EFFECT OF pH AND IONIC STRENGTH ON PERMEATE FLUX DURING SEPARATION OF *Lactobacillus plantarum* BY USING HOLLOW FIBER CROSSFLOW MICROFILTRATION

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BORANG PENGESAHAN STATUS TESIS◆

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SESI PENGAJIAN: 2009/2010

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APRIL 2010

I declare that this dissertation entitled "*Effect of PH and Ionic Strength on Permeate Flux During Separation of Lactobacillus plantarum by Using Hollow Fiber Crossflow Microfiltration* "is the result of my own research except as cited in the references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

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Date : ....

Special dedication for:

My beloved mother, Hamidah Binti Hussain

My beloved father, Abdullah Bin Sulaiman

Beloved Siblings

and

My Lovely Friends

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ABSTRACT

Microfiltration is a separation process used for cell harvesting in downstream process. Current research focused on the factor affecting the process of cell separation from fermentation broth to be recycled into the fermenter. The efficiency of solute separation by microfiltration can be influenced by solution pH and ionic strength. The objectives of this research is to study the effects of pH solution which is pH 4.5, 5.5, 6.5, 7.5, 8.5 and ionic strength on permeate flux of separation of *Lactobacillus plantarum* bacteria. For this research, the 0.2, 0.4, 0.6, 0.8 and 1.0 M of salt concentration are also used. At pH 8.5, permeate flux is the highest due to the electrostatic repulsion between the *Lactobacillus plantarum* bacteria and the surface of the membrane. The lowest permeate flux is at 1.0 M of ionic strength due to compaction of membrane and results in reduction of effective permeability. As conclusion, flux can be affected by pH solution and addition of salt. Increase in pH solution resulted in increase in permeate flux and addition of salt decreases permeate flux.

ABSTRAK

Penapis mikro adalah satu proses pemisahan yang digunakan di dalam process pemisahan hiliran. Kajian terkini lebih mengfokuskan kepada faktor yang mempengaruhi proses pemisahan sel daripada campuran penapaian untuk dikitar semula ke dalam penapai. Kecekapan pemisahan bahan larut oleh penapis mikro dipengaruhi oleh pH dan kekuatan ionik. Tujuan kajian ini adalah untuk mengkaji kesan pH larutan iaitu pH 4.5, 5.5, 6.5, 7.5, 8.5 dan kekuatan ionik kepada arus resapan pemisahan bacteria *Lactobacillus plantarum* . Dalam kajian ini, 0.2, 0.4, 0.6, 0.8 dan 1.0 molar kepekatan garam juga digunakan. Pada pH 8.5, arus resapan pemisahan *Lactobacillus plantarum* adalah paling tinggi disebabkan penolakan elektrostatik antara bacteria *Lactobacillus plantarum* dan permukaan penapis. Pada 1.0 molar kekuatan ionik, arus resapan adalah paling rendah disebabkan kepadatan penapis dan menyebabkan pengurangan resapan efektif. Sebagai kesimpulan, arus resapan boleh dipengaruhi oleh pH larutan dan penambahan garam. Arus resapan lebih tinggi apabila pH larutan bertambah dan penambahan garam mengurangkan arus resapan.

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LIST OF SYMBOLS

µm	-	Micrometer
Psig	-	Pound-force per square inch gauge (pressure)
°C	-	Degree celcius
Ml	-	Milliliter
Rpm	-	Revolutions per minute
M	-	Molarity
J	-	Permeate flux
A	-	Area
Cm^3	-	Centimeter cube
NaOH	-	Sodium hydroxide
HCl	-	Hydrocloric Acic
n	-	Moles
KH_2PO_4	-	Potassium dihydrogen phosphate
$KHC_8H_4O_4$	-	Potassium hydrogen phatalate

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Fermentation broths are complex aqueous mixtures of cells, soluble extracellular products, intracellular products and converted substrate or unconvertible components of a process called fermentation. As with other chemical process, fermentation for producing products is also aimed at minimizing production costs. Besides minimizing the cost, in order to improve fermentation efficiency and production rate, reusing cells hold promises (Hoek, 2003).

Production of sorbitol is one of the fermentation processes which using *Lactobacillus plantarum* in order to convert the glucose and produces the sugar alcohol, sorbitol. In context of sorbitol production, the *Lactobacillus plantarum* bacteria possess some relevant characteristics. It is a food grade microorganism belonging to the group of lactic acid bacteria and largely found as the dominant species in the last step of natural food raw material fermentation. There are a few microorganism have been suggested as potential sorbitol producers, but *Lactobacillus plantarum* bacteria is the best choose in order to achieve high level sorbitol production by fermentation (Ladero *et al.*, 2007).

Sorbitol is also referred as D-glucitol, is naturally found in many fruits, such as berries, cherries and apple. The worldwide production of sorbitol is estimated to be larger than 500000 tonnes per year and the market is continuously increasing. This polyol has a relative sweetness of around sixty percent compared to sucrose.

Based on these properties, sorbitol is widely used in a range of food products such as confectionery, chewing gums, candy, desserts, ice cream, diabetic foods as sweetener, humectants, texturizer and softener. In addition, sorbitol is the starting material for the production of pharmaceutical compounds such as sorbose and ascorbic acid (Ladero *et al.*, 2007).

In order to minimize the production costs, improve efficiency and production rate, usage of membrane separation to separate the bacteria cell is the best way to achieve the goals because membrane nowadays have gained wide acceptance and made significant inroads against competing technologies in many areas because of flexibility and performance reliability, cost competitiveness and environmental awareness. Besides that, the advantages of using membrane including good process ability, inexpensive production and low operating cost. In short, it offers low capital cost, low energy consumption, ease of operation and cost effectiveness (Sarif, 2005). There are four types of membrane process. They are microfiltration, ultrafiltration, nanofiltration and reverse osmosis (Ghosh, 2006).

1.2 Problem Statement

The percent of cell retention from fermentation broth that can be recycled back to the bioreactor may be affected by the pH of the fermentation broth used because the efficiency of membrane is influenced by pH (Ghosh, 2003). The percent might be too low or zero to be recycled if the pH can cause the pore size of membrane bigger because of the permeation of the cell through the membrane. The membrane morphology may be affected by the pH of the fermentation broth because Rubia states that pH can have significant effect on both fouling and rejection because of the changing of pores size of membrane. The membrane pore size can decrease and increase due to the changing of pH. If the pH of the fermentation broth causes the membrane pores size bigger, the bacteria in the fermentation broth can pass through the membrane, the product may be contaminated and the bacteria cell cannot be recycled back to be used for other fermentation process will cause wastes of money.

Besides that, it can also causes the wasting of time to culture the bacteria for some days before the fermentation process, compared with recycling the bacteria by harvesting from fermentation broth by using microfiltration process. It will also cause the waste of money when the membrane should be replaced so many times because of the fouling, affected by the pH of the fermentation broth.

Membrane fouling is one of the critical phenomena governing the performance of microfiltration separation because fouling causes flux decline. pH and ionic strength are some of factors that can affect the membrane separation (Ghosh, 2003). The different pH and ionic strength causes the different in permeate flux. Hence, the fouling can causes money and time consuming. Because of that, remedies should be done to increase the flux and avoid fouling.

1.3 Objectives

1. To study the effect of pH and ionic strength on membrane flux during separation of *Lactobacillus plantarum* bacteria.

2. To study the effects of fermentation broth pH on permeate flux of separation of *Lactobacillus plantarum* bacteria.

1.4 Scope of Study

In order to achieve the objectives, the following scopes have been identified.

1. Study of the culture process of *Lactobacillus plantarum* bacteria

2. The study of separation of *Lactobacillus plantarum* separation by using hollow fiber cross flow microfiltration

3. The study of pH and ionic strength effect on permeate flux during separation of *Lactobacillus plantarum* bacteria. The range of pH that is used is between 4.5 until 8.5. They are 4.5, 5.5, 6.5, 7.5 and 8.5. For ionic strength, the range which is used between 0.2 M until 1.0 M of ionic strength. They are 0.2, 0.4, 0.6, 0.8 and 1.0 (Yun, J. 1999)

1.4 Rationale and Significance

Microfiltration is a separation process used for cell harvesting in downstream process. Current research focused on the factor affecting the process of cell separation from fermentation broth in order to identify the amount of bacteria cell that can be recycled back into fermentation tank (Kaghazchi *et al.*, 2000). The study of fermentation broth pH effects on the *Lactobacillus plantarum* is to determine whether microfiltration membrane separation is suitable for the separation of *Lactobacillus plantarum* and to determine the effects of the fermentation pH on the permeate flux during *Lactobacillus plantarum* separation.

pH and ionic strength are two of factors that can affect the membrane separation (Ghosh, 2003). The remedies is one of the way to enhance the flux and increase the profits because the study of pH and ionic strength effects on *Lactobacillus plantarum* separation can help in determination of the optimum pH and ionic strength that used be used for separation process of *Lactobacillus plantarum*

from the fermentation broth in order to achieve high permeate flux and one hundred percent of *Lactobacillus plantarum* bacteria retention. Besides that, it can avoid fouling of the membrane, money and time consuming.

CHAPTER 2

LITERATURE REVIEW

2.1 Sorbitol

Sorbitol also referred as glucitol, $C_6H_{14}0_6$ as shown in figure 2.1 (Chun *et al.*, 1988) classified as sugar alcohols have existed as commercial products for more than 60 years. It is can be naturally found in many fruit. Today, sorbitol is used in food, confectionary, oral care, pharmaceutical and industrial applications because of their unique physical and chemical properties which is as the starting material for the production of sorbose and ascorbic acid.

Sorbitol is suitable for a variety of products reduced in calories, sugar or fat and has been safely used for almost half a century. Sorbitol has relative sweetness of round sixty percent compared to sucrose with one-third fewer calories. In products, it not only fulfils a role as sweetener, but also as a humectants, texturizer and softener. It is also non-cariogenic and because of its benefits, it may be useful to people with diabetes (Kellen *et al.*, 2007).

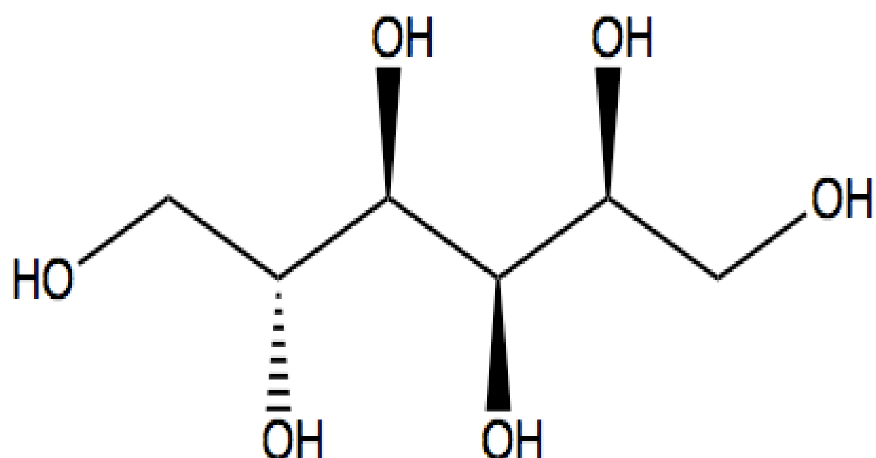

Figure 2.1: Chemical structure of sorbitol

2.2 Sorbitol Production by Fermentation

Several industrial processes have been described for the production of sorbitol as potential sorbitol produces, including fermentation process. Ladero states that production of sorbitol can achieved in bacteria. However, only few microorganisms have been described for the production of sorbitol. But, compared to the others, *Lactobacillus plantarum* bacteria can be utilized in fermentation process to achieve high level production of sorbitol from glucose (Ladero *et al.*, 2007).

2.2.1 pH of Medium

At the end of fermentation, pH was equal to 6.5 in order to maintain the pH growth of *Lactobacillus plantarum*. The pH is a key parameter which has to be taken into account when optimizing the separation process of fermentation broth by

microfiltration because of the broth pH, which is generally determined by the fermentation conditions that can affect the filtration performance (Milcent, 2001).

2.3 *Lactobacillus plantarum* Bacteria

Lactobacillus plantarum is a 0.3 µm in diameter and 8 µm long, rod shaped bacteria as shown in figure 2.2 (Ferrer, 2009). It is one of lactic acid, gram positive, nonsporulated and anaerobic bacteria which able to synthesis sorbitol, sugar alcohol from glucose by fermentation process where the growth and fermentation pH of the bacteria is at 6.5 (Patra *et al*., 1997).

Sabaitis (1976) states that the isoelectric point of *Lactobacillus plantarum* is about 3.75. The behavior of the lactic acid bacteria is depends on its surface properties. This is because of the cell surface of *Lactobacillus plantarum* that can adapt in responses to environmental change, like in low pH and ionic strength solution (Rodriguez *et al*., 2004).

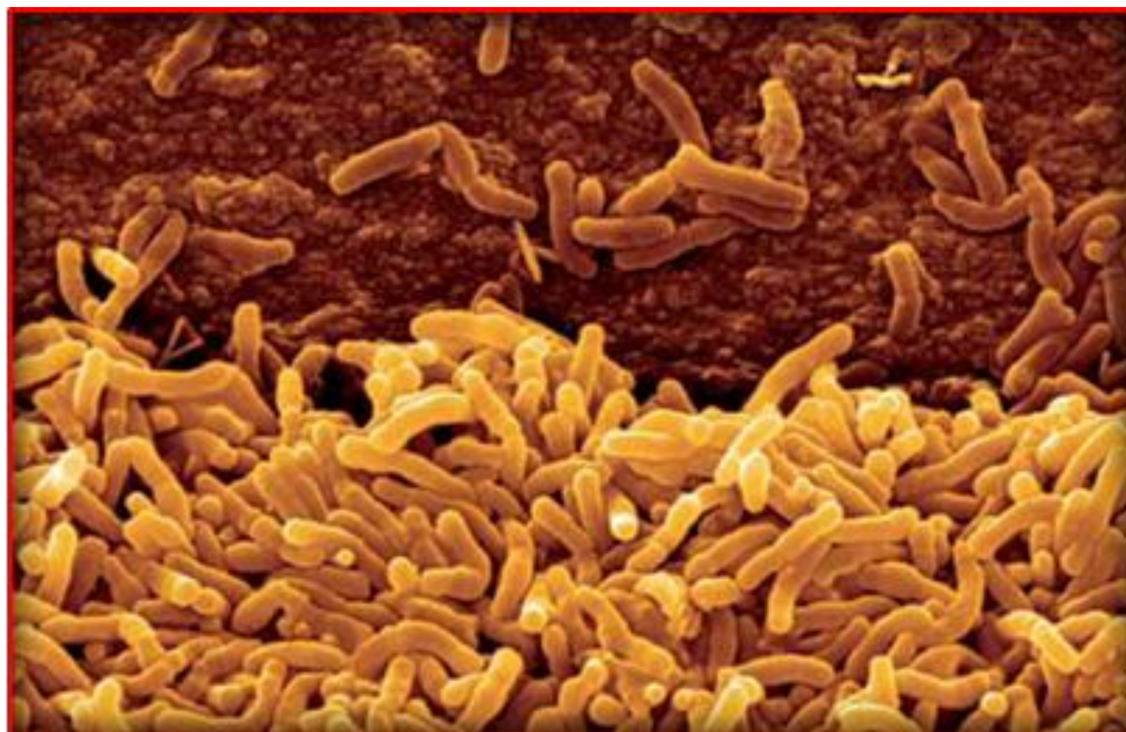

Figure 2.2: *Lactobacillus plantarum* bacteria

2.3.1 Isoelectric Point of *Lactobacillus plantarum*

Isoelectric point is the pH at which a particular molecule or surface properties carries no net charge. The net charge on the molecule is affected by pH of their surrounding environment and can become more positive or negatively charged. Sabaitis states that the isoelectric point of *Lactobacillus plantarum* is at pH 3.75. Even though the *Lactobacillus plantarum* bacteria can adapt to environmental change, the different of pH can affect the surface charge of the bacteria if the pH is lower or higher that the isoelectric point of the bacteria (Manttari *et al*., 2006).

