Extraction and Concentration of Glutathione from Yeast

by

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Glutathione is a tripeptide present in most mammalian tissue. It has a great variety of significant physiological functions, such as regulating cell growth and division; helping DNA synthesis and repair. Glutathione can be used in food additive and cosmetic industries at present.

This study aimed to extract glutathione from yeast using hot-water and to concentrate glutathione by membrane separation processes, including ultrafiltration and nanofiltration. Glutathione recovery efficiency by hot-water extraction was 73.8%. Supernatant obtained by hot water extraction was first passed through a ultrafiltration membrane to remove macromolecules, and then glutathione in this permeate was concentrated using a nanofiltration membrane. Glutathione recovery efficiency in nanofiltration step was 99.7%. In addition, the performance of the membranes used in this study was tested, including the permeation flux of supernatant and aqueous glutathione solution by ultrafiltration and nanofiltration and the rejection of glutathione by these two processes.

This study also investigated the effect of hot water extraction time and temperature on the glutathione yield, and exposure to light on the stability of glutathione extracted was also studied.

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First, I would like to express my deepest appreciation and gratitude to my supervisor Dr. Xianshe Feng for providing me with such a great opportunity to study here. His valuable advice and guidance led me to overcome difficulties in my research. His responsible and patient attitudes have a great influence on me. It was never possible to complete this thesis without his support and encouragement.

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I wish to thank the group members in our lab. They never hesitated to provide their supports, suggestions and assistance during my Master study.

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Chapter 1

Introduction

1.1 Background

Glutathione is a tripeptide with abundant thiol compound in living organisms. Glutathione has various clinical applications because of its important physiological functions. For example, it is an important antioxidant for preventing DNA, proteins and other biomolecules from oxidative damage. Nowadays, glutathione has gradually expanded its application into food and cosmetic industries. It can be used as food additives and cosmetic compositions. Glutathione can be produced by enzymatic catalysis, chemical synthesis or microbial fermentation, but these methods have obvious disadvantages, including low yield and high cost.

Membrane separation technology is a viable separation technique. It usually consumes less energy and have better separation efficiency than conventional separation processes. A membrane can be considered as a semi-permeable barrier that rejects certain molecules to pass through the membrane. Membrane separation processes include ultrafiltration, nanofiltration and reverse osmosis.

Ultrafiltration has the characteristics of molecular level separation, no phase change, no solvent pollution, no damage to biological activity and simple operation. It has been used increasingly for the separation and purification of proteins and other components. Ultrafiltration can be used to separate small molecule glutathione from macromolecular proteins and carbohydrates in the yeast extract, reducing the

interference of these substances to subsequent purification.

Nanofiltration is a process between reverse osmosis and ultrafiltration. It is often used for treating waste water. Nanofiltration is becoming widely used in food processing, e.g. dairy production.

1.2 Objectives

The objectives of this study are to extract glutathione from yeast using hot-water extraction, followed by ultrafiltration and nanofiltration for glutathione concentration. Supernatant obtained by hot-water extraction was first subjected to ultrafiltration to remove macromolecules, and then glutathione in this permeate was concentrated using a nanofiltration membrane. This study also investigated the effect of hot water extraction time and temperature on the glutathione yield, and exposure to light on the stability of glutathione extracted was also studied.

1.3 Thesis Outlines

This thesis consists of five chapters as follows:

Chapter 1 introduces the background of this study and describes the objectives of the research.

Chapter 2 presents a literature review of glutathione and membrane separation technology. This chapter briefly introduces different membrane processes to give a fundamental understanding, especially ultrafiltration and nanofiltration. This chapter also reviews the mass transport mechanisms in these membrane processes. The

membrane contamination, resistance model and osmotic pressure model are also briefly introduced.

Chapter 3 presents the experimental procedure of hot-water extraction, the determination of glutathione concentration, and membrane separation experiments. This chapter also describes the procedure to evaluate the effects of hot water extraction time and temperature on the glutathione yield produced, as well as the stability of glutathione when exposed to light.

Chapter 4 shows the experimental data obtained. The research findings are discussed and explained.

Finally, Chapter 5 describes general conclusions of this research. Based on the thesis work, recommendations for future studies are provided.

Chapter 2

Literature Review

Nowadays, membrane processes have a widespread applications because they have advantages in energy savings and friendly environmental impact (Elimelech and Phillip, 2011). In addition, membrane processes are commonly more effective than most traditional separation processes (Noble, 1987).

This literature review covers the fundamental principles of membrane separation technologies, including membrane transport models and ultrafiltration and nanofiltration. Moreover, membrane fouling is a significant issue for liquid separation, and therefore, membrane contamination and its control are also described in this chapter (Guo *et al.*, 2012).

2.1 Glutathione

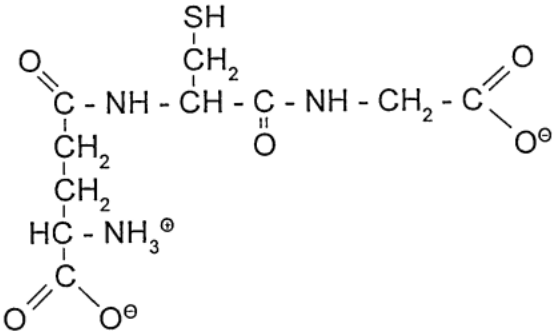
2.1.1 Introduction of Glutathione

Glutathione is an important antioxidant in living organisms (Meister and Anderson, 1983). It's a tripeptide which consists of L-glutamate, L-cysteine and glycine as shown in Fig. 2.1. Glutathione is widely found in plants and animals, and it is extremely high in yeast, wheat germ and animal liver (Meister, 1988). The structure of glutathione contains a reactive thiol group (R-SH) which is easily oxidized. Glutathione is stable in solid state, while it is easily oxidized to oxidized glutathione in aqueous solution. The oxidized form, glutathione disulfide (GSSG), is shown in Fig. 2.2. The oxidized

glutathione is a dimer bonded by a disulfide bond, and it is obtained by dehydrogenating two molecules of glutathione. Glutathione disulfide can be reduced to glutathione by the enzymic glutathione reductase (Johnson and Voegtlin, 1927). Equation (2.1) shows this chemical reaction (McGraw-Hill, 1995; Nagy and Ashby, 2007).

$$2GSH \underset{+2H}{\overset{-2H}{\rightleftharpoons}} GSSG \tag{2.1}$$

Glutathione can exist in reduced state and oxidized state in cells, tissues and plasma (Anderson, 1998). In healthy cells and tissue, over 90% of glutathione exist under the reduced form and less than 10% exists under the oxidized form. The GSSG-to-GSH ratio is often considered as an indicator of oxidative stress (Finley *et al.*, 1981; Kidd, 1997).



Glutathione (GSH)

Figure 2.1 The structure of glutathione (Anderson, 1998).

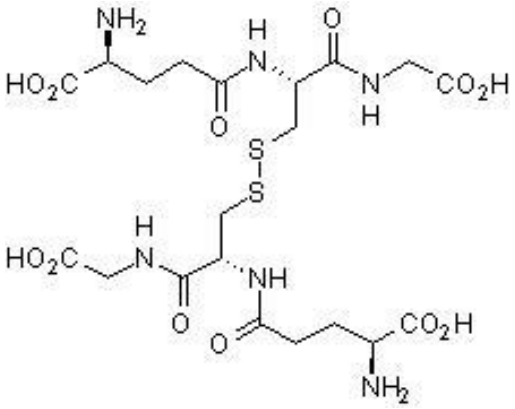


Figure 2.2 The structure of glutathione disulfide.

Glutathione has many important cellular functions, as shown in Fig. 2.3. They are related to protection against oxidative stress, amino acid transport, enzyme activity, xenobiotic and endogenous toxic metabolic detoxification, and nitrogen and sulfur metabolisms (Penninckx, 2002; Penninckx *et al.*, 1980; Sies, 1999).

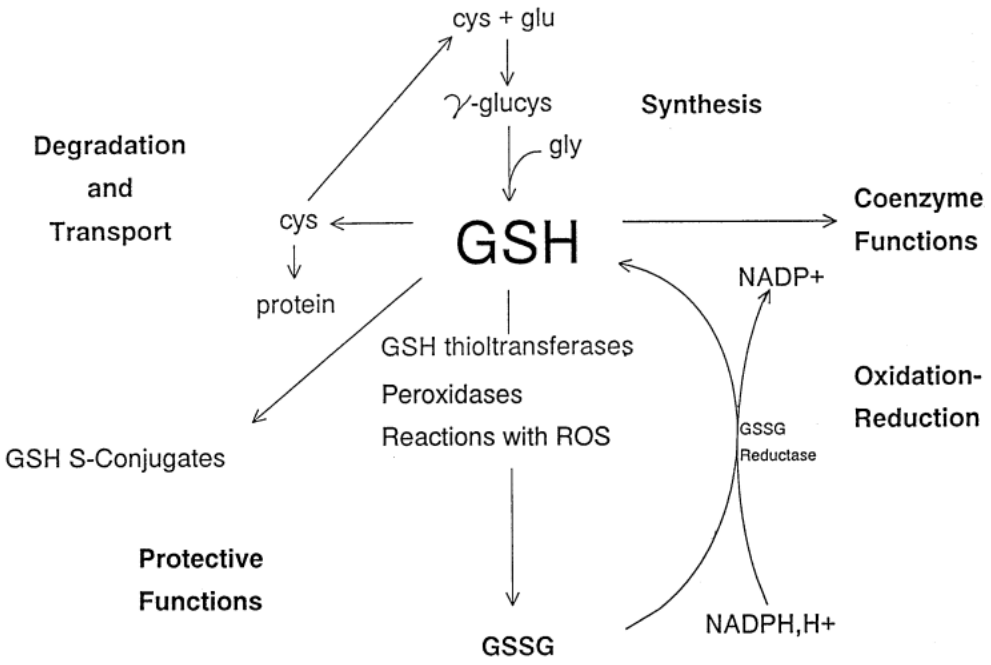


Figure 2.3 Summary of glutathione metabolism (Anderson, 1998).

Glutathione has a variety of physiological functions, which can be summarized in three main aspects: antioxidant, defence against molecule and immunity booster (Dröge and Breitkreutz, 2000; Penninckx and Elskens, 1993). These physiological functions make glutathione an important biochemical drug to treat lots of diseases, such as liver cirrhosis, HIV infections and pancreatic inflammations (Li *et al.*, 2004; Wu *et al.*, 2004). Nowadays, glutathione has been used in cosmetic industries and in food additives.

2.1.2 Glutathione Production

Glutathione was originally recovered by solvent extraction from plant or animal tissue, but this method gave an expensive end-product. Glutathione can also be produced by chemical approach, microbial fermentation and enzymatic reaction (Musatti *et al.*, 2013; Rollini *et al.*, 2010). Mead and Harington (1935) proved that glutathione can be chemically synthesized; however, the end-product was a racemic mixture which needed an optical resolution to separate the active L-form from the D-isomer. Enzymatic reaction method can result in a high accumulation of glutathione; however, the high cost limits their industrial application (Ohtake *et al.*, 1989). Fermentative production of glutathione is the most popular approach employed on industrial scales (Wei *et al.*, 2003). The yield is relatively low with this method, but the sugar materials used as substrates make this process cheaper than enzymatic production (Sakato and Tanaka, 1992). Cell density and intracellular glutathione content need to be increased to improve the glutathione yields (Ubiyvovk *et al.*, 2011). However, by-products, oxygen supply and other issues may prevent cell growth in high-cell-density

cultivation (Murata and Kimura, 1990; Nie *et al.*, 2005). In addition, an advanced separation technique is necessary to obtain glutathione with a high recovery rate. Some techniques have been used to separate glutathione from fermentation broth, including copper-salt method, affinity chromatography and ion-exchange chromatography (Liang *et al.*, 2009). The copper-salt method produces a large amount of H_2S in the separation process, which is a significant environmental problem. The affinity chromatography method is unsatisfactory due to high toxicity of copper residue. Although ion-exchange chromatography has been used to separate glutathione from fermentation broth, the cost of the preparation of high-purity glutathione is high because of its such inherent disadvantages as complexity, multi-steps, and being time-consuming. In addition, the concentration of glutathione in fermentation broth is often very low, which restricts the application of this method. Therefore, biotechnological process optimization is required. Experimental designs were used to find the best conditions of agitation rate, initial pH, temperature, glucose concentration and inoculum concentration for glutathione production by *S. cerevisiae* (Penninckx, 2000).

2.1.3 Extraction of Glutathione from Yeast

In the past, the yeast *S. cerevisiae* is known as microbial starters used in the production of alcoholic beverages and food. In these traditional processes, the substrates are converted into carbon dioxide, ethanol and biomass. Nowadays, yeast and its derivatives have been used as nutritive additives and as food ingredients to form a variety of industrial food products. Glutathione is the major (95%) nonprotein thiol

compound in *S. cerevisiae* where it plays several important roles in response to nutritional and oxidative stress (Lee *et al.*, 2001; Stephen and Jamieson, 1996).

Hot water extraction is a conventional method to extract glutathione from yeast. Reduced glutathione from fermentation broth of *S. cerevisiae* was extracted with ethanol without disruption of cells (Xiong *et al.*, 2009). However, this process has no obvious advantage for glutathione extraction from yeast over the hot water extraction. Aqueous two-phase systems including polymer-polymer (PEG-Dextran) and polymer-salt (PEG-salt) have also been used to extract glutathione from yeast (Wu *et al.*, 2010).

2.2 Membrane Separation Technology

2.2.1 Overview of Membrane Separation Technology

As an emerging technology, membrane separation technology has been used in a broad range of applications, from food processing to petrochemical industries. Although there are some differences among all membrane processes, there is one thing in common: the membrane is a semi-permeable barrier, and it can pass certain components while rejecting other components under the driving force of a pressure, concentration, electric potential or temperature gradient across the membrane. This process is shown in Fig. 2.4.

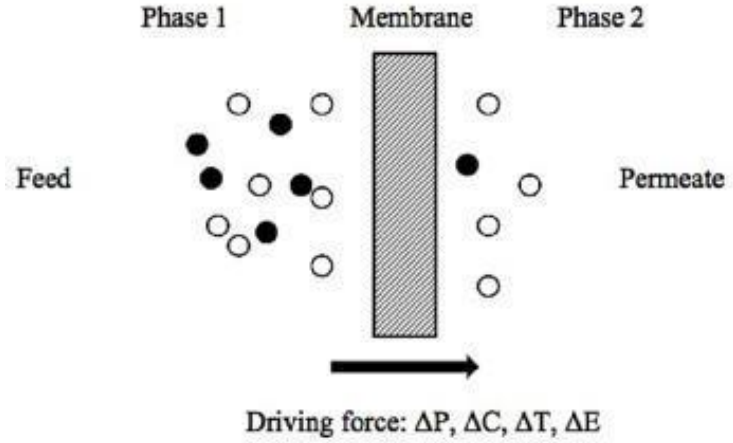


Figure 2.4 Schematic of a two-phase system separated by membrane (Mulder, 1991).

The membrane can be made from either ceramic or polymeric materials in general. Based on structure, separation manner, preparation and geometry, membranes can be categorized accordingly. Fig. 2.5 shows the classification of synthetic membranes.

