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# An investigation into the impact of cell metabolic activity on biofilm formation and flux decline during cross-flow filtration of cellulose acetate ultrafiltration membranes

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A Thesis

entitled

# An Investigation into the Impact of Cell Metabolic Activity on Biofilm Formation and Flux Decline during Cross-flow Filtration of Cellulose Acetate Ultrafiltration Membranes

By

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Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Master of Science Degree in Civil Engineering

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An Abstract of

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Membrane filtration is an effective technique used in water treatment to remove particles, organic pollutants, inorganic compounds, and microorganisms to accomplish a biologically safe and consistently high quality drinking water. One significant challenge to membrane separation technologies is membrane fouling causing pressure drop, flux decline and eventually significant cost of membrane replacement. Specifically, membrane biofouling is considered a major problem due to the capabilities of microorganisms to adapt their growth rate, multiply, and relocate even if they were 99.99% removed from the feed stream. The objective of this research was to determine the impact of metabolic activity of the pure culture of biofoulants on the membrane biofilm metabolic activity, biofilm formation rate, and operational flux decline.

In this study, the metabolic activity of *Pseudomonas fluorescens* in active, inactive, and different growth phases were investigated during cross-flow filtration using a cellulose acetate ultrafiltration (UF) membrane at different sampling times (4, 11, and 24 hours) of filtration. In accordance with previous biofilm studies, ATP was used to determine the metabolical activity of the biomass. Dehydrogenase activity assessment of the membrane biofilm using CTC was also carried out on intact biofilms.

Our results showed that after 10-12 hours of filtration, the biofilm ATP levels reach an equilibrium concentration (avg. 8 amol/cell) and do not appear to be related to biofoulant ATP levels from cells harvested in the late exponential growth phase regardless of initial ATP level. However, the bacterial growth phase affected the ATP activity of cells. Membrane biofilms formed from biofoulants in the lag and stationary phase of growth contained similar levels of ATP (avg. 1.8 amol/cell), and the exponential phase cells resulted in significant higher activity. Flux decline does not appear to be related to metabolic activity of the biofoulant or biofilm following 24 hours of filtration. Notably, there was much less (ca. 2.5%) flux decline when the biofoulant cells were inactive. Since metabolic activity determines the substrate conversion rate and the biofilm growth rate, the knowledge of the metabolic activity of the biofoulants and membrane biofilm is essential for understanding the biofilm accumulation mechanism for applying appropriate countermeasures to control membrane biofouling.

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This thesis would not have been possible without the love, support, and encouragement I received from my family. Finally, I would like to thank all those who have helped put this thesis in its final form.

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# List of Abbreviations

ATP …….. Adenosine triphosphate

CA …….. Cellulose Acetate

CTC …….. 5-cyano-2,3-ditolyl tetrazolium chloride

Da …….. Dalton

DI …….. Deionized Water

EPS ..……...….. Extracellular Polymeric Substances

INT …….. 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride

LB ……..……..... Luria-Bertani

LPS ……..……..... Lipopolysaccharides

MF ……..…….. Microfiltration

MWCO ……...…….. Molecular Weight Cut-off

NF ….……...…….. Nanofiltration

NOM ……...….. Natural Organic Matter

PG ……..... Picogreen

RFU ….…… Relative fluorescence units

RO ….…….. Reverse Osmosis

SD ……. Standard Deviation

SMP …….... Soluble Microbial Products

TOC ……...…… Total Organic Carbon

UF ……..……. Ultrafiltration

# Chapter 1

## 1.1. Overview of membrane filtration

There are approximate 1.2 billion people in developing countries that have inadequate access to water and 2.6 billion people lack basic sanitation (United Nations, 2006). In addition, millions of people die annually from diseases transmitted through unsafe water, and as the world population is expanding, the demand for potable water is increasing (Shannon *et al.*, 2008). Therefore, investigating innovative and effective methods for water treatment resulting in high quality water is necessary. Membrane separation is an attractive option for drinking water production and wastewater treatment since it produces biologically safe and consistently high quality drinking water by removing microorganisms, organic and inorganic compounds. For example, none of the available disinfection techniques (e.g., chlorine disinfection, UV irradiation) are optimum since some organisms are resistant to these techniques; however membranes can remove them (Thomas and Dillon, 1989; Jarrol *et al.*, 1981). Furthermore, membrane filtration technology is more sustainable with a smaller footprint compared to conventional water treatment due to fewer additions of chemicals to raw water in the water treatment process and lower energy requirements for operation and maintenance. In addition, costs with membrane systems can be less than or equivalent to conventional systems for some applications (Owen *et al.*, 1995).

Membrane filtration is a pressure- or vacuum-driven separation process in which particulate matter is rejected by an engineered barrier primarily through a size exclusion mechanism (USEPA, 2001). A membrane is a thin film made of synthetic or inorganic materials that is capable of separating two phases and restricts the transport of targeted chemical or biological species. The permeate outlet is the water that passes through the membrane, and the concentrate outlet is the rejected flow of waste. Membrane flux is defined as the throughput of a membrane filtration process expressed as flow per unit of membrane area.

Membrane filtration has a wide application in various scopes. Since 1960, reverse osmosis (RO) membranes have been used for the desalination of water. And, since late 1980 nanofiltration (NF) use has been more widespread for softening and removal of total organic carbon (TOC) (USEPA, 2001). Other membrane application areas range from industrial effluent cleaning, color removal, sludge dewatering, wastewater disinfection, suspended solids and pathogen removal to medical applications such as urea and other toxin removal from the blood stream in an artificial kidney (Tan and Sudak, 1992; Castelas, 1992).

Membranes are capable of removing different sized materials based on their pore size. As shown in the membrane spectrum in Figure 1.1, microfiltration (MF) can separate the colloidal and particle contaminants, ultrafiltration (UF) is capable of separation of macromolecules, whereas nanofiltration (NF) and reverse osmosis (RO) are being used in separation of ionic and organic particles.