2.3.2 Cell Surface Properties

The electric charge is consequence of chemical composition of the surface layer protein conveys hydrophobicity to the *Lactobacillus plantarum* cell surface. This suggests that cell surfaces of *Lactobacillus plantarum* may adapt in response to environmental change like in pH or ionic strength. *Lactobacillus plantarum* is also a strong electron donor and weak electron acceptor. In other words, *Lactobacillus plantarum* bacteria have strong basic and weak acidic character (Pelletier *et al*., 1997).

2.4 Culture Medium of *Lactobacillus plantarum* Bacteria

The function of Man Rogossa and Sharpe (MRS) agar and broth is to provide a medium that would support the good growth of *Lactobacillus plantarum*. The ammonium citrate that contained in both the MRS agar and broth inhibits most microorganisms, but allows for the growth of *Lactobacillus plantarum*. The dipotassium phosphate and sodium acetate are buffer agents to maintain the pH of the agar and broth, tween 80 is an emulsier, manganese and magnesium sulfates are sources of ions and sulfate, peptone and meat extracts are nutrient sources for growth that contain nitrogen, vitamins, minerals and amino acids. In addition, dextrose is

the fermentable carbohydrate as carbon and energy source for the *Lactobacillus plantarum* bacteria (Briggs, 1960).

Table 2.1: Description of lactobacillus species

Species	Description	References
L. casei	Cell surface of Lactobacillus can adapt and response to pH and ionic strength	Rodriguez et al., 2005
L. casei	Lactobacillus strain slightly negatively charged at alkaline pH solution and positively charged with decreasing pH	Pelletier *et al.*, 1997
L. plantarum	Suggested for use when bacteria need to adapt efficiently to environmental change	Koupion *et al.*, 2007
L. plantarum	MRS broth culture maintained at pH 6	Todorov, 1999
L. plantarum	The cell harvested and washed with phosphate buffer pH 6.5	Rivas *et al.*, 2008

2.5 Streaking Technique

Agar streak plates are an essential tool in culture process. The streaking technique which is used allows bacteria and fungi to grow on a solidified agar surface to produce discrete colonies. These colonies can be used to help identifying the organism, purify the strain free of contaminants, and produce a pure genetic clone. In order to obtain well isolated discrete colonies, the quadrant streak technique should be used because it allows sequential dilution of the original microbial broth or colonies on a plate (Thiel, 1999)

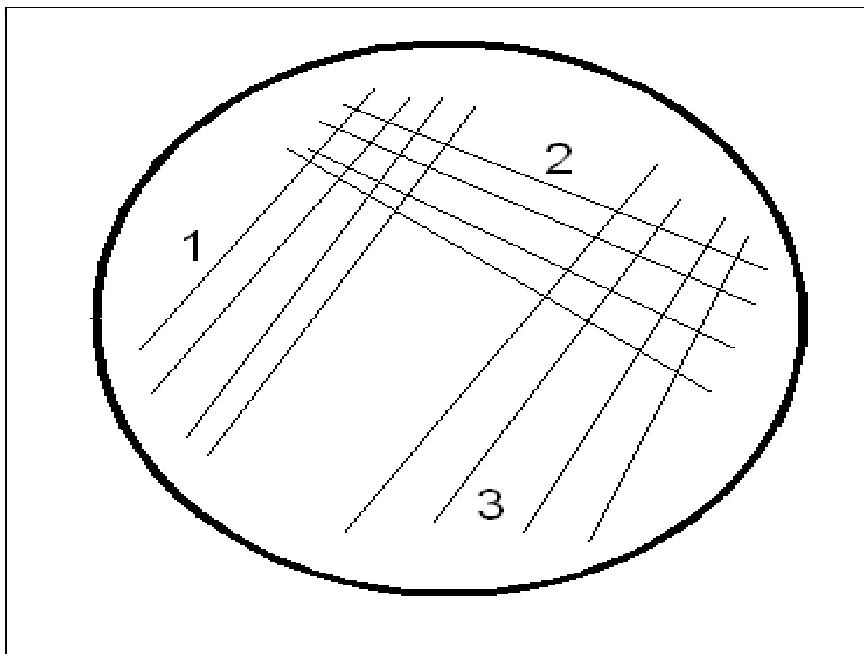

Figure 2.3: Streaking Technique

2.6 Membrane

A membrane can be described as a thin barrier between two bulk phases that permits transport of some components but retain others (Sarif, 2005). In order to allow the transport of material through a membrane, a driving force is necessary. The transport of material through a membrane could be driven by convection or by diffusion.

A membrane may be made from organic polymers or inorganic material such as glass, metals and ceramics or even liquids. The examples of polymeric or organic membranes including those made from polysulfone, cellulose, cellulose acetate, polyethersulfone and polyamide. But, the inorganic membranes can be made from ceramics, glass and stainless steel (Ghosh, 2006).

There are many ways to classify a membrane. From a structural point of view of membranes, basically membrane can be classified as symmetric or asymmetric and from a morphological point of view, membranes can be classified into two categories which are porous or dense (Sarif, 2005). Porous membrane has

tiny pores or pore networks and on the other hand, dense membrane do not have any pores (Ghosh, 2006).

2.7 Membrane Separation Process

Membrane separation involves partially separating a feed containing a mixture of two or more components by use of a semipermeable barrier, the membrane through which one or more of the species moves faster than another or other species. The transport of material through a membrane could be driven by convection or by diffusion or indeed by a combination of the two.

Convection based transport takes place due to transmembrane pressure and diffusion based transported utilizes the concentration difference of the transported species across the membrane as the driving force. Pressure driven membrane based bioseparation process can be classified into four types based on the size of the permeable species. They are microfiltration, ultrafiltration, nanofiltration and reverse osmosis process (Ghosh, 2006).

2.8 Microfiltration

Microfiltration (MF) is used for separation of fine particles or micron-sized particles such as bacteria from fluids. The separation limit of microfiltration falls within 0.02 to 10µm, which is placed coarse filtration and ultra filtration (Young *et al.*, 1999). Microfiltration membranes are asymmetric, porous and retain particles by a purely sieving mechanism. In term of pressure, the transmembrane pressure ranges usually used for microfiltration ranges from 1 to 50 psig.

In addition, most microfiltration membranes capture particles by surface filtration which is the surface of the membrane. The applications of microfiltration in biotechnology include cell harvesting from bioreactors during fermentation

process. A microfiltration process can be operated either in a dead-end mode or cross-flow mode. But, for most applications, cross flow microfiltration is preferred (Ghosh, 2006).

2.8.1 Cross Flow Microfiltration

Cross flow microfiltration is a pressure driven membrane process in which the fluid to be filtered flows parallel to the membrane surface (Young *et al.*, 1999). The configuration of cross flow microfiltration helps to reduce the formation of filter cake can allow a better permeate flux because cross flow microfiltration has a filtration surface which is continuously swept by flowing liquid. The shear of the flowing liquid along the tube wall minimizes the buildup of the solids on the microfiltration surface and hence, minimizes the fouling of membrane. Thus, cross flow microfiltration affords the possibility of nearly steady state operation.

The cross flow micro filtration modules contain multiple porous tubes, which have a nominal pore size of 0.2 microns. With this small pore size, large colloidal particles, and bacteria can be filtered from a fermentation process, but not molecular level substances (Moka *et al.*, 2001)

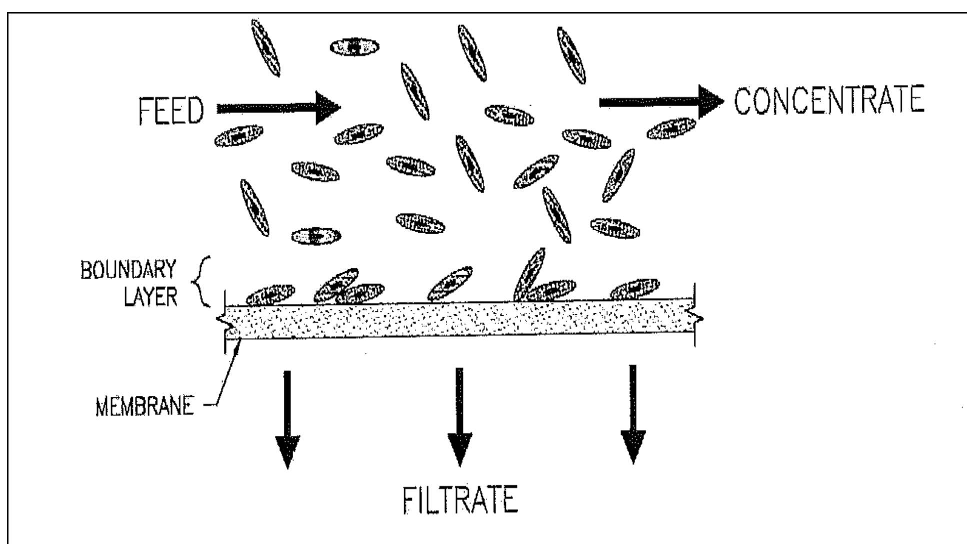

Figure 2.4: Cross flow mechanism

2.8.2 Polysulfone Hollow Fiber Membrane Module

Membrane is housed within devices called membrane modules. Polysulfone hollow fiber membrane module is one of the types of membrane modules used in membrane system that is made by polymer which is polysulfone (Ghosh, 2006). Hollow fiber membrane is tube like in appearance. Flat sheet and hollow fiber are the most common membrane modules. But, the hollow fiber has more advantages than flat sheet membranes, because it has more advantages that flat sheet membrane, because it have higher permeation area or module volume ratio. Another advantage of hollow fiber is that it is self supporting and do not require a support or spacers to separate the membrane (Faria *et al*., 2002).

In addition to that, the fibers may be bundled in one of several arrangements. A typical hollow fiber module may consist of several hundred to over 10000 fibers. Hollow fibers membrane modules may operate in an inside-out or outside-in mode. In inside-out mode, feed solution enters the center of the fiber (lumen) and is filtered radially through the fiber wall. The filtrate then can be collected in the center of the fiber. During outside-in operation, feed solution passes from outside the fiber to the inside, where filtrate is collected in the center of the fiber (Drioli *et al*., 2006).

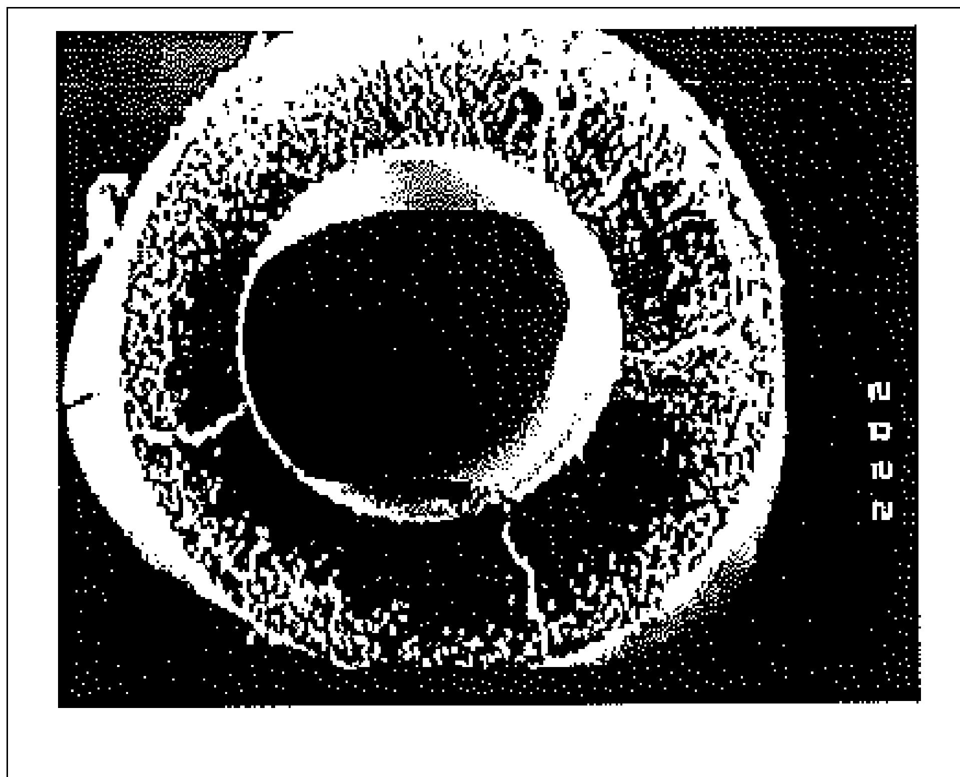

Figure 2.5: Hollow fiber membrane module

2.8.3 Polymer Membrane

Almost all membranes are constructed of polymers since they are significantly less expensive than membranes constructed of other materials. Mechanical strength is one of the considerations of polymer membrane since a polymer membrane can withstand larger transmembrane pressure, allowing for greater operational flexibility and the use of higher pressures (Drioli *et al.*, 2006).

2.8.4 Symmetric Membrane

An isotropic (symmetric) membrane is one of classification of a membrane. An isotropic membrane has a uniform composition and structure throughout or morphology at all position within it (Ghosh, 2006).

2.8.5 Microporous Membrane

A microporous membrane is very similar in structure and function to a conventional filter. It has a rigid, highly voided structure with randomly distributed, interconnected pores. However, these pores differ from those in a conventional filter by being extremely small, on the order of 0.01 to 10 μm in diameter. All particles larger than the largest pores are completely rejected by the membrane. Particles which are smaller than the largest pores, but larger than the smallest pores are partically rejected, according to the pore size distribution of the membrane.

Thus, separation of solutes by microporous membrane is mainly a function of molecular size and pore size distribution. In general, only molecules that differ considerably in size can be separated effectively by microporous membranes, for example in microfiltration (Baker, 2004). Figure 2.6 (Baker, 2004) shows the mechanism of the microporous membrane.

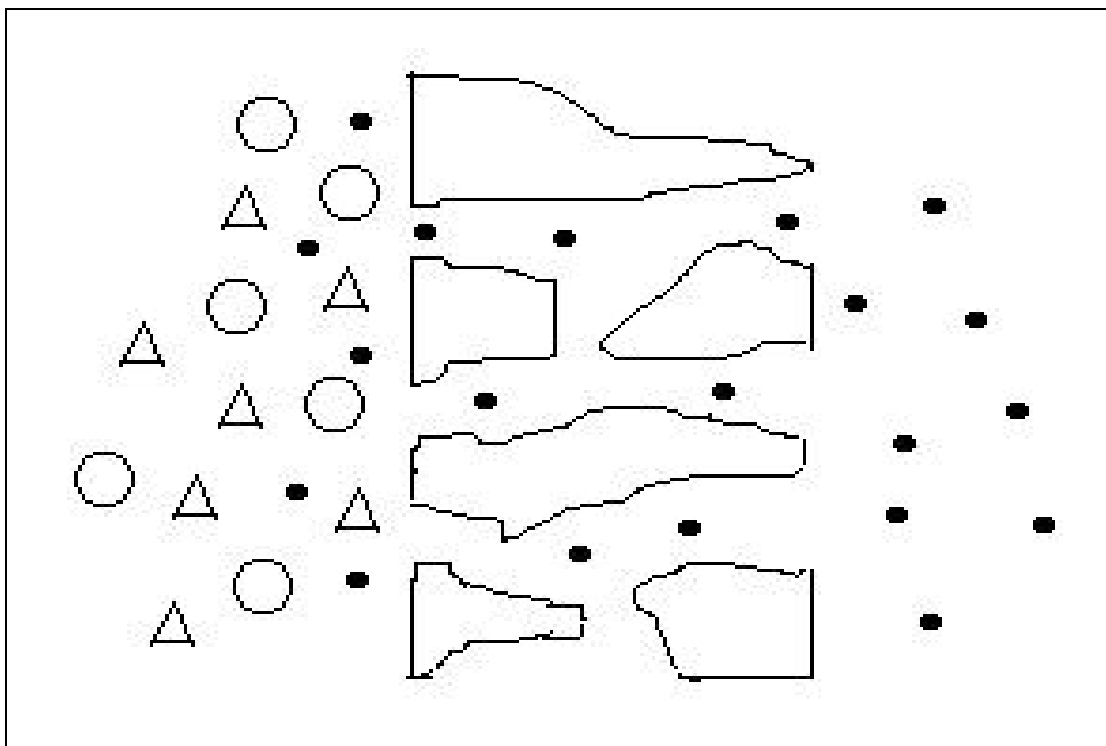

Figure 2.6: Microporous membrane

2.9 Chemical Stability of Membrane

In the field of microfiltration, the chemical stability of polymeric membrane materials is limited with respect to pH and organic liquids. Another important factor is the ease of cleaning, especially in high fouling applications involving microfiltration. Fouling leads to a drastic decrease of flux through the membranes and periodic cleaning is necessary (Mulder, 1996).

