90.6	91.8	92.6	94.7
1.5	93.2	87.8	96.0	88.7	89.5	90.4	91.3	93.4
2.0	92.8	88.2	96.5	91.5	88.9	88.0	90.8	93.0

[a] Three injection of each sample(n=3)
[b] Calculated by means of the overall flux

Table 14: Rejection coefficient of fermentation broth of *B. subtilis* MSH1 after UF treatment.

TMP	Type of membrane			
	PES10	HT10	PES30	HT30
	Rejection coefficient	Rejection coefficient	Rejection coefficient	Rejection coefficient
0.5	0.97	0.98	0.95	0.96
1.0	0.97	0.98	0.94	0.96
1.5	0.97	0.97	0.93	0.95
2.0	0.96	0.97	0.93	0.94

The UF technique applied was able to recover surfactin in the retentate, thus achieving a good degree of purity. The niche of UF compared to other downstream processing techniques is its ability to segregate the interested molecules based on molecular weight without phase changes. In this present study, surfactin was completely rejected by all membranes, and achieving good range of recovery and purity of 83% - 94% and 78% - 95%, respectively. The effect of increasing TMP on permeates flux, rejection coefficient (*R*) of surfactin by membrane, total recovery and purity of surfactin in the final fraction of all membranes (HT10, HT30, PES10 and PES30) were shown in Table 11 to Table 14.

Insignificant differences ($P < 0.05$) were observed when increasing TMP towards *R* of surfactin, the method applied in this study was able to achieve almost complete rejection of surfactin by all membrane ($R \sim 1$). Very limited differences in the rejection coefficient (*R*) on both membranes were observed due to the size of surfactin micelles was bigger than the MWCO of membranes, making surfactin unable to permeate and thus can be retained completely. The possible explanation for this behavior because of the size of surfactin micelles was bigger than the MWCO and this is in agreement with previously works (Kaya *et al.,* 2011; Isa *et al.,* 2008). Even though the molecular size of surfactin monomer varying from 994 Da to 1050 Da, at concentrations above 15 mg/L (Wei *et al.,* 2003) surfactin able to form micelles within size varying from 30 kDa to 100 kDa (Lin *et al.,* 1997; Yakimov *et al.,* 1997). At critical micelle concentrations (CMC), surfactin molecules readily associate to form supramolecular structures with nominal molecular diameters of up to two to three orders of magnitude larger than single unassociated molecules (Lin *et al.,* 1997) and makes it able to be

retained completely by any membrane within the size of surfactin micelle structure. Surfactin micelles were sufficiently retained by using membrane with MWCO of less than 100 kDa and this is in agreement with findings previously discussed. The effectiveness of UF membrane applied is due to fact that the surfactin micelles were big enough to be rejected by at least 30 kDa membrane. It was agreed that the size of the surfactin micelles was above 30 kDa, as reported in previous studies (Lin *et al.,* 1997; Wei *et al.,* 2003).

According to Table 11 and 13, both membranes were able to achieve high recovery and purity of surfactin from fermentation broth although the membrane material had an effect on the recovery and purity of surfactin. Through comparison of all membranes used in this study, the use of HT30 provided significantly higher ($P > 0.05$), although no mere than 5% reduction in the recovery of surfactin in the final fraction were obserbed in comparison to PES10 and HT10 membranes.

Approximately, 88% to 96% of surfactin being recovered under various TMP. However, the total recovery of surfactin was unable to achieve 100% due to lost of surfactin through membrane fouling. Mulder (1991) stated, surfactin molecules able to form CMC and form a kind of dynamic membrane on top of membrane surface besides the possibility of surfactin monomers through permeate pores and present in permeates however its concentration were too low makes it is undetectable by HPLC. On the other hand, results obtained suggest at least 13% of impurities component were able to pass through the membrane achieving good purity on surfactin final fraction. This study indicated that HT membranes able to effectively permeate impurities compared to PES membrane achieving better purity. PES retained lower purity of surfactin, also the flux decreased quite significantly over time (Figure 14 (a) -(d)) comparing to HT membrane because this membrane was affected more by concentration polarization (Table 15) due to hydrophobic interactions between PES and the aggregated protein contaminants in solutions. This result was in agreement with previous study, however with different mode of UF (Isa *et al.,* 2007). The gradual and limited flux decline with HT 30 membrane implies that much weak adsorption of the micelles occurs and pore radius is not significantly reduced although

the membrane is comparatively hydrophobic (Song, 1998; Cornelis *et al.,* 2005).

6.4.4. The Flux of UF Process

Figure 14 (a) Effect of TMP at 0.5 bar on the permeate flux on all membranes for *B. subtilis* ATCC 213332.

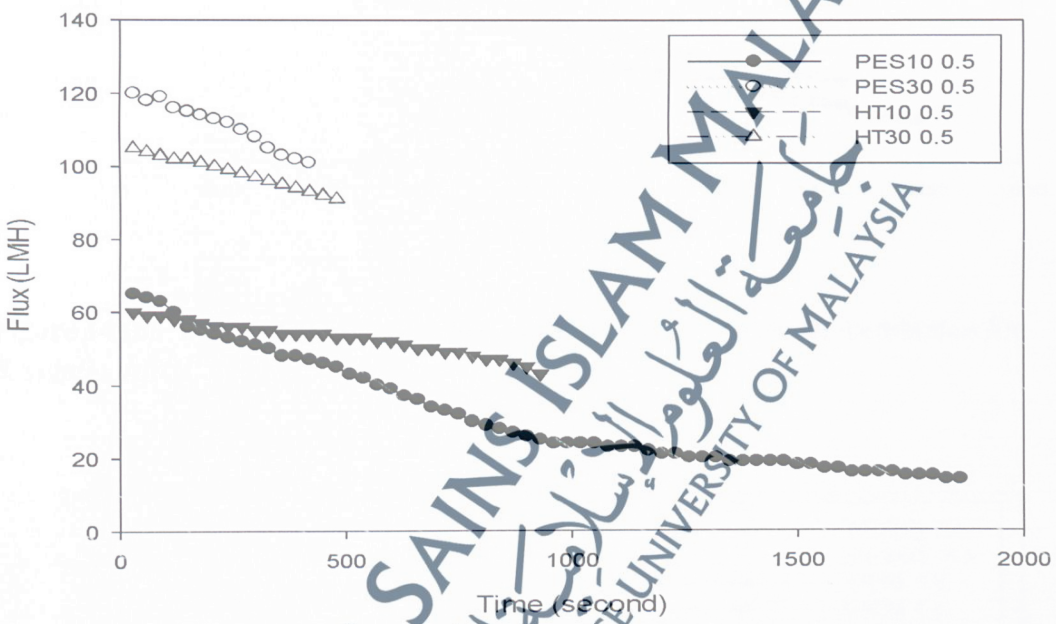


Figure 14 (b) Effect of TMP at 1.0 bar on the permeate flux on all membranes for *B. subtilis* ATCC 213332.

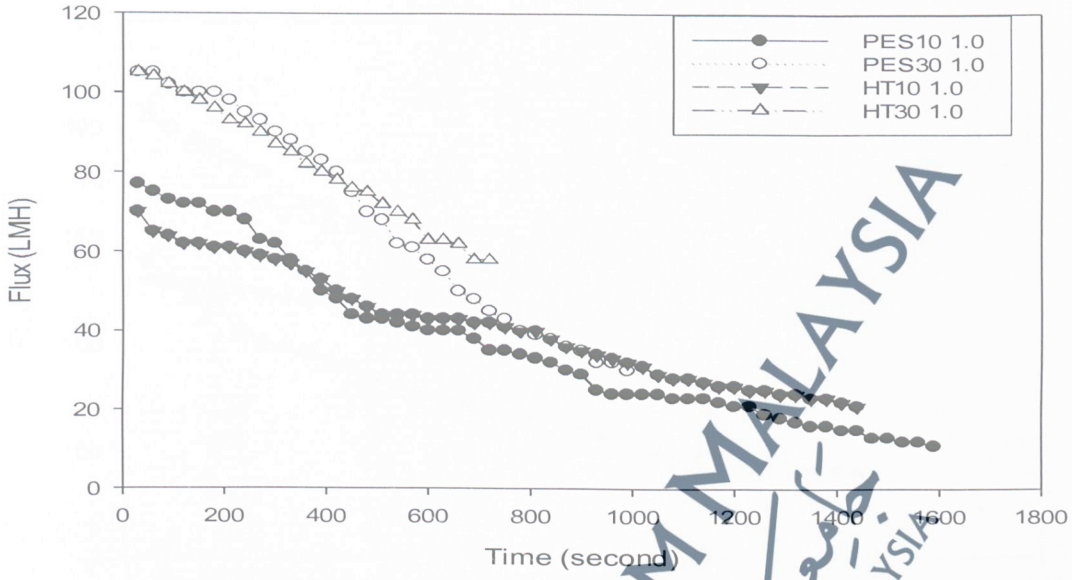


Figure 14 (c) Effect of TMP at 1.5 bar on the permeate flux on all membranes for *B. subtilis* ATCC 213332.