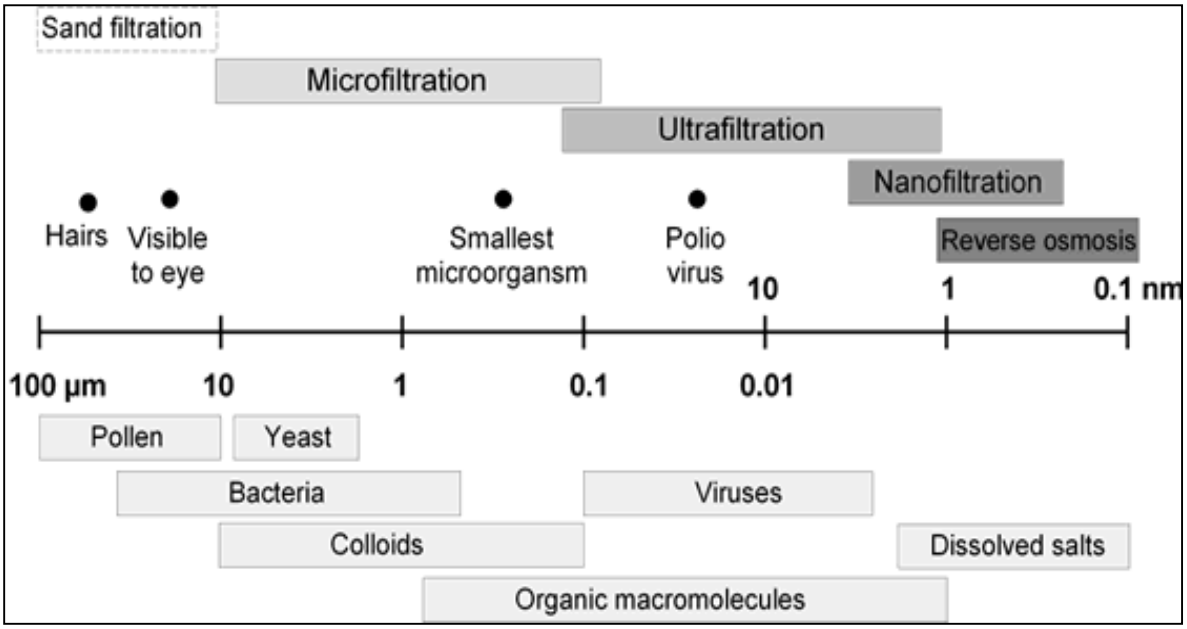
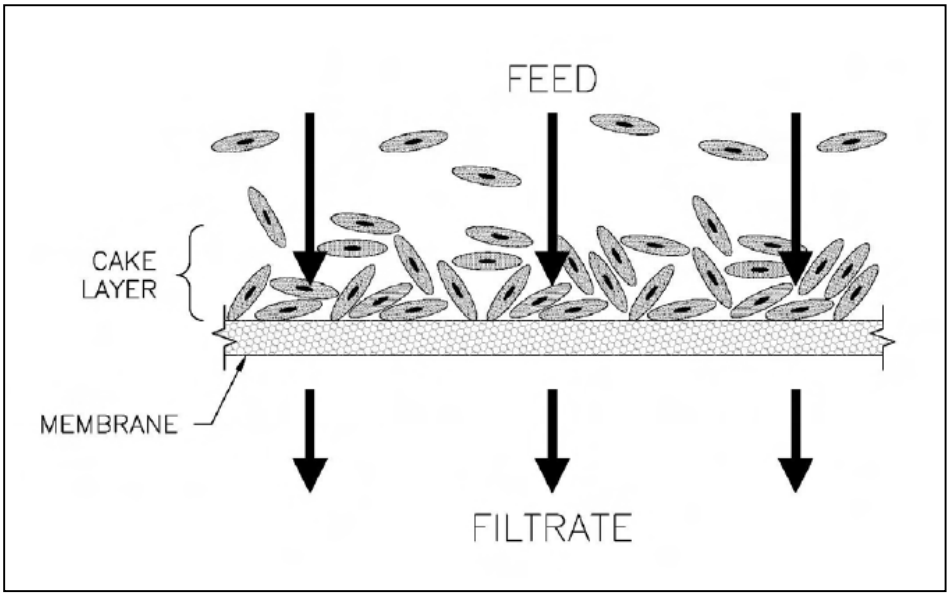



**Figure 1-1: Membrane filtration spectrum based on pore size and removal capabilities (Modified from MEMOS, 2010)**

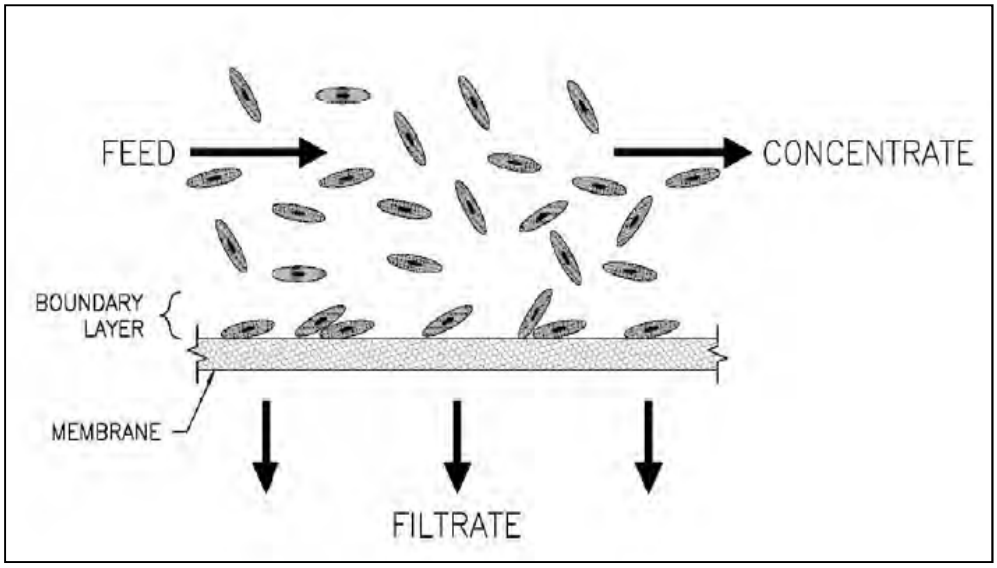
Ultrafiltration (UF) membranes can be used for separation of large macromolecules such as proteins and starches and all types of microorganism such as bacteria and virus (Aptel and Buckley, 1996). Membranes are classified by molecular weight cut-off (MWCO) which is defined as the molecular weight of the smallest molecules that can pass through the membrane pores. Ultrafiltration targets particles and molecules that range from 1,000 to 500,000 Daltons (Da) in molecular weight (Cheryan, 1998). UF membrane is an effective "stand alone" physical treatment process and has the capability of disinfection and removing water turbidity at relatively low pressure. UF membrane can be considered as a substitute for conventional drinking water treatment by simplifying treatment process by eliminating coagulation, flocculation and sedimentation processes (Ericsson and Tragardh, 1997).