2.10 Electrically Charged Membrane

Electrically charged membrane can be dense or microporous, but are most commonly very finely microporous, with the pore walls carrying fixed positively or negatively charged ions. A membrane with fixed negatively charged ions is referred to as a cation exchange membrane.

Separation with charged membrane is achieved mainly by exclusion of ions of the same charges as the fixed ions of the membrane structured and to a much lesser extent by the pore size. The separation is affected by the charge and concentration of the ions in the solution (Baker, 2004).

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2.11 Permeate Flux

Flux referred as the throughput of material through a membrane whether as permeate or retentate. The permeate flux in a separation process determines its productivity if it is high or low productivity. The permeate flux depends on primarily on the properties of the membrane and feed solution. In addition, permeate flux also can be affected by membrane fouling. At constant transmembrane pressure, the permeate flux will decrease with time due to fouling (Ghosh, 2006).

2.12 Flux Decline

The effectiveness of membrane separation is greatly affected by fouling. Fouling can cause the decline in permeate flux. Flux decline is the decreasing in flux with time due to the accumulation or cake deposited on the membrane surface and the pore blocking by particles during membrane separation (Suk *et al.*, 1999).

2.13 Fouling

Fouling is an accumulation of substances on the membrane surface and or within the membrane pore which results in deterioration of membrane performance. It is an undesirable phenomenon which is usually caused by adsorption and deposition of material on the membrane (Ghosh, 2006). Membrane surface plays the role of a particle collector in a microfiltration. But, the formation of cake can cause negative effect on the performance and the production rate (Polyakov, 2005).

2.13.1 Inorganic Fouling

Inorganic fouling or scaling is caused by the accumulation of inorganic precipitates such as metal hydroxides and scales on membrane surface or within pore structure. Precipitates are formed when the concentration of chemical species exceeding their saturation concentrations. For microfiltration, inorganic fouling due to concentration polarization is much less profound, but can exists most likely due to interactions between ions and other fouling materials via chemical bonding (Otoyo *et al.*, 1999).

2.13.2 Microbial Fouling

Microbial fouling is a result of formation of biofilms on membrane surfaces. Once bacteria attaché to membrane, they start to multiple and produce extracellular polymeric substances to form a viscous, slimy, hydrated gel. Extracellular polymeric substances typically consist of heteropolysaccarides and have high negative charge density. This gel structure protects bacterial cells from hydraulic shearing and from chemical attacks (Otoyo *et al.*, 1999).

2.13.3 Organic Fouling

Organic fouling is profound in membrane filtration with source water containing relatively high natural organic matters. Surface water typically contains higher natural organic matters than ground water, with exceptions. For source water high in natural organic matters, organic fouling is believed to be the most significant factor contributed to flux decline (Otoyo et al., 1999).

2.13.4 Colloid Fouling

Algae, bacteria, and certain natural organic matters fall into the size range of particle and colloids. In most cases, particles and colloids do not really foul the membrane because the flux decline caused by their accumulation on the membrane surface is largely reversible by hydraulic cleaning measures such as backwash and air scrubbing. A rare case of irreversible fouling by particles and colloids is that they have smaller size relative to membrane pore size. Therefore, those particles and colloids can enter and be trapped within the membrane structure matrix, and not easily be cleaned by hydraulic cleaning (Otoyo *et al.*, 1999).

2.14 Transmembrane Pressure

Transmembrane pressure is considered as force which drives liquid flow through a cross flow membrane. During separation process, the feed side of the membrane is under higher pressure than the permeate side. The pressure difference which is the transmembrane pressure will force liquid through the membrane and can affects the separation flux (Mulder, 1996). The transmembrane, feed, retentate and permeate pressure of membrane separation can be related as:

Transmembrane pressure = [(feed pressure + retentate pressure)/2] – permeate pressure

(**Equation 2.1**)

2.15 Effect of pH and Ionic Strength on Membrane

Efficiency of solute separation by microfiltration can be influenced by solution pH and ionic strength (Ghosh, 2006). Ghosh states that permeate flux coefficient depend not only on solute and membrane properties but also on operating and environmental parameters such as pH and ionic strength. The transmission of charged solutes is particularly sensitive to pH and salt concentration.

It is also found that the effect of solution chemistry which is pH and ionic strength can be modeled on solution flux terms of the effect of ions shielding charge on the membrane surface. Theoretically, increase ionic strength will neutralize membrane charge, thus changing membrane morphology, as reflected in a reduction of membrane permeability. Besides that, pH can also affect the effective permeability and the solute rejection. It is the dominant effect of pH and ionic strength solution on membrane separation process (Mattaraj, 1997).

Table 2.2: Effect of pH and Ionic Strength on Membrane Morphology

Parameter	Description	Reference
pH	It was found that the effect of solution chemistry (pH and ionic strength) can be modeled on solution flux in term of the effect of ions shielding charge on membrane surface.	Mattara, 1997
pH and ionic strength	Permeate flux and solute rejection decreased significantly at low pH.	Rubia *et al*., 2006
pH and ionic strength	Electrokinetic effects (membrane and solute charge, pH, ionic strength) can have significant effect on both fouling and rejection of charged species.	Jones *et al*., 2000
pH and ionic strength	The zeta potential dependent on two parameters, the surface charge of membrane and the ionic strength of solution. The surface charge may be strongly dependent on pH.	Rodriguez *et al*., 2009

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2.16 Backwashing

The purpose of backwash process is to remove contaminants accumulated on the surface and wash accumulated particles out through the discharge line. Backwashing is almost exclusively associated with hollow fiber microfiltration processes. In general, a backwash cycle is triggered when a performance based benchmark is exceeded such as increase in transmembrane pressure and flux decline. Ideally, the backwash process restores the transmembrane pressure to its clean level. For most systems, backwashing is fully automatic. If backwashing is incapable of restoring the flux, then membranes are chemically cleaned.

The variables that should be considered in cleaning microfiltration membranes includes the frequency and duration of cleaning, chemicals and their concentrations, cleaning and rinse volumes, temperature of cleaning, recovery and reuse of cleaning chemicals, neutralization and disposal of cleaning chemicals. However, most membranes exhibit a gradual increase in transmembrane pressure after each backwash, indicating accumulation of foulants that cannot be removed by the backwash process alone and should be addressed through chemical cleaning (Drioli *et al.*, 2006).

2.17 Chemical Cleaning

The purpose of chemical cleaning is to control membrane fouling. Chemical cleaning can be conducted on microfiltration because accumulation of foulant makes it necessary. The goal of chemical cleaning is to restore the transmembrane pressure of a membrane system to its clean level. Once the cause of membrane fouling is identified, various cleaning chemicals can be used to removed fouling material from the membrane and restore membrane flux. Chemicals commonly used for cleaning microfiltration fall into five categories. They are caustic, oxidants of disinfectants, acids, chelating agents and surfactants (Otoyo *et al.*, 1999).

2.17.1 Caustic

Caustic is typically used to clean membrane fouled by organic and microbial foulant. The function of caustic is two- fold. First, hydrolysis and the second is solubilization. A very important function of caustic is to increase negative charges of humic substances. Therefore, they are easier to be removed from membranes. The typical caustic chemical is sodium hydroxide (Otoyo *et al.*, 1999).

2.17.2 Oxidant

The second type is of chemicals is oxidants. Most common oxidants used for membrane cleaning include chlorine and hydrogen peroxide. There are three purpose of using oxidants. They are to enhance cleaning efficiency, control of excess oxidation to membrane and other module components and reduce the health hazards of cleaning operation (Otoyo *et al*., 1999).

2.17.3 Acids and Chelating

The third type of chemicals used for cleaning is acids and chelating. Acids are used primarily for removing scales and metal dioxides from fouling layers. When membrane is fouled by iron oxides, citric acid is very effective because it not only dissolves iron oxides precipitates, but also form complex with iron. So, the removal of iron can also improve the cleaning of membranes fouled by organic foulants (Otoyo *et al*., 1999).

2.17.4 Surfactant

The fourth chemical is surfactant. Surfactants are compounds that have both hydrophilic and hydrophobic and hydrophobic structures. They can form micelles with fat, oil and proteins in water and help to clean the membrane fouled by these materials. Some surfactants may also interfere in hydrophobic interactions between bacteria and membranes. In addition, surfactant can disrupt functions of bacteria cell walls. Therefore, surfactant affects fouling dominated by the formation of biofilms (Otoyo *et al*., 1999).

Sometimes, the foulants cannot be removed through chemical cleaning or backwashing and the problem experienced in all membrane systems. Thus, it will require membrane replacement (Drioli *et al*., 2006). .

2.18 Total Organic Carbon Analyzer

Total organic carbon analyze is a complete analytical system capable of measuring total carbon, total organic carbon and total inorganic carbon in solid and liquid samples and analyzing most of any sample type and concentration with a precision unmatched by other analytical techniques (Schumacher, 2002).

2.18.1 Carbon Analysis

Carbon analysis by total organic carbon analyzer involves total carbon (TC) and total inorganic carbon (TIC). The various carbon forms, are distinguished by the manner in which the carbon dioxide is formed, either combustion or acidification. Theoretically, the total carbon (TC) is considered as the sum of the inorganic total carbon (TIC) and total organic carbon (TOC) components.

Total carbon (TC) = Total inorganic carbon (TIC) + Total organic carbon (TOC)

(**equation 2.3**)

Specifically, total carbon can always be measured directly by the combustion of a sample while total inorganic carbon can always be measured directly by the acidification of a sample. However, the measurement of total organic carbon for most sample types requires either a pre-treatment step or the separate analyses of total carbon (TC) and total inorganic carbon (TIC) in order to obtain total organic carbon (TOC) by difference.

Total organic carbon (TOC) = Total carbon (TC) –Total inorganic carbon (TIC)

For homogeneous liquids, the pretreatment method is most commonly used. This step involves acidifying the sample and purging it of all total inorganic carbon (TIC). The resulting solution is then analyzed by combustion to obtain the total organic carbon (TOC) result (Schumacher, 2002)

CHAPTER 3

METHODOLOGY

3.1 Introduction

The purpose of this study is to study the effect of pH and ionic strength on the separation of *Lactobacillus plantarum* bacteria by using the hollow fiber cross flow microfiltration. The experiment was conducted starting from the culture process of *Lactobacillus plantarum* bacteria in Man's Rogosa and Sharpe (MRS) agar and broth (Kachouri *et al*., 2005). The second step is the preparation of buffer solution. There are two types of buffer solution which are used to control the pH. They are the acetate and phosphate buffer solution. To control the ionic strength of solution, certain amount of sodium chloride (NaCl) is added into the pH 6.5 of the buffer solution where the 6.5 pH value is the pH of the *Lactobacillus plantarum* bacteria growth and used for production of sorbitol from glucose by fermentation (Harigan, 1998). The next step is the *Labtobacillus plantarum* bacteria cell harvesting by using centrifuge and diluted with the buffer solution and after that, the step of separation process of the bacteria cell, by using microfiltration. Before the separation process, the washing step of the membrane system was conducted. The membrane system was washed by using 0.1 M of sodium hydroxide (NaOH) followed by using distilled water. During separation process, *Lactobacillus plantarum* bacteria cell was separated and volume at permeate was measured to determine the permeate flux for every pH and ionic strength. The last step of the experiment is sample analyzing by using total organic carbon analyzer (TOC).

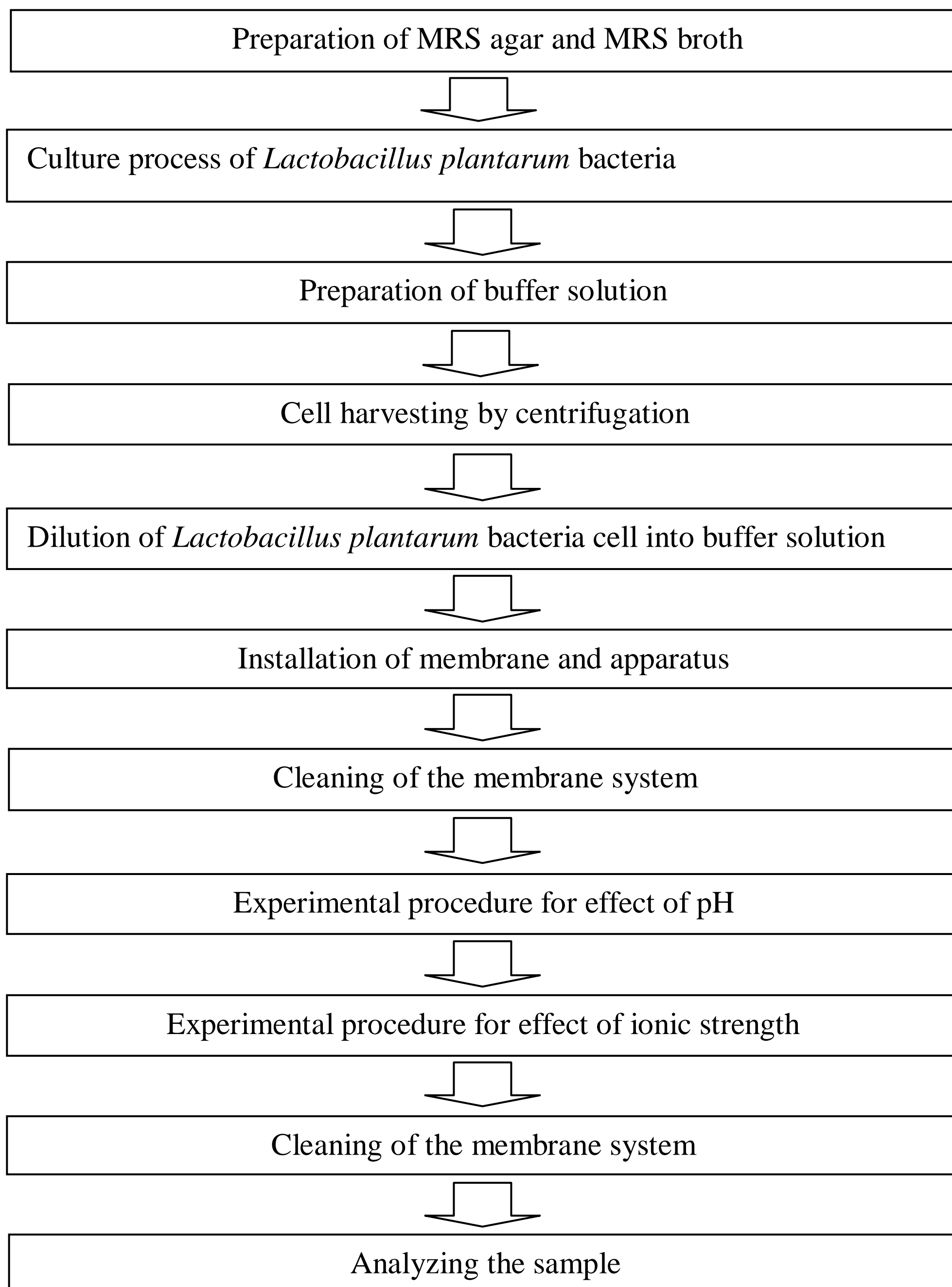

Figure 3.1: Overall process flow

Figure 3.1 shows the overall process of this study "Effect of pH and Ionic Strength on Hollow Fiber Cross Flow Microfiltration during Separation of *Lactobacillus plantarum*".