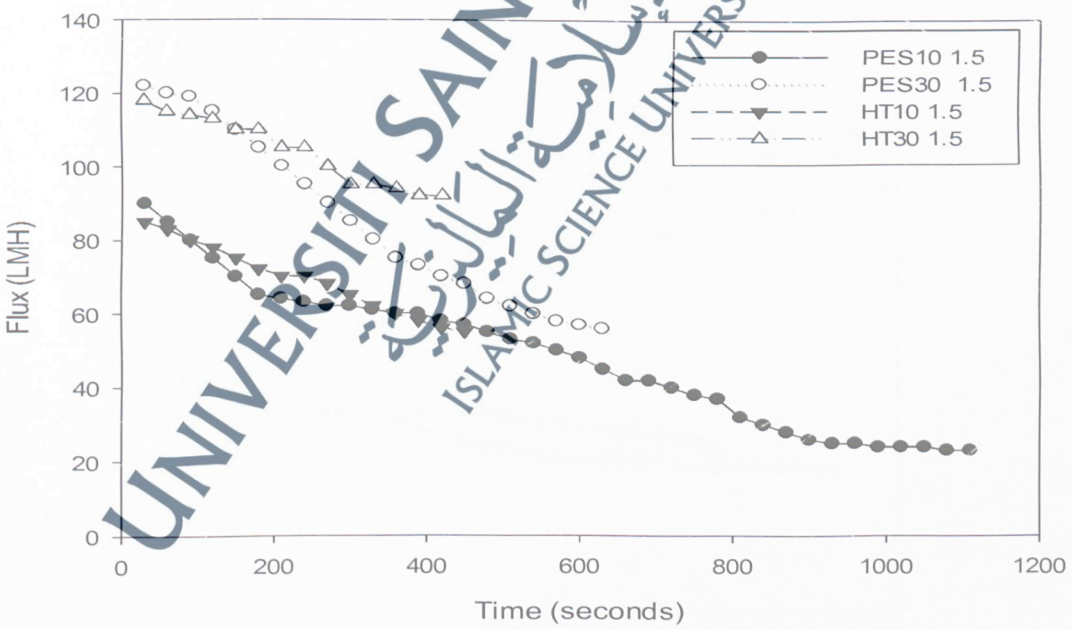


Figure 14 (d) Effect of TMP at 2.0 bar on the permeate flux on all membranes for *B. subtilis* ATCC 213332.

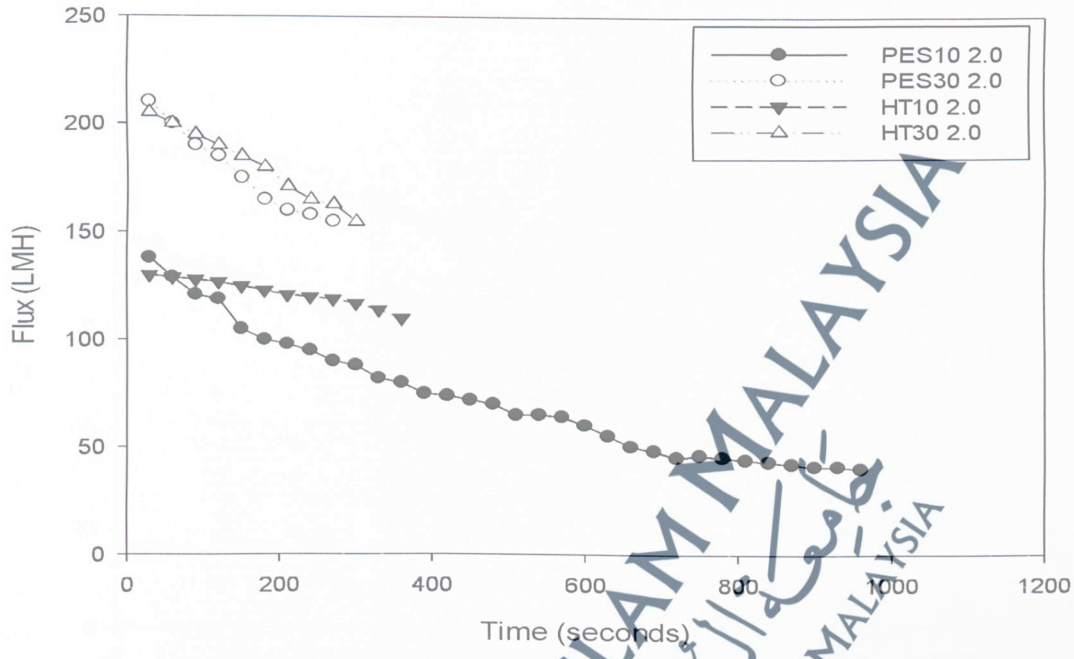


Figure15 (a) : Effect of TMP at 0.5 bar on the permeate flux on all membranes for *B. subtilis* MSH1

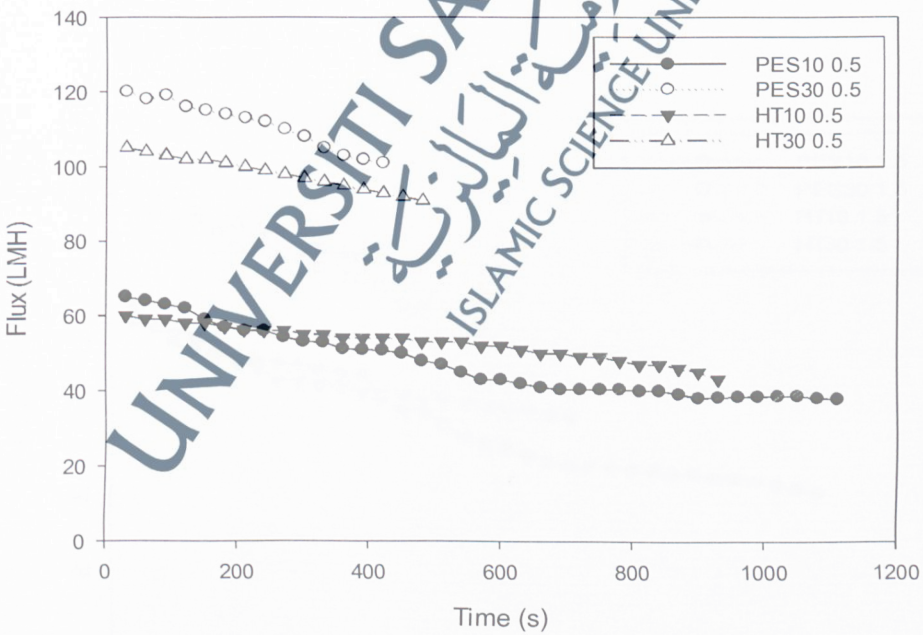


Figure 15 (b) : Effect of TMP at 1.0 bar on the permeate flux on all membranes for *B. subtilis* MSH1.

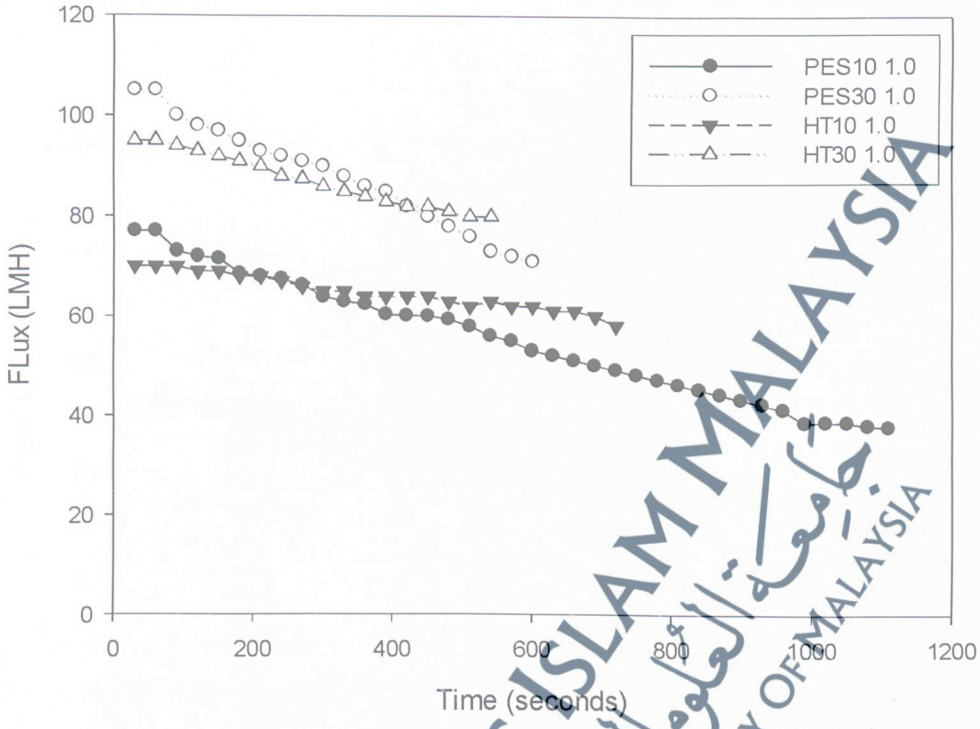


Figure 15 (c) : Effect of TMP at 1.5 bar on the permeate flux on all membranes for *B. subtilis* MSH1.

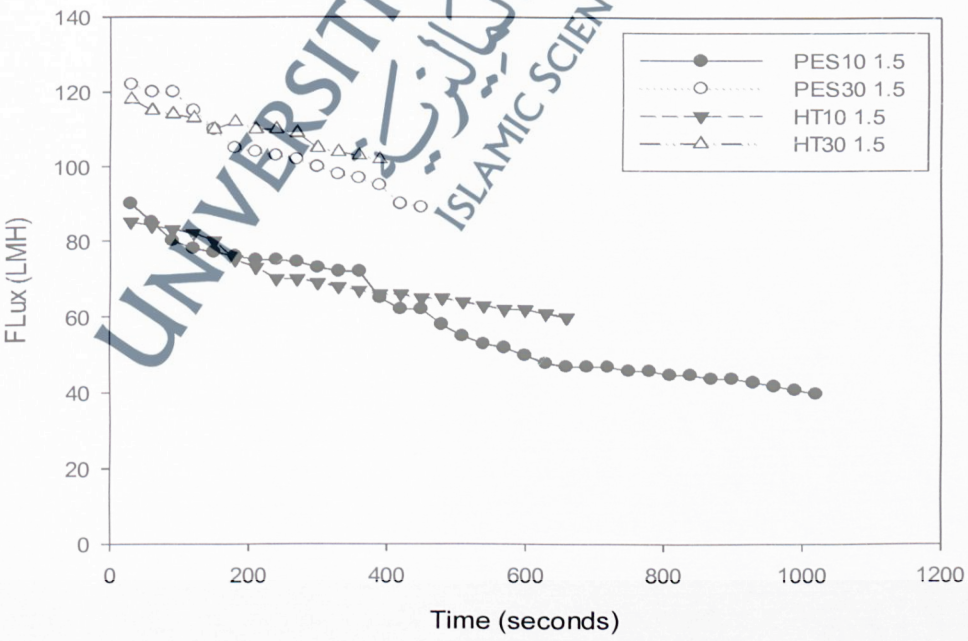


Figure 15 (d) : Effect of TMP at 2.0 bar on the permeate flux on all membranes for *B. subtilis* MSH1.