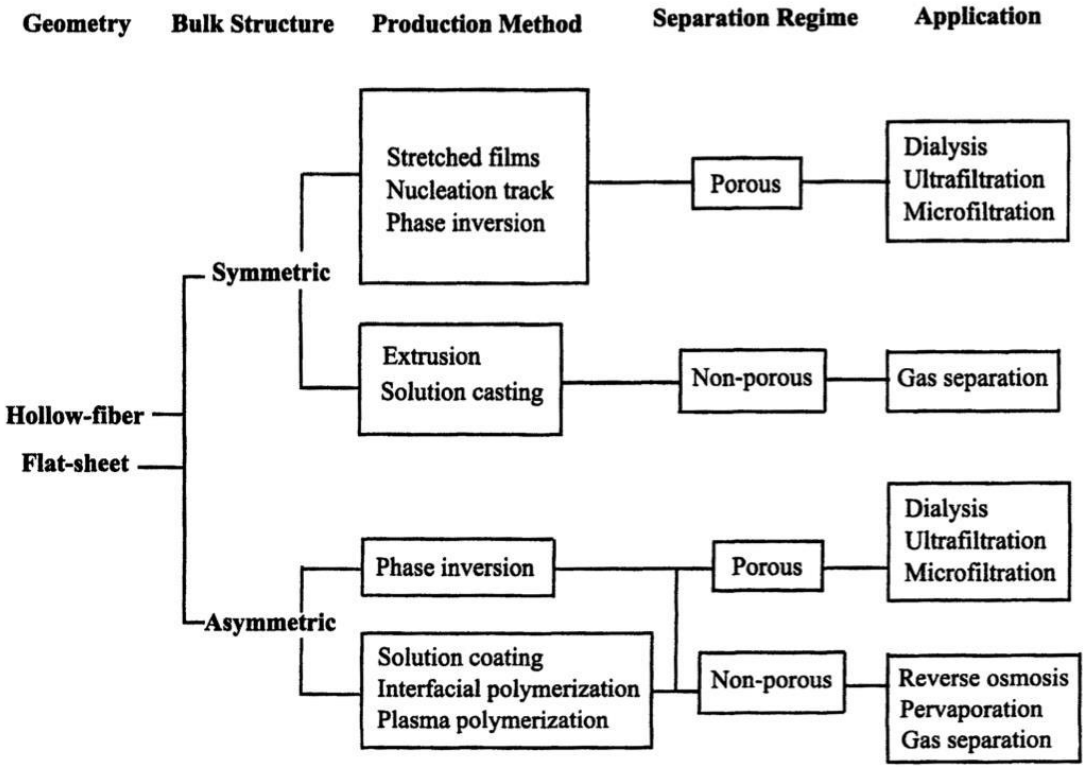


Figure 2.5 Classification of synthetic membranes (Pinnau and Freeman, 1999).

Membranes can be symmetric or asymmetric. Separation regime was often determined by membrane structure and morphology. Symmetric membranes often have a thickness of 10-200 μm with uniform structures. The mass transport resistances of these membranes are proportional to their membrane thicknesses. Asymmetric membranes consist of a thin skin layer with a thickness of 0.1-0.5 μm and a 50-150 μm thick porous support layer. Therefore, the membrane has a high selectivity and high permeance because the resistance of the membrane is mainly determined by the top thin skin of the asymmetric membrane (Mulder, 1991). Fig. 2.6 illustrates the structures of symmetric and asymmetric membranes.

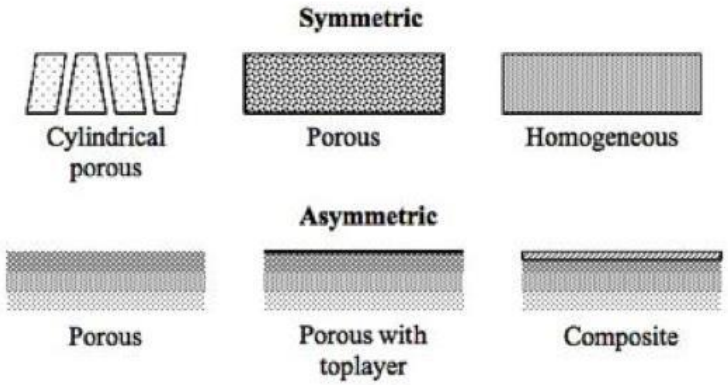


Figure 2.6 Schematic of membrane cross-sections (Mulder, 1991).

Microfiltration, ultrafiltration, electrodialysis and reverse osmosis are four well-established membrane separation processes. Conventional filtration, microfiltration, ultrafiltration and reverse osmosis are related processes that differ mainly in the membrane pore size, as shown in Fig. 2.7. Microfiltration, ultrafiltration and reverse osmosis are pressure-driven processes. Microfiltration membranes filter bacteria and

colloidal particles which have a diameter from 0.1 to 10 µm. Ultrafiltration membranes have been used to filter macromolecules, including proteins from solutions. The mechanism of reverse osmosis is quite different. The membranes pores of reverse osmosis membranes are very small with a diameter rating of 3 to 5 Å, within the broad range of thermal motion of polymer chains. Electrodialysis separate ions from aqueous solutions under an electrical potential difference.

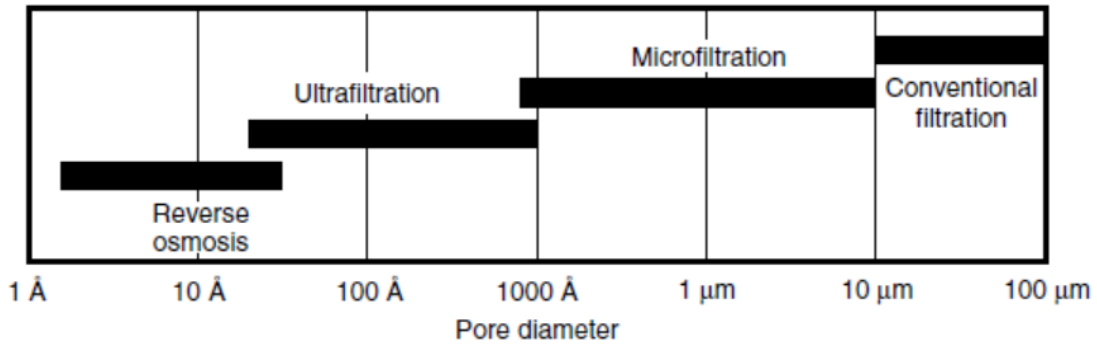


Figure 2.7 Pore diameters of reverse osmosis, ultrafiltration, microfiltration and conventional filtration (Baker, 2004).

Gas separation and pervaporation are two developing membrane separation processes. In gas separation, a gas mixture at a given pressure is passed through the surface of a membrane which is permeable to one component of the feed gas and the permeate is enriched in this species. The process is illustrated in Fig. 2.8. Pervaporation is a relatively new process, where a liquid mixture first contacts one side of a membrane, and then the permeate is removed as a vapor from other side. Fig. 2.9 shows a simple pervaporation process.

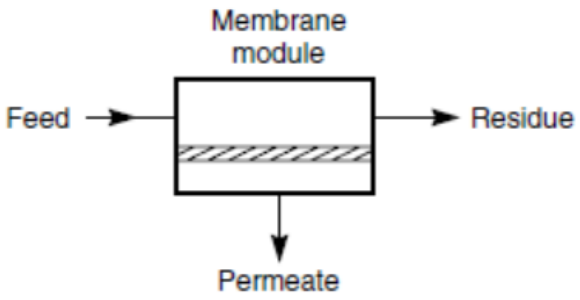


Figure 2.8 Schematic of the gas separation process (Baker, 2004).

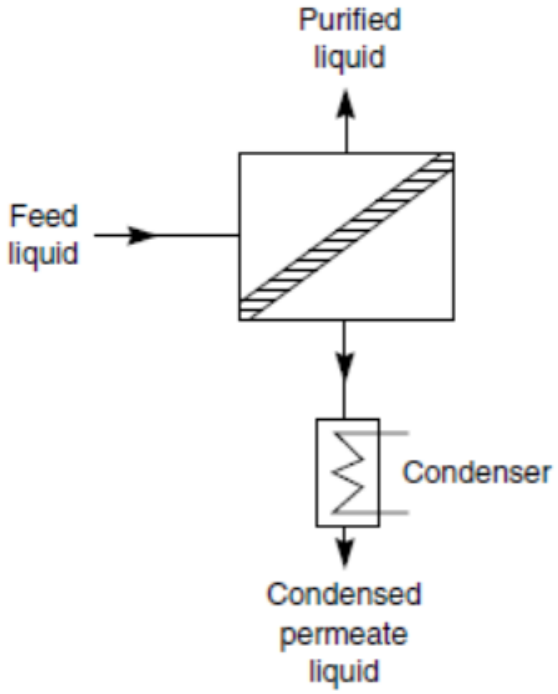


Figure 2.9 Schematic of the pervaporation process (Baker, 2004).

2.2.2 Mass transport mechanism through membranes

The most important characteristics of membranes is the control of the permeation rate of different species. In general, there are two models to describe the mass transport through membranes. One is the pore-flow model, and the other is the solution-diffusion model, as shown in Fig. 2.10 (Baker, 2004). In the solution-diffusion model, permeants

first dissolve in the membrane, then diffuse through the membrane by the concentration gradient based on Fick's law of diffusion (Wijmans and Baker, 1995). The permeants are separated due to the differences in the solubility and diffusivity of permeants in the membrane. In the pore-flow model, permeation occurs by pressure-driven convective flow through pores, and separation occurs due to the exclusion of permeants from the pores. The pore-flow model is applied for porous membranes, such as ultrafiltration (Tu *et al.*, 2005). The solution-diffusion model is applied in the non-porous membrane such as gas separation, pervaporation and reverse osmosis. Nanofiltration is between ultrafiltration and reverse osmosis, and therefore the mass transport mechanism in nanofiltration is a transition between solution-diffusion and pore-flow model.

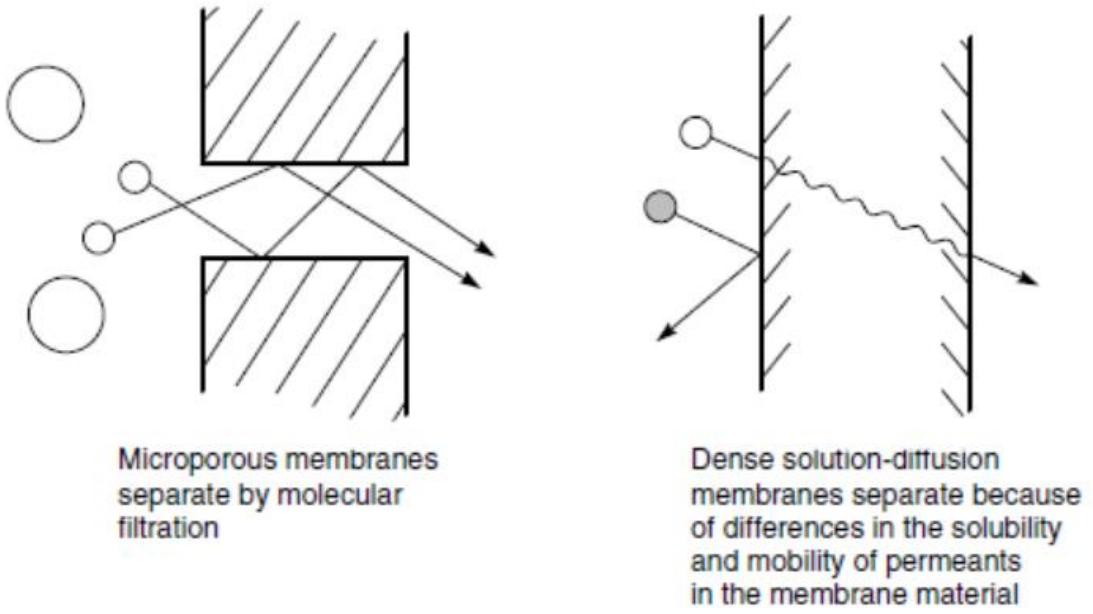


Figure 2.10 Schematic of membrane transport mechanism (Baker, 2004).

The difference between the two mechanisms lies in the relative sizes and

permanence of pores. For membranes that use the solution-diffusion model to describe the mass transfer mechanism, the pores in the membrane are very small spaces among polymer chains resulting from thermal motion of polymer molecules. These pores fluctuate because they open and close when permeating molecules pass through the membrane. In contrast, the pore-flow model is usually used to describe membranes with pores fixed and relative large (Hwang, 2010). In general, the pores of the transition between pore-flow and solution-diffusion model is around 5-10 Å in diameter.

Membrane transport processes are organized into three general groups as shown in Fig. 2.11. First, mass transport occurs by pore-flow, and the membranes have pore sizes larger than 10 Å (e.g., ultrafiltration and microfiltration). Second, membranes with spaces between the polymer chains less than 5 Å, and these membranes are considered to have no visible pores (e.g., reverse osmosis membrane). Mass transport in these types of membranes is described by the solution-diffusion model. Third, membranes with pore diameters between 5-10 Å, for which the mass transport is an intermediate between the pore-flow and solution-diffusion models. For example, nanofiltration is a transition between ultrafiltration and reverse osmosis.

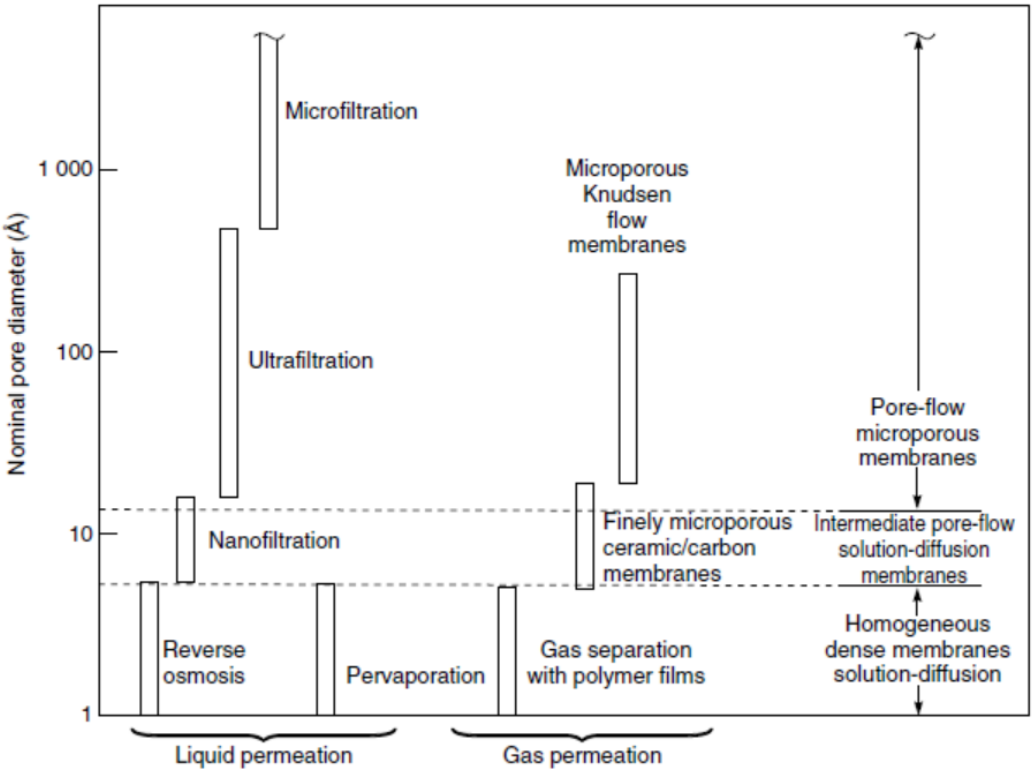


Figure 2.11 Membrane transport models for various membrane separation processes (Baker, 2004).