Membranes can also be classified into two operation filtrations including dead-end filtration and cross-flow filtration, as shown in Figure 1-2. Particles retained by the

filter in dead-end filtration during filtration forming a cake layer resulting in an increased resistance to filtration. This requires frequent cleaning or replacement of membrane filters. Cross-flow filtration is a pressure-driven separation process where the feed stream runs tangential to the membrane, and the permeate flow is perpendicular to the feed flow, establishing a pressure differential across the membrane. This causes some of the particles to pass through the membrane and remaining particles continue to flow across the membrane. In contrast to dead-end filtration, the use of a tangential flow prevents accumulation of solute and colloids forming up a cake layer by a high velocity gradient in the boundary layer proximal to the membrane surface, which assists in reducing the fouling and polarization effects (Chen and Kim, 2006).




Dead-end filtration




Cross-flow filtration

**Figure 1-2: Dead-end and cross-flow filtration in the membrane process (Modified from USEPA, 2001)**

Membranes can be manufactured from a variety of materials including inorganic membranes such as sintered metals and ceramics, organic membranes made up polymers such as cellulose acetate (CA), cellulose diacetate (CDA), cellulose triacetate (TAC), polyamide (PA), and other aromatic polyamides. Cellulose acetate (CA) is an environmentally friendly non-toxic substance for making membranes and can be available at low cost (Bhongsuwan, 2008). Cellulose acetate membrane filters composed of cellulose di- and triacetate are demonstrating low static charge and high strength (Braganca and Rosa, 2003). Cellulose acetate (CA) membranes are hydrophilic, high throughput, uniform pore structures with very low protein binding capacity making them suitable for aqueous media and for use in pressure filtration devices.

One of the serious problems in membrane filtration is membrane fouling, causing increase of pressure drop, decrease of normalized flux, significant increase of operational

energy costs to maintain the design flow rate and ultimately causing membrane replacement. The average replacement cost for the 8-inch membranes is about 2200 USD per element for spiral wound membranes and 8800 USD per element for hollow-fiber membranes (Avlonitis *et al.*, 2003). Fouling can block the feed water side of membranes, making it necessary to increase the trans-membrane feed pressure up to 3-4 bars compared to less than 1 bar in clean membranes (Veza *et al.*, 2008).

Properties of the membrane fouling layer are dependent on the membrane properties including surface charge, hydrophobicity, roughness, and hydraulic conditions such as permeate flux, and cross-flow velocity (Frank and Belford, 2003; Gorenflo *et al.*, 2005). On the other hand, feed water solution chemistry such as pH, ionic strength and foulant characteristics affect the membrane fouling. The physicochemical characteristics of the foulant such as surface charge and molecular conformation have a direct effect on the rate of foulant accumulation (Flemming and Schaule, 1988) and eventually flux decline.

## 1.2. Biofouling

As mentioned before, fouling causes permeate membrane flux decline and increased pressure drop which becomes one of the most limiting factors in membrane technology. In addition, membrane fouling has a negative impact on process performance and causes the use of costly cleaning procedures or membrane replacement in severe conditions to maintain desired flow rate and quality.

In spite of the cross-flow component, a proportion of the feed water colloids and microorganisms entering the module are transported to the membrane surface where they

adsorb, forming a thin fouling layer. Fouling mechanisms of membranes can be categorized into four groups: (i) crystalline fouling (mineral scaling) originated from deposition of minerals due to excess of the solution products, (ii) organic fouling initiated of dissolved humic acids, oil, and grease deposition, (iii) colloidal fouling from deposited clay, silt, particulate substances and debris, and last but not least, (iv) microbiological fouling (biofouling) derived from adhesion and accumulation of microorganisms and biomass on a surface to a level that causes operational problems (Flemming, 1997).

Although natural organic matter (NOM) is generally recognized as the main foulant in water treatment (Jermann *et al.*, 2007), biofouling is still considered a major problem in membrane separation systems (Escobar *et al.*, 2005) since the first three types of fouling can be controlled by reduction of foulant concentration in the feed water (Flemming, 1997). Aquatic microorganisms have an affinity to form a biofilm in membrane filtration processes, having bacterial cell growth and produce soluble microbial and organic materials (SMP) as their by-products. SMP is defined as the collection of organic compounds resulting from substrate metabolism and biomass decay during digestion of organic substrates (Noguera *et al.*, 1994). In addition, microorganisms usually produce acidic by-products that can cause the most damage by concentrating at the membrane surface (Al-Ahmad *et al.*, 2000).

Biofouling is hard to control due to presence of microorganisms in nearly all water systems, and even if their number is low, microorganisms can easily grow and multiply in an environment with available nutrients. Even in low nutrient systems, bacteria have a strategy of adhesion and colonization to survive (Marshall, 1985). Bacteria are capable of colonizing on almost any surface and can survive under extreme

conditions such as temperature from -12° C to 110° C and pH values between 0.5 and 13 (Characklis and Marshall, 1990). Biofouling can occur despite the use of pre-treatment systems such as addition of disinfectants (e.g. chlorine) since a biocide may kill the biofilm microorganisms but it usually cannot remove the biofouling layer (Flemming *et al.*, 1997). Compared to planktonic cells, bacteria embedded in a biofilm are up to 1000-times more resistant to conventional disinfectants (Fux *et al.*, 2003).

Although a number of physical factors such as surface roughness (Herzberg and Elimelech, 2007) and surface charge (Emerson and Camesano, 2004); chemical factors such as ionic strength (Otto *et al.*, 1999) and alkalinity (Kayaoglu *et al.*, 2005) and biological factors influence bacterial adhesion and biofilm formation on the membrane, the initial step of biofilm formation plays a critical role and has an important impact on the structure and physico-chemi-biological properties of the established biofilm (Bryers and Characklis, 1981; Samuelsson and Kirchman, 1990). In addition, the type of substrate and its initial concentration also affect the biofilm properties (Speitel and DiGiano, 1987).