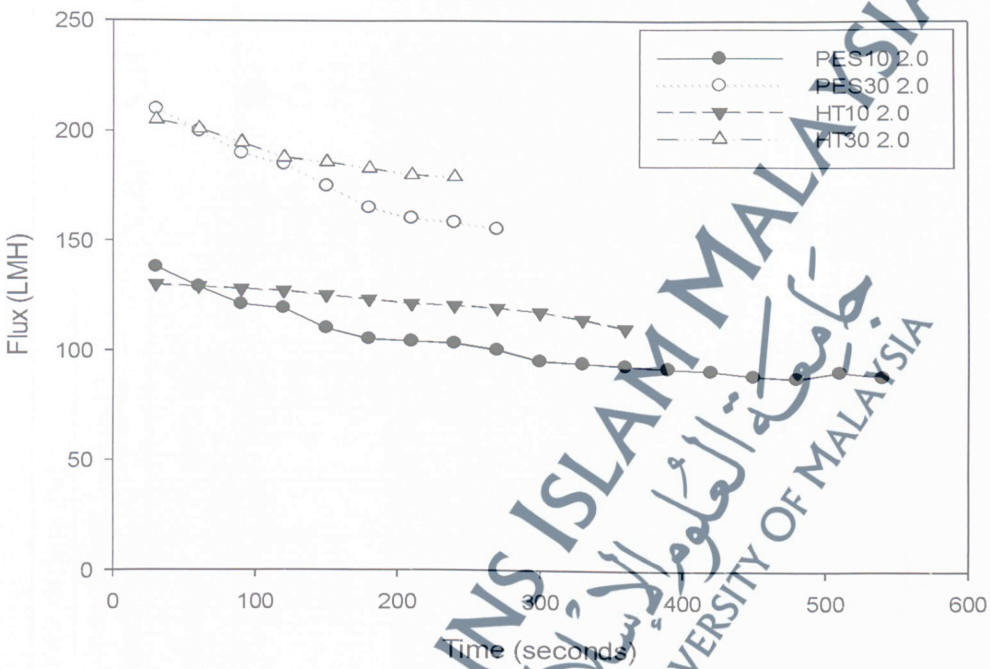


Table 15: The flux decline results and VRF values of all membranes for filtration of raw fermentation broth of *B. subtilis* ATCC 2132 at various TMP.

Type of membrane	TMP (bar)	Flux			Flux decline (%)			V_f (L)	V_c (L)	VRF (V_f/V_c)
		J_w	J_s	J_f	Total	Concentration polarisation	Fouling			
					(100-RF)	(FR-RF)	(100- FR)			
PES10	0.5	51.5	38.6	43.2	25.1	8.9	16.2	250.0	24.0	10.4
	1.0	57.1	41.0	46.3	28.2	9.3	18.9	250.0	23.0	10.9
	1.5	66.6	40.0	48.5	39.9	12.8	27.1	250.0	23.0	10.9
	2.0	167.6	89.0	119.0	46.9	17.9	29.0	250.0	24.0	10.4
PES30	0.5	125.2	101.2	109.4	19.1	6.5	12.6	250.0	24.0	10.4
	1.0	88.8	71.2	76.9	19.8	6.5	13.3	250.0	23.0	10.9
	1.5	124.7	89.0	100.5	28.6	9.2	19.4	250.0	24.0	10.4
	2.0	225.6	149.1	180.3	33.9	13.8	20.1	250.0	26.0	9.6
HT10	0.5	54.6	43.1	46.8	21.0	6.8	14.2	250.0	25.0	10.0
	1.0	75.9	57.9	64.3	23.7	8.4	15.3	250.0	23.0	10.9
	1.5	91.6	58.2	70.4	36.5	13.4	23.1	250.0	22.0	11.4
	2.0	187.1	110.0	135.4	41.2	13.6	27.6	250.0	24.0	10.4
HT30	0.5	108.3	91.0	96.5	16.0	5.1	10.9	250.0	24.0	10.4
	1.0	97.1	79.8	86.0	17.8	6.4	11.4	250.0	23.0	10.9
	1.5	131.1	102.0	112.6	22.2	8.1	14.1	250.0	24.0	10.4
	2.0	248.3	178.2	204.1	28.2	10.4	17.8	250.0	25.0	10.0

Table 16: The flux decline results and VRF values of all membranes for filtration of raw fermentation broth of *B. subtilis* MSH1 at various TMP.

Type of membrane	TMP (bar)	Flux			Flux decline (%)			V_t (L)	V_c (L)	VRF (V_t/ V_c)
		J_w	J_s	J_f	Total	Concentration polarisation	Fouling			
					(100-RF)	(FR-RF)	(100- FR)			
PES10	0.5	53.0	38.6	43.2	27.2	8.7	18.5	250.0	23.0	10.9
	1.0	69.2	41.0	46.3	40.8	7.7	33.1	250.0	23.0	10.9
	1.5	99.2	40.0	48.5	59.7	8.6	51.1	250.0	24.0	10.4
	2.0	147.6	89.0	119.0	39.7	20.3	39.4	250.0	23.0	10.9
PES30	0.5	91.0	71.2	77.6	21.8	7.0	14.7	250.0	23.0	10.9
	1.0	127.2	89.0	100.5	30.0	9.0	21.0	250.0	25.0	10.0
	1.5	180.2	101.2	108.5	43.8	4.1	39.8	250.0	25.0	10.0
	2.0	225.6	149.1	180.3	33.9	13.8	40.1	250.0	26.0	9.6
HT10	0.5	52.0	43.1	46.8	17.1	7.1	10.0	250.0	25.0	10.0
	1.0	77.2	57.9	64.3	25.0	8.3	16.7	250.0	24.0	10.4
	1.5	91.0	58.2	70.4	36.0	13.4	22.6	250.0	24.0	10.4
	2.0	140.2	110.0	135.4	21.5	18.1	33.4	250.0	23.0	10.9
HT30	0.5	98.1	79.8	86.0	18.7	6.3	12.3	250.0	24.0	10.4
	1.0	110.0	91.0	96.5	17.3	5.0	12.3	250.0	23.0	10.9
	1.5	134.5	102.0	112.6	24.2	7.9	16.3	250.0	22.0	11.4
	2.0	241.0	178.2	204.1	26.1	10.7	25.3	250.0	24.0	10.4

Cross flow UF mode was chosen in this study because the tangential flow of feed solution along the membrane will prolonged the time of the depositions on the membrane surface by sweeping effects, theoretically results will be in less fouling and continues of high flux can be maintained (Isa *et al.,* 2008). However, the permeate flux decay is affected by a number of factor such as TMP, temperature, feed surfactant concentration, membrane pore size, membrane material chemistry and the dynamics of the filtration process (Akay and Wakeman, 1994). Figure 14 (a) - (d) to Figure 15 (a) - (d) show the permeate flux decay for crude fermentation broth of *B. subtilis* MSH1 and *B. subtilis* ATCC 213332 under various processing condition (TMP, MWCO membrane materials) in order to asses which UF system the most suit for downstream processing.

As shown in Figure 14 (a) - (d) to Figure 15(a) - (d), flux were dramatically decreased with increasing TMP in all the experiments even the flux was high at initial stage. The UF process leading to long-term flux decline can be seen on all membranes however with different profiles as a result of different surface interaction between membrane surfaces with solute. The expected possible interactions were CP, adsorption of surfactin molecules and other small impurities onto the membrane surface (Song, 1998). Interestingly, the flux of permeate seems to be more stable at low TMP (0.5 bar and 1.0 bar) whereas at high TMP (1.5 bar and 2.0 bar), an obvious pattern of flux decline can be seen. Flux decline is an unavoidable deleterious phenomenon in the filtration process (Huaiqun *et al.,* 2006) eventhough we have high interest to maintain the highest possible flux value.

The accumulation of molecules on the nearby membrane surface may lead to membrane fouling. The highest total flux declines were obtained for the raw fermentation broth of *B. subtilis* ATCC 21332 at TMP of 1.5 bar with reduction of 54.9% at the end of filtration time. Higher flux declines were observed for the fermentation broth of *B. subtilis* ATCC 21332 compared to *B. subtilis* MSH1 for all TMP values due to the higher surfactin concentration in fermentation broth of *B. subtilis* ATCC 21332.

6.3.5. Fouling Behavior

According to Kaya *et al.* (2011), the flux decline occurs because of the accumulation of molecules in solutes on the membrane surface due to CP lead to membrane fouling. Flux decline clearly observed with increasing TMP although the higher pressure generated higher initial flux which later caused increased of CP due to high TMP that elevates the initial permeate flux by pushing more solute toward a clean membrane. At a later stage, the higher TMP pulls more particles towards the membrane surface by providing faster permeate velocity thus contributes to the rapid generation of the deposited cake layer which causes a dramatic increase of resistance onto the membrane surface. Higher pressure will significantly compresses the pre-built cake layer so it will becomes denser and contributed to the rapid flux decline.