2.2.3 Ultrafiltration

Ultrafiltration (UF) is a pressure-driven membrane separation process, and the separation is mainly based on size exclusion. Normally, UF can retain molecules with diameters in the order of 100 nm. UF was primarily used to remove macromolecules and particles from wastewater in the early days, and nowadays it has extended its applications to processing of biological macromolecules (Qi *et al.*, 2012). Colloids, bacteria, viruses and macromolecules with a molecular weight of 1000 to 300,000 Da can be rejected by UF membranes (Mulder, 1991).

For UF membranes, the selectivity and permeability are highly related to the materials and pore structure of the membrane. Generally speaking, the materials of UF membrane should have a high mechanical strength, thermal stability, chemical resistance, and the ability to form flat sheet or hollow fiber membrane easily. Commercial UF membranes are made from different polymers, including polysulfone (PS), polyethersulfone (PES), polypropylene (PP). The transmembrane pressure of UF membrane is usually in the range of 0.1-0.5 MPa (Ghosh, 2009).

2.2.4 Nanofiltration

Nanofiltratiom is principally used in water treatment, including wastewater treatment and drinking water production, and it can be also used in peptide and amino acid separation (Lau *et al.*, 2012; Martin-Orue *et al.*, 1998; Tsuru *et al.*, 1994). It has the advantages of high rejection of multivalent salts, low operating and maintenance costs, and high flux (Bowen and Mukhtar, 1996; Hilal *et al.*, 2004). The permeability and selectivity of pressure-driven membrane process mainly depend on membrane pore properties (including porosity, pore size and pore-size distribution). As mentioned before, ultrafiltration and microfiltration are porous and are usually used for low-pressure operations and reverse osmosis membranes are nonporous. Nanofiltration membranes are between a loose reverse osmosis membrane and a tight ultrafiltration membrane. Molecular weight cut off (MWCO) refers to the molecular weight of the molecule which is 90% retained by the membrane. The molecular weight cut off of a nanofiltration membrane is often in the range of 100-1000 Da (Miner, 2005; Oatley *et*

al., 2012).

2.3 Membrane Contamination

2.3.1 Membrane Fouling and Concentration Polarization

Membrane performance is usually inevitably affected by membrane contamination during membrane separation processes. The contamination is often reflected in deterioration of membrane selectivity and flux decline over time. Membrane contamination is a severe problem, and fouling is the most significant problem of membrane contamination.

Membrane fouling is a significant issue for a flux decline. It is caused by the deposition of solutes on the membrane surface or inside membrane pores (Fane *et al.*, 2011). The mechanism of membrane fouling depends on the nature of foulants. In general, there are four types of fouling: clogging of the pores by colloids, adsorption of solutes from feed solution to the surface of the membrane, gel-layer formed by microorganisms or macromolecules, deposition of insoluble solids or salts due to chemical precipitation or crystallization. Membrane fouling will cause an increase in mass transport resistance, and then reduce solvent permeation flux at the given operating pressure. Severe membrane fouling can reduce the lifetime of the membrane. The flux change of a UF membrane during its lifetime is illustrated in Fig. 2.12. In the initial period of each operation cycle, the flux decreases significantly because of concentration polarization. The flux decline between the cycles is a result of the membrane fouling.

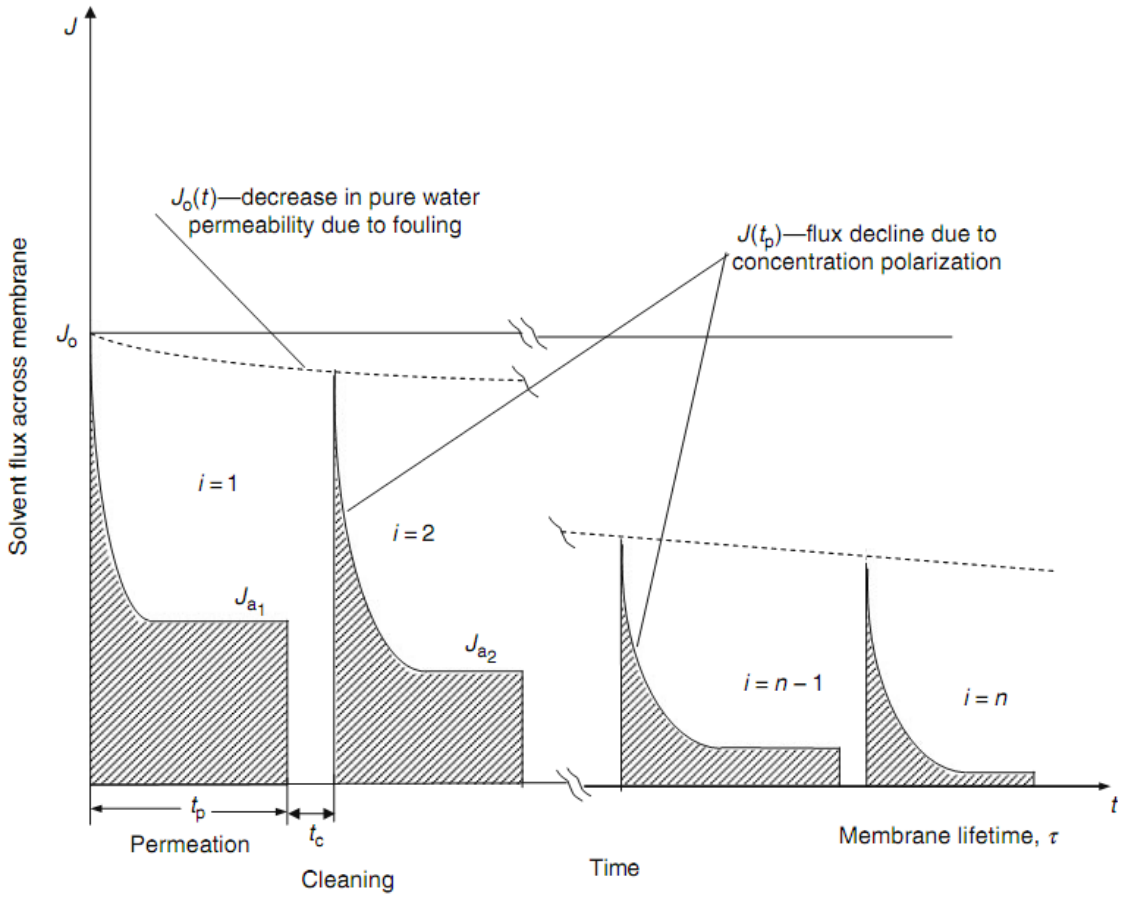


Figure 2.12 Flux-time plot in large-scale ultrafiltration systems (Goosen *et al.*, 2009).

Generally speaking, membrane fouling is a complicated physicochemical phenomenon in which many mechanisms are participated simultaneously.

Membrane fouling can initially result from concentration polarization. In membrane separation processes, the solvent permeates through the membrane but the solute is partly retained. The rejected solutes gradually accumulate on the membrane surface over time, and a concentration gradient of the solute forms in the boundary layer until a steady state is reached. Concentration polarization can significantly influence membrane performance in reverse osmosis, but this phenomenon is often well controlled in industrial systems. In addition, concentration polarization seriously affects

membrane performance in ultrafiltration. Concentration gradients on both sides of the membrane is formed because of concentration polarization as shown in Fig. 2.13.

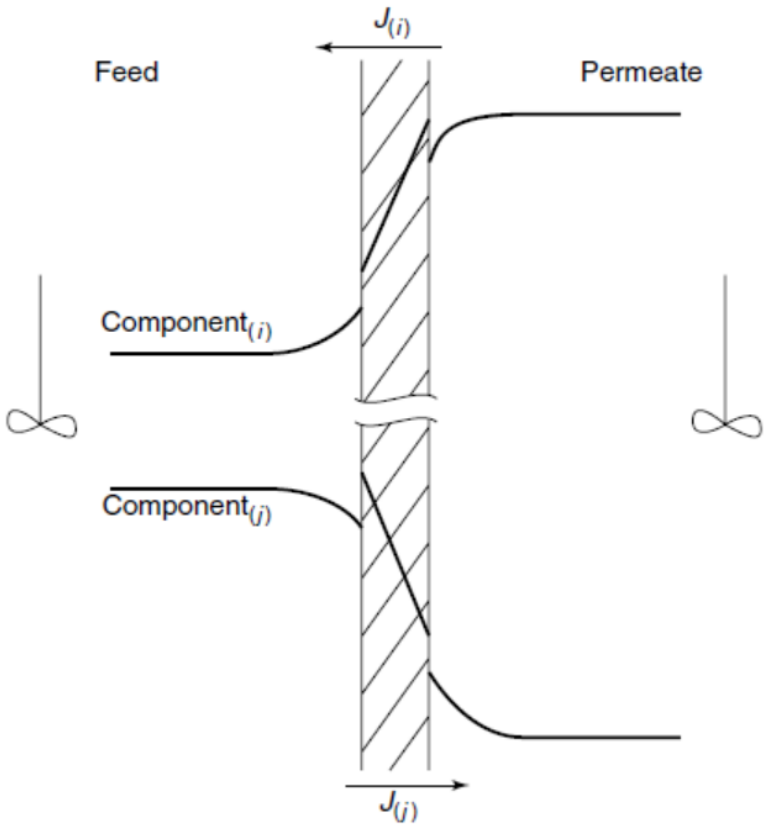


Figure 2.13 Schematic of the concentration polarization gradients formed (Baker, 2004).

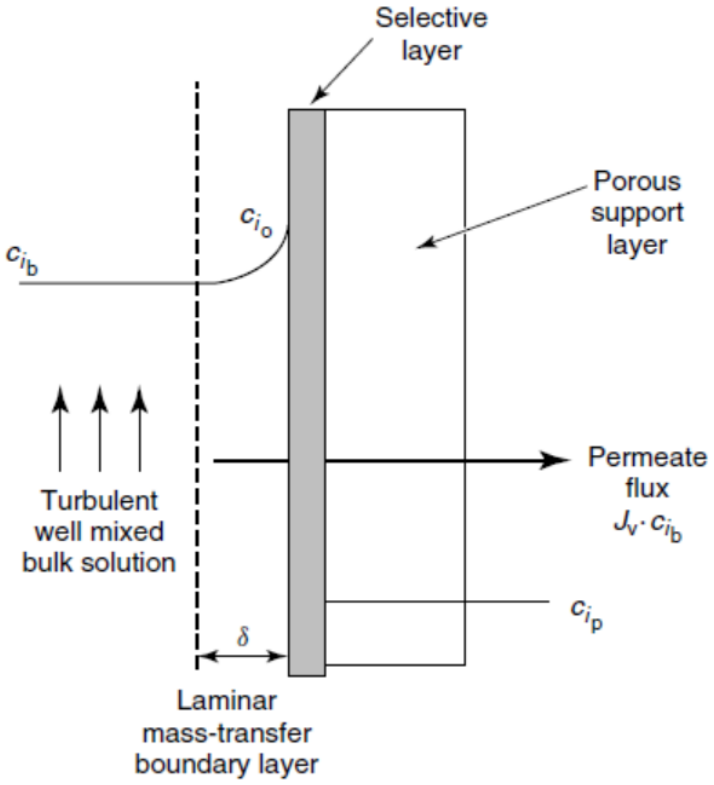


Figure 2.14 Concentration profile formation in boundary layer (Baker, 2004).

The concentration gradient build-up can be described in mathematical form. The steady-state salt gradient for a reverse osmosis membrane process is shown in Fig. 2.14. The salt flux across the membrane is the product of the permeate salt concentration c_{ip} and permeate volume flux J_v. At steady state, the net flux within the boundary layer is equal to the permeate flux $J_v c_{ip}$. In the boundary layer, this net flux is equal to the convective flux towards the membrane $J_v c_i$ subtract the diffusive flux away from the membrane which can be described by Fick's law ($D_i dc_i / dx$). Thus, transport of salt within the boundary layer can be expressed by the equation:

$$J_v c_i - D_i dc_i / dx = J_v c_{ip} \tag{2.1}$$

where D_i is the salt diffusion coefficient.

The mass balance equation (2.1) can be integrated to

$$\frac{c_{i_o} - c_{i_p}}{c_{i_b} - c_{i_p}} = \exp\left(J_v \delta / D_i \right) \tag{2.2}$$

where c_{i_o} is the solute concentration in feed solution at the membrane surface, c_{i_b} is the bulk solution concentration, δ is the boundary layer thickness. Replacing the concentration term by E defined as c_{i_p} / c_{i_b} and E_o defined as c_{i_p} / c_{i_o}, equation (2.2) can be written as

$$\frac{1/E_o - 1}{1/E - 1} = \exp\left(J_v \delta / D_i \right) \tag{2.3}$$

c_{io} / c_{i_b} is called the concentration polarization modulus, and it can measure the extent of concentration polarization. No concentration polarization occurs when the value of c_{io} / c_{i_b} is equal to 1, but as the value deviates further from 1, concentration polarization effects membrane flux and selectivity more significantly. From equations (2.2) and (2.3), c_{io} / c_{i_b} can be written as the following:

$$\frac{c_{i_o}}{c_{i_b}} = \frac{\exp(J_v \delta / D_i)}{1 + E_o \left[\exp\left(J_v \delta / D_i \right) - 1 \right]} \tag{2.4}$$

Equation (2.4) shows that the degree of concentration polarization can be determined by these factors: the thickness of the boundary layer δ, the permeate volume flux J_v, the diffusion coefficient D_i and E_o which is the membrane enrichment. Fig. 2.15 shows the effect of these factors on the concentration gradients in boundary layer. Among these factors, the boundary layer thickness δ can be changed most easily. Reduce the thickness of the boundary layer by increasing turbulent mixing can minimize concentration polarization.