3.2 Equipments/ Apparatus

In order to complete the study, there are some equipments are required. They are:

1. Autoclave (HVE-50)
2. pH meter (HM 30P)
3. Electric Balance Shimadzu (A W220)
4. Magnetic stirrer
5. Hot plate (ERLA)
6. Incubator shaker
7. Centrifuge (eppendorf 5810R)
8. Volumetric flask
9. Conical flask
10. Beaker
11. Measuring cylinder
12. Glass rod
13. Aluminium foil
14. Cotton
15. Micro pipette
16. Vortex
17. Hollow fiber cross flow microfiltration, 0.2 µm (QUIXSTAND)

3.3 Reagents

In order to complete the study, there are some reagents or chemical are required. They are:

1. Peptone
2. Meat extract
3. Yeast extract
4. Glucose
5. Tween 80
6. Potassium dihydrogen phosphate
7. Sodium acetate
8. Tri-ammonium citrate
9. Tetrahydrated magnesium sulphate
10. Tetrahydrated manganese sulphate
11. Potassium hydrogen phatalate
12. Sodium hydroxide
13. Potassium dihydrogen phosphate
14. Hydrochloric acid
15. Deionized water

3.4 Preparation of MRS Agar and MRS Broth

Man's Rogosa and Sharpe (MRS) agar and broth used for *Lactobacillus plantarum* bacteria culture process were prepared according to the method recommended by Harrigan (1998). The ingredients, 10.0 grams of peptone, 10.0 grams of meat extract, 5.0 grams of yeast extract, 20.0 grams of glucose, 2.0 grams of dikalium hydrogen phosphate, 2.0 grams of tri-ammonium citrate, 0.2 gram of tetrahydrated magnesium sulphate and 0.05 grams of tetrahydrated manganese sulphate were dissolved in 1000 ml of deionized water. The solution was heated and stirred so that the solution homogenous. To maintain the 6.5 pH of the MRS agar and broth, the pH is adjusted by using 0.1 M sodium hydroxide and 0.1 M of hydrochloric acid. When the solution is homogenous, the conical flask was closed by using cotton and followed aluminium foil. Then, the medium was sterilized by using autoclave at 121°C for 15 minutes to avoid contamination. Figure 3.2 (a) and (b) shows the MRS agar and broth after sterilization.

Figure 3.2 (a): MRS agar for culture process of *Lactobacillus plantarum*

Figure 3.2 (b): MRS broth for culture process of *Lactobacillus plantarum*

3.5 Culture Process of *Lactobacillus plantarum* Bacteria

After sterilization, the MRS agar was poured into a sterile petri dish and solidified. The next step is the inoculation of *Lactobacillus plantarum* on the solidified agar in agar plate by using streaking technique. After inoculation, the inoculated agar was incubated at 35°C for 3 days. After the three days incubation, the sample was inoculated into 100 ml broth and incubated again in incubator shaker at 35°C, 300 rpm for 3 days (Harrian, 1976).

Figure 3.3 (a): MRS broth before incubation

Figure 3.3 (b): MRS broth after incubation

3.6 Preparation of Buffer Solution

There are two types of buffer solution used to control the pH and ionic strength. They are the 0.1 M acetate and phosphate buffer solution. For the buffer solution preparation, each of 120 grams of sodium hydroxide, 408.27 grams of potassium dihydrogen phosphate and 612.66 grams of potassium hydrogen phatalate were diluted in 1000 ml deionized water to get 2 liters of 1.5 M solution. The next step is the pH adjusting by adding the 1.5 M of potassium hydrogen phatalate and 1.5 M of sodium hydroxide to prepare pH 4.5 and pH 5.5 acetate buffer solutions by using the pH meter. Later, 130 ml of the each acetate buffer solution was taken and diluted again with 2 liters of deionized water to get 0.1 M of the acetate buffer solution. For the phosphate buffer solution preparation, the same method was used. In order to get the pH 6.5, 7.5 and 8.5 of phosphate buffer solution, the potassium dihydrogen phosphate solution and sodium hydroxide solution were mixed and the pH adjusted. Next, 130 ml of the adjusted pH were taken and diluted again with 2 liters of deionized water to get 0.1 M of phosphate buffer solution (Renzo, 2008).

For the 0.2 M, 0.4 M, 0.6 M, 0.8 M and 1 M of sodium chloride solution, each of 23.38, 37.40, 70.13, 93.50 and 116.88 grams was diluted in 2 liters of pH 6.5 of phosphate buffer solution. Lastly, all the buffer solutions were autoclaved to avoid precipitation (Renzo, 2008).

Figure 3.4 (a): pH meter used for preparation of buffer solution

Figure 3.4 (b): Buffer solution

3.7 Cell Harvesting by Centrifugation

After the three days incubation in the incubator shaker, 40 ml of 100 ml was taken out and centrifuged at 4°C, 6000 rpm for 10 minutes. The supernatant was decanted and the pellet was washed by using deionized water for two times and recentrifuged at 4°C, 6000 rpm for 10 minutes. Again, the pellet was taken and diluted with the buffer solution which was prepared (Kachouri *et al.*, 2005).

3.8 Cleaning of the Membrane System

Before the separation process takes place, the membrane system was washed by using 0.1 M of sodium hydroxide first, followed by using the deionized water for 30 minutes, at the transmembrane pressure of 0.4 bar and 300 rpm to remove any particles left inside the membrane system and to avoid fouling of the membrane. This washing step was repeated after every separation process (Otoyo *et al.*, 1999).

3.9 Experimental Procedure for Effect of pH

For the experimental procedure for effect of pH, the 2 liters of buffer solution that contain *Lactobacilllus plantarum* bacteria cell was filled in the 2 liters tank and close tightly. The transmembrane pressure was set up at 4.0 bar and 300 rpm. The pump was switched on. Next, the experiment was run. Starting from the pump switched on, the time was set up. The volume of the sample form permeate was taken for every five minutes and measured by using the measuring cylinder. The experiment was repeated for every pH. They are 4.5, 5.5, 6.5, 7.5 and 8.5 (Jones *et al.*, 2000).

Figure 3.5: Mixture of *Lactobacillus plantarum* bacteria cell and buffer solution

Figure 3.6: Membrane system for separation process

Figure 3.7: The solution of before and after separation process

3.10 Experimental Procedure for Effect of Ionic Strength

For the experimental procedure for effect of ionic strength, the 2 liters of buffer solution that contain sodium chloride and *Lactobacilllus plantarum* bacteria cell was filled in the 2 liters tank. Again, the tank was closed tightly and the pump was switched on. Next, the experiment was run. Starting from the pump switched on, the time was set up. The volume of the sample form permeate was taken for every five minutes and measured by using the measuring cylinder. The experiment was repeated for 0.2 M, 0.4 M, 0.6 M, 0.8 M and 1.0 M of sodium chloride concentration (Jones *et al*., 2000).

3.11 Analyzing Sample

Sample analyzing was started by preparation of carbon (TC) and inorganic carbon (IC) solution. Total carbon was prepared by adding 2.125 grams of hydrogen phatalate into 1 liter of deionized water and diluted in volumetric flask. Inorganic carbon solution was prepared by adding 3.50 grams of sodium hydrogen carbonate and 4.41 grams of sodium carbonate into 1 liter of deionized water and diluted. Both solutions are used as the calibration solution for the total organic carbon analyzer. As all switches of total organic carbon analyzer were switched on, the calibration solution was poured into a sample bottle and the total organic carbon analyzer was run to get the reading, followed by the reading of sample (Schumacher, 2002).

Figure 3.8: Total organic carbon analyzer

3.12 Determination of Permeate Flux

The analysis of the experiment is about the determination of the permeate flux of the separation of *Lactobacillus plantarum* bacteria cell. Shahbazi (2005) states that the permeate flux, J can be determined by using equation 3.1.

$$\text{Permeate Flux, } J = V / (A \times t) \qquad \textbf{(Equation 3.1)}$$

Where:

V = Permeate Volume, cm^3

A = Membrane Area, cm^2

t = Time, min

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Effect of pH and Ionic Strength

In order to determine the effect of pH and ionic strength on separation of *Lactobacillus plantarum* bacteria from buffer solution, the experiment was carried out for different pH and ionic strength. The pH which is used is at 4.5, 5.5, 6.5, 7.5 and 8.5. For the study of effects of ionic strength, the experiment was conducted for 0.2, 0.4, 0.6, 0.8 and 1.0 M of salt concentration.

For the determination of the permeate flux, the volume of sample at the permeate was collected for every five minutes and the permeate flux was calculated. The calculation of the permeate flux is based on volume of sample (cm^3), time (minute) and the membrane area (110 cm^2).

4.2 Effect of pH 4.5

Table 4.1: Change of permeate flux with time (pH 4.5 buffer solution)

Time, t (min)	Volume, v (cm^3)	Flux, J (cm/min)
0	0.00	0.0000
5	26.9	0.0489
10	26.6	0.0485
15	26.5	0.0484
20	26.4	0.0482
25	26.4	0.0480
30	26.3	0.0480
35	26.3	0.0478
40	26.2	0.0478
45	26.1	0.0476
50	26.1	0.0475
55	26.1	0.0474
60	26.0	0.0475
65	26.0	0.0473
70	26.0	0.0473
75	26.0	0.0473
80	26.0	0.0473
85	26.0	0.0473
90	26.0	0.0473
95	26.0	0.0473
100	26.0	0.0473
105	26.0	0.0473
110	26.0	0.0473
115	26.0	0.0473
120	26.0	0.0473

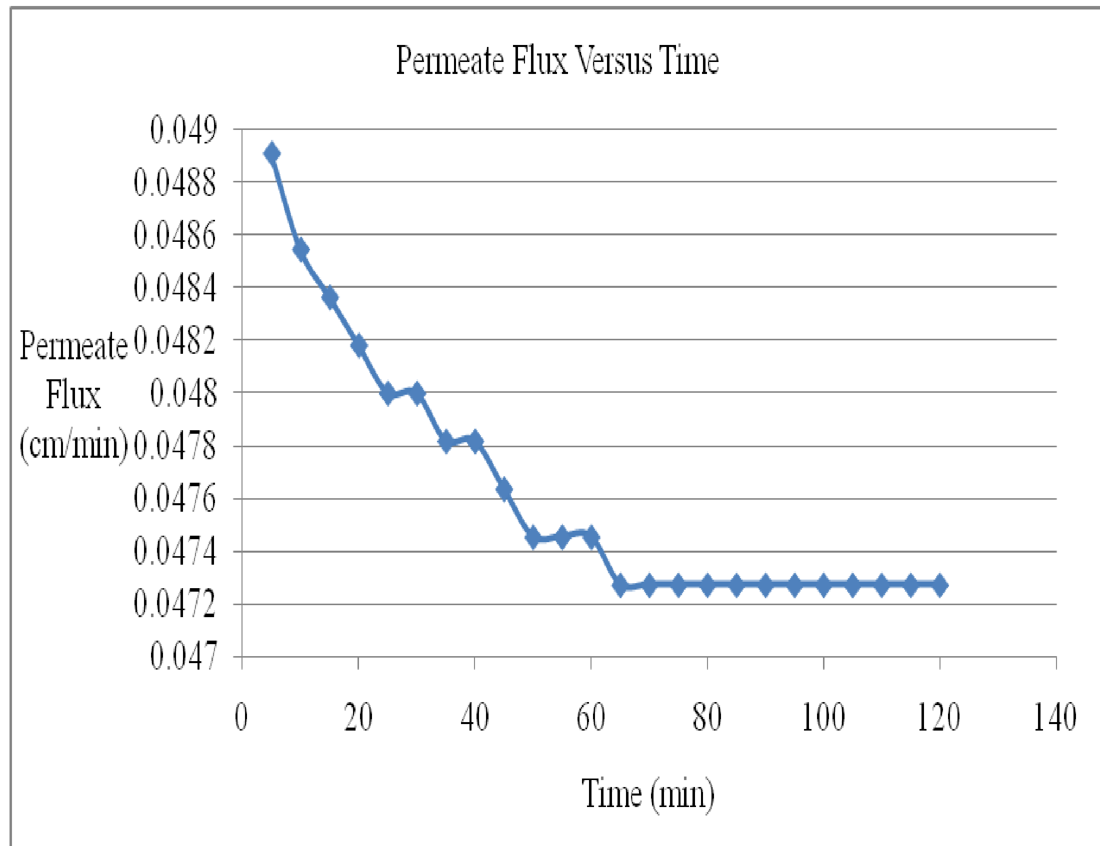

Figure 4.1: Graph of permeate flux versus time for pH 4.5

Figure 4.1 shows the results of the permeate flux in centimeter per minute versus time in minute for acidic, pH 4.5 solution. From the figure, it can be seen that the permeate flux starts to decrease from the 5th minute until the 60th minute and start to be constant from 65th minute to 120th of the experiment . The graph also shows that the greatest decreasing is between the 5th minute to 10th minute of the experiment which is about 0.74 % and the permeate flux is constant at 0.047273 cm.min^{-1}.

4.3 Effect of pH 5.5

Table 4.2: Change of permeate flux with time (pH 5.5 buffer solution)

Time, t (min)	Volume, v (cm^3)	Flux, J (cm/min)
0	0.00	0.0000
5	28.0	0.0505
10	27.8	0.0504
15	27.7	0.0500
20	27.5	0.0498
25	27.4	0.0492
30	27.1	0.0491
35	27.0	0.0491
40	27.0	0.0491
45	27.0	0.0491
50	27.0	0.0491
55	27.0	0.0491
60	27.0	0.0491
65	27.0	0.0491
70	27.0	0.0491
75	27.0	0.0491
80	27.0	0.0491
85	27.0	0.0491
90	27.0	0.0491
95	27.0	0.0491
100	27.0	0.0491
105	27.0	0.0491
110	27.0	0.0491
115	27.0	0.0491
120	27.0	0.0491

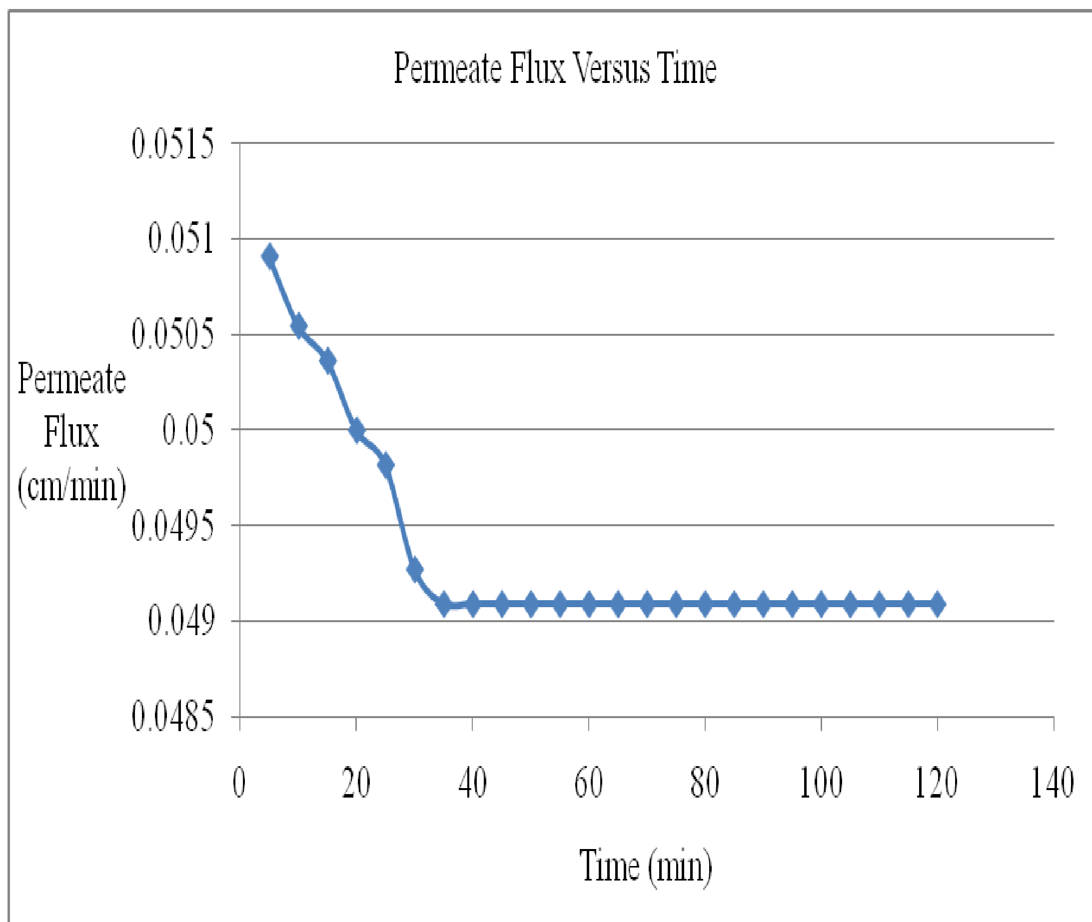

Figure 4.2: Graph of permeate flux versus time for pH 5.5

The result for effect of pH 5.5 solution is shown by the figure 4.2. From the graph, permeate flux decreases from the start of the experiment until the 25th minute of the experiment and start to be constant from the 30th to 120th minute of the experiment. The greatest decrease is between the 5th to 10th minute, which is about 3.92%. The permeate flux is constant at 0.049091 cm.min^{-1}.