When using low TMP (0.5 bar and 1.0 bar), a gradual flux decline was observed for all membranes because of the slow formation of cake layer. At low TMP the permeate flux is sustained at it's high value for a considerable time before the flux were drop (Figure 14 (a)-(d) to 15(a)- (d)) because of the wettability of the membrane was only sufficient to minimize surfactin and impurities deposition to take places on membrane surface. By times, the concentration of feed in reservoir increasing as the volume decreased and hence speed up the rate of surfactin deposition which later resulted to permeate flux decay.

Concentration polarization (CP) layer starts to develop as a result of increased deposition of surfactin and impurities on top of membrane surface and forming a dynamic membrane layer which act as secondary layer (Mulder, 1991). These phenomena resulted to decrease of flux throughout the UF process. As the concentration of the surfactin on the membrane and within the pores increases over time, the permeate flux started to decay and effect of membrane material on permeate flux became unimportant. The fouling occurs when substances in fermentation solutions coating the membrane surface which resulted to pore blockage and causes serious gradual permeate flux decline. In any membrane system, it is vital that CP is reduced as much as possible as it has a very strong impact on permeate flux operating cost.

The improvement of flux decline and cake formation could be minimized by the pre-treatment of the raw broth through as acid precipitation, salting out (Chen *et al.*, 2007)

or removal of large particle by centrifugation and microfiltration. In this study, the pretreatment of raw fermentation broth was conducted by removal of biomass by centrifugation and removal of any large particle using 0.2 mm membrane filtration.

Howell and Velicangil (1987) divided the UF phase into three phase intervals through evaluation of permeate flux pattern which are: (1) first few seconds (a quasi-steady-state concentration polarization layer is set up), (2) solute adsorption), (3) and long term (gel layer formation). Thus, the quick development of concentration polarization occurring at the first few seconds of the process could explain the flux decline with PES 10 and PES 30 membrane at the high TMP, and this factor primarily affects the flux behavior throughout the process. However, the time required for the process to establish quasi-steady-state is very short which less than 10 seconds according therefore can be ignored (Howell and Velicangil, 1987). The gradual flux decline with HT 30 membrane is a result of concentration polarization as well as weak adsorption of surfactin micelles and other small impurities onto the membrane surface (Chen *et al.,* 2008a). Permeate concentration starts to decay only after the establishment of a slow decaying plateau region. Overall, the flux decreases with increasing concentration in feed because the molecules in concentrated solutions accumulated near the membrane surface more rapidly.

6.3.6. Hydrophilicities of Membrane

HT and PES membranes were selected due to their different hydrophilicities. According to Chen *et al.,* (2008), the contact angles of water for PES membrane were greater than Cellulose Ester (similar material with HT membranes) with 62.9° and 56.4°, respectively. This characteristic make HT membrane more hydrophilic than PES, resulting in earlier attachment of dissolved amino acids (Chen *et al.,* 2008). Thus, the steady-state flux of PES membrane is higher compared to HT membrane under identical conditions.

The flux decline using PES membranes was greater than HT concur the existence of negative charge molecules in molecular structure of surfactin. Surfactin is a negatively charge molecules because of the presence of negatively charge amino acid in fact, the the zeta potential value for surfactin were −5mV to −30mV at pH 6–11 with surfactin concentration vary from 0.2–2.0 g/L (Chen *et. al.,* 2006). The possible interaction for

hydrophilic membrane surface (HT10 and HT30) due to strong irreversible adsorption of the polar surfactin head occurs on the membrane surface thus surface wettability reduced. While on hydrophobic membrane, strong adsorption of hydrophobic tails occurs improves the wettability. Strong adsorption of the monomers within PES membrane could make the pore radius reduced leading to flux decline. Depending on the hydrophilicity of the membrane, the net effect is flux decrease or increase (Kaya *et al.,* 2011). This mechanism stated the HT has a very low protein binding feature which ensures that maximum recovery can be retained for final surfactin fractions.

The use of HT30 provided better recovery and purity of final surfactin fraction followed by HT10, PES30 and PES10. The understanding of molecular structure, shape and size, as well as the interaction between the solute and membrane is important in order to optimize recovery and purification from complex fermentation broth. HT30 membrane is the more suitable membrane for single step UF for simple and highly cost effective downstream processing method which can offer high recovery and purity of surfactin from complex fermentation broth of *B. subtilis.*

6.3.7. Membrane Cleaning

Table 17: The efficiency of membrane cleaning using different cleaning solution on PES10 membrane.

Concentration of surfactin	Type of cleaning	Cleaning solution (flux recovery and cleaning time)			
		Deionized water	NaOH (pH10)	NaOH (pH12)	NaOH (pH14)
B. subtilis MSH1	Flushing	20 min (61%)	20 min (67%)	10–15 min (100%)	10–15 min (100%)
B. subtilis MSH1	Back-flushing	20 min (59%)	20 min (65%)	10–15 min (100%)	10–15 min (100%)
B. subtilis ATCC 21332	Flushing	20 min (52%)	20 min (57%)	10–15 min (100%)	5–10 min (100%)
B. subtilis ATCC 21332	Back-flushing	20 min (43%)	20 min (52%)	10–15 min (100%)	10–15 min (100%)

Table 18: The efficiency of membrane cleaning using different cleaning solution on HT10 membrane.

Concentration of surfactin	Type of cleaning	Cleaning solution (flux recovery and cleaning time)			
		Deionized water	NaOH (pH10)	NaOH (pH12)	NaOH (pH14)
B. subtilis MSH1	Flushing	20 min (74%)	20 min (82%)	15–20 min (100%)	15–20 min (100%)
B. subtilis MSH1	Back-flushing	20 min (69%)	20 min (74%)	15–20 min (100%)	15–20 min (100%)
B. subtilis ATCC 21332	Flushing	20 min (60%)	20 min (71%)	15–20 min (100%)	15–20 min (100%)
B. subtilis ATCC 21332	Back-flushing	20 min (57%)	20 min (65%)	15–20 min (100%)	15–20 min (100%)

Table 19: The efficiency of membrane cleaning using different cleaning solution on PES30 membrane

Concentration of surfactin	Type of cleaning	Cleaning solution (flux recovery and cleaning time)			
		Deionized water	NaOH (pH10)	NaOH (pH12)	NaOH (pH14)
B.subtilis MSH1	Flushing	20 min (75%)	20 min (86%)	10-15 min (100%)	10-15 min (100%)
B.subtilis MSH1	Back-flushing	20 min (69%)	20 min (79%)	10-15 min (100%)	10-15 min (100%)
B.subtilis ATCC 21332	Flushing	20 min (70%)	20 min (82%)	10-15 min (100%)	10-15 min (100%)
B.subtilis ATCC 21332	Back-flushing	20 min (64%)	20 min (75%)	10-15 min (100%)	10-15 min (100%)

Table 20: The efficiency of membrane cleaning using different cleaning solution on HT30 membrane

Concentration of surfactin	Type of cleaning	Cleaning solution (flux recovery and cleaning time)			
		Deionized water	NaOH (pH10)	NaOH (pH12)	NaOH (pH14)
B. subtilis MSH1	Flushing	20 min (83%)	15–20 min (89%)	5–10 min (100%)	5–10 min (100%)
B. subtilis MSH1	Back-flushing	20 min (75%)	15–20 min (82%)	5–10 min (100%)	5–10 min (100%)
B. subtilis ATCC 21332	Flushing	20 min (76%)	15–20 min (81%)	5–10 min (100%)	5–10 min (100%)
B. subtilis ATCC 21332	Back-flushing	20 min (74%)	15–20 min (86%)	5–10 min (100%)	5–10 min (100%)

One of the major problems in the application of membrane filtration technique is the formation of CP on the membranes surfaces during the UF operation. This CP is not desirable because it will reduce the flux of permeate and increasing filtration time. The cleaning protocols recommended by membrane manufactures consist of a series of acid-alkaline cleaning cycles, and it depends on the feed and the membrane materials (Li *et al.,* 2005). It was found that the increasing of TMP resulted to inefficient cleaning process. Thus, periodical chemical cleaning with *in situ* back-flushing is one possible way to restore and maintain the flux, preserve and prolong the membrane usage. Four types of cleaning solutions were tested, which were deionized water, NaOH solutions at pH 10, 12 and 14. The effects of flushing and back-flushing on the flux and surfactin rejection with PES 10, PES30 HT10 and HT30 are shown in Table 16-19, respectively, at different initial surfactin concentration (*B. subtilis* MSH1= 226.17 ± 1.83 mg/L;. *B. subtilis* ATCC 21332 = 447.06 ± 1.25 mg/L).

After 20 min flushing and back-flushing using deionized water for both broth, approximately 43% to 83% of the fluxes could be recovered on the basis of pure water flux. On the other hand, more time needed approximately at least 20 min for flushing and back-flushing to restore the flux for *B. subtilis* ATCC 21332 comparing to *B. subtilis* MSH1. The results in terms of flux recovery and the cleaning time required for all membrane are all compiled in Table 16-19 for comparison. In comparison, the fouled membrane has too little contact with the cleaning solution to achieve maximum removal in short time. For this study, we were observed that the cleaning duration the most important parameter during the cleaning process of membrane after each filtration with the optimal cleaning efficiency is reached at 15 min of cleaning (Cabero *et al.,* 1999). It was found, deionized water alone is not satisfactory solution in order to restore the membrane performance especially for high feed concentrations of surfactin.