The first step in biofilm formation on a membrane interface is the transport and attachment of suspended bacterial cells. The permeate flux generates a convective drag force perpendicular to the membrane for both bacteria and nutrients to transport toward the membrane surface and according to accumulation of concentrated layers of biofilm and relatively high nutrients, cross-flow membrane filtration presents a different environment for biofilm. In addition to the convective force, it has been suggested that the flagella mediated bacterial motility plays an important role to overcome repulsive forces in the initial biofilm formation and attachment (O'Toole and Kolter., 1998). As

bacteria approach the membrane surface, surface-bacteria interactions such as electrostatic and hydrophobic interactions begin to play a role in the attachment process (Kang *et al*., 2004) and there will be more bacterial attachment with hydrophobic, non-polar surfaces (Donlan, 2002). On the other hand, hydrophobicity of the cells also contributes to increase attachment and hydrophobic bacterial cell surface has an affinity to hydrophobic surfaces (Bullitt and Makowski, 1995).

The general development of a biofilm can be described in three phases (Characklis, 1990) as schematically illustrated in Figure 3-1. First phase in biofilm formation is an initiation phase which represents a primary biofilm formation plateau after a rapid, short, and unstable biofilm formation proportional to the feed water cell concentration. This step consists of adsorption of organic molecules to the membrane surface that forms a "conditioning" organic surface and increases subsequent microbial adhesion. It is a relatively rapid layer formation and can occur about 2 hours after filtration (Al-Ahmad *et al.*, 2000). The second step in biofilm development is a logarithmic growth phase corresponds to the microorganisms' adhesion on the conditioned surface, attached cell growth and biofilm accumulation with a mature biofilm formation. The last stage in biofilm development is a plateau phase with the limitation of biofilm growth that occurs when cell attachment and biofilm growth reaches equilibrium with the surface cell detachment due to fluid shear forces. This phase is mainly dependent on the nutrient concentration and biofilm stability and is independent of the feed water cell density.

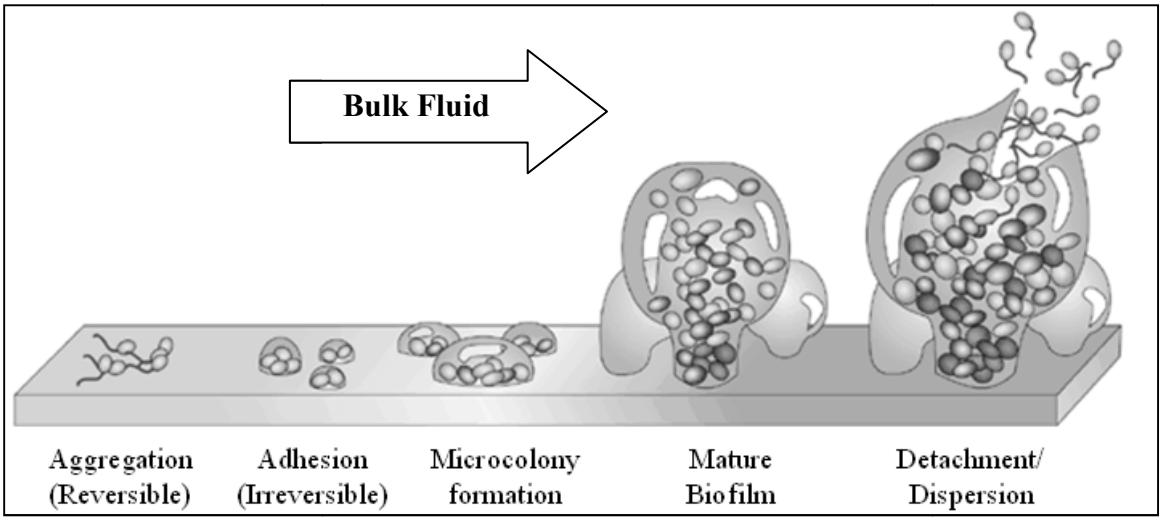



**Figure 1-3: Biofilm formation consists of three major steps including induction and primary biofilm formation, microorganism adhesion on the conditioned surface and biofilm accumulation, and plateau phase with the limitation of biofilm growth with the surface cell detachment due to fluid shear forces. (Modified from Davies, 2003)**

It has been determined that there is a variable bacterial density in a biofilm (Rittmann and Manem, 1992), and there are several citations confirming that the biofilm spatial structure is heterogeneous (non-homogenous), multi-layered and complex (LaMotta, 1976; Alleman *et al.,* 1982; Watanabe *et al.,* 1982). The heterogeneous and porous structure of the biofilm matrix gives the opportunity for the nutrients to penetrate the biofilm pores and reach deep layers of the biofilm and has many physiological consequences for microbial growth and biofilm accumulation. In addition, the "conditioning" layer generated with adsorbed nutrient macromolecules or suspended bacterial cells can also increase attachment of cells to the membrane surface (Walker and Marsh, 2004; Olofsson *et al.*, 2003).

## 1.3. Extracellular polymeric substances (EPS)

Bacteria embedded in the biofilm provide their own microenvironment by forming complex layers of microorganisms set in a matrix of extracellular polymeric substances (EPS) that consist of polysaccharides as the dominant component which represents up to 65% of extracellular materials (Horan and Eccles, 1986). In addition to the polysaccharide that is produced either for adhesion of bacteria to the surface (biofilm formation) or cohesion to other bacteria (microbial aggregation), there are other components such as proteins, nucleic acids, lipopolysaccharides (LPS) (only present in gram-negative bacteria) and complex mixture of biopolymers (Flemming and Wingender, 2001). In general, EPS contain high molecular weight compounds and have both adsorptive and adhesive properties, covering the bacterial cells to entrap nutrient species and hinder transport of biocides that could efficiently kill bacteria.

The first step in bacterial adhesion is the immediate attachment of bacteria onto the membrane surface which is a reversible process (Ofek and Doyle, 1994). The biofilm formation begins after the initial interaction between the bacteria and the substratum. Thereafter, the growth of biofilm process consists of the transport of organic and inorganic cells and eventually the production of EPS that makes the attachment more firmly adhered and an irreversible process (Beech, 2004).