4.4 Effect of pH 6.5

Table 4.3: Change of permeate flux with time (pH 6.5 buffer solution)

Time, t (min)	Volume, v (cm3)	Flux, J (cm/min)
0	0.00	0.0000
5	45.8	0.0833
10	45.8	0.0833
15	45.5	0.0827
20	44.5	0.0818
25	44.0	0.0809
30	44.0	0.0800
35	43.9	0.0800
40	43.8	0.0798
45	43.6	0.0796
50	43.5	0.0793
55	43.0	0.0791
60	43.5	0.0782
65	43.0	0.0782
70	43.0	0.0773
75	42.8	0.0773
80	42.8	0.0773
85	42.5	0.0773
90	42.5	0.0773
95	42.5	0.0773
100	42.5	0.0773
105	42.5	0.0773
110	42.5	0.0773
115	42.5	0.0773
120	42.5	0.0773

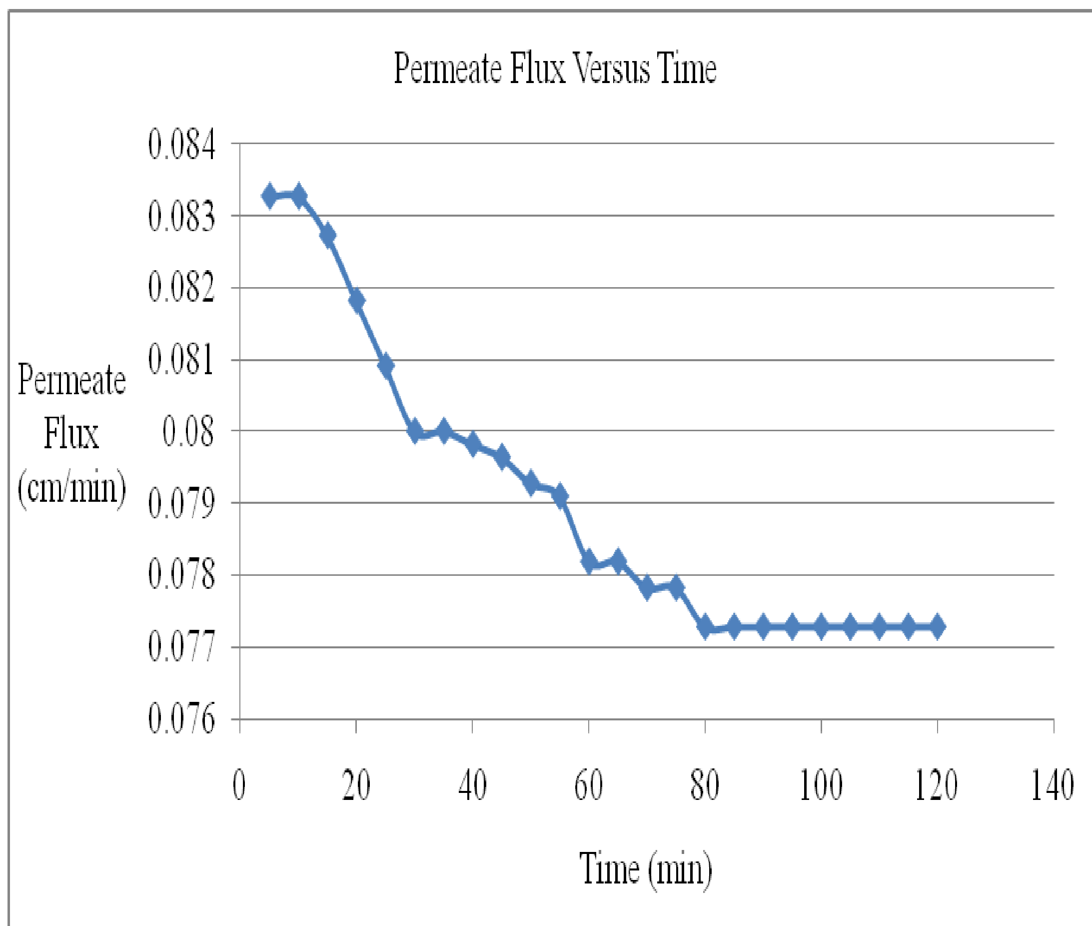

Figure 4.3: Graph of permeate flux versus time for pH 6.5

Figure 4.3 shows the permeate flux versus time for effect of pH 6.5 solution which is the pH of fermentation broth for production of sorbitol. The result for effect of pH 6.5 solution is shown by the figure 4.3. The permeate flux is constant from the 5th to 10th minute of the experiment and start to decrease from the 15th to 65th and constant again at the 70th to 120th minute of the experiment. The greatest decrease is between the 20th to 25th minute, which is about 1.12%. The permeate flux is constant at 0.077273 $cm.min^{-1.}$

4.5 Effect of pH 7.5

Table 4.4: Change of permeate flux with time (pH 7.5 buffer solution)

Time, t (min)	Volume, v (cm3)	Flux, J (cm/min)
0	0.00	0.0000
5	46.5	0.0845
10	46.0	0.0836
15	46.0	0.0836
20	45.2	0.0822
25	45.0	0.0818
30	45.0	0.0818
35	44.9	0.0816
40	44.8	0.0815
45	44.5	0.0809
50	44.5	0.0809
55	43.8	0.0796
60	43.8	0.0796
65	43.3	0.0787
70	43.1	0.0784
75	43.0	0.0782
80	43.0	0.0782
85	43.0	0.0782
90	43.0	0.0782
95	43.0	0.0782
100	43.0	0.0782
105	43.0	0.0782
110	43.0	0.0782
115	43.0	0.0782
120	43.0	0.0782

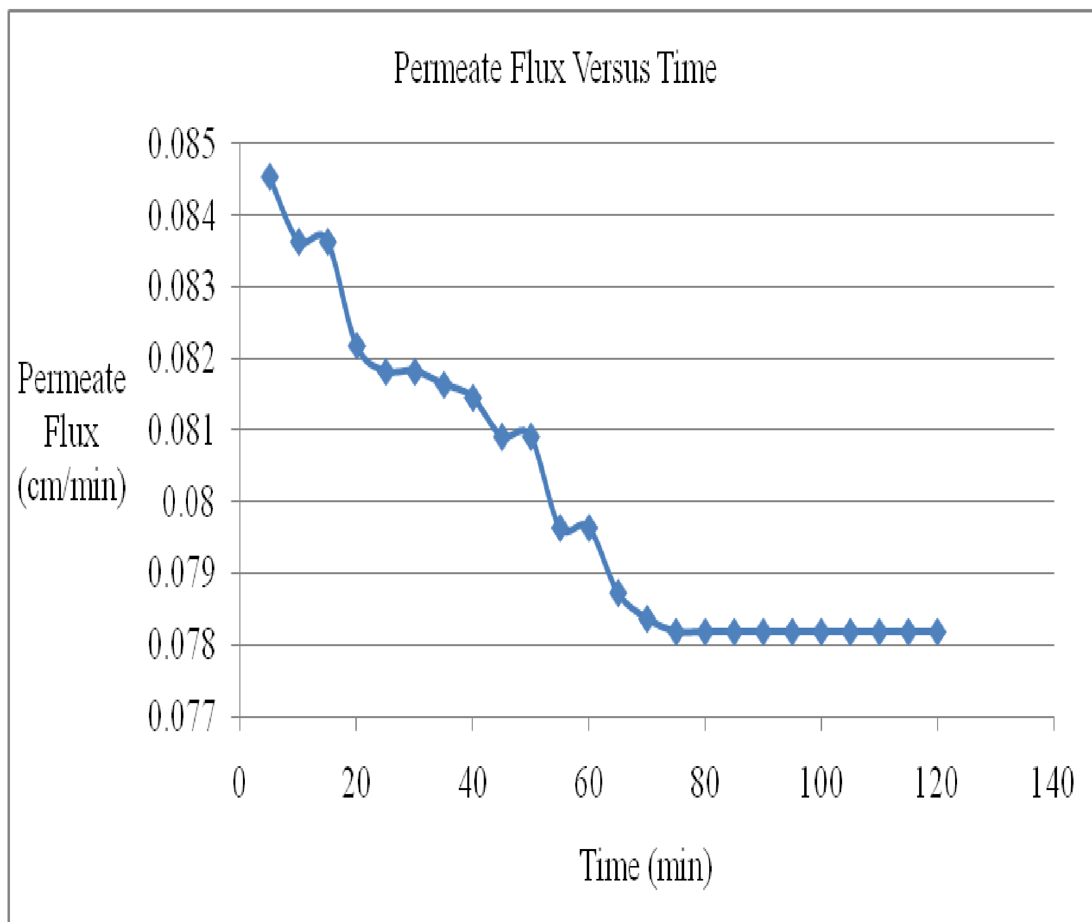

Figure 4.4: Graph of permeate flux versus time for pH 7.5

The result for effect of pH 7.5 is shown by the figure 4.4. From the graph, it shows that the permeate flux decreases from the 5th minute until the 70th minute of the experiment and start to be constant from the 75th to 120th minute of the experiment. The graph also shows that the greatest decrease of permeate flux is between the 5th to 10th minute, which is about 1.74%. The permeate flux is constant at 0.078182 cm.min^{-1}.

4.6 Effect of pH 8.5

Table 4.5: Change of permeate flux with time (pH 8.5 buffer solution)

Time, t (min)	Volume, v (cm^3)	Flux, J (cm/min)
0	0.00	0.0000
5	79.0	0.1436
10	79.0	0.1436
15	78.8	0.1433
20	78.0	0.1418
25	77.2	0.1407
30	77.0	0.1400
35	77.0	0.1400
40	76.9	0.1398
45	76.8	0.1396
50	76.4	0.1389
55	76.4	0.1389
60	76.0	0.1382
65	75.9	0.1380
70	75.7	0.1376
75	75.4	0.1371
80	75.3	0.1369
85	75.0	0.1364
90	75.0	0.1364
95	75.0	0.1364
100	75.0	0.1364
105	75.0	0.1364
110	75.0	0.1364
115	75.0	0.1364
120	75.0	0.1364

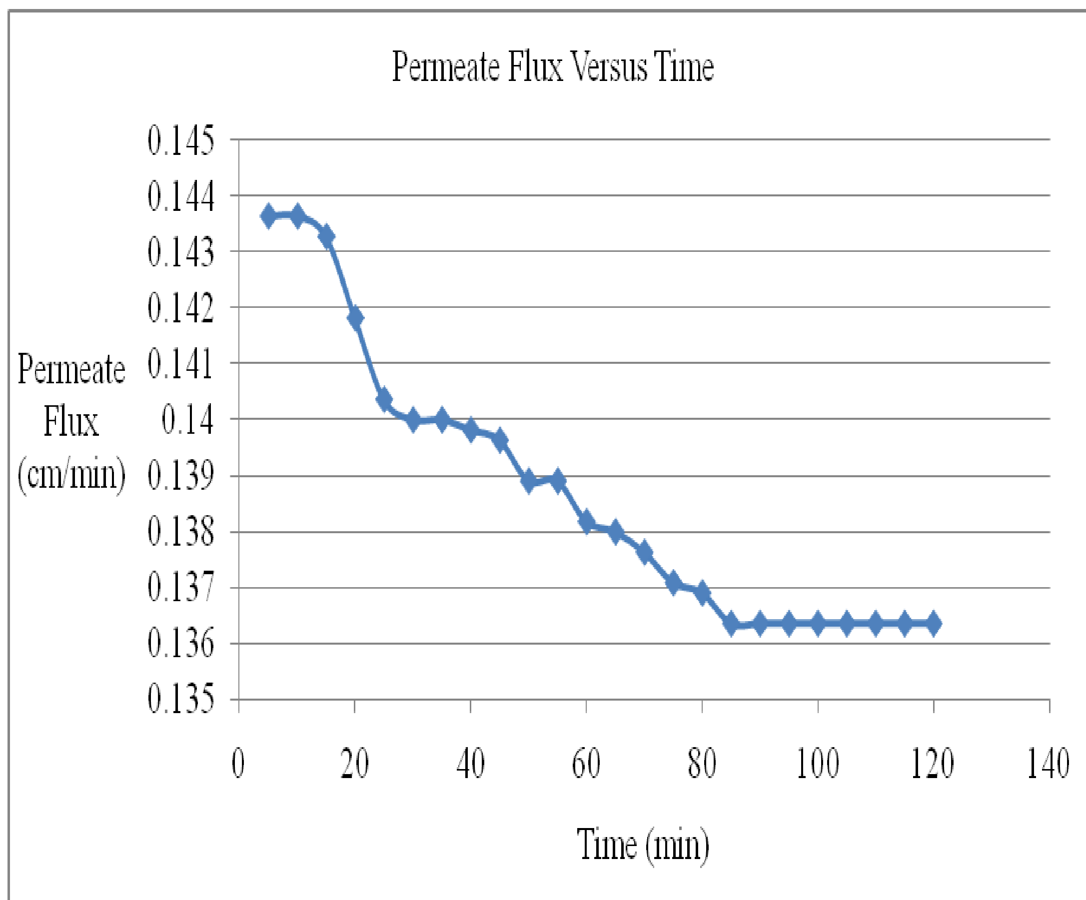

Figure 4.5: Graph of permeate flux versus time for pH 8.5

Figure 4.5 shows the result for the study of pH 8.5 solution effect on *Lactobacillus plantarum* separation process. It is also can be seen that at the 5th to 45th minute of the experiment, the permeate flux decreases and constant from the 85th to 120th minute. The graph also shows that the greatest decrease of permeate flux is at the 15th and 20th minute, which is about 1.02% and the permeate flux is constant at 0.136364 $cm.min^{-1}$.