Previous research have reported the effects of high-pH NaOH solution on flux recovery and cleaning time cycle for the membranes fouled with proteins (Cabero *et al.,* 1999; Munoz-Aguado *et al.,* 1999). Comparison of Table 16-19 was indicates that the performance of fluxes able to restored when the used membrane is cleaned using NaOH, by flushing or back-flushing. However, it was find that more time needed to

restore the membrance performance using deionized water compared to NaOH as cleaning solution at various surfactin concentrations. Besides, the cleaning performance using NaOH at pH 12 is equivalent to that using NaOH at pH 14. Of the four cleaning solutions used, the flux recovery decreases in the order NaOH pH 14 > NaOH pH 12 > NaOH pH 10 > water. By comparing all of the cleaning solution used, the cleaning performance of using NaOH at pH14 is not significantly differ ($P > 0.05$) with NaOH at pH 12. It seems that NaOH solutionat pH 12 and Ph 14 appears to be the most suitable one for this purpose because surfactin deposited on the membrane surface would be destroyed by NaOH. Besides, it was noticed that flux recovery by flushing is generally higher than that by back-flushing, particularly at higher feed concentrations of surfactin. It is because PES membrane is mainly fouled during cross-flow UF by weak adsorption/gel layer formation, rather than pore blocking. These findings can reflect the gradual decline behavior of the flux with time as previously discussed. Simultaneously considering the factors of cost reduction, NaOH at pH 12 appears to be the most suitable cleaning solution for cleaning purpose because the CP on the membrane surface could be removed efficiently as NaOH at pH14.

6.3.8. Surfactin Product Characterization

6.3.8.1. Surface Tension Measurement

Figure 16: Surface tension of surfactin standard, purified surfactin (HT10), purified surfactin (PES10), purified surfactin (HT30) and purified surfactin (PES30) from fermentation broth *B. subtilis* ATCC 21332.

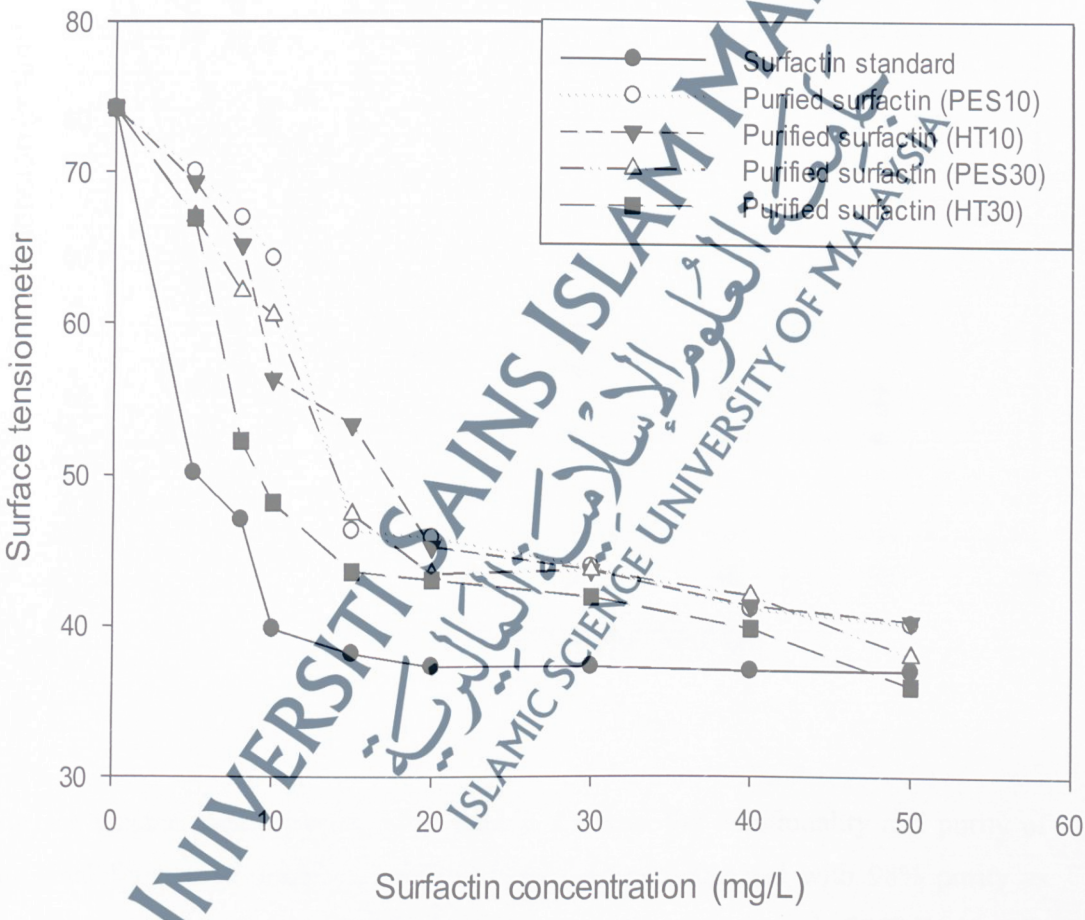
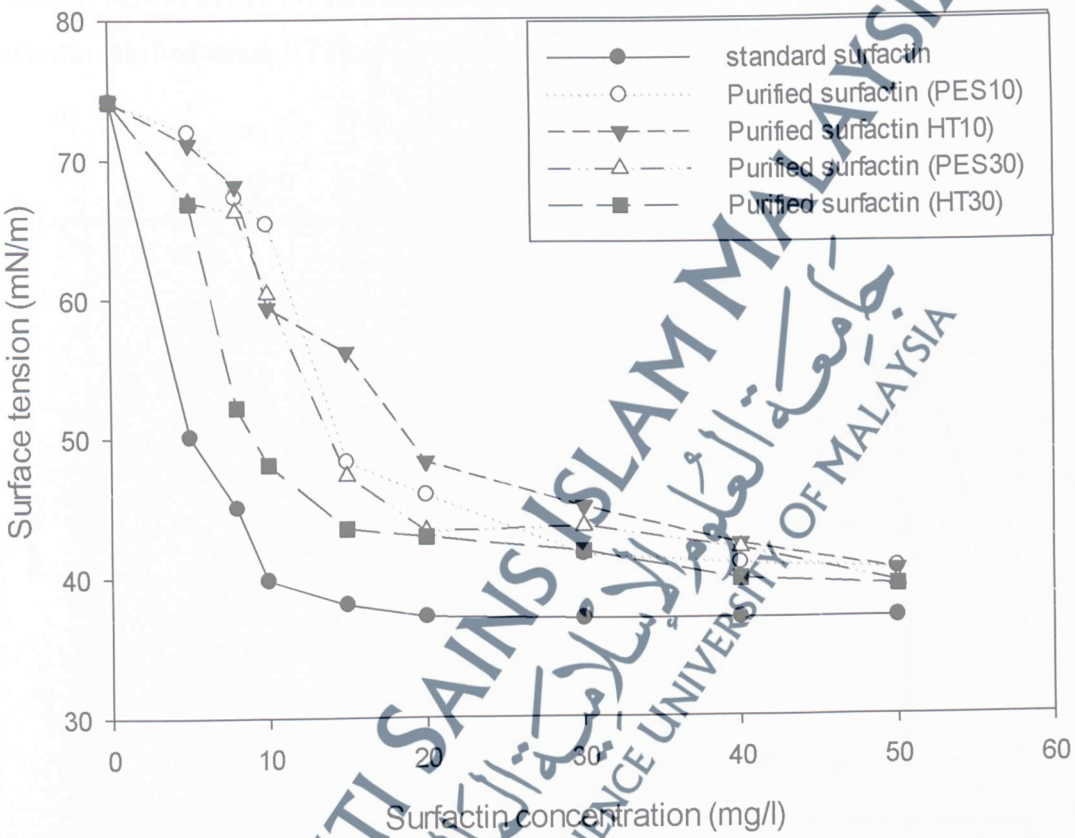


Figure 17: Surface tension of surfactin standard, purified surfactin (HT10), purified surfactin (HT30), purified surfactin (PES10), and purified surfactin (PES30) from fermentation broth *B. subtilis* MSH1.