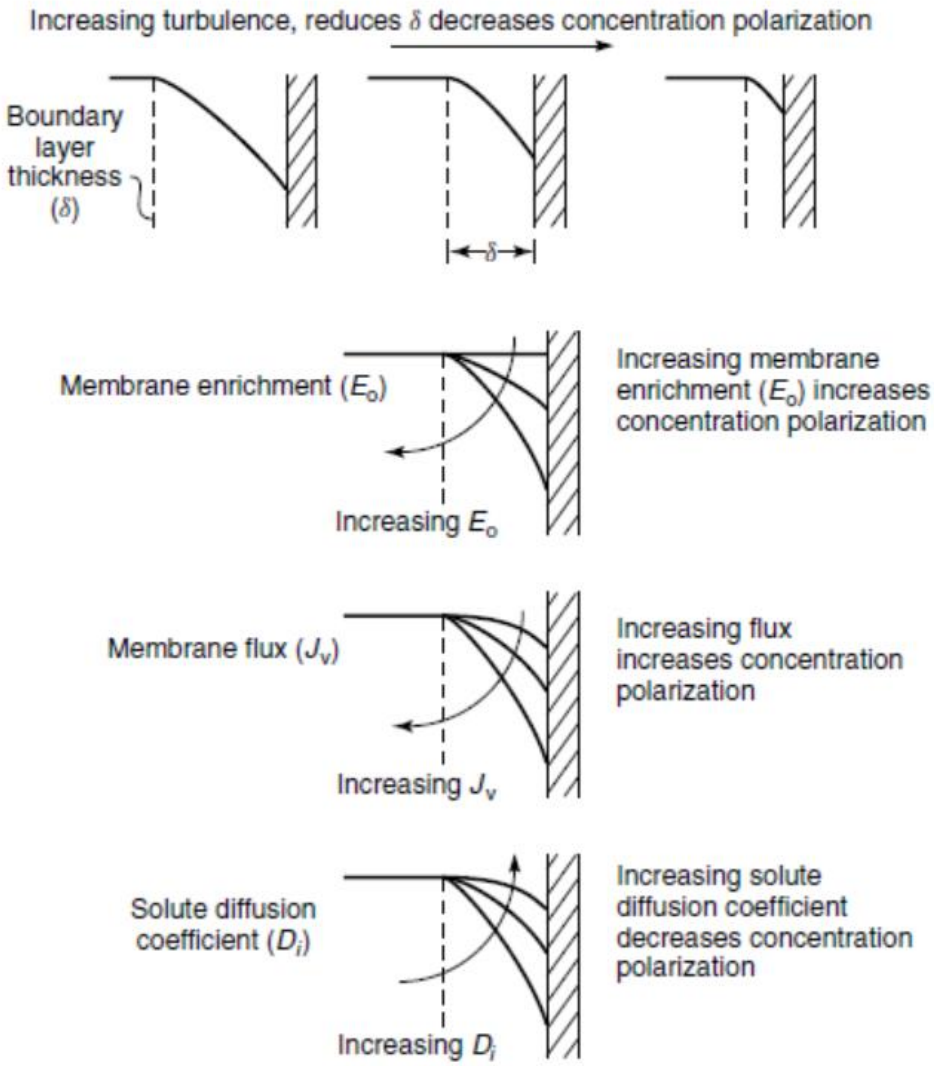


Figure 2.15 The effect of changes in these factors on concentration gradients in boundary layer (Baker, 2004).

In general, concentration polarization has negative influence on permeation flux, and the importance of concentration polarization varies for different membrane processes. For example, ultrafiltration and microfiltration are affected significantly by concentration polarization. In addition, membrane processes with dense membrane are affected much less significantly by concentration polarization.

2.3.2 Resistance-in-series Model

Besides the resistance of the membrane itself (R_m), gel-layer formation (R_g), concentration polarization (R_{cp}) and internal pore blocking (R_{in}) will yield additional mass transfer resistances. The resistance-in series model is usually used to understand the fouling behavior. The relationship between resistance and permeate flux can be described as follows:

$$J = \frac{\Delta P}{\mu R_{tot}} = \frac{\Delta P}{\mu(R_m + R_g + R_{cp} + R_{in})} \tag{2.5}$$

where μ is the viscosity of the permeate solution, ΔP is the transmembrane pressure.

When pure water permeates the membrane, the relationship is given by:

$$J = \frac{\Delta P}{\mu R_m} \tag{2.6}$$

Therefore, the membrane resistance R_m can be calculated from pure water permeation data. Thus, the fouling resistance can also be calculated based on equation (2.5). The typical flux-pressure relationship for a solution and pure water are shown in Fig. 2.16.

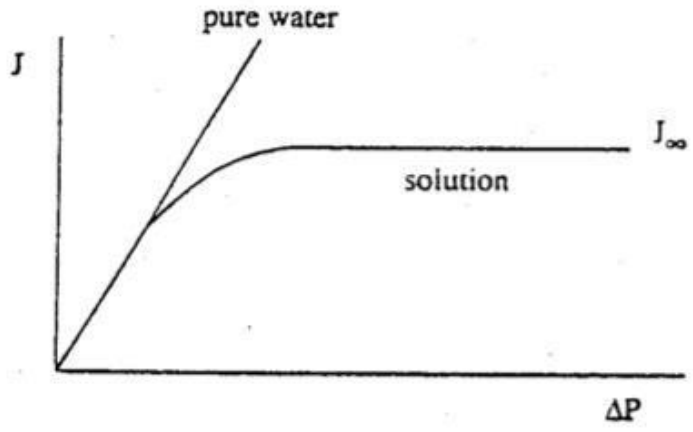


Figure 2.16 Flux-pressure curves for a solution and pure water permeation (Baker, 1991).

2.3.3 Osmotic pressure Model

The osmotic pressure of a solution increases exponentially with the concentration of solute, and this relationship can be described as

$$\pi = a \cdot c^n \tag{2.7}$$

where a is a constant; n is an exponential factor with a value not less than 1. The value of a and n depend on type of polymer and molecular weight. For dilute low molecular weight solutions, the osmotic pressure has a linear relationship with concentration. For concentrated polymer solutions, the value of n is 2 or greater.

The water flux, J_w, is related to concentration gradients and the pressure across the membrane, and it can be described as:

$$J_w = A(\Delta p - \Delta \pi) \tag{2.8}$$

where Δp is the pressure difference across the membrane; $\Delta \pi$ is the osmotic

pressure difference across the membrane, and it is determined by the concentration at the membrane surface rather than the bulk concentration; A is the water permeability constant.

2.3.4 Methods to Membrane Fouling Control

There are four major approaches for membrane fouling control (Hilal et al., 2005).

Pretreatment of the feed solution is usually used, particularly in membrane distillation and pervaporation in which membrane fouling is not severe. This method is often used to remove and filter particles which can result in pore blocking of a membrane or preventing macromolecules and particles from depositing on the surface of the membrane. In general, pH pre-adjustment, chemical clarification and pre-filtration are usually used as pretreatment (Peuchot and Aim, 1992).

Membrane surface modification can reduce adhesive fouling on the membrane by changing surface properties, such as surface charge, pore size distribution and hydrophilicity/hydrophobicity. Approaches to modify membrane surface properties include grafting and surface coating with a polymer layer which has antifouling properties (Nunes et al., 1995).

Improvement of operating conditions can also reduce membrane fouling. These operation parameters include pressure on the feed side, concentration of feed solution and temperature (Winzeler and Belfort, 1993).

Among all the methods to membrane fouling control, membrane cleaning is used most frequently. Three cleaning approaches can be used based on the foulants and

resistance of membranes: Mechanical cleaning, hydraulic cleaning and chemical cleaning. Hydraulic cleaning is often used in cross-flow filtration processes; mechanical cleaning is usually applied in tubular systems; chemical cleaning with chemicals such as alkali, acids and enzymes is the most widespread cleaning approach in membrane fouling control (Mulder, 1991).

Chapter 3

Experimental

3.1 Materials

Sodium hydroxide (97%, Caledon Laboratories), tris(hydroxymethyl)amino-methane (99.8%, Sigma-Aldrich), ethylenediaminetetraacetic acid (99%, Sigma-Aldrich), hydrochloric acid (37%, Sigma-Aldrich), 5,5'-dithiobis 2-nitrobenzoic acid (Sigma-Aldrich), glutathione (Sigma-Aldrich) are used in the study. The UF membrane used was supplied by Sepro Membranes (membrane PES-10), and the NF membrane used was supplied by Koch Membrane System Inc. (membrane SelRO MPF-34). Dry yeast was supplied by Lallemand company. Deionized water and air were supplied by University of Waterloo.

The lab equipment used included: Shimadzu 1240 UV-Vis spectrophotometer, Eppendorf 5804 Centriguge, Model 501 Thermostatic Bath, Mettler Toledo PM200 scale, Precision Scientific Thelco Model 28 heater, and a magnetic stirrer.

3.2 Hot-water Extraction of Glutathione from Yeast

Hot water extraction disrupted yeast cells, leading to the release of many water-soluble proteins, including glutathione.

10g of dry yeast was mixed with 100g of 78°C hot water. The yeast suspension is then held for 10 min with mixing. Thereafter, the yeast suspension was cooled immediately with ice and then centrifuged at 10,000 rpm for 20 minutes. The

supernatant (yeast extract) was collected and weighed and analyzed. The supernatant was dried in an oven to get the dry matter, and then the weight of the dry matter was measured. The dry matter content and glutathione content in the supernatant (yeast extract) were then determined.

3.3 Determination of Glutathione

5,5'-dithiobis 2-nitrobenzoic acid (DTNB) reacts at pH 8 with SH-groups, giving one mol yellow colored anion per mol SH-compound reacted. Therefore, only the reduced form of glutathione was measured by this method. Glutathione mentioned after that refers to the reduced form. The concentration of glutathione was determined with a UV-Vis spectrophotometer at 412 nm (Akerboom and Sies, 1981; Eyer and Podhradský, 1986).

The following was carried out to prepare the calibration. The Buffer consisted of 50 mM tris, 3 mM EDTA and 38 mM HCl, pH 8. The stock DTNB reagent was made up of 0.0396g DTNB and 8 ml Buffer, followed by addition of 2 ml 0.1N NaOH, ending up with a pH 8. 0.8 ml of DTNB was diluted with Buffer to 100 ml, for use as DTNB reagent. 30.7g of glutathione was mixed with 10 ml 0.1N HCl under agitation, for 5 min. The solution was diluted with 0.1N HCl to prepare 1, 1.8, 2, 2.4, 2.6, 3 mM solutions of glutathione. These standard glutathione solutions were each added to 4.9ml of DTNB reagent. The resulting mixtures were shaken thoroughly and incubated for 10 min at room temperature, and absorbances were determined at a wavelength of 412nm. The calibration curve was constructed by plotting the absorbance versus concentration

of glutathione.

To determine the glutathione content of a sample, 0.4g sample (dry yeast products or liquid yeast extract) was added to 10ml 0.1N HCl in a centrifuge tube, followed by rigorous shaking to suspend the sample. The solution is mixed regularly during the digestion period of 30 to 60 minutes, and then centrifuged at 8000 rpm for 5 min. 0.1ml of supernatant from the centrifuge tube was mixed with 4.9ml of DTNB reagent. After thorough shaking, the absorbance of the sample was measured at 412nm after standing at room temperature for 10 min. The concentration of glutathione was determined from using glutathione calibration curve. As a result, the concentrations of glutathione in all solutions were obtained.

3.4 Purification and Concentration of Glutathione

The UF membrane used was polyethersulfone membrane, which was supplied by Sepro Membranes with a MWCO of 10 kDa. The NF membrane used was made up of polysulfone on polypropylene (supplied by Koch Membrane System Inc.) with a molecular weight cut-off 200 Da. The membranes were soaked in deionized water overnight before use. The experimental setup for membrane separation is shown in Fig. 3.1. The membranes were mounted in a stainless steel test cell with an effective working volume of 250 ml and an effective permeation area of 14.85 cm^2. The feed solution was agitated by a magnetic stirrer. The transmembrane pressure for permeation was supplied by pressurized nitrogen gas.

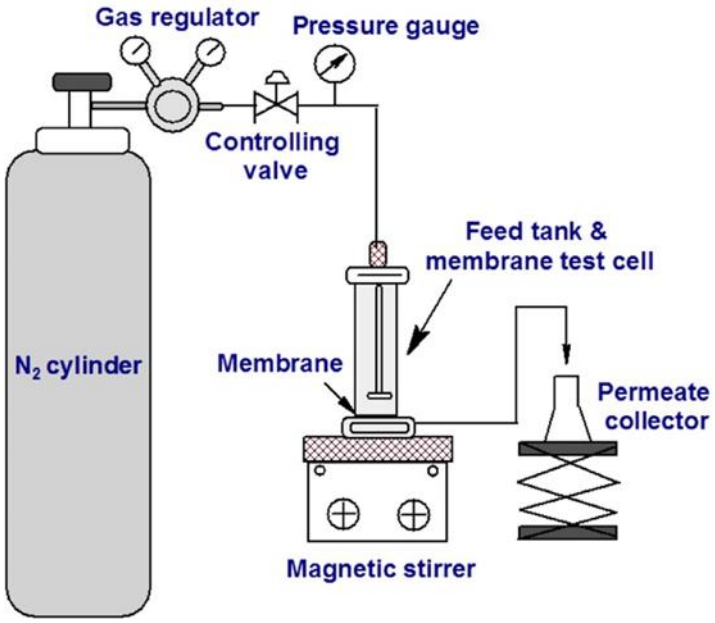


Figure 3.1 Schematic diagram of dead-end teat set-up (Wu et al., 2016).

The separation involved two process steps: UF purification and NF concentration. Supernatant from hot water extraction was first passed through a UF membrane under a gauge pressure of 0.2 MPa at room temperature (23°C) to remove macromolecules; about 4 h was required to pass 81 g of the supernatant. Then, the UF permeate was subjected to NF under a gauge pressure of 0.8 MPa at room temperature (23°C) to concentrate the glutathione, as shown in Fig. 3.2. The permeate flux gradually decreased during the NF concentration process. The UF permeate, UF retentate, NF permeate, NF retentate were dried in an oven to get the dry matter, and then these dry matters were weighed respectively.

Aqueous glutathione solutions with the same concentrations and volumes as the supernatant from hot water extraction were also tested with the UF membrane to compare the flux and rejection characteristics of the solutions. This aqueous glutathione solution was also passed through UF and NF membrane under the same pressure and

temperature as that of supernatant to test the permeation flux and glutathione rejection.

Concentrations of glutathione in UF permeate, UF retentate, NF permeate, and NF retentate in the supernatant as well as the aqueous glutathione solution were analyzed using the spectrophotometer as described previously.

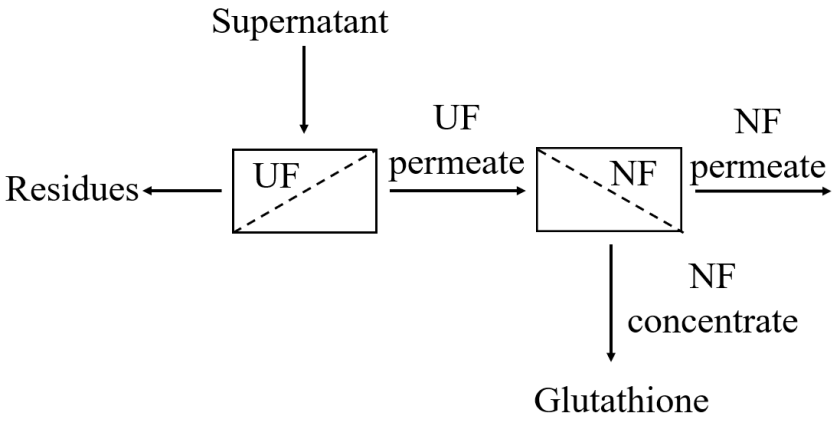


Figure 3.2 Scheme showing the membrane separation process.

The separation performance of a membrane is evaluated in terms of two parameters: membrane selectivity and permeation flux. The permeation flux is determined as

$$J = \frac{Q}{A\Delta t} \tag{3.1}$$

where Q is the volume of permeate (L) collected over a time interval Δt (h), and A is the effective area of the membrane (m^2).