4.7 Effect of pH

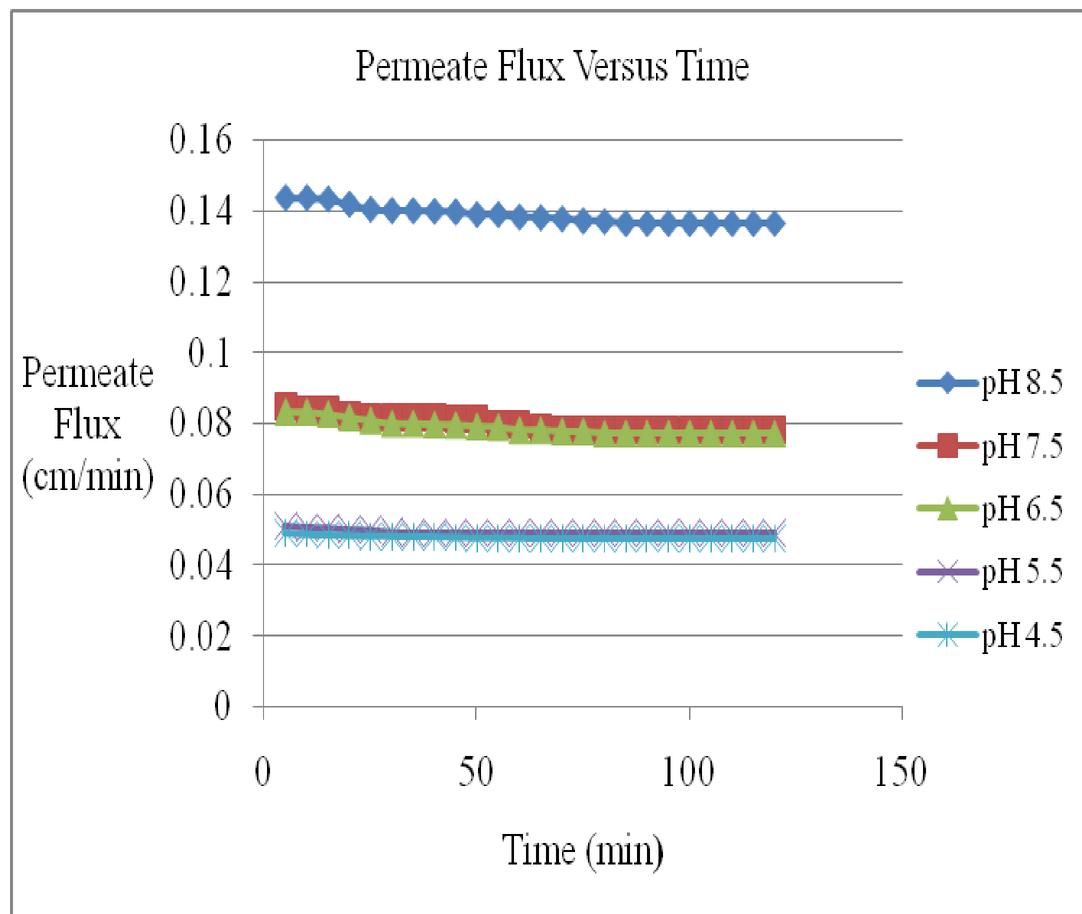

Figure 4.6: Graph of permeate flux versus time at different pH

The figure 4.6 shows the graph of permeate flux versus time at different pH. From the graph, at pH 8.5, the permeate flux of separation of *Lactobacillus plantarum* is the highest and at pH 4.5, the permeate flux is the lowest. This is due to the electrostatic repulsion between the *Lactobacillus plantarum* bacteria and the surface of the membrane. The two factors, isoelectric point and the morphology of the membrane cause the permeate flux is highest at pH 8.5.

The first factor which is the isoelectric point between of the *Lactobacillus plantarum* bacteria is 3.75 and negatively charge in alkaline solution. Second, due to the morphology of polymer membrane, the polymer chain of membrane which is negatively charge, start to repel and cause the membrane pores size bigger when there is increasing in pH solution. Hence, because of the same charge between the bacteria and the surface of the membrane, the repulsion occur between the bacteria

and the surface of the membrane that cause the bacteria cannot pass through the pores of the membrane and retained. The repulsion between the bacteria and the surface of the membrane is called electrostatic repulsion. In addition to that, because of the pores size of the membrane are bigger in alkaline solution, the buffer solution can pass through the membrane causes the permeate flux is highest at pH 8.5.

At pH 4.5, the permeate flux is lowest. This is because of the pH of the buffer solution is close to the isoelectric point of the *Lactobacillus plantarum* bacteria, cause the bacteria is less negatively charged, causes more bacteria deposits close to the surface of the membrane and causes the lowest in permeate flux compared to the permeate flux at pH 5.5, 6.5 and 7.5. Because of the higher in the pH, the bacteria surface is more negatively charge, the electrostatic repulsion increases cause higher in permeate flux.

At pH 6.5, which is the pH of the fermentation media based on the production of sorbitol that the *Lactobacillus plantarum* bacteria should be hundred percent retained on membrane, the graph shows that the permeate flux decline is small and membrane do not foul easily. So, at that pH, the hollow fiber membrane is able to separate the *Lactobacillus plantarum* bacteria .

4.8 Effect of 0.2 M of Ionic Strength

Table 4.6: Change of permeate flux with time (0.2 M of ionic strength)

Time, t (min)	Volume, v (cm^3)	Flux, J (cm/min)
0	0.00	0.0000
5	22.8	0.0413
10	22.7	0.0413
15	22.7	0.0407
20	22.4	0.0405
15	22.3	0.0405
30	22.3	0.0405
35	22.3	0.0404
40	22.2	0.0404
45	22.2	0.0400
50	22.0	0.0400
55	22.0	0.0398
60	21.9	0.0398
65	21.9	0.0396
70	21.8	0.0396
75	21.8	0.0396
80	21.8	0.0396
85	21.8	0.0396
90	21.8	0.0396
95	21.8	0.0396
100	21.8	0.0396
105	21.8	0.0396
110	21.8	0.0396

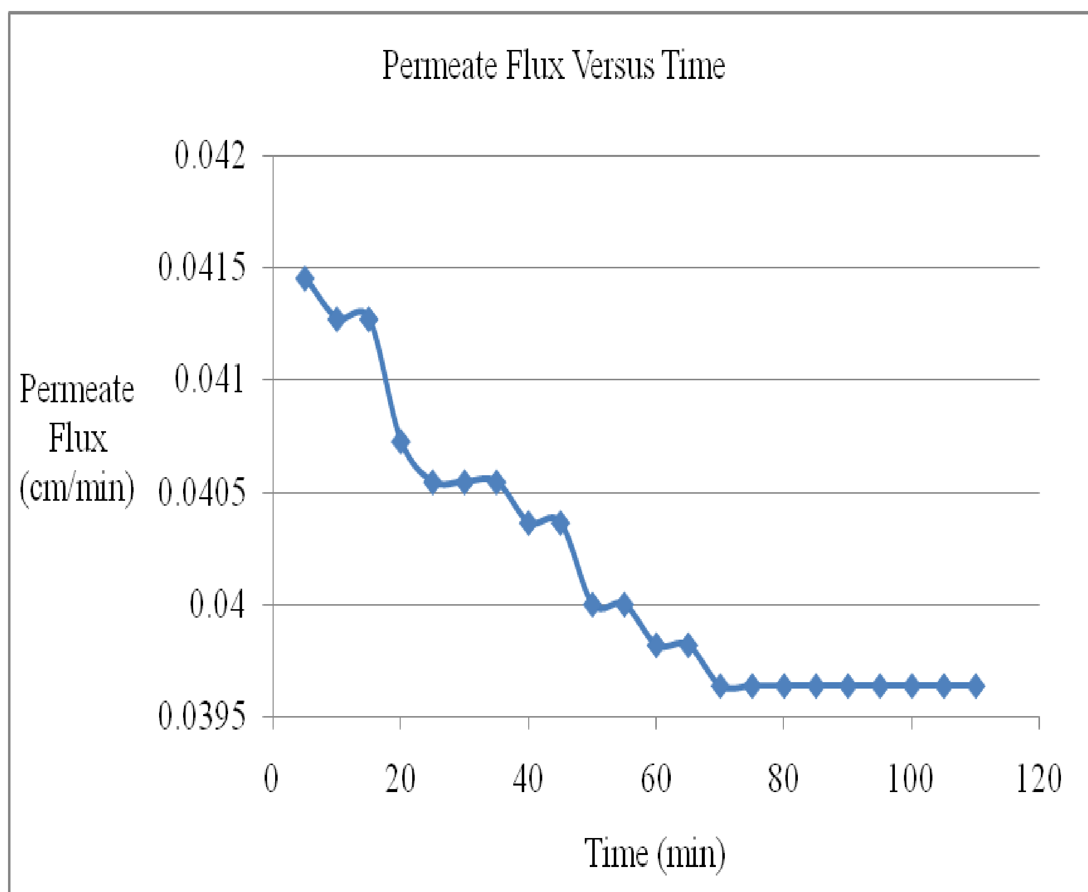

Figure 4.7: Graph of permeate flux versus time for 0.2 M of ionic strength

Figure 4.7 shows the graph of permeate flux versus time for 0.2 M of ionic strength. It shows that from the 5th to 60th minute of the experiment, the permeate flux decrease and from the 65th minute, the permeate flux start to constant because of the constant volume of sample at the permeate collected during the experiment. The greatest decrease in permeate flux is at 15th to 20th minute and 20th to 25th minute of the experiment which is about 1.32%. The result also shows that the permeate flux is constant at 0.039636 cm min^{-1}.

4.9 Effect of 0.4 M of Ionic Strength

Table 4.7: Change of permeate flux with time (0.4 M of ionic strength)

Time, t (min)	Volume, v (cm^3)	Flux, J (cm/min)
0	0.00	0.0000
5	22.5	0.0409
10	22.5	0.0409
15	22.4	0.0407
20	22.3	0.0405
25	22.2	0.0404
30	22.2	0.0400
35	22.0	0.0400
40	22.0	0.0400
45	22.0	0.0396
50	21.8	0.0396
55	21.8	0.0395
60	21.7	0.0395
65	21.7	0.0393
70	21.6	0.0391
75	21.5	0.0391
80	21.5	0.0391
85	21.5	0.0391
90	21.5	0.0391
95	21.5	0.0391
100	21.5	0.0391
105	21.5	0.0391
110	21.5	0.0391

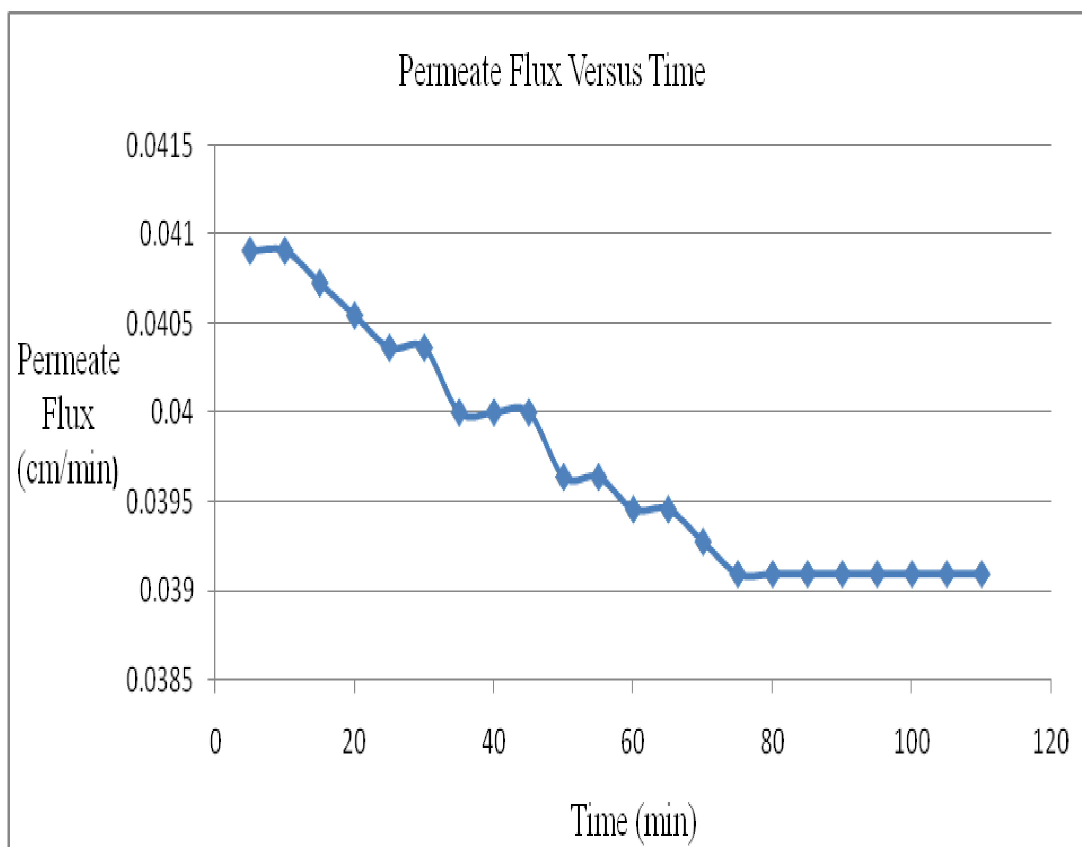

Figure 4.8: Graph of permeate flux versus time for 0.4 M of ionic strength

The result for effect of 0.4 M of ionic strength is shown in figure 4.8. The figure shows that the permeate flux of the separation of *Lactobacillus plantarum* decreases from the 5th to 65th minute of the experiment and it starts to constant from the 70th to 110th minute of the experiment. From the graph, the greatest decrease is found between the 20th and 25th minute which is about 0.45%. The result also shows that the permeate flux is constant at 0.039091 cm/min^{-1}.

4.10 Effect of 0.6 M of Ionic Strength

Table 4.8: Change of permeate flux with time (0.6 M of ionic strength)

Time, t (min)	Volume, v (cm^3)	Flux, J (cm/min)
0	0.00	0.0000
5	22.5	0.0409
10	22.5	0.0409
15	22.4	0.0407
20	22.3	0.0405
25	22.2	0.0404
30	22.2	0.0404
35	22.0	0.0400
40	22.0	0.0400
45	22.0	0.0400
50	21.8	0.0396
55	21.8	0.0396
60	21.7	0.0395
65	21.7	0.0395
70	21.6	0.0393
75	21.5	0.0391
80	21.5	0.0391
85	21.5	0.0391
90	21.5	0.0391
95	21.5	0.0391
100	21.5	0.0391
105	21.5	0.0391
110	21.5	0.0391

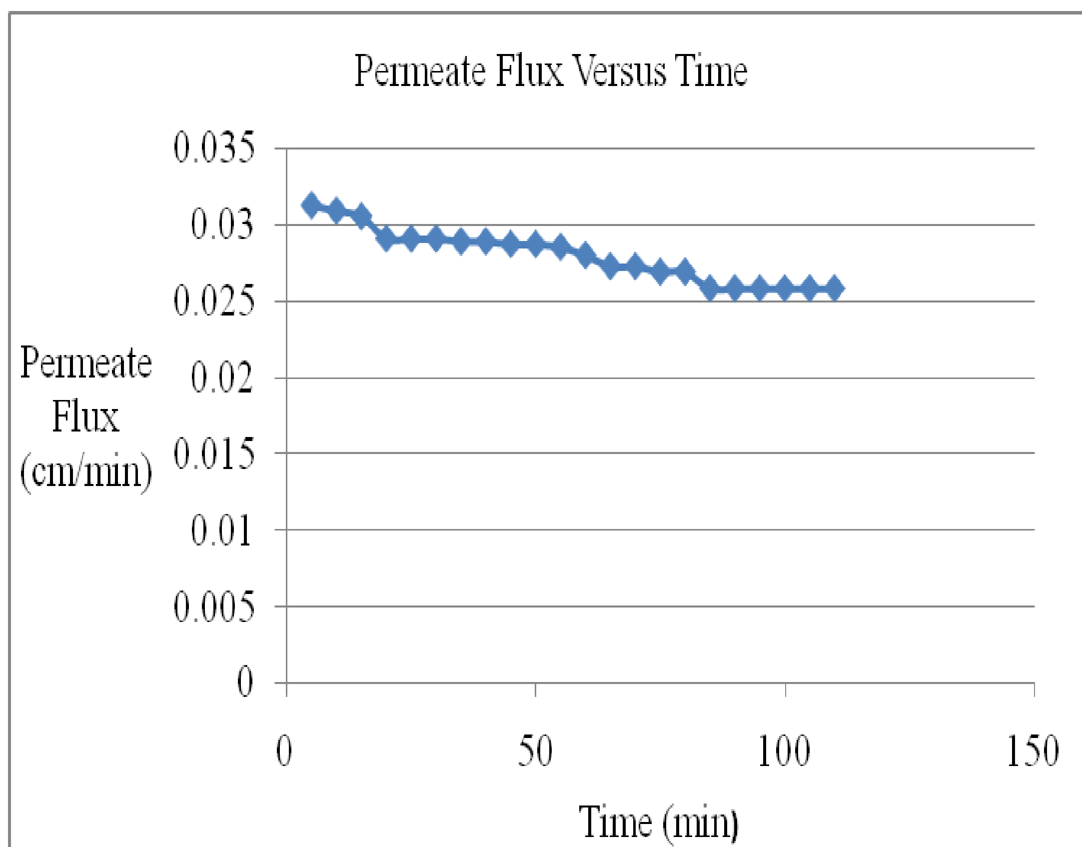

Figure 4.9: Graph of permeate flux versus time for 0.6 M of ionic strength

Figure 4.9 shows the result shows for the effect of 0.6 M of ionic strength on permeate flux of the *Lactobacillus plantarum* bacteria. From the graph, it shows that the permeate flux decreases from the 15th to 70th minute of the experiment and constant from the 75th to 110th minute of the experiment. The greatest decrease is found between the 15th and 20th minute of the experiment. The graph also shows that the permeate flux of the separation of *Lactobacillus plantarum* bacteria based on the effect of 0.6 M of ionic strength is constant at 0.039091cm.min^{-1}.