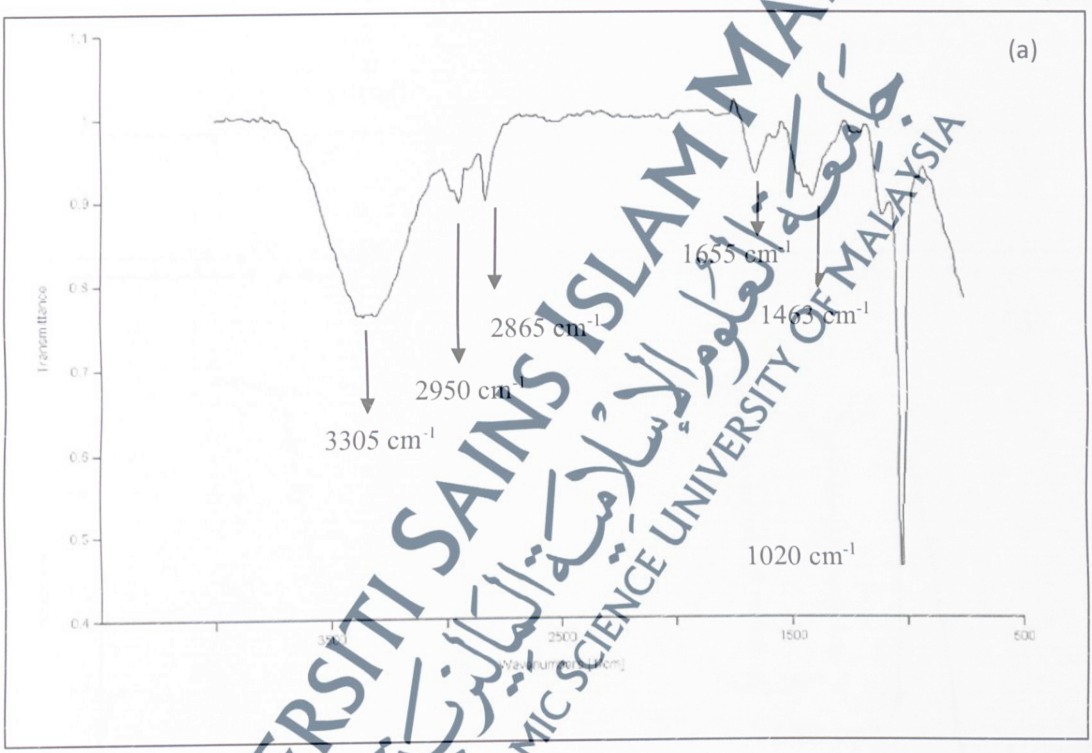


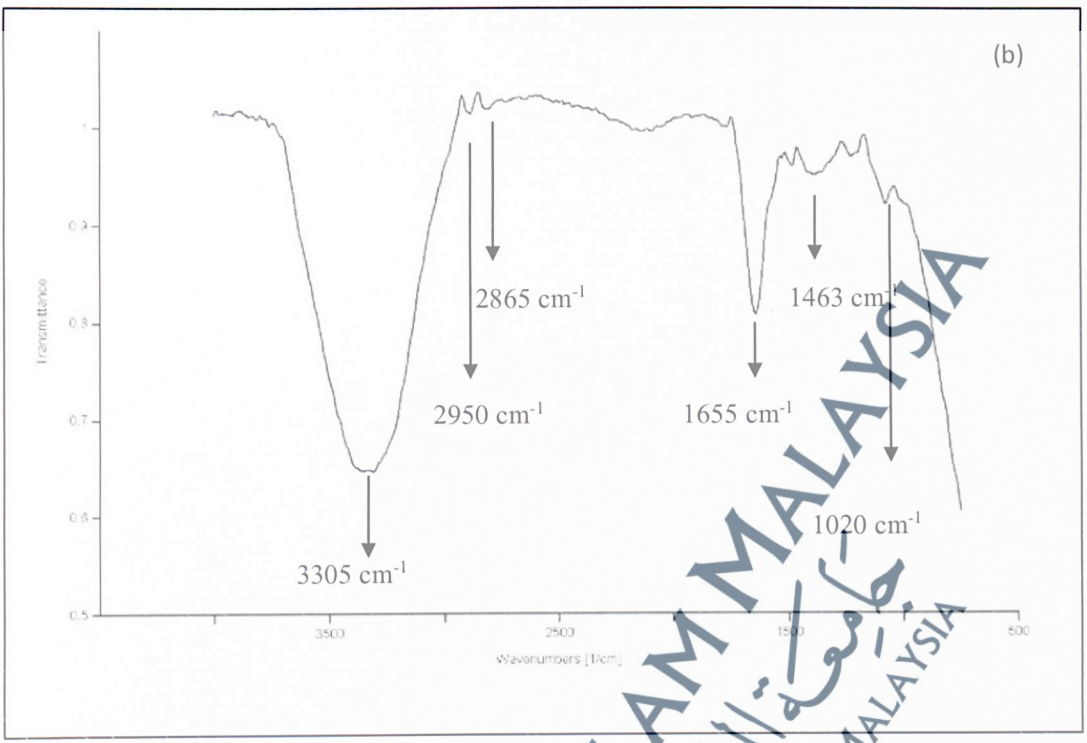
Surface tension measurements were used to evaluate the functionality and purity of the final fraction of surfactin samples using surfactin standard with 98% purity as reference sample under similar and controlled conditions (Isa *et al.,* 2008;Wei *et al.,* 2003). Figure 16 and Figure 17 show the surface tension profiles of the purified surfactin for all membranes in comparison to surfactin standard. The results show close proximity in terms of the surface activity of purified surfactin in relation to surfactin standard, which indirectly shows purity of surfactin samples and this corresponds to the earlier results on purity measurements as shown in Table 11 and Table 13. In addition, Figure 16 and Figure 17 shows the purified surfactin behaves as

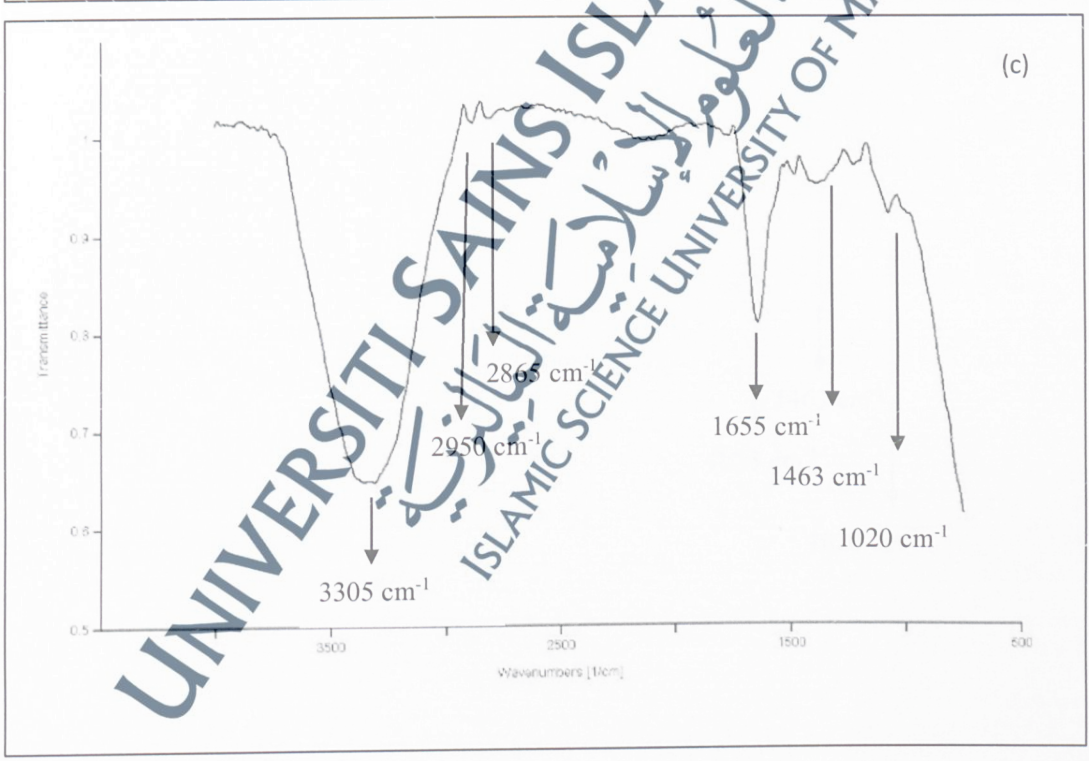
a very powerful biosurfactant and the presence of some contaminants did not affect the original surfactin functionality (Shepard *et al.*, 1987).

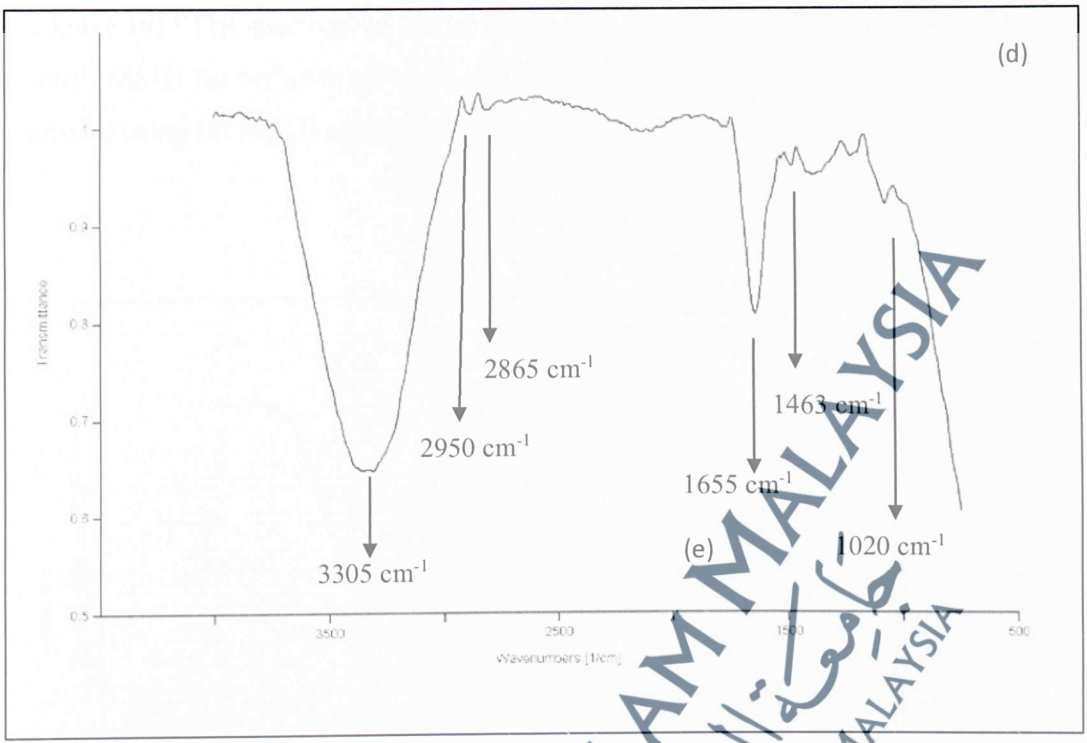
6.3.8.2. Structural Analysis Using FTIR

Figure 18: FTIR spectrum of final sufactin fraction from fermentation broth of *B. subtilis* ATCC 21332 (a) surfactin standard; (b) surfactin purified using PES10; (c) surfactin purified using HT10;(d) surfactin purified using PES30; (e) surfactin purified using HT30.