The rejection coefficient is a measure of membrane selectivity, and it is defined as

$$R = (1 - \frac{c_p}{c_f}) \times 100\% \tag{3.2}$$

where c_p and c_f are the glutathione concentrations in the feed and the permeate,

respectively.

3.5 Effects of Hot-water Temperature and Extraction Time on Glutathione Extracted

This experiment aimed to investigate the influence of hot water temperature and extraction time on the amount of glutathione in the supernatant obtained.

The hot-water extraction of glutathione from yeast was carried out at different temperatures (67, 70, 73, 75, 79 and 85°C) for different durations (2, 4, 8,10, 12, 16, 20, 30, 40 and 60min); this was done by taking the samples using a pipette at different instant, and the samples were cooled immediately with ice. After centrifugation, the supernatant was subjected to UV-Vis spectrophotometer to determine glutathione content.

3.6 Effect of Light on Stability of Glutathione in Supernatant

It was found that the glutathione might undergo certain changes in its content in the samples (Zhu, 2011). To investigate the effect of light on the stability of glutathione in the supernatant, 40 ml of supernatant was placed in a transparent bottle (with light), and another 40 ml was placed in a bottle which was in a black plastic bag (without light). The absorbance of the samples was monitored to determine how the concentration of glutathione in supernatant in the two bottles varied with time.

Aqueous glutathione solutions at the same initial concentration of glutathione in the supernatant from hot water extraction were prepared. 40 ml of aqueous glutathione

solution was placed in a transparent bottle, and another 40 ml was placed in a bottle which was in a black plastic bag. Their absorbance variations, if any, with time were monitored respectively.

Chapter 4

Results and Discussion

4.1 Extraction and Concentration of Glutathione from Yeast

The absorbance of the solution (the mixture of 0.4g dry yeast and 10 ml 0.1N HCl) was 0.870. The concentration of glutathione in the solution can be calculated from the calibration curve of aqueous glutathione solution (Fig.A.1) and the value of the concentration was 3.382 mM. In addition, the volume of the solution was 10 ml and consequently the moles of glutathione in the solution was

$$n = c \cdot V = 3.382 \times 10^{-3} \times 10 \times 10^{-3} = 3.382 \times 10^{-5} \text{ mol}$$

The mass of glutathione in the solution was

$$m = n \cdot M = 3.382 \times 10^{-5} \times 307.32 = 0.01039 \text{ g}$$

Therefore, glutathione content in the dry yeast was

$$\frac{0.01039}{0.4} \times 100\% = 2.6\%$$

The absorbance of the solution (the mixture of 0.4g supernatant from hot water extraction and 10 ml 0.1N HCl) was 0.080. The concentration of glutathione in the solution can be calculated from the calibration curve of aqueous glutathione solution (Fig.A.1) and the value of the concentration was 0.3082 mM. Therefore, the glutathione content in supernatant (liquid yeast extract) was

$$\frac{0.3082 \times 10^{-5} \times 307.32 \times 100\%}{0.4} = 0.2368\%$$

It means that the concentration of glutathione in supernatant was 2.368 mg/ml. In

addition, 10 g of dry yeast and 100 g of water were used to get 81 g of supernatant in hot water extraction process. Therefore, glutathione recovery efficiency in this step can be calculated as follows

W_{ye} = Weight of liquid yeast extract harvested (g) = 81g

GSH_{ye} = Glutathione content in liquid yeast extract (%) = 0.2368%

W_y = Weight of dry yeast used for glutathione extraction (g) = 10g

GSH_y = Glutathione content in dry yeast (%) = 2.6%

Glutathione recovery efficiency (%) = $(W_{ye} \times GSH_{ye}) / (W_y \times GSH_y) \times 100\%$ = [(81 × 0.2368%) / (10 × 2.6%)] × 100% = 73.8%

3.1 g of dry matter was obtained after drying 81 g of supernatant, and consequently the dry matter content of the supernatant was

$$\frac{3.1}{81} \times 100\% = 3.83\%$$

The mass fraction of glutathione on dry basis was

$$\frac{2.368 \times 81}{3.1} = 61.87 \text{ mg glutathione/ g dry matter}$$

Next, 81 g of supernatant was passed through a UF membrane for about 4 h under a gauge pressure of 0.2 MPa. As a result, 76g of UF permeate and 5 g of UF retentate were obtained.

The absorbance of the solution (the mixture of 0.4g UF permeate and 10 ml 0.1N HCl) was 0.066. The concentration of glutathione in the solution can be calculated from the calibration curve of aqueous glutathione solution (Fig.A.1) and the value of the concentration was 0.2537 mM. Therefore, the glutathione content in UF permeate was

$$\frac{0.2537 \times 10^{-5} \times 307.32 \times 100\%}{0.4} = 0.1949\%$$

It means that the concentration of glutathione in UF permeate was 1.949 mg/ml.

2.57 g of dry matter was obtained after drying 76 g of UF permeate. Therefore, the mass fraction of glutathione on dry basis was

$$\frac{1.949\times 76}{2.57} = 57.64 \text{ mg glutathione/ g dry matter}$$

The absorbance of the solution (the mixture of 0.4g UF retentate and 10 ml 0.1N HCl) was 0.087. The concentration of glutathione in the solution can be calculated from the calibration curve of aqueous glutathione solution (Fig.A.1) and the value of the concentration was 0.3354 mM. Therefore, the glutathione content in UF retentate was

$$\frac{0.3354\times 10^{-5}\times 307.32\times 100\%}{0.4} = 0.2577\%$$

It means that the concentration of glutathione in UF retentate was 2.577 mg/ml.

0.229 g of dry matter was obtained after drying 5 g of UF retentate. Therefore, the mass fraction of glutathione on dry basis was

$$\frac{2.577\times 5}{0.229} = 56.27 \text{ mg glutathione/ g dry matter}$$

Then, 76 g of UF permeate was concentrated using a NF membrane for about 25 h under a gauge pressure of 0.8 MPa. 47g of NF permeate and 29 g of NF retentate were obtained.

The absorbance of the solution (the mixture of 0.4g NF permeate and 10 ml 0.1N HCl) was 0.001. The concentration of glutathione in the solution can be calculated from the calibration curve of aqueous glutathione solution (Fig.A.1) and the value of the concentration was 0.0008 mM. Therefore, the glutathione content in NF permeate was

$$\frac{0.0008\times 10^{-5}\times 307.32\times 100\%}{0.4} = 0.00006\%$$

It means that the concentration of glutathione in NF permeate was 0.006 mg/ml.

0.387 g of dry matter was obtained after drying 47 g of NF permeate; therefore, the mass fraction of glutathione on dry basis was

$$\frac{0.006\times 47}{0.387} = 0.726 \text{ mg glutathione/ g dry matter}$$

The absorbance of the solution (the mixture of 0.4g NF retentate and 10 ml 0.1N HCl) was 0.171. The concentration of glutathione in the solution can be calculated from the calibration curve of aqueous glutathione solution (Fig.A.1) and the value of the concentration was 0.6623 mM. Therefore, the glutathione content in NF retentate was

$$\frac{0.6623\times 10^{-5}\times 307.32\times 100\%}{0.4} = 0.5088\%$$

It means that the concentration of glutathione in NF retentate was 5.088 mg/ml.

1.826 g of dry matter was obtained after drying 29 g of NF retentate. Therefore, the mass fraction of glutathione on dry basis was

$$\frac{5.088\times 29}{1.826} = 80.81 \text{ mg glutathione/ g dry matter}$$

Therefore, glutathione recovery efficiency in NF concentration step was

$$\frac{29\times 0.5088\%}{76\times 0.1949\%}\times 100\% = 99.6\%$$

Hot water extraction conditions and operation conditions for ultrafiltration and nanofiltration were shown in Table 4.1, Table 4.2 and Table 4.3, respectively.

Table 4.1 Hot-water extraction conditions

Hot-water temperature (°C)	Extraction time (min)	Concentration of glutathione in supernatant obtained (mg/ml)
78	10	2.368

Table 4.2 Operation conditions for ultrafiltration

Feed solution	Gauge pressure (MPa)	Glutathione concentration in feed (mg/ml)	Temperature (°C)
Supernatant from yeast	0.2	2.368	23

Table 4.3 Operation conditions for nanofiltration

Feed solution	Gauge pressure (MPa)	Glutathione concentration in feed (mg/ml)	Temperature (°C)
UF permeate from supernatant	0.8	1.949	23

Fig. 4.1 shows the flow chart of the process described above. These results demonstrated that glutathione in the supernatant was highly concentrated and proved that membrane separation was a promising way to recover glutathione from yeast solution.

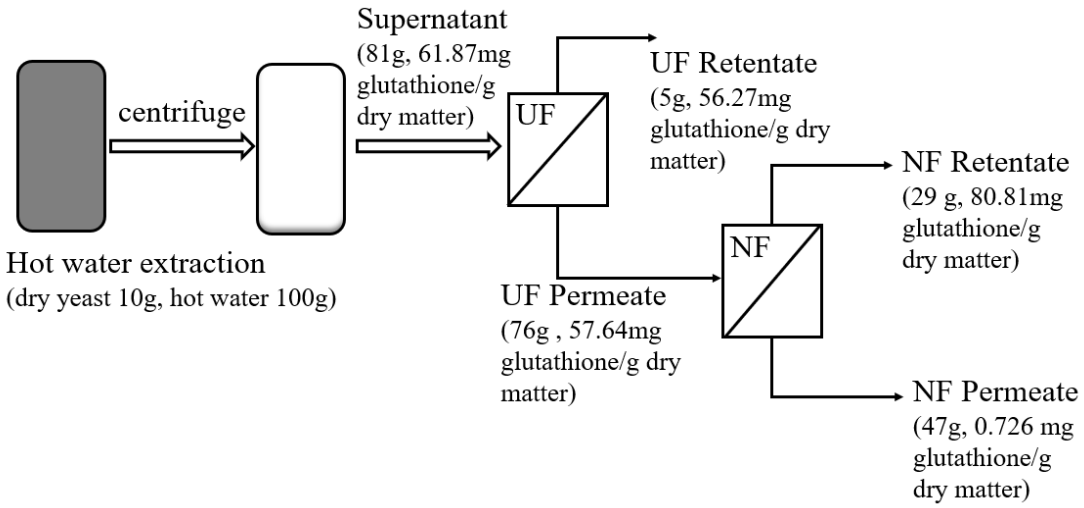


Figure 4.1 Extraction and concentration of glutathione from yeast.

4.2 Ultrafiltration Performance

81 g of supernatant obtained from hot water extraction was passed through an UF membrane for about 4 h. Volume of accumulated permeate increased with time, and concentration of glutathione in UF permeate increased with time, as shown in Fig 4.2. Function of volume of accumulated permeate versus time was made by polynomial fitting. The first derivative of this function was used to obtain the change of flux with time. The molecular weight of water was much smaller than that of glutathione. Therefore, at the beginning of the process, water was easier to pass through the membrane, which caused the concentration of glutathione in permeate was relatively low at the beginning, and then the concentration gradually increased to 1.949 mg/ml.

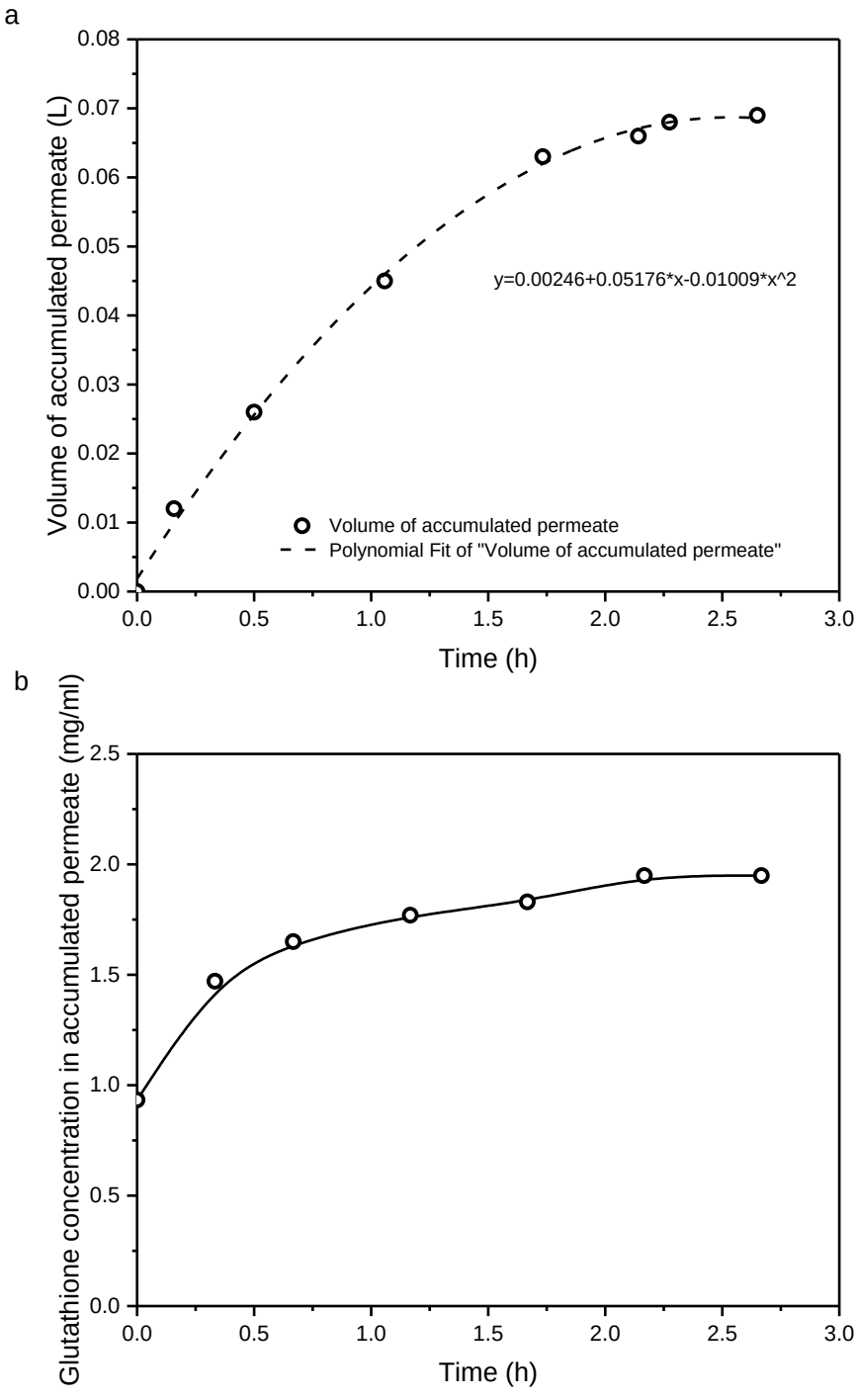


Figure 4.2 (a) Change of volume of accumulated permeate with time by UF; (b) change of glutathione concentration in UF permeate with time.

Volume concentration ratio (VCR) was the ratio of volume of feed to volume of residue. As UF went on, the volume of residue decreased, so VCR increased from 1 to 6.6, as shown in Fig. 4.3 (a). Glutathione recovery efficiency in permeate refers to the mass of glutathione in permeate as a percentage of the mass of glutathione in feed. Fig. 4.3 (b) shows that glutathione recovery efficiency in permeate increased as VCR increased. More and more glutathione went into the permeate as the volume of residue decreased, and consequently glutathione recovery efficiency in permeate increased as VCR increased.