The rate of EPS formation within the biofilm depends predominantly on the availability of carbon substrates both inside and outside the cell (Sutherland, 2001). Therefore, an increase in the available carbon substrate can increase the synthesis of EPS. On the other hand, bacterial growth rate could be a factor in the amount of EPS formation. For instance, the slow bacterial growth in biofilms would also enhance the

EPS production (Sutherland, 2001). Furthermore, under nutrient-starvation conditions, EPS degrading enzymes, used to convert the EPS to a food source, may be excreted that cause local destruction of the biofilm matrix and cells release (Allison *et al.*, 1998).

EPS is a critical and primary component of biofilm since 90% of the biofilm's organic carbon content can be attributed to EPS material (Characklis and Marshall, 1990). Consequently, composition and quantity of EPS can greatly influence and control the physical properties of the biofilm including density, porosity, strength, cohesiveness and the biofilm's biological properties such as metabolic activity (Zhang and Bishop, 2001; Decho, 2000). It has been shown that in strains like *Pseudomonas putida* and *Escherichia coli*, the activity of the cells embedded in the center of the biofilm decreased as the biofilm grew larger, indicating a lack of nutrients in the interior of the cluster due to the EPS matrix (Sternberg *et al.*, 1999). Several authors concluded that more EPS will occur with lower growth rate of microorganisms, but there are some studies that found no correlation between the EPS formation and microorganisms' growth rate (Laspidou and Rittmann, 2002). For instance, many diatoms and other phototrophic organisms such as cyanobacteria produce different amounts of EPS depending on the physiological state of the cells (Hoagland *et al.*, 1993).

Although approximately a quarter of EPS consists of proteins (Allison *et al.*, 1998), the function of proteins in the EPS matrix is not well understood. It is evident that proteins are associated with the primary adhesion process during cell attachment and biofilm development (Danielsson *et al.*, 1977). Moreover, proteins serve as an external enzyme in the exopolymer matrix breaking up macromolecules for the intracellular functions (Sinsabaugh *et al.*, 1991).

EPS not only encourages individual cells to colonize the membrane surface, but also it makes possible the interaction between different species within a biofilm that give metabolic and physiological capabilities to the biofilm community. For instance, direct interactions between the exopolymer matrices through EPS affect diffusion and availability of nutrients (Gilbert *et al.*, 1997). EPS in a biofilm can bind nutrients essential for biofilm growth and adherent microorganisms embedded in EPS are often cultivated in nutrient-rich growth media, regardless of their natural habitat making them significantly different from planktonic bacterial cells (Costerton *et al.*, 1994).

It is not only the amount of EPS but also the spatial distribution of EPS surrounding the biofilm that may affect the membrane permeability during filtration (Yun *et al.*, 2006). The highly distributed and more uniform polysaccharide may result in a greater hydraulic barrier and more flux loss. The matrix can also act directly as a carbon and energy source for the embedded bacterial cells and can be effective on their viability and metabolic activity (Allison, 2003). On the other hand, the viability of the microorganisms can be effective on amount and type of EPS production. For instance, dead cells form a relatively more porous cake layer than the living cells due to the lack of slime-like EPS produced (Herzberg and Elimelech, 2007).

### 1.4. Biofilm metabolic activity

A biofilm is a complex aggregation of microorganisms embedded on the surface. Bacteria that are attached to surfaces frequently have metabolically different characteristics from their planktonic counterparts. This physiological difference can be as a result of the bacteria in biofilms are often situated in close proximity to other

microorganisms and they are influenced by synergistic, mutualistic, or competitive interactions among biofilm members (Melo *et al.*, 1992). These possible interactions make the bacteria in biofilm less susceptible to toxic substances compared to free-living bacteria.

Metabolic activity determines the substrate conversion rate and the biofilm growth rate. Generally, the ability of performing all cell functions necessary for survival is considered as the benchmark for cell viability. Various criteria are used for viability assessment including reproduction, membrane integrity, respiration, enzyme activity, and membrane potential (Breeuwer and Abee, 2000). In addition, there are some basic requirements for a viable microorganism to survive including an intact cytoplasmic (plasma) membrane as a barrier between the cytoplasm and the extracellular environment, energy generation for cell metabolism, biosynthesis of proteins, nucleic acids, polysaccharides, and other cell components, DNA transcription and RNA translation, and finally cell growth and reproduction (Breeuwer and Abee, 2000).

There are different methods and techniques to observe growth and viability of the organisms. Colony counting, the classical procedure for determining the number of viable cells, consists of multiple processes that require several days of culturing to form visible colonies. Moreover, the viable plate count method has some problems due to clumping and aggregation of neighboring cells. Therefore, this method is being replaced with new techniques based on the evaluation of cellular metabolic activity. Variations in respiratory activity during a specific time can be reflected as the metabolic behavior of the microorganisms in an environment (Roszak and Colwell, 1987). Different approaches have been used to measure respiratory activity, such as evaluating electron transport and

using the reduction in tetrazolium colored salts as a measure of electron transport (Hatzinger *et al.*, 2003). Tetrazolium dyes can act as an electron acceptor (Altman, 1976) and therefore, the reduction in tetrazolium sodium salts, such as XTT and 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), during bacterial activity metabolism can be used as colorimetric indicator for quantifying cell viability.

CTC is a colorless, membrane-permeable compound with relatively rapid reduction to formazan that produces a stable red-fluorescing precipitate in the cell when it is reduced by the electron transport system of bacterial cell and can be observed and quantified using optical detection methods such as direct microscopy under UV lighting (Gruden *et al.*, 2003). CTC has the main advantage over the other fluorostains such as 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) that forms a fluorescent formazan that is relatively easy to visualize by epifluorescence microscopy against an opaque background (Rodriguez *et al.*, 1992). Moreover, CTC is insoluble in immersion oil and sustains its fluorescence when mounted on a microscope slide (Yu *et al*., 1995). However, no correlation is determined between the amount of active respiring and growing cells and the amount of cells that reduce sufficient salts to be detected as active CTC positive (Jorgensen *et al.*, 1994). CTC has been used to determine the number of respiring *Pseudomonas fluorescens* in pure culture (Bovill *et al.*, 1994; Jørgensen *et al.*, 1994), water samples (Joux *et al.*, 1997; Bartscht *et al.*, 1999) and biofilms (Stewart *et al.*, 1994; Huang *et al.*, 1995). In addition, CTC has been applied successfully to study respiratory activity within eukaryotic (Stellmach, 1984) and prokaryotic (Kaprelyants and Kell, 1993).