(e)

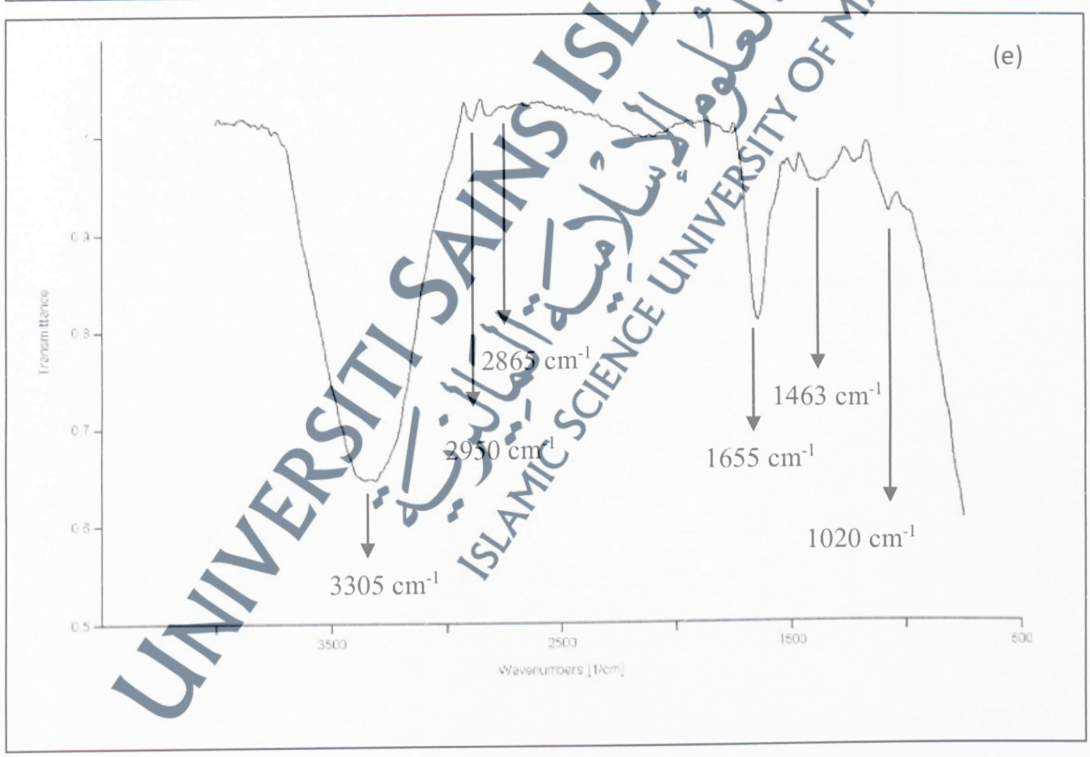
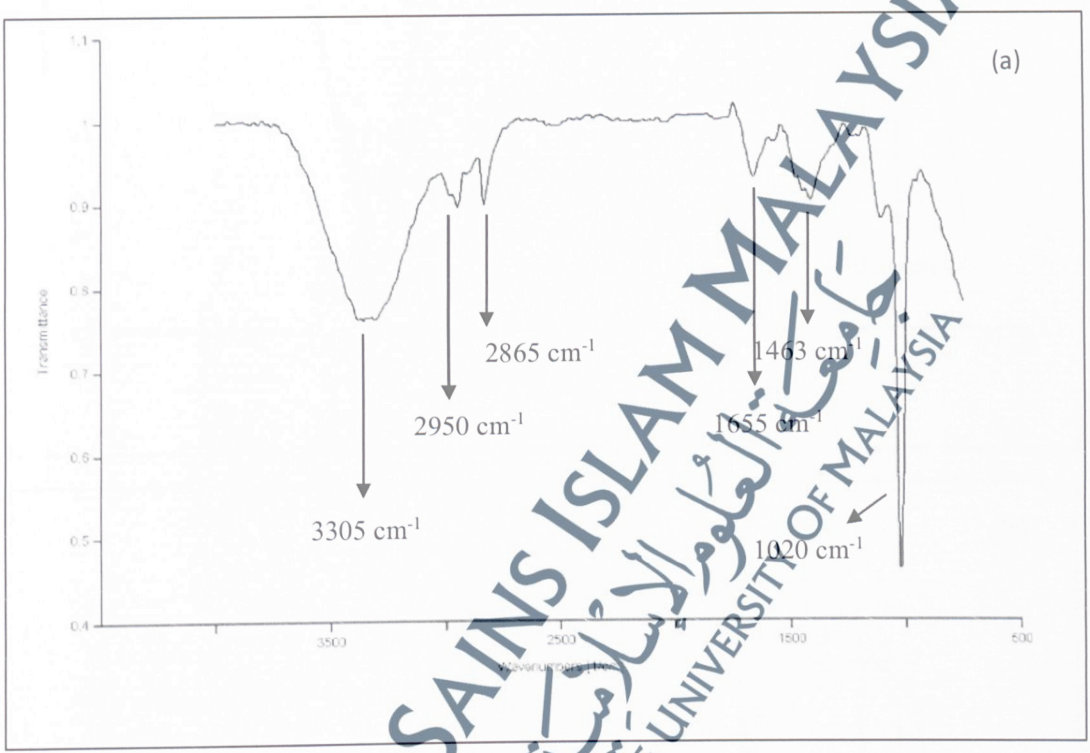
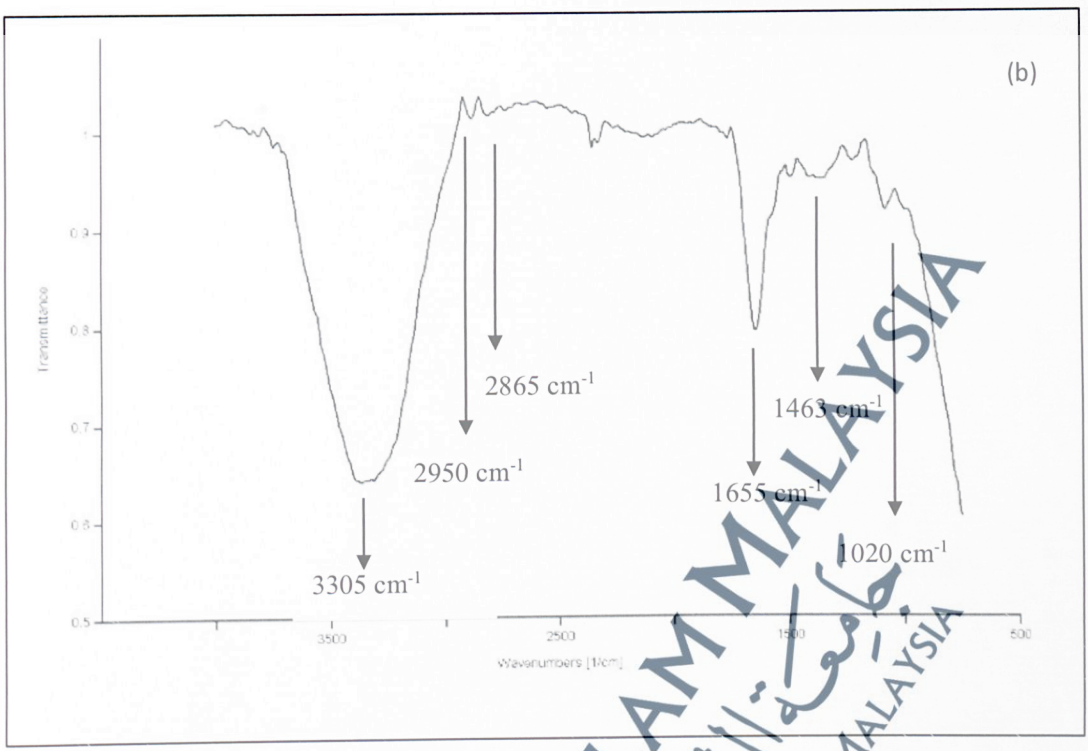
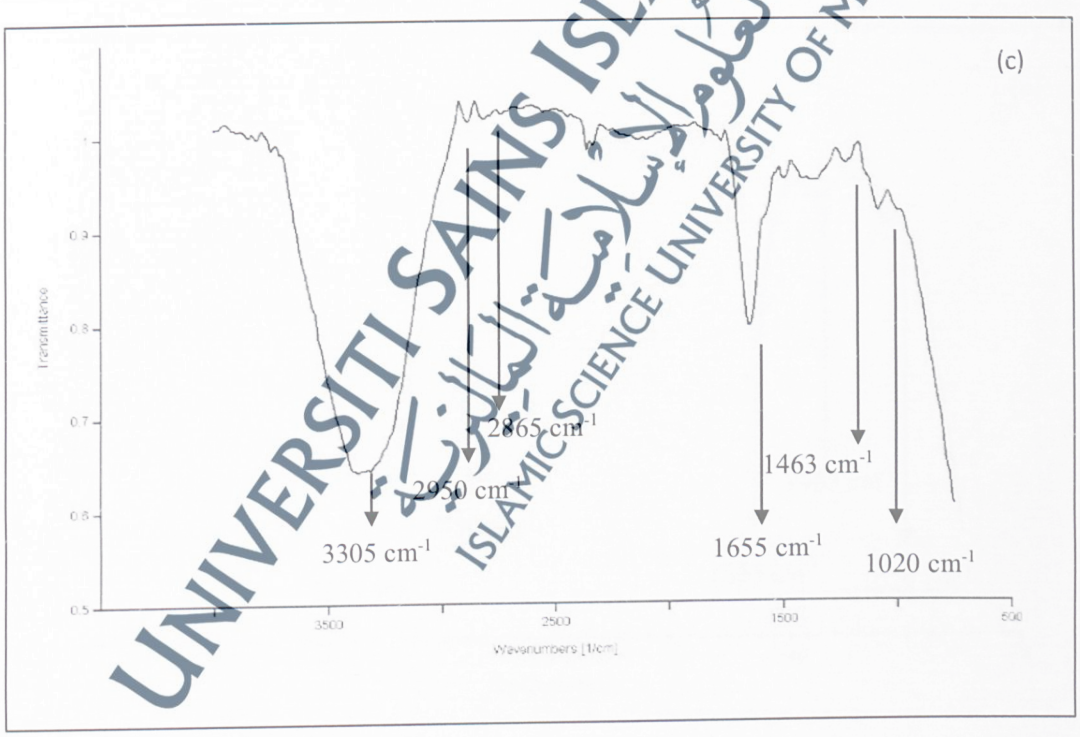
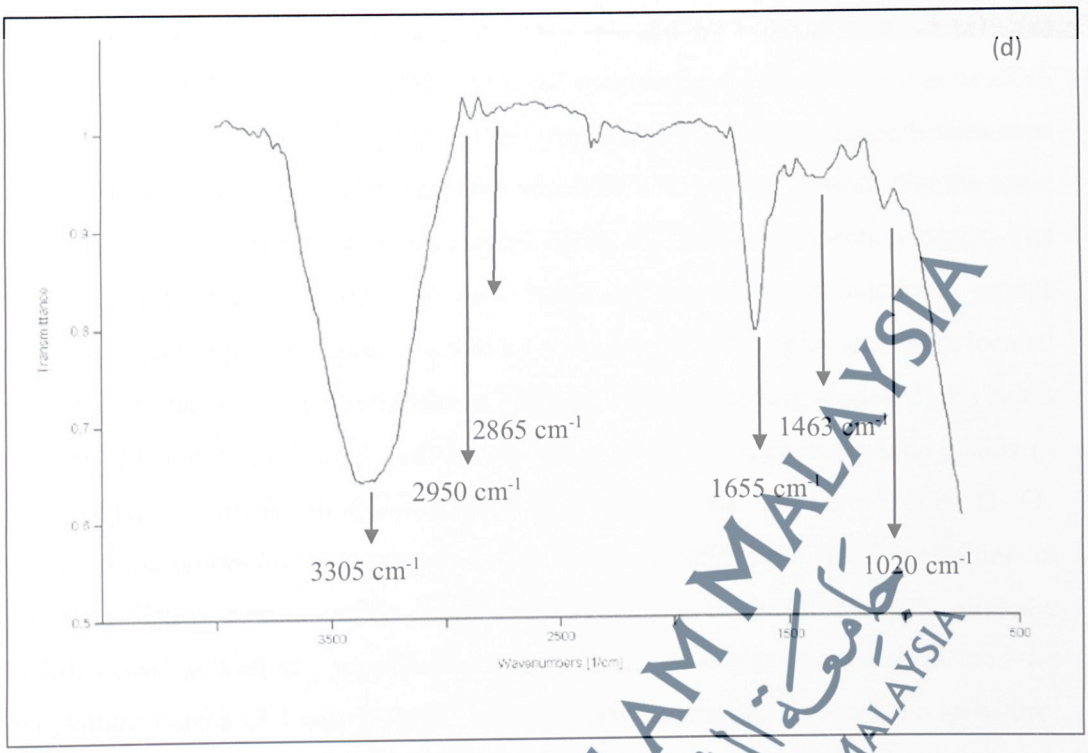