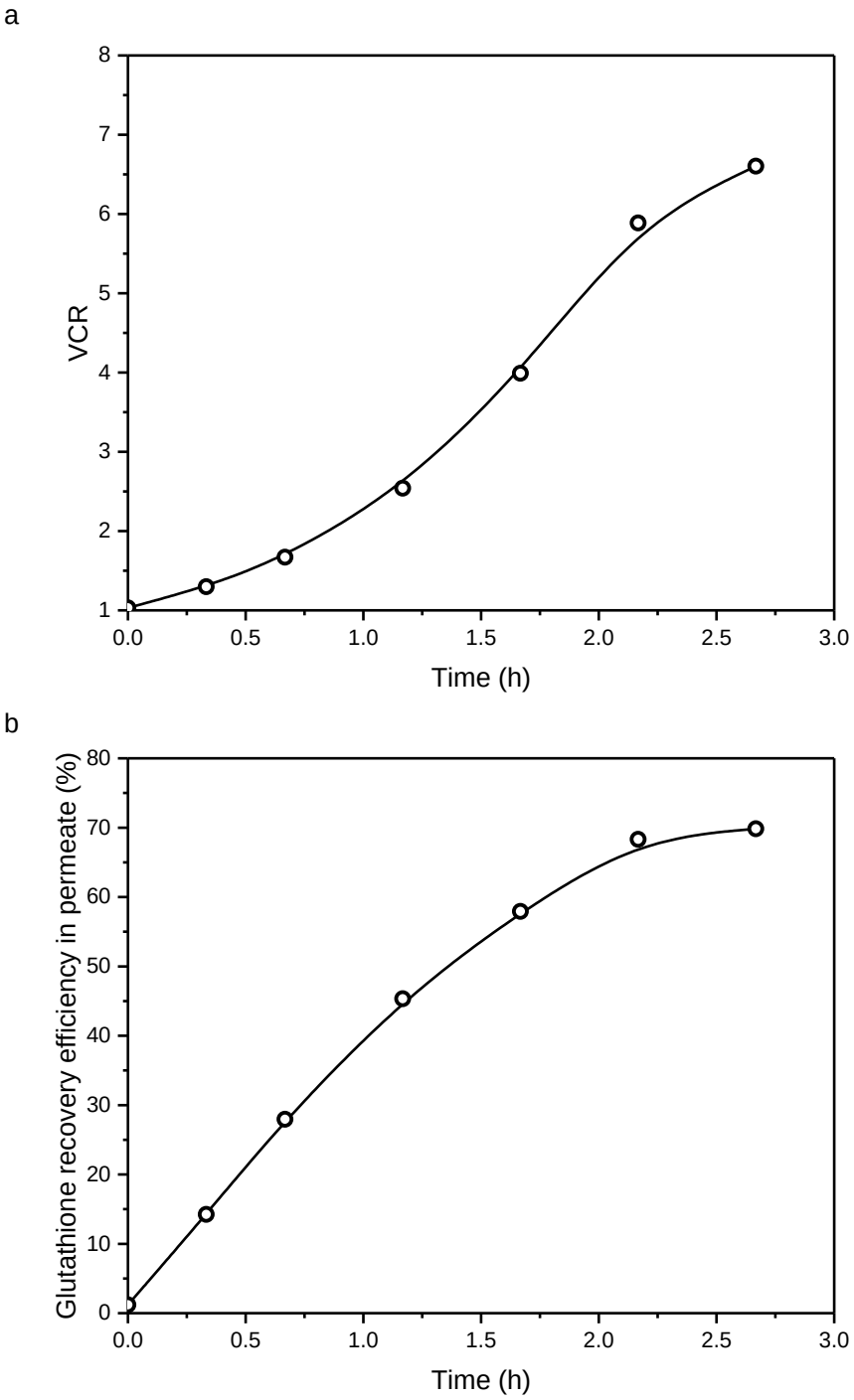


Figure 4.3 (a) Change of volume concentration ratio (VCR) with time; (b) change of glutathione recovery efficiency in UF permeate with time.

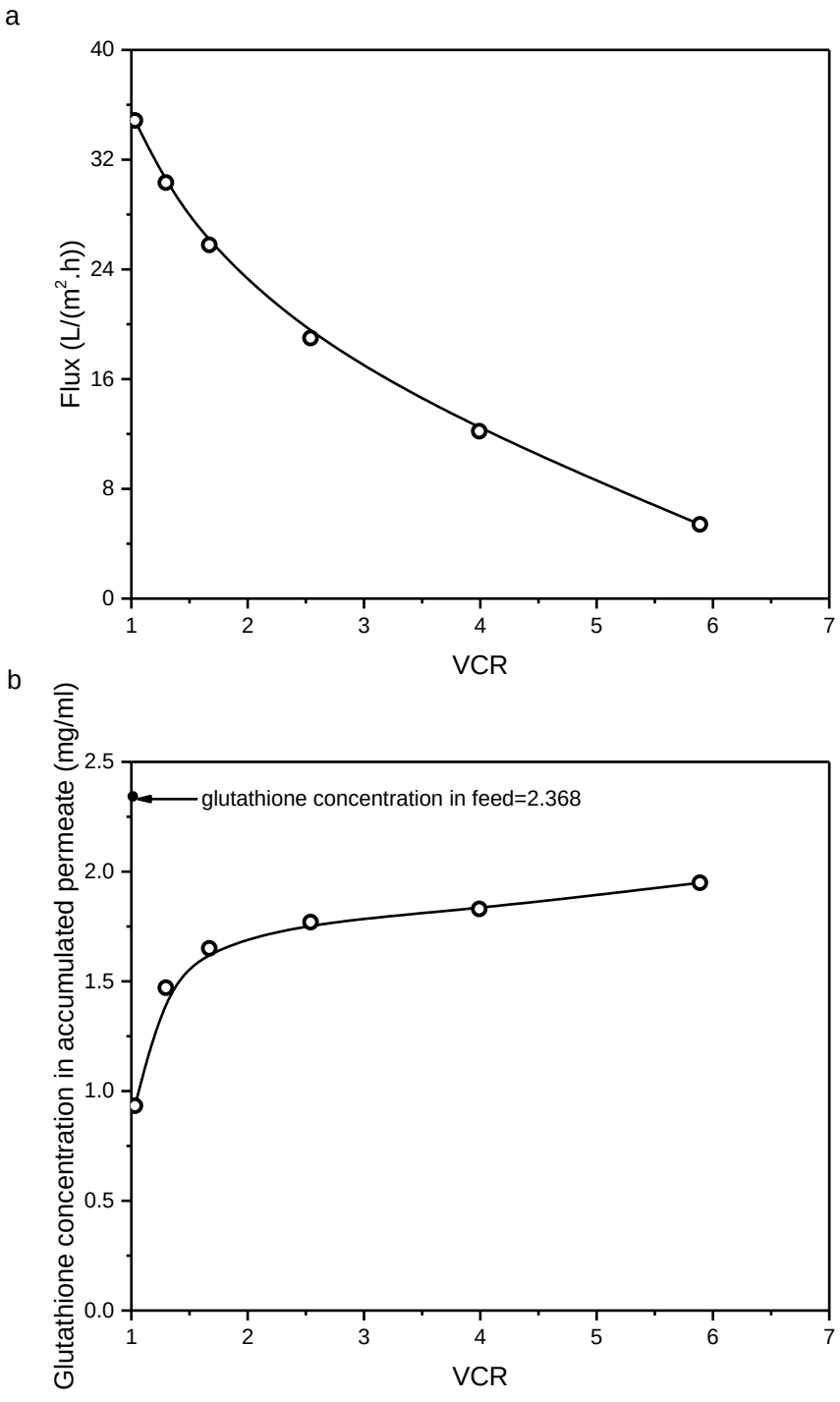


Figure 4.4 (a) Change of flux with VCR by UF; (b) change of glutathione concentration in accumulated permeate with VCR.

Fig. 4.4 (a) shows that the flux decreased as VCR increased. Macromolecules in supernatant, such as proteins, were rejected by UF membrane; however, low molecular weight components can pass through UF membrane freely. The decline in flux was caused by the concentration of retentate and concentration polarization.

Solvent (water) and solutes which contains some macromolecules are carried towards the membrane surface when the solution is permeating the membrane. The water molecules permeate the membrane, but the larger solute molecules accumulate at the membrane surface. Due to their size, the rate at which the retained solute molecules can diffuse from the surface of the membrane back to the bulk solution is quite low. Thus, the concentration of macromolecules at the membrane surface are much higher than those in the feed solution. This phenomenon is called concentration polarization.

Because of concentration polarization, first, the osmotic pressure difference across the membrane increased, and consequently the water flux decreased, as mentioned before. Second, macromolecules retained by UF membrane formed a gel layer on the membrane surface. Then, it becomes a barrier for the solution to pass through the membrane, which caused the decline in flux. For ultrafiltration, concentration polarization is a key factor determining the performance of the membrane, and it can cause membrane fouling because of deposition of retained macromolecules and colloids on the membrane surface, which is illustrated in Fig. 4.5.

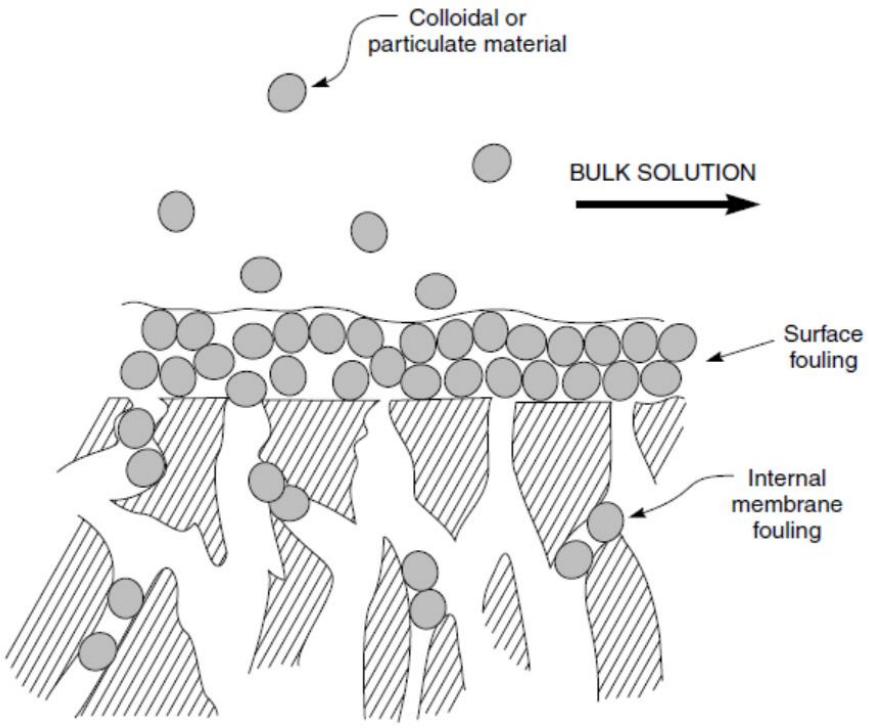


Figure 4.5 Schematic diagram of fouling on a UF membrane (Baker, 2004).

Glutathione concentration in UF permeate increased as VCR increased, as shown in Fig. 4.4 (b). It was caused by two reasons. On one hand, glutathione had a strong interaction with the retained macromolecules in supernatant, such as Van der Waals' force, electrostatic interactions, and polar interactions. Glutathione can be adsorbed to macromolecules by these interactions. As UF went on, the concentration of macromolecules at the surface of the membrane increased. Therefore, the concentration of glutathione at the surface of the membrane also increased. Then, the concentration of glutathione in the permeate increased.

0.192 g of glutathione was added to 80.81 g of water to make 81 g of aqueous glutathione solution with glutathione concentration of 2.368 mg/ml, which was the same as the concentration in supernatant passed through the UF membrane. This

aqueous glutathione solution was passed through the UF membrane, 78.8g of UF permeate with glutathione concentration of 2.361 mg/ml was obtained. The permeation flux of aqueous glutathione solution kept at 80.81 L/(m^2.h), as shown in Fig. 4.6. It was much higher than the initial flux of supernatant (34.9 L/(m^2.h)). These results proved that glutathione indeed had some interactions with macromolecules in supernatant.

On the other hand, as mentioned before, the water flux is related to concentration gradients and pressure across the membrane. The osmotic pressure of a solution increases exponentially with the concentration. Concentration gradients increased due to the macromolecules accumulated on the membrane surface, and consequently the osmotic pressure difference increased. In addition, the pressure difference across the membrane was kept at a gauge pressure of 0.2 MPa. Therefore, the water flux decreased. Water passed through the membrane more and more slowly, which caused the increase of glutathione concentration in UF permeate.

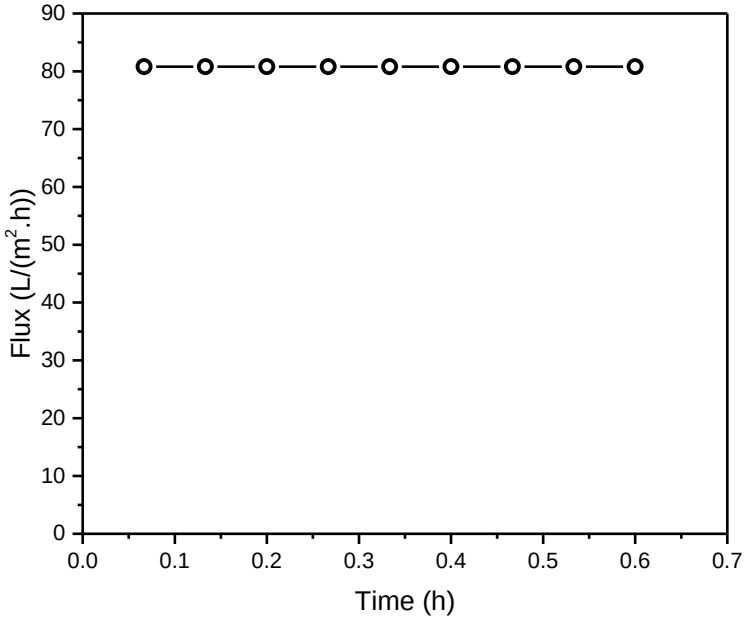


Figure 4.6 The decrease in flux over time by UF. (for aqueous glutathione solution)

For supernatant, glutathione concentration in UF feed, UF permeate and UF retentate was 2.368, 1.949 and 2.577 mg/ml, respectively. Therefore, the final rejection of glutathione by the UF membrane was 17.7%. For aqueous glutathione solution, glutathione concentration in UF feed and UF permeate was 2.368 and 2.361 mg/ml, respectively, and consequently the glutathione rejection was 0.3%. The molecular weight cut-off of the UF membrane was 10 kDa, which means the molecular weight of the molecule that is 90% retained by the membrane was 10,000 g/mol. The molecular weight of glutathione is 307.32 g/mol. Theoretically, the UF membrane can reject neither glutathione nor water. The glutathione rejection for supernatant should also be almost zero, and the concentration of glutathione in UF retentate and UF permeate should be the same. However, the concentration of glutathione in UF retentate was

higher than that in UF permeate. It is because the membrane also rejected glutathione while retaining macromolecules, since glutathione had some interactions with macromolecules in supernatant, as mentioned before.