The main parameter in study of biofilm metabolism is the estimation of active biomass or biofilm activity, which has a direct influence on the substrate degradation rate. There are several studies that have focused on cell mass and activity determination (Atlas, 1982; Geesey and White, 1990; Sonnleitner *et al.,* 1992), but most of the methods are based on free cell cultures and their application to biofilm analysis requires removal procedure that can be one of limiting steps in biofilm analysis.

## 1.5. Adenosine triphosphate (ATP) as a measure of biomass activity

A promising approach to measure a specific cell constituent including biomass activity should meet the following requirements: (a) the measured compound should be available in all living cells and must be absent in all dead cells, (b) it must be found in uniform concentrations in all cells, regardless of environmental stresses, (c) the technique should be capable of measuring sub-microgram quantities, and finally (d) the measurement procedures must be reasonably quick and easy to use for various samples (Holm-Hansen, 1973).

There are several techniques and biochemical tests to estimate the biomass activity which measure either certain specific enzymes or specific products of the bacterial metabolism. Among several methods used for determination of biomass, estimation of adenosine triphosphate (ATP) is one of the most useful and remarkable methods. ATP is a fundamental biomolecule present in all viable and living cells whether their metabolism is aerobic or anaerobic and is degraded immediately after the cell death (White *et al.,* 1979). It is relatively stable in many studied organisms forming biomass (Patterson *et al.*, 1970; Holm-Hansen, 1970) and dependent on physiological state, its

concentrations are quite uniform relative to cell carbon for many bacteria (Atlas and Bartha, 1987). The ATP is utilized by the cells to drive enzymatic reactions that produce other chemicals such as DNA, RNA, protein, and polysaccharides that are needed for metabolism, growth and viability of the cells.

ATP is a complex molecule that contains the nucleoside adenosine and a tail consisting of three phosphates. It functions as a chemical energy carrier from the catabolic reactions of metabolism to the different cellular processes that require energy. The available energy in the ATP molecules is stored in the chemical bonds between the two final phosphate groups and during the energy release in a biochemical process the ATP is converted into ADP and inorganic phosphate as the following reaction:

$$ATP + H_2O \longrightarrow ADP + P_i$$

The generation of ADP from ATP is carried out with an electron transport system that requires oxygen as the terminal electron acceptor and the rate of conversion of ATP to ADP has a direct proportion to the energy requirements of increased cellular activity (Engel *et al.,* 1975).

Furthermore, it is relatively simple for quantitative measurement of ATP at micro-level. The principle of ATP determination is based on the measurement of the quantity of light (bioluminescence) produced when luciferin is oxidized in the presence of ATP and the enzyme luciferase. Although ATP determination has a complex analytical procedure, but one of its important advantage is that ATP amount remains constant after freezing which can be useful for prolonged samples conservation (Atlas, 1982). Moreover, the quantity of ATP in a bacterial cell is not significantly variable for a particular strain

(Stanley, 1989) and so the amount of activity can be estimated from a specific ATP concentration.

Previous studies provide numerous procedures for ATP extraction (Lundin and Thore, 1975). In addition, there are still questions on extractant interference with ATP, instability of ATP, lack of reproducibility, and the requirement for internal standard conditions in some methods. For instance, the increased ionic strength in the sample has an adverse effect on the ATP release reaction; high salt concentrations in the sample have a general inhibitory effect on the luciferase and will decrease sensitivity (Denburg and McElroy, 1970); the presence of organic and inorganic materials in samples may also interfere with the analysis by catalysis of non-enzymatic hydrolysis of ATP (Tobin *et al.*, 1978).

Since the ATP is the primary energy molecule and energy required for all biological reactions is directly or indirectly provided by ATP, any changes in cellular activity and integrity should be reflected in the ATP quantities. It has been studied that ATP has a very good correlation with the number of viable cells in their exponential growth phase (Nuzback *et al.,* 1983) and a stable level of ATP has been measured in the endogenous conditions (Kang *et al.*, 1983). In addition, ATP is found in all living cells whether with aerobic or anaerobic metabolism and can be measured at the same time, while standard plating techniques require different media to detect aerobic and anaerobic bacteria and often underestimates the actual microorganisms present.

As nucleotides are present in all cells, ATP cannot differentiate between prokaryotes and eukaryotes. In prokaryotes (bacteria and cyanobacteria), ATP is produced both in the cell wall and in the cytoplasmic matrix. For bacterial activity

characterization, several studies have been carried out based on the quantifying the ATP (Strange *et al.,* 1963; Holm-Hansen and Booth, 1966). The quantity of ATP depends on the physiological state of the organisms and consequently on the biomass growth rate (Chiu *et al.,* 1973; Atlas, 1982). Prediction of biofilm production can be achieved by analysis of biofilm formation rate (Hammes *et al.*, 2005) which is based on ATP analysis of the biofilm in monitoring biofilm and extraction and measurement of biomass ATP activity (Fiksdal and Bjorkoy, 2007).

## 1.6. Research objective

In summary, since biofouling results from interactions of living microorganisms, it could be considered as the most difficult and complicated fouling process to control and probably the most complex and least understood. In order to control and develop effective anti-biofouling strategies in membrane filtration, it is essential to understand the mechanisms of biofouling, specifically during its early stages to determine the factors that impact the rate of biofilm development.

There is a lack of studies on the initial step of biofilm formation and particularly in the biological characteristics such as metabolic activity of the established biofilm. In addition, the knowledge of the metabolic activity of the membrane biofilm may assist in understanding the biofilm accumulation mechanisms to apply appropriate countermeasure and control of membrane biofouling.

The hypothesis in this study is that the activity of initial bacterial cells or biofoulant will impact membrane biofilm formation and biomass activity and consequently membrane performance (i.e. flux decline). In addition, bacterial cell growth

phase conditions could be a significant factor in membrane biofouling and biomass activity. Therefore, **the objective of this study was to investigate the effects of biofoulant activity in ultrafiltration membrane biofouling and the consequent effects on permeate flux.** Short-term biofouling experiments with a mono-culture biofilm were carried out using a laboratory-scale ultrafiltration membrane filtration test unit and the activity of the pure culture of biofoulant, feed water, and biofilm accumulated on the membrane surface were investigated with both ATP and CTC-stained bacterial intensity measurement.