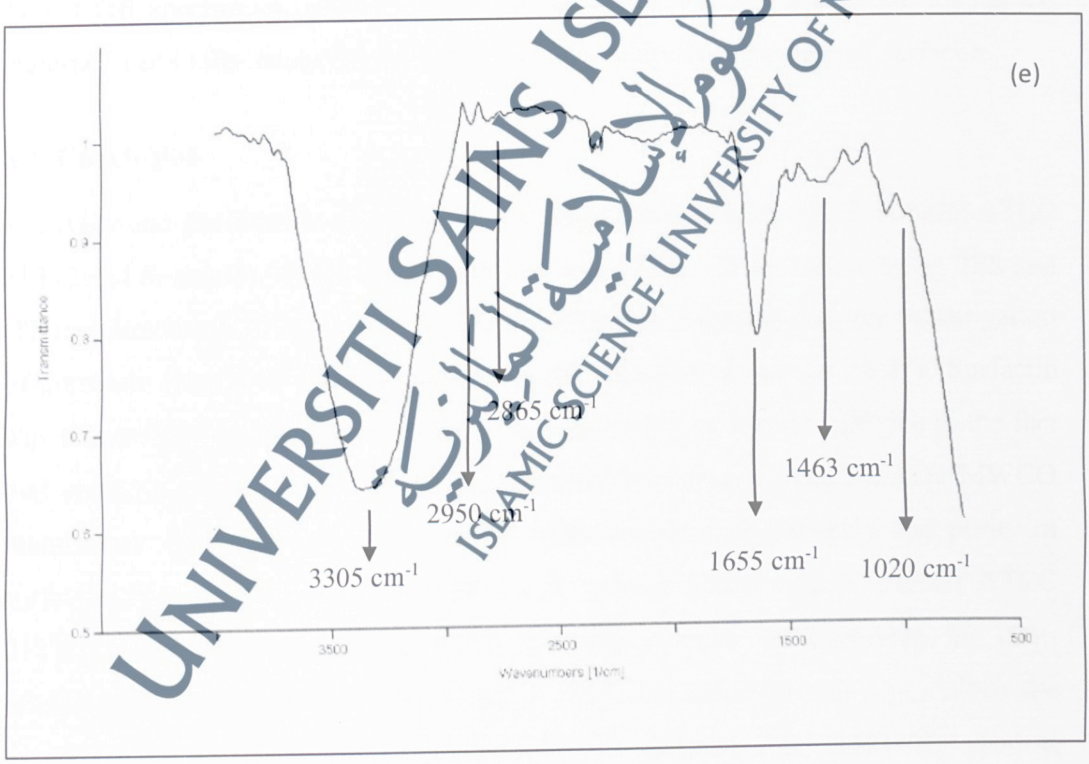
Figure 19: FTIR spectrum of final sufactin fraction from fermentation broth of *B. subtilis* MSH1 (a) surfactin standard; (b) surfactin purified using PES10; (c) surfactin purified using HT10; (d) surfactin purified using PES30; (e) surfactin purified using HT30 *B. subtilis* MSH1.











The molecular composition of surfactin was evaluated by FTIR. Figure 18 (a) - (e) and Figure 19 (a) - (e) presents the infra-red spectrogram purified surfactin by of *B. subtilis* ATCC 21332 and *B. subtilis* MSH1 respectively by using all membranes used in this study and compare with surfactin standard. The spectra showed that the same adsorption bands differed in relative areas under the various absorption bands. The most important characteristic absorption bands corresponding to functional groups typically forming part of surfactin could be observed for all samples and were located at: 1) bands characteristic of peptides at 3305 cm^{-1} (NH stretching mode); 2) the bands at 2956–2924cm^{-1}, 2869 cm^{-1} and at 1463 cm^{-1}, 1377cm^{-1} reflect aliphatic chains (–CH3, –CH2–) of the fraction; 3) the bands between 1020 and 1030 cm^{-1} reflect C– O–C vibrations (surfactin head rings); 4) the bands at 1655 cm^{-1} (C=O stretching in proteins). These results imply that the biosurfactant produced contains aliphatic hydrocarbons as well as a peptide like moiety. This FTIR spectrogram confirmed the lipopeptide nature of biosurfactants, which could correspond to surfactin structure. The FTIR spectrogram shows almost similar structure which means the membrane material used in this study did not affect the final molecular structure of surfactin.

6.4. Conclusion

Recovery and purification of surfactin from fermentation broth of *B. subtilis* ATCC 21332 and *B. subtilis* MSH1 by a single-step cross flow UF technique using PES and HT membranes of 10 kDa and 30 kDa MWCO was evaluated through investigation of permeate flux, *R* of surfactin and purity of surfactin at various TMP's. Surfactin was successfully retained by all membrane achieving *R* of almost 1.00 due to the fact that surfactin micelles were unable to permeate by using at least 30 kDa MWCO membranes. All membranes used in this study lead to high recovery and purity of surfactin from raw fermentation broth of *B. subtilis* MSH1 and *B. subtilis* ATCC 21332, results obtained in this study can further assist in improving the cost-effectiveness of downstream processing of surfactin which in turn can reduce the overall cost in downstream processing of surfactin. In addition, product characterization analysis was conducted to evaluate the functionality and purity of surfactin final fraction by using surface tension and FTIR analysis with use of surfactin standard as reference under similar and controlled conditions. Results showed close proximity of surface activity in relation to surfactin standard which

indirectly indicated the presence of impurities in the final fraction did not affect the original surfactin functionality. FTIR spectra confirmed that the UF retentate contains aliphatic hydrocarbons as well as a peptide-like moiety, which correspond to the structure of surfactin. Among all of the membranes used, it was found that HT30 membrane is most suitable for the downstream processing of surfactin because it provides better purity of surfactin final fraction, higher flux rates are attained besides have minimize effect of concentration polarization in comparison to all membrane. It was found that the variation of TMP had no significant effect on recovery and purity of surfactin final fraction however it affect the permeate flux. The flux of filtration can be maintained by using lower TMP that is an important characteristic in larger scale of process. Selective separation of surfactin from fermentation broths could be achieved by a single step UF process at the laboratory scale and shows potential to be scaled-up for industrial application.