4.3 Nanofiltration Performance

Glutathione in UF permeate from supernatant was concentrated using the NF membrane for about 78 h under a gauge pressure of 0.8 MPa. Fig. 4.7 (a) shows the change of volume of accumulated NF permeate with time. Fig. 4.7 (b) shows the change of concentration of glutathione in NF permeate and NF residue with time. Glutathione concentration in NF permeate was almost 0, and the concentration in NF residue increased with time, from 1.949 to 10.71mg/ml. Function of volume of accumulated permeate versus time was also made by polynomial fitting. The first derivative of this function was used to obtain the change of flux with time.

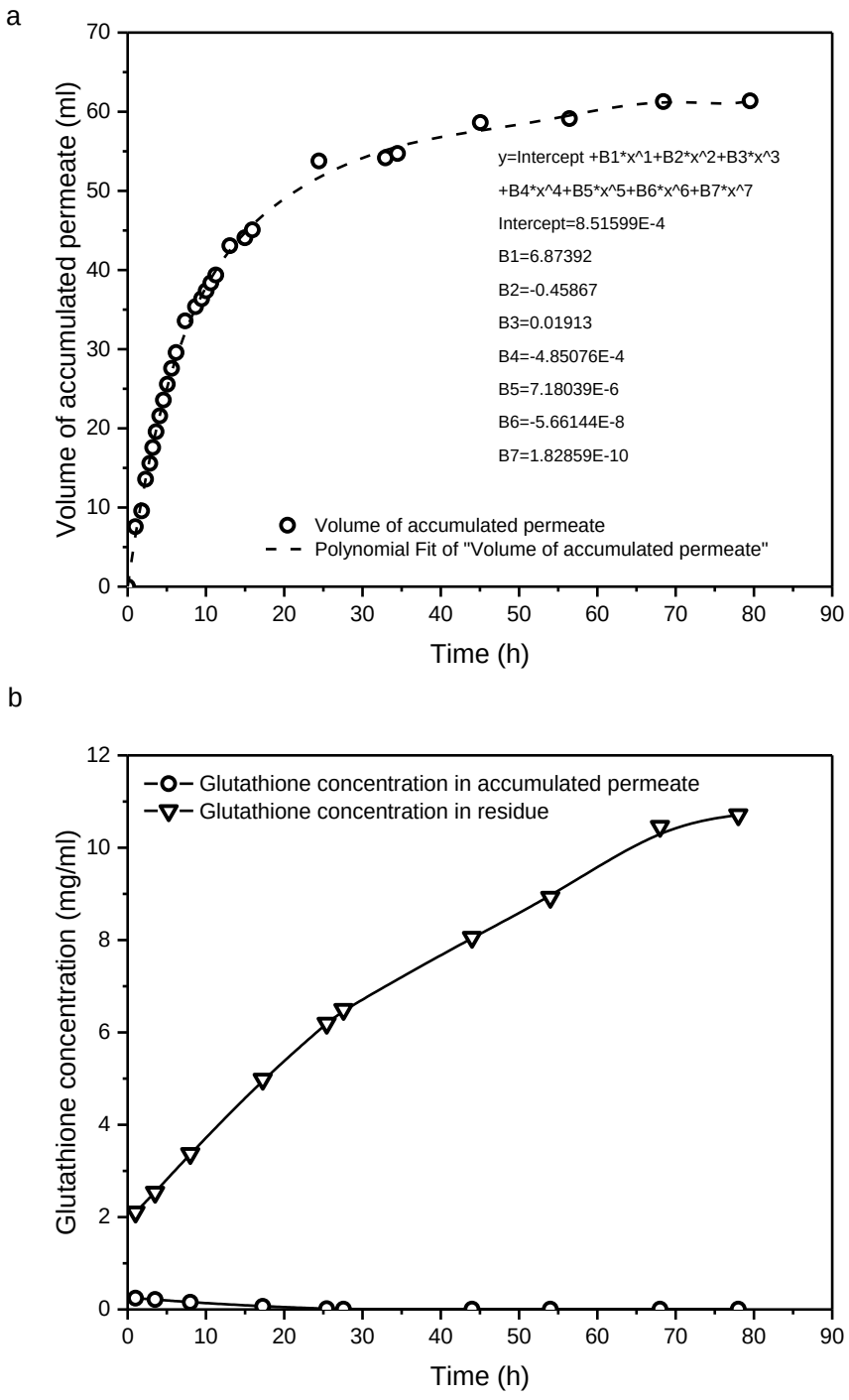


Figure 4.7 (a) Change of volume of accumulated permeate with time by NF; (b) change of glutathione concentration in NF permeate and NF residue with time.

As NF went on, the volume of residue decreased, and VCR increased to 5.5 after 78 h, as shown in Fig. 4.8 (a). Glutathione retention efficiency in residue refers to the mass of glutathione in residue as a percentage of the mass of glutathione in feed. Fig. 4.8 (b) shows that glutathione retention efficiency in residue was almost 100%, as shown in Fig. 4.8 (b). Fig. 4.9 (a) shows that the flux decreased as VCR increased, from 4.05 to 0.05 L/(m^2.h). At first, the concentration of retentate and concentration polarization caused the decline in flux. As the NF process went on, some other substances in solution, such as some peptides and amino acids, were also deposited on the membrane surface. It caused membrane fouling, which result from the deposition of other retained solutes on the membrane. Thus, membrane fouling is another reason for the decline of flux in NF systems. In addition, the operation pressure for NF was high, and consequently a dense layer of deposition was formed on the membrane surface, as shown in Fig. 4.10. It caused the resistance of the cake layer increased, and consequently the flux decreased. Glutathione concentration in NF residue increased as VCR increased, and glutathione concentration in NF permeate decreased as VCR increased, as shown in Fig. 4.9 (b). It is because the NF membrane can reject glutathione. The deposition can also reject glutathione, partly causing the decrease of glutathione concentration in NF permeate. In addition, glutathione had some interactions with other substances in residue, and consequently it can be attached to other molecules in NF residue. In the end, the concentration of glutathione in NF permeate decreased to almost 0 mg/ml, and the concentration in NF residue increased to 10.71 mg/ml.

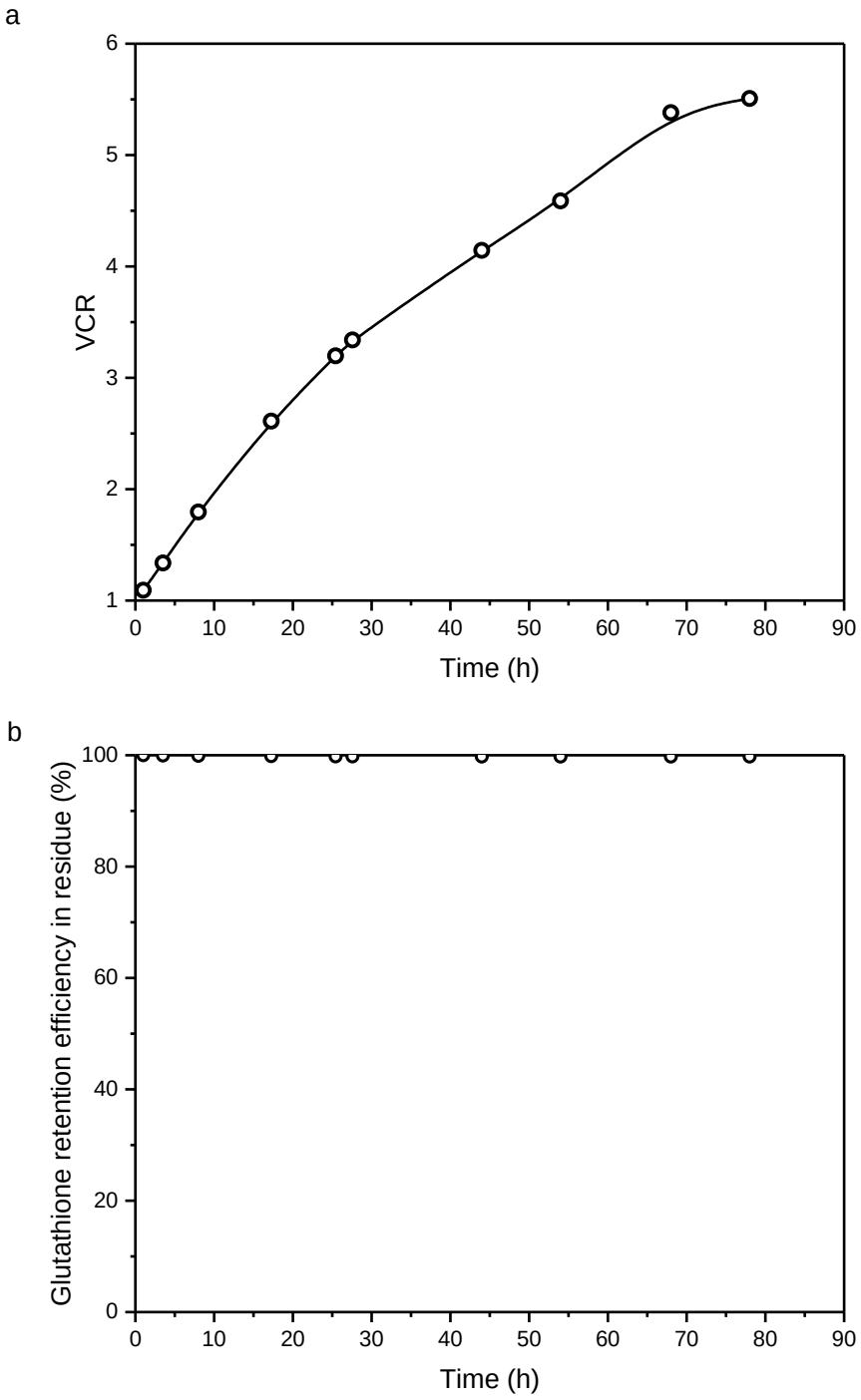


Figure 4.8 (a) Change of volume concentration ratio (VCR) with time; (b) change of glutathione retention efficiency in NF residue with time.

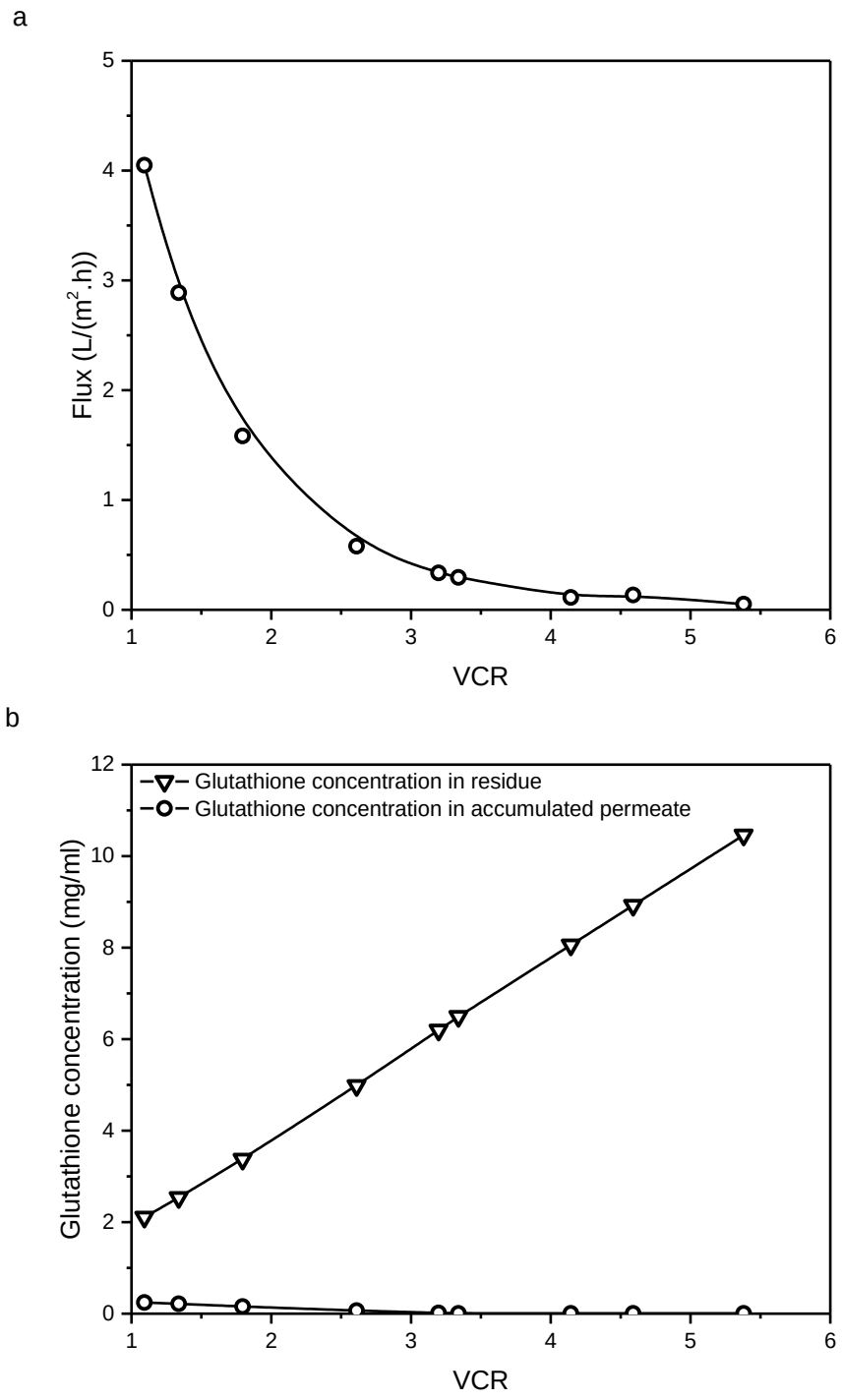


Figure 4.9 (a) Change of flux with VCR by NF; (b) change of glutathione concentration in residue and accumulated permeate with VCR.

76g of UF permeate from aqueous glutathione solution with glutathione concentration of 2.361 mg/ml was passed through the NF membrane. 72g of NF permeate and 3.784 g of NF retentate were obtained. The glutathione concentration in NF retentate was 47.4mg/ml, and that in NF permeate was almost 0 mg/ml.

Glutathione concentration in the NF feed, NF permeate and NF retentate was 1.949, 0.006 and 5.088 mg/ml respectively when feed solution was UF permeate from supernatant. Therefore, the rejection of glutathione by the NF membrane was 99.7% when feed solution was UF permeate from supernatant. The rejection of glutathione was almost 100% when the feed solution was the UF permeate from aqueous glutathione solution. It is because the molecular weight cut-off of the NF membrane was 200 Da, which means the molecular weight of the molecule that is 90% retained by the membrane was 200 g/mol. Therefore, this NF membrane is appropriate to reject glutathione with molecular weight of 307.32 g/mol.

Figure 4.10 Photograph of the used NF membrane after cleaning by water washing.