# Chapter 2

# Materials and Methods

## 2.1. Cross-flow filtration experiments

Cellulose acetate ultrafiltration membranes with a molecular weight cut-off of 20,000 Da (General electric water and process technology, Minnetonka, MN) were used for the cross-flow filtration. Cross-flow experiments were carried out using membrane sheets with an area of 267.4 $cm^2$ (19.1 cm x 14 cm) mounted on the cell membrane (Osmonics Sepa CF, Minnetonka, MN) with an effective membrane area of 155 $cm^2$. A schematic diagram of the membrane filtration system has been shown in Figure 2.1.

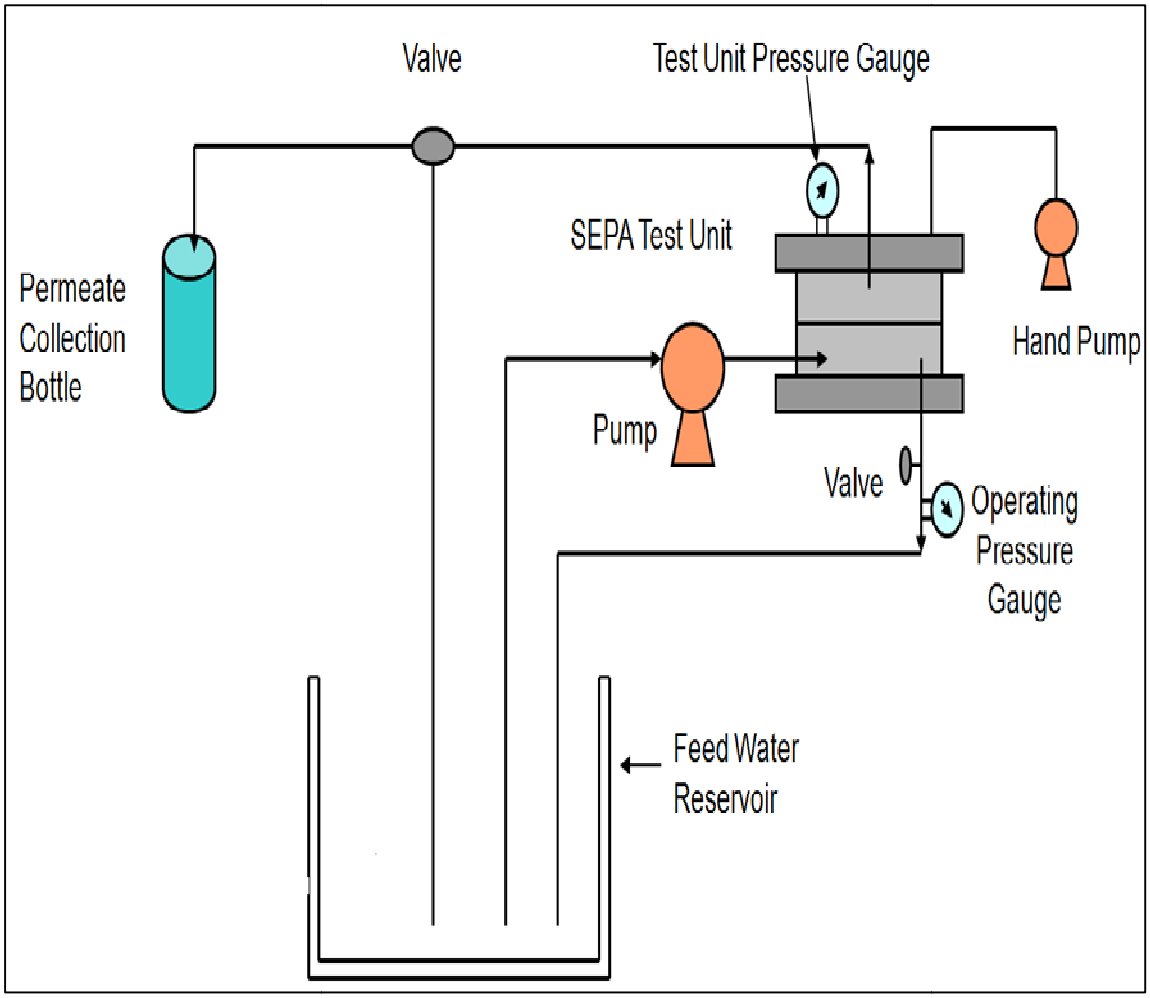



**Figure 2.1: Cross-flow membrane filtration system pumping feed water through UF membrane and circulating concentrated and permeate water in the reservoir. Trans-membrane pressure could be adjusted with the operating pressure valve.**

Before each experiment, the cross-flow unit was sterilized by recirculation of 0.5% (V/V) of bleach, 0.015% (V/V) Micro liquid laboratory cleaner (International products corp., Trenton, NJ) and the Works disinfectant (Lawrenceville, GA) for two hours. The unit was rinsed twice by recirculation of tap water for 10 minutes. Trace organic matter was eliminated by recirculation of 5 mM EDTA for 45 minutes then rinsed again. In the last step, the unit was sterilized by recirculation of 1% ethanol (V/V)

for an hour followed by a final rinse of the unit three times with de-ionized (DI) water to eliminate chemical residues.

### 2.1.1. Active and inactive cells in feed water

The feed water was circulated from the reservoir to the cell membrane and both the concentrate and permeate outlets were recycled to the reservoir. The cross-flow experiment was performed for different filtration durations (4, 11, and 24 hours) to understand biofilm activity changes during the first day of filtration. Cross-flow filtration was operated at a constant pressure of 172.36 kPa (25 psi) and a constant water temperature of 27° C using a cooling fan. All membranes were pre-compacted for 12 hours to reach a constant flux before starting the filtration period. The permeate flux (J) was measured during the filtration experiment and the final permeate flux was evaluated as normalized flux with respect to initial flux ($J_0$). It is also important to consider that there are higher flux values at the high temperature due to decrease in water viscosity as a function of temperature. Therefore, the permeated flux was normalized based on the initial flux and a constant temperature. The initial flux for all of the experiments was adjusted to 26 $L.m^{-2}.hr^{-1}$.