4.11 Effect of 0.8 M of Ionic Strength

Table 4.9: Change of permeate flux with time (0.8 M of ionic strength)

Time, t (min)	Volume, v (cm^3)	Flux, J (cm/min)
0	0.00	0.0000
5	14.5	0.0264
10	14.4	0.0262
15	14.4	0.0262
20	14.3	0.0260
25	14.3	0.0260
30	14.2	0.0258
35	14.1	0.0256
40	13.8	0.0252
45	13.8	0.0252
50	13.5	0.0245
55	13.5	0.0245
60	13.5	0.0245
65	13.5	0.0245
70	13.5	0.0245
75	13.5	0.0245
80	13.5	0.0245
85	13.5	0.0245
90	13.5	0.0245
95	13.5	0.0245
100	13.5	0.0245
105	13.5	0.0245
110	13.5	0.0245

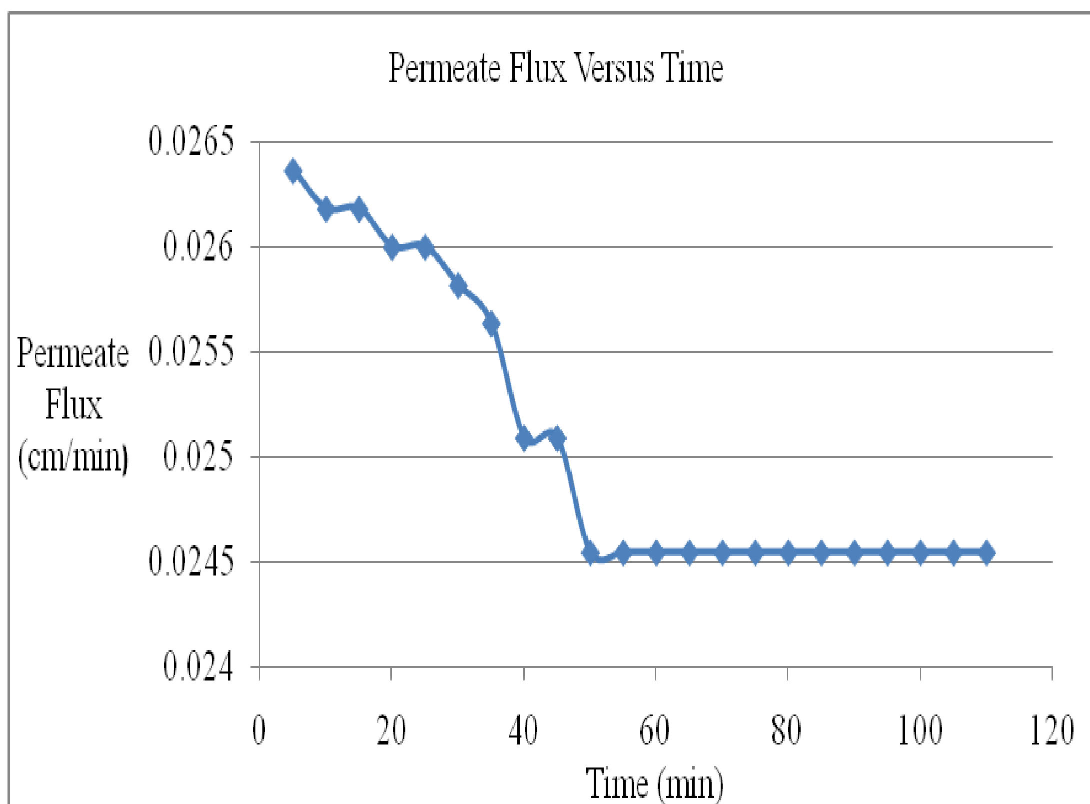

Figure 4.10: Graph of permeate flux versus time for 0.8 M of ionic strength

Figure 4.10 shows the result of permeate flux for the effect of 0.8 M of ionic strength on the separation of *Lactobacillus plantarum* bacteria. From the results, it shows that the permeate flux of the experiment decreases from the 5th to 45th minute of the experiment. The graph also shows that the permeate flux is constant from the 50th to 110th minute of the experiment and the greatest decrease is found between the 35th to 40th minute which is about 2.13%. From the graph, it is also found that the permeate flux of the experiment is constant at 0.024545 cm.min^{-1}.

4.12 Effect of 1.0 M of Ionic Strength

Table 4.10: Change of permeate flux with time (1.0 M of ionic strength)

Time, t (min)	Volume, v (cm^3)	Flux, J (cm/min)
0	0.00	0.0000
5	13.4	0.0243
10	13.3	0.0242
15	13.0	0.0236
20	13.0	0.0236
25	13.0	0.0236
30	13.0	0.0236
35	13.0	0.0236
40	13.0	0.0236
45	13.0	0.0236
50	13.0	0.0236
55	13.0	0.0236
60	13.0	0.0236
65	13.0	0.0236
70	13.0	0.0236
75	13.0	0.0236
80	13.0	0.0236
85	13.0	0.0236
90	13.0	0.0236
95	13.0	0.0236
100	13.0	0.0236
105	13.0	0.0236
110	13.0	0.0236

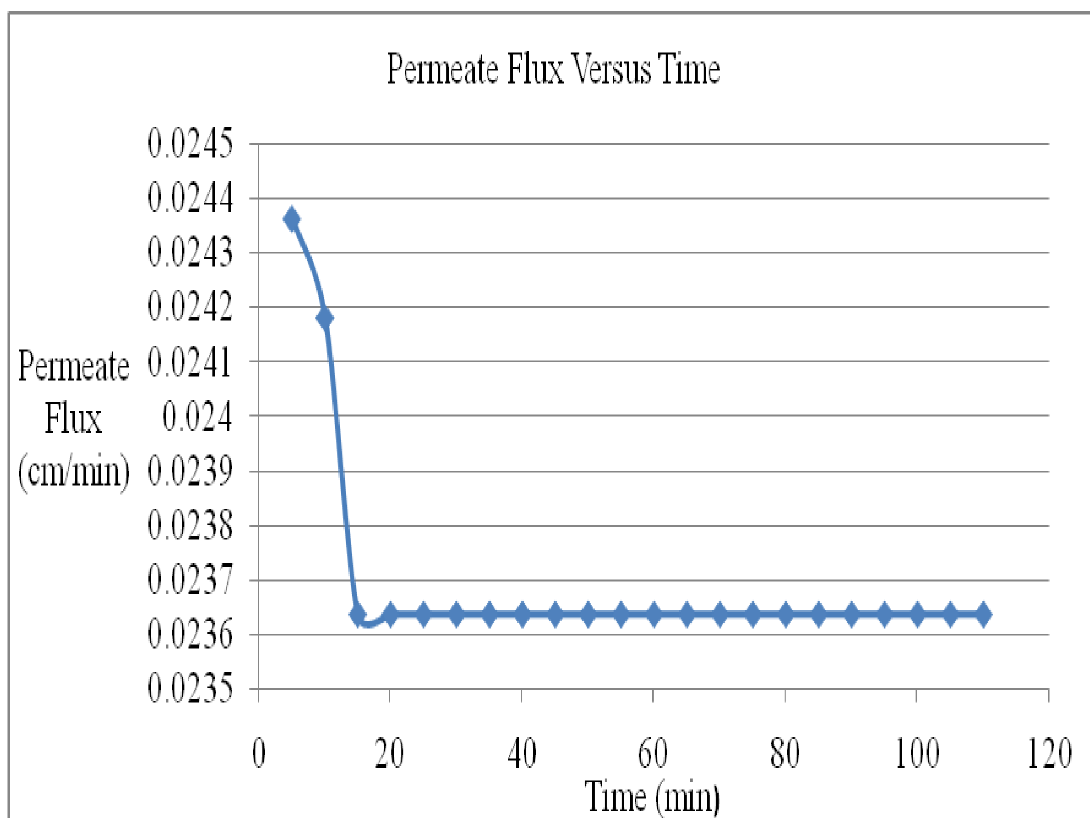

Figure 4.11: Graph of permeate flux versus time for 1.0 M of ionic strength

Figure 4.11 shows the graph pf permeate flux versus time for 1.0 M of ionic strength. From the graph, it can be clearly seen that the permeate flux of the separation of *Lactobacillus plantarum* bacteria decreases from the 5th to 10th minute of the experiment and starts to constant from the 15th minute until the 110th minute of the experiment. The graph also shows that the greatest decrease of permeate flux is between the 10th and 15th minute of the experiment which is about 2.26% and the permeate flux is constant at 0.023636 cm.min^{-1}.

4.13 Effect of Ionic Strength at pH 6.5

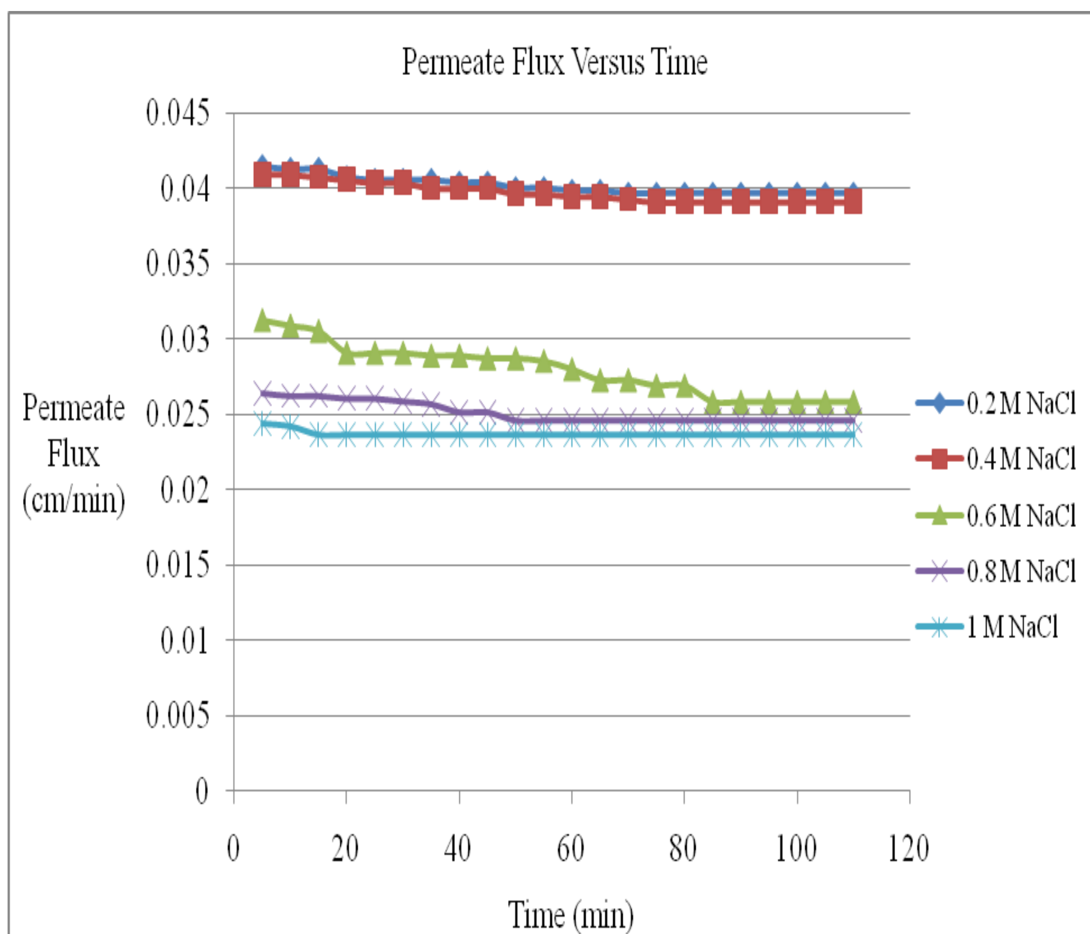

Figure 4.12: Graph of permeate flux versus time at different ionic strength

Figure 4.11 shows the relationship between the permeate flux and time for the separation of *Lactobacillus plantarum* bacteria. At 0.2 M of ionic strength, the permeate flux is the highest and at 1.0 M of ionic strength, the permeate flux is the lowest. This is due to effect of the membrane pores size which is bigger when the sodium chloride concentration is decrease. Besides that, it is because of the *Lactobacillus plantarum* bacteria is negatively charged at pH 6.5 which is higher than its isoelectric point since its isoelectric point is at pH 3.75, causes the electrostatic repulsion between the bacteria cell and the surface of membrane.

At 1.0 M of ionic strength, the permeate flux is lowest at high ionic strength compared to 0.8 M, 0.6 M, 0.4 M of ionic strength. This is due to the compaction of membrane and results in reduction of effective permeability because the addition of

sodium chloride concentration reduced membrane permeability due to effects of positively charged (Na^+) shielding on negatively charged membrane, thus changing membrane morphology. So, higher the ionic strength, the permeate flux decreases.

4.14 Concentration of *Lactobacillus plantarum*

Table 4.11: Concentration of *Lactobacillus plantarum* (pH 6.5)

Time, t (min)	Volume, v (cm3)	Flux, J (cm/min)	Concentration (mg/L)
0	0.00	0.0000	791.3
5	45.8	0.0832	0.000
10	45.8	0.0832	0.000
15	45.5	0.0827	0.000
20	44.5	0.0818	0.000
25	44.0	0.0809	0.000
30	44.0	0.0800	0.000
35	43.9	0.0800	0.000
40	43.8	0.0798	0.000
45	43.6	0.0796	0.000
50	43.5	0.0793	0.000
55	43.0	0.0791	0.000
60	43.5	0.0781	0.000
65	43.0	0.0782	0.000
70	43.0	0.0773	0.000
75	42.8	0.0773	0.000
80	42.8	0.0773	0.000
85	42.5	0.0773	0.000
90	42.5	0.0773	0.000
95	42.5	0.0773	0.000
100	42.5	0.0773	0.000
105	42.5	0.0773	0.000
110	42.5	0.0773	0.000
115	42.5	0.0773	0.000
120	42.5	0.0773	0.000

Table 4.13 shows the concentration of *Lactobacillus plantarum* bacteria in the permeate sample when pH 6.5 of buffer solution used and detected by using total organic analyzer (TOC). From the results, it is found that the concentration of Lactobacillus plantarum bacteria cell is about 791.3 mg/L before the separation process but from the 5th minute until the 120th minute, there is no bacteria cell in the permeate sample, which is 0.000 mg/L.