4.4 Effects of Hot-water Temperature and Extraction Time on Glutathione Extracted

Effects of Hot-water Temperature and Extraction Time on Glutathione Extracted was investigated using 10 g of dry yeast and 100 g of hot water with different temperature. The concentration of glutathione in supernatant increased over time and then decreased under each temperature, as shown in Fig. 4.11. The concentration reached its maximum when extraction time was 10 to 12 min. At 73°C, the concentration of glutathione changed most significantly over time. Below 73°C, the maximum concentration of glutathione increased with temperature. Above 73°C, the maximum concentration of glutathione decreased with the increase of temperature, as shown in Fig 4.12. The maximum concentration of glutathione was 2.487 mg/ml, which obtained when extraction time was 12 min and hot water temperature was 73°C.

Glutathione in yeast diffused outward slowly when the temperature was low. When the temperature was high, glutathione diffused rapidly from yeast; however, the extracted glutathione oxidized fast at the same time. Therefore, there was a proper temperature at which most glutathione was extracted. In the beginning, the longer the extraction time, the more glutathione was extracted. Later, the content of glutathione in yeast was too little to be extracted, and the extracted glutathione was continuously oxidized. Therefore, the concentration of glutathione in supernatant first increased and then decreased over time under each temperature.

The previous experiment of hot water extraction was carried out at 78°C with the extraction time of 10 min, and the concentration of glutathione was 2.368 mg/ml, which

was not quite different from the maximum concentration of glutathione. When extraction time was over 12 min, the concentration of glutathione was higher at relatively low temperature under the same extraction time. For example, when the extraction time was 25 to 60 min, the highest concentration of glutathione was obtained at 67°C under the same extraction time.

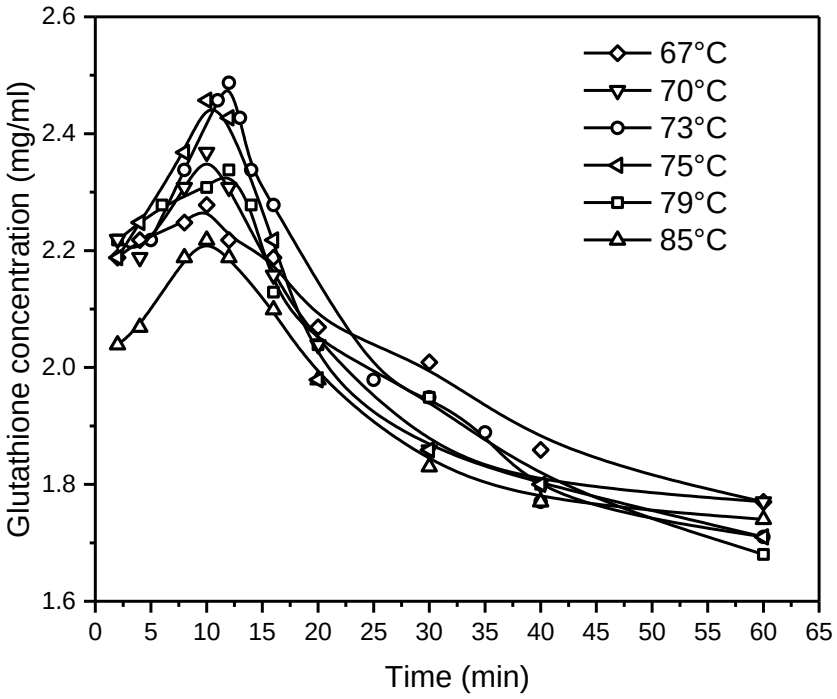


Figure 4.11 Effect of heating time on glutathione concentration under different temperature.

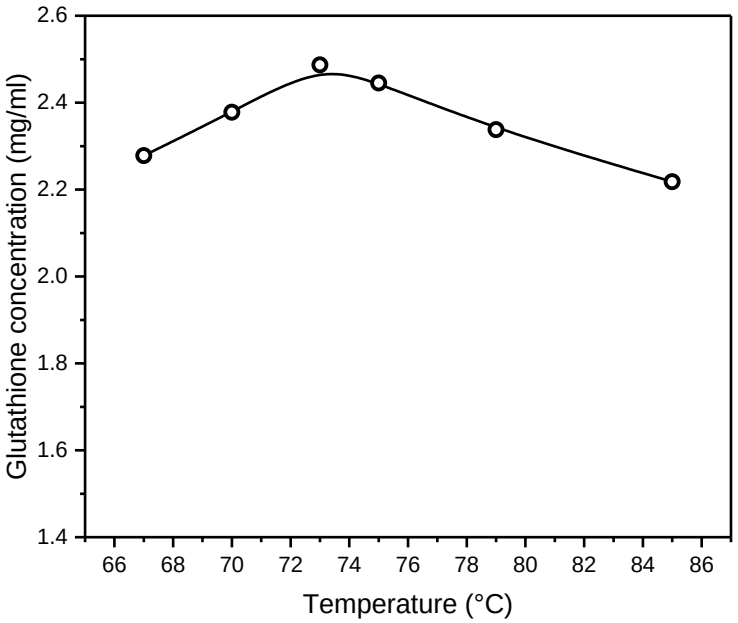


Figure 4.12 The maximum concentration of glutathione under different temperature.

4.5 Effect of Light on Stability of Glutathione in Supernatant

A researcher found that light has an effect on the stability of glutathione in aqueous glutathione solution (Zhu, 2011). He found that the content of glutathione in the solution stored in a brown bottle decreased slower than that stored in a transparent bottle. Therefore, this study investigated the effect of light on the stability of glutathione in supernatant extracted from yeast as well as in aqueous glutathione solution.

The concentrations of glutathione in both supernatant and aqueous glutathione solution decreased over time. In addition, the concentrations of glutathione in these solutions decreased slower and slower over time, and they did not decrease in the end, as shown in Fig. 4.13. As mentioned before, glutathione can be oxidized to glutathione

disulfide in aqueous solution. It is a reversible reaction and consequently the concentration of glutathione in the solution did not change when the reaction reached equilibrium (McGraw-Hill, 1995; Nagy and Ashby, 2007). In addition, antioxidants like sodium hyposulfite and ascorbic acid were added to these solutions, but no significant improvements in preserving the reduced form of glutathione were observed.

The initial concentration of glutathione in both supernatant extracted from yeast and aqueous glutathione solution was 2.368 mg/ml, which was the same as the concentration of glutathione in supernatant that passed through the UF membrane. Concentration of glutathione in supernatant with light decreased faster over time than that without light, and so is it in aqueous glutathione solution, as shown in Fig. 4.13. It suggests that light can promote the oxidation of glutathione in aqueous solution.

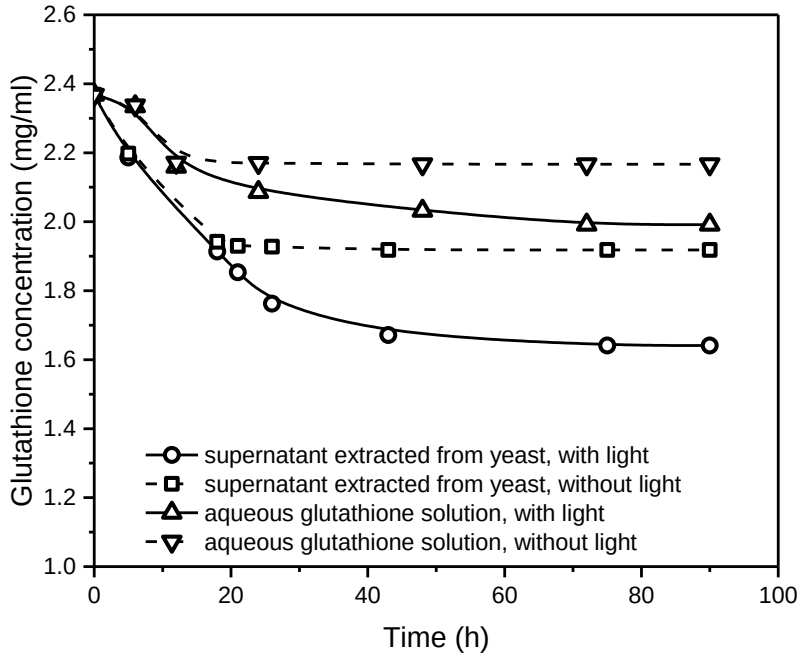


Figure 4.13 Effect of light on stability of glutathione in supernatant extracted from yeast and aqueous glutathione solution.

The concentration of glutathione in supernatant decreased faster over time than that in aqueous glutathione solution whether there was light or not, as shown in Fig. 4.13. It suggests that other substances in supernatant, such as proteins, nucleic acids, peptides, polypeptides and amino acids (e.g. lysine, threonine and tryptophan) may have an effect on the stability of glutathione. It is partly because some chemical bonds in proteins and amino acids can be broken when exposed to light (Witt, 2008).

In this study, there was a loss of glutathione during ultrafiltration and nanofiltration, and the remain ratio of glutathione after these processes can be calculated. The content of glutathione in supernatant, UF permeate, UF retentate, NF permeate and NF retentate was shown in Table 4.4.

Table 4.4 Glutathione content in supernatant, UF permeate, UF retentate, NF permeate and NF retentate

Sample	Glutathione content mg glutathione/g sample	Sample weight/g
Supernatant	2.368	81
UF permeate	1.949	76
UF retentate	2.577	5
NF permeate	0.006	47
NF retentate	5.088	29

Therefore, the remain ratio of glutathione after ultrafiltration was

$$\frac{76\times1.949+2.577\times5}{81\times2.368}\times100\%=83.9\%$$

In the experiment of ultrafiltration, UF permeate was collected by a glass beaker which was not in a black plastic bag. In other words, the experiment of ultrafiltration was carried out with light. It took about 12 h from the start of ultrafiltration to the measurement of the absorbance of UF permeate and UF retentate. Fig. 4.13 shows that the remain ratio of glutathione in supernatant with light was about 85% after 12 h, which is consistent with the results of the ultrafiltration experiment.

The remain ratio of glutathione after nanofiltration was

$$\frac{0.006\times47+5.088\times29}{76\times1.949}\times100\%=99.8\%$$

In the experiment of nanofiltration, NF permeate was collected by a small glass beaker in a black plastic bag, and NF retentate was in the stainless membrane cell. Therefore, the experiment of nanofiltration was carried out without light. The feed solution of the experiment of nanofiltration was UF permeate which contained much less macromolecules than supernatant. As a result, the constituents in NF feed was more similar to the aqueous glutathione solution than the supernatant. Fig. 4.13 shows that the content of glutathione in aqueous glutathione solution without light did not decrease after 12h, and consequently there was no loss of glutathione during nanofiltration. This is consistent with the results of the nanofiltration experiment.

Chapter 5

Conclusions and Recommendations

5.1 Conclusions

This study dealt with the extraction of glutathione from yeast using hot water, followed by ultrafiltration and nanofiltration to concentrate glutathione. The effects of hot water temperature, extraction time and light on the content of glutathione in supernatant were investigated. The performance of membranes used in this study was also tested. It showed that glutathione recovery efficiency was high by this method. The following conclusions can be drawn from this study.

(1) The glutathione content in dry yeast was 2.6%. Glutathione recovery efficiency from hot water extraction was 73.8%. Glutathione recovery efficiency in the NF concentration step was 99.6%. It demonstrated that glutathione in the supernatant was highly concentrated and proved that membrane separation was a promising way to recover glutathione from aqueous yeast solution.

(2) The permeation flux decreased over time by UF mainly because of the concentration of retentate and concentration polarization. The concentration of glutathione in UF permeate increased over time because glutathione had some interactions with the retained macromolecules in the supernatant. The rejection of glutathione by UF membrane was 17.7%.

(3) The permeation flux decreased over time by NF due to the concentration of retentate, concentration polarization and membrane fouling. The concentration of

glutathione in NF permeate gradually decreased to almost 0 mg/ml. The rejection of glutathione by NF membrane was 99.7%. The ultrafiltration and nanofiltration membrane exhibited an outstanding performance for purification and concentration of glutathione.

(4) The concentration of glutathione in supernatant first increased and then decreased over time under each temperature. The maximum concentration of glutathione in supernatant was 2.487 mg/ml under 73°C when heating time was 12 min.

(5) Light can promote the oxidation of glutathione in aqueous solution. In addition, other substances in the supernatant extracted from yeast may have an effect on the stability of glutathione.

5.2 Recommendations

Based on this research, the followings are recommended for further study:

(1) Pure water flux of the ultrafiltration and nanofiltration membrane can be tested. The membranes should be cleaned by water washing after use and then the pure water flux should be tested again. Membrane contamination degree can be obtained by comparing these fluxes.

(2) A larger range of hot water temperature should be carried out to find the most accurate temperature that can give the maximum concentration of glutathione in supernatant.

(3) Other factors may also influence the stability of glutathione in supernatant, such as pH, so the influence of other factors should also be investigated.

(4) The composition of the supernatant should be explored because some substances in supernatant have interactions with glutathione.

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Appendix A

A.1 Calibration Curve of Glutathione (GSH)

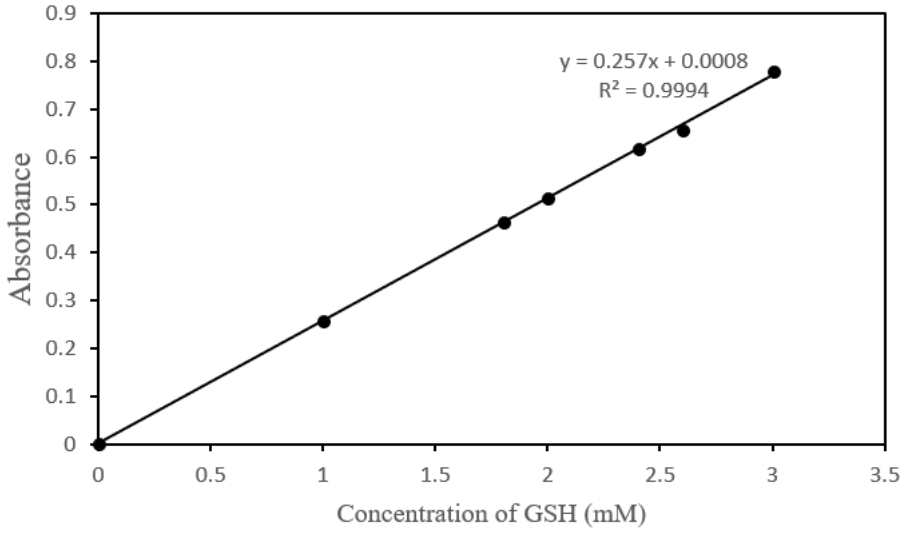

Figure. A.1 UV-Vis Spectrophotometer calibration curve for glutathione aqueous solution at 412 nm.

A.2 Sample Calculations

Permeation flux

Using aqueous glutathione solution passing through a UF membrane as an example,

Feed: aqueous glutathione solution

Effective membrane area (A): 14.85 cm^2

Time interval: 20 min

Quantity of permeate collected (Q): 40 ml

Permeate flux: $J = \frac{Q}{A\Delta t} = \frac{40\times10^{-3}}{14.85\times10^{-4}\times\frac{20}{60}} = 80.81 L/m^2.h$

Glutathione rejection

Using supernatant passing through a UF membrane as an example,

Concentration of glutathione in the feed (C_f): 2.368 mg/ml

Concentration of glutathione in the permeate (C_p): 1.949 mg/ml

$$R = (1-\frac{c_p}{c_f})\times100\% = \left(1-\frac{1.949}{2.368}\right)\times100\% = 17.7\%$$