Based on the results, it is proved that the hollow fiber crossflow microfiltration is able to separate and retain 100% of *Lactobacillus plantarum* on the membrane for pH 6.5 of solution which is equal to the pH of fermentation broth for production of sorbitol. This is because of the size of *Lactobacillus plantarum* which is bigger that the pore size of the membrane. Besides that, it is because of the electrostatic repulsion between the negatively charged *Lactobacillus plantarum* bacteria cell because of the pH of solution which is higher than its isoelectric point and negatively charged of membrane surface. Because of the same charge, the bacteria cell cannot pass through the membrane pores and retain on the membrane.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

This study which entitled as "Effect of pH and Ionic Strength on Hollow Fiber Cross flow Microfiltration on Separation of *Lactobacillus plantarum*" has been successfully done. From the result obtained, it can be conclude that the objectives of this study have been achieved. The objectives of this study are to study the effect of pH and ionic strength on permeate flux during separation of *Lactobacillus plantarum*.

Nowadays, microfiltration separation has been used for cell harvesting in downstream process. Current research focuses on the factor that affecting the process of cell separation from fermentation in order to recycle the bacteria (Kaghachi *et al.*, 2000). By doing this research, the economical profits can be increased and decreases wastes because of the price of pure bacteria and the membrane system are quite expensive. So, the study of the effect of pH is one of remedies on how to decreases wastes and increases profits. This study indicates that the hollow fiber cross flow microfiltration can be used for separation of *Lactobacilllus plantarum* bacteria from fermentation broth at the pH 6.5 because at that pH, it causes less flux decline and do not foul membrane easily.

It is also conclude that the pH and plays important roles in affecting the performance of the membrane and separation process. Based on the study, it is found that the fouling and flux can be affected by pH solution and because the

permeate flux is higher when the pH solution is increased. For the effect of ionic strength, the permeate flux is highest when the salt concentration is decreased. Based on the study, the permeate flux is highest when there is no usage of salt in solution. This is because of addition of salt can cause membrane compaction and reduce the effective permeability. In addition to that, when 1.0 M of salt concentration is used, the flux decline increase and the permeate flux decrease rapidly.

5.2 Recommendations

The study of "Effect of pH and Ionic Strength on Hollow Fiber Cross flow Microfiltration on Separation of *Lactobacillus plantarum*" is an important study that should be done to see the effect of some factors that can affect the performance and production to avoid wastes and increases the production and economical profits. This is because there will be the wastes of money and time if the membrane have to be replaced so many times because of the fouling. Besides that, this study can be applied to any fermentation process that involving the usage of bacteria to determine the suitable usage of membrane that can be used to recycle the bacteria for other fermentation because by doing this study, it can also decrease the usage of money and time to replaced the bacteria since it can be recycled.

In order to enhance the study, there are some recommendations that should be considered. In this study, the result of the experiment can be clearly seen if the analysis method is added in order to approve that there is no bacteria in the permeate sample because of its size which is bigger than the pores size of membrane and because of the properties of the *Lactobacillus plantarum* including its isoelectric point.

Besides that, the study also can be enhanced by using the fermentation broth instead of using the buffer solution because may be there are other factors that can affect the membrane performance besides the pH such as the properties of chemical

or sorbitol itself in the fermentation broth of sorbitol production during the separation of the *Lactobacillus plantarum* bacteria cell.

In addition, one more recommendation is about the addition of Scanning Electron Microscopic (SEM) method so that the effect of fouling can be seen clearly on membrane as a proof of calculated permeate flux to show the cause of the decreasing of permeate flux or fouling.

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APPENDIX A

SOLUTION PREPARATION

A.1 MRS Agar and Broth Preparations

Table A.1: MRS broth ingredients

Ingredients	Quantity (g)
Peptone	10.0
Meat extract	10.0
Yeast extract	5.0
D-glucose	20.0
Tween 80	1.0
Potassium Dihydrogen Phosphate	2.0
Sodium Acetate	5.0
Tri-ammonium citrate	2.0
$MgSO_4.7H_2O$	0.2
$MnSO_4.4H_2O$	0.05

The ingredients are dissolved in 1000 ml deionized water. Next, the pH is adjusted by using 0.1M NaOH and 0.1 M HCl in order to maintain pH 6.5. MRS agar is prepared by adding 8.2875 g agar in the ingredients above.

A.2 Buffer Solution Preparation

Based on equation:

$MI.VI = M2.V2$ (**Equation A.1**)

$M1\ (2L) = (0.1)\ (30\ L)$

$M1 = 1.5\ M$

A.2.1 Sodium Hydroxide Solution

MW of NaOH = $40 g.mol^{-1}$

Where $M = n/V$ (**Equation A.2**)

$1.5 = n/2L$

$N = 3\ mol$

Hence $M_{NaOH} = 3\ mol \times 40\ g.mol^{-1}$

$M_{NaOH} = 120\ g$

A.2.2 Potassium Dihydrogen Phosphate

MW of KH_2PO_4 = $136\ g.mol^{-1}$

Where $M = n/V$

$1.5 = n/2L$

$N = 3\ mol$

Hence $M_{KH2PO4} = 3\ mol \times 136.09\ g.mol^{-1}$

$M_{KH2PO4} = 408.27\ g$

A.2.3 Potassium Hydrogen Phatalate

MW of $KHC_8H_4O_4$ = 204.22 g.mol^{-1}

Where $M = n/V$

$1.5 = n/2L$

$N = 3$ mol

Hence $M_{KHC8H4O4}$ = 3 mol x 204.22 g.mol^{-1}

$M_{KHC8H4O4}$ = 612.66 g

A.3 Preparation of Salt Solution

Salt solution is used to study the effect of ionic strength on separation of *Lactobacillus plantarum*

A.3.1 Preparation 0.2 M of NaCl (2 L)

Based on equation, M = n/V and the MW of NaCl is 58.44 g/mol

For 0.2 M of NaCl, n = 0.2 mol/L x 2L = 0.4 mol

Hence, mass of NaCl = 0.4 mol x 58.44 g/mol = 23.37 g

A.3.1 Preparation 0.4 M of NaCl (2 L)

For 0.2 M of NaCl, n= 0.4 mol/L x 2L = 0.8 mol

Hence, mass of NaCl = 0.8 mol x 58.44 g/mol = 37.40 g

A.3.2 Preparation 0.6 M of NaCl (2 L)

For 0.6 M of NaCl, n= 0.6 mol/L x 2L = 1.2 mol

Hence, mass of NaCl = 1.2 mol x 58.44 g/mol = 70.13 g

A.3.3 Preparation 0.8 M of NaCl (2 L)

For 0.8 M of NaCl, n= 0.8 mol/L x 2L = 1.6 mol

Hence, mass of NaCl = 1.6 mol x 58.44 g/mol = 93.50 g

A.3.3 Preparation 1.0 M of NaCl (2 L)

For 1.0 M of NaCl, n= 1.0 mol/L x 2L = 2.0 mol

Hence, mass of NaCl = 2.0 mol x 58.44 g/mol = 116.88 g

APPENDIX B

RESULTS OF PERMEATE FLUX

B.1 Results

Table B.1: pH 4.5 of buffer solution

Time, t (min)	Volume, v (cm^3)	Flux, J (cm/min)
0	0.00	0.000000
5	26.9	0.048909
10	26.6	0.048545
15	26.5	0.048364
20	26.4	0.048182
25	26.4	0.048000
30	26.3	0.048000
35	26.3	0.047818
40	26.2	0.047818
45	26.1	0.047636
50	26.1	0.047455
55	26.1	0.047455
60	26.0	0.047455
65	26.0	0.047273
70	26.0	0.047273
75	26.0	0.047273
80	26.0	0.047273
85	26.0	0.047273
90	26.0	0.047273
95	26.0	0.047273
100	26.0	0.047273
105	26.0	0.047273
110	26.0	0.047273
115	26.0	0.047273
120	26.0	0.047273

Table B.2: pH 5.5 of buffer solution

Time, t (min)	Volume, v (cm^3)	Flux, J (cm/min)
0	0.00	0.000000
5	28.0	0.050545
10	27.8	0.050364
15	27.7	0.050000
20	27.5	0.049818
25	27.4	0.049273
30	27.1	0.049091
35	27.0	0.049091
40	27.0	0.049091
45	27.0	0.049091
50	27.0	0.049091
55	27.0	0.049091
60	27.0	0.049091
65	27.0	0.049091
70	27.0	0.049091
75	27.0	0.049091
80	27.0	0.049091
85	27.0	0.049091
90	27.0	0.049091
95	27.0	0.049091
100	27.0	0.049091
105	27.0	0.049091
110	27.0	0.049091
115	27.0	0.049091
120	27.0	0.049091

Table B.3: pH 6.5 of buffer solution

Time, t (min)	Volume, v (cm3)	Flux, J (cm/min)
0	0.00	0.000000
5	45.8	0.083273
10	45.8	0.083273
15	45.5	0.082727
20	44.5	0.081818
25	44.0	0.080909
30	44.0	0.080000
35	43.9	0.080000
40	43.8	0.079818
45	43.6	0.079636
50	43.5	0.079273
55	43.0	0.079091
60	43.5	0.078182
65	43.0	0.078182
70	43.0	0.077273
75	42.8	0.077273
80	42.8	0.077273
85	42.5	0.077273
90	42.5	0.077273
95	42.5	0.077273
100	42.5	0.077273
105	42.5	0.077273
110	42.5	0.077273
115	42.5	0.077273
120	42.5	0.077273

Table B.4: pH 7.5 of buffer solution

Time, t (min)	Volume, v (cm3)	Flux, J (cm/min)
0	0.00	0.000000
5	46.5	0.084545
10	46.0	0.083636
15	46.0	0.083636
20	45.2	0.082182
25	45.0	0.081818
30	45.0	0.081818
35	44.9	0.081636
40	44.8	0.081455
45	44.5	0.080909
50	44.5	0.080909
55	43.8	0.079636
60	43.8	0.079636
65	43.3	0.078727
70	43.1	0.078364
75	43.0	0.078182
80	43.0	0.078182
85	43.0	0.078182
90	43.0	0.078182
95	43.0	0.078182
100	43.0	0.078182
105	43.0	0.078182
110	43.0	0.078182
115	43.0	0.078182
120	43.0	0.078182

Table B.6: pH 8.5 of buffer solution

Time, t (min)	Volume, v (cm^3)	Flux, J (cm/min)
0	0.00	0.000000
5	79.0	0.143636
10	79.0	0.143636
15	78.8	0.143273
20	78.0	0.141818
25	77.2	0.140364
30	77.0	0.140000
35	77.0	0.140000
40	76.9	0.139818
45	76.8	0.139636
50	76.4	0.138909
55	76.4	0.138909
60	76.0	0.138182
65	75.9	0.138000
70	75.7	0.137636
75	75.4	0.137091
80	75.3	0.136909
85	75.0	0.136364
90	75.0	0.136364
95	75.0	0.136364
100	75.0	0.136364
105	75.0	0.136364
110	75.0	0.136364
115	75.0	0.136364
120	75.0	0.136364

Table B.6: 0.2 M of NaCl and pH 6.5 of buffer solution

Time, t (min)	Volume, v (cm^3)	Flux, J (cm/min)
0	0.00	0.000000
5	22.8	0.041273
10	22.7	0.041273
15	22.7	0.040727
20	22.4	0.040545
15	22.3	0.040545
30	22.3	0.040545
35	22.3	0.040364
40	22.2	0.040364
45	22.2	0.040000
50	22.0	0.040000
55	22.0	0.039818
60	21.9	0.039818
65	21.9	0.039636
70	21.8	0.039636
75	21.8	0.039636
80	21.8	0.039636
85	21.8	0.039636
90	21.8	0.039636
95	21.8	0.039636
100	21.8	0.039636
105	21.8	0.039636
110	21.8	0.039636

Table B.7: 0.4 M of NaCl and pH 6.5 of buffer solution

Time, t (min)	Volume, v (cm^3)	Flux, J (cm/min)
0	0.00	0.000000
5	22.5	0.040909
10	22.5	0.040909
15	22.4	0.040727
20	22.3	0.040545
25	22.2	0.040364
30	22.2	0.040000
35	22.0	0.040000
40	22.0	0.040000
45	22.0	0.039636
50	21.8	0.039636
55	21.8	0.039455
60	21.7	0.039455
65	21.7	0.039273
70	21.6	0.039091
75	21.5	0.039091
80	21.5	0.039091
85	21.5	0.039091
90	21.5	0.039091
95	21.5	0.039091
100	21.5	0.039091
105	21.5	0.039091
110	21.5	0.039091

Table B.8: 0.6 M of NaCl and pH 6.5 of buffer solution

Time, t (min)	Volume, v (cm^3)	Flux, J (cm/min)
0	0.00	0.000000
5	22.5	0.040909
10	22.5	0.040909
15	22.4	0.040727
20	22.3	0.040545
25	22.2	0.040364
30	22.2	0.040364
35	22.0	0.040000
40	22.0	0.040000
45	22.0	0.040000
50	21.8	0.039636
55	21.8	0.039636
60	21.7	0.039455
65	21.7	0.039455
70	21.6	0.039273
75	21.5	0.039091
80	21.5	0.039091
85	21.5	0.039091
90	21.5	0.039091
95	21.5	0.039091
100	21.5	0.039091
105	21.5	0.039091
110	21.5	0.039091

Table B.9: 0.8 M of NaCl and pH 6.5 of buffer solution

Time, t (min)	Volume, v (cm^3)	Flux, J (cm/min)
0	0.00	0.000000
5	14.5	0.026364
10	14.4	0.026182
15	14.4	0.026182
20	14.3	0.026000
25	14.3	0.026000
30	14.2	0.025818
35	14.1	0.025636
40	13.8	0.025091
45	13.8	0.025091
50	13.5	0.024545
55	13.5	0.024545
60	13.5	0.024545
65	13.5	0.024545
70	13.5	0.024545
75	13.5	0.024545
80	13.5	0.024545
85	13.5	0.024545
90	13.5	0.024545
95	13.5	0.024545
100	13.5	0.024545
105	13.5	0.024545
110	13.5	0.024545

Table B.10: 1.0 M of NaCl and pH 6.5 of buffer solution

Time, t (min)	Volume, v (cm^3)	Flux, J (cm/min)
0	0.00	0.000000
5	13.4	0.024364
10	13.3	0.024182
15	13.0	0.023636
20	13.0	0.023636
25	13.0	0.023636
30	13.0	0.023636
35	13.0	0.023636
40	13.0	0.023636
45	13.0	0.023636
50	13.0	0.023636
55	13.0	0.023636
60	13.0	0.023636
65	13.0	0.023636
70	13.0	0.023636
75	13.0	0.023636
80	13.0	0.023636
85	13.0	0.023636
90	13.0	0.023636
95	13.0	0.023636
100	13.0	0.023636
105	13.0	0.023636
110	13.0	0.023636

Table B.11: Concentration of *Lactobacillus plantarum* at permeate

Time, t (min)	Volume, v (cm3)	Flux, J (cm/min)	Concentration (mg/L)
0	0.00	0.000000	791.3
5	45.8	0.083273	0.000
10	45.8	0.083273	0.000
15	45.5	0.082727	0.000
20	44.5	0.081818	0.000
25	44.0	0.080909	0.000
30	44.0	0.080000	0.000
35	43.9	0.080000	0.000
40	43.8	0.079818	0.000
45	43.6	0.079636	0.000
50	43.5	0.079273	0.000
55	43.0	0.079091	0.000
60	43.5	0.078182	0.000
65	43.0	0.078182	0.000
70	43.0	0.077273	0.000
75	42.8	0.077273	0.000
80	42.8	0.077273	0.000
85	42.5	0.077273	0.000
90	42.5	0.077273	0.000
95	42.5	0.077273	0.000
100	42.5	0.077273	0.000
105	42.5	0.077273	0.000
110	42.5	0.077273	0.000
115	42.5	0.077273	0.000
120	42.5	0.077273	0.000