All of the cross-flow filtration experiments were carried out using feed water composed of DI water and the following buffering chemicals; 0.05 mM sodium bicarbonate ($NaHCO_3$), 0.01 mM calcium chloride ($CaCl_2$), 0.2 mM sodium chloride (NaCl), 0.094 mM ammonium chloride ($NH_4Cl$), 0.045 mM potassium dihydrogen phosphate ($KH_2PO_4$), and 0.06 mM magnesium sulfate ($MgSO_4$). For experiments with active bacterial cells, the carbon source for the bacterial cell solution in the feed water

was 0.95 mM sodium acetate trihydrate ($NaC_2H_3O_23H2O$). During the filtration experiments using inactive bacteria 0.2 mM sodium azide ($NaN_3$) was added instead of sodium chloride to prevent any possible bacterial cell growth (Herzberg and Elimelech, 2007). In this study, biofouling was investigated under fixed and same biological (i.e. dissolved organics, type of carbon and energy source); physical (i.e. cross-flow velocity, applied pressure, temperature, initial permeate flux); and chemical (i.e. membrane type, pH, ionic strength) conditions and the effects of cell deposition and biofilm growth on the membrane were characterized with different levels of biofoulant activity.

### 2.1.2. Development of characteristic growth curve

In order to study behavior of the model microorganism, the bacterial growth curve was measured at 27° C in Luria-Bertani (LB) broth. For measuring ATP activity, cell solution samples (100 μL) were taken at different times during the incubation period and were analyzed for the cell number and intracellular activity. According to Beer-Lambert law, the absorbance is linearly proportional to concentration, and this is the basis of the use of turbidity as a rapid and non-destructive method for monitoring the cell concentration related to each sample. The samples were analyzed in triplicate and LB broth was used as the control sample (blank sample without inoculated cells) in all of the measurements.

As previously indicated, bacterial cells have different levels of activity during their growth curve. Therefore, the filtration experiments were carried out with bacterial cells in three different growth conditions (i.e. lag, late exponential and stationary phase). 24 hours filtration was chosen as the filtration duration based on the earlier studies since

there was more biofilm formed. This filtration study was carried out to elucidate the impact of varying levels of activity (rather than just active and inactive).

## 2.2. Bacterial strain and growth conditions

A Gram-negative aerobic bacterium, *Pseudomonas fluorescens Migula* (ATCC # 12842) was used as the microorganism to produce the membrane biofouling. These rod-shaped bacteria have the optimal growth temperature of 27° C, pH 7, and use glucose as their carbon source. Members of *Pseudomonas* genus are one of the most ubiquitous bacterial species in the environment and *Pseudomonas fluorescens* is well known to be good biofilm producer due to its short generation time and resistance to heat fluctuation (Pereira and Vieira, 2001; Simoes *et al.*, 2008).

For each experiment, cultures were pre-grown on LB broth for 24 hours to obtain fresh cells. Then, they were grown on LB agar and harvested at different times depending on the objective of the experiment. For the filtration experiments with active cells, 10 mL of *Pseudomonas fluorescens* with the optical density at 600 nm of $OD_{600}$=0.1 representing approximately $10^4$ cells added to the reservoir feed water. For the experiments with inactive cells, one mL of formaldehyde solution supplemented with 87.5 mM sodium dihydrogen phosphate ($NaH_2PO_4$) was added to the cell solution and incubated at room temperature for two hours to fix the bacteria.

## 2.3. Cellular ATP determination

Cells produce ATP continuously while they are alive and when processes for ATP production are inhibited, all available ATP is consumed (Lundin, 1982). Without ATP,

bacteria become dormant and unable to maintain cell integrity and eventually die. Generally, the average ATP content of one bacterial cell is about $10^{-15}$ g (1 fg) (Crombrugge and Waes, 1991; Stanley, 1989). Since the molecular weight of ATP is equal to 507.18 g/mol, the ATP content per cell is approximately 2 attomoles ($2x10^{-18}$ moles).

The intracellular adenosine triphosphate (ATP) concentration was measured using an ATP colorimetric/fluorometric assay kit (Biovision, Mountain View, CA) as the kit was reported to be successfully used in various ATP measurements (Su and Hong, 2010; Wei *et al.*, 2011) and adhered closely to the instructions provided by Biovision. To measure the amount of ATP in a sample containing viable cells, the cell wall needs to be first ruptured by adding lysing agent. Cell solutions were lysed in ATP assay buffer based on their concentration ($1x10^6$ cells in 100 µl of ATP assay buffer). This breaks apart the cell wall and membrane and releases intracellular contents including ATP into the solution. ATP measurements were performed by extracting 50 µL of the sample and adding 50 µL of the ATP extract mixture, incubating at room temperature in the dark for 30 minutes and the amount of light produced was measured by a spectrophotometer in relative fluorescence units (RFU) which is proportional to the amount of ATP (Venkateswaran *et al.,* 2003).

For the membrane biofilm analysis, the biomass was removed from the membrane surface. A section of the biofouled membrane with an area of 2 $cm^2$ (10 mm x 20 mm) was cut and the biomass was scraped off the membrane. In order to counteract flocculation of the bacterial cells, 0.1 % (V/V) tween-80 was added to DI water in the 6-well plate. The membrane was immersed in the tween-80 solution and agitated for 5

minutes at 25 rpm. Bacteria that were still attached to the membrane were scraped off the membrane by using a utility knife sterilized with 95% (V/V) ethanol. This process was repeated until all biomass was collected from the membrane.

To avoid light interference, all procedures were carried out in a poorly lit room and all of the materials and solutions were stored in the dark. In order to minimize the light interference of adjacent wells, black microplates were used and to have well-mixed solution, the microplate was mixed for 10 seconds before each reading. Excitation and emission wavelength in the spectrophometer were 535 nm and 587 nm, respectively.

In order to have sample readings in the linear range of the standard curve, the samples were concentrated with centrifugation. On the other hand, the fluorescent intensity of ATP assays was determined by its concentration which depends on the secretion and hydrolysis rates (Krasnyi *et al.*, 2010). ATP secretion by the cells is activated during centrifugation (Pederson *et al.*, 1999). In addition, ATP is one of the microbial growth characteristics that can be affected with the change in temperature (Kahru *et al.*, 1987). It has been studied that there is a reverse correlation between temperature and the rate of ATP hydrolysis, so the intensity of fluorescence in the presence of ATP linearly increases with the temperature decrease (Krasnyi *et al.*, 2010). Therefore, in order to increase the amount of hydrolyzed ATP, the centrifugation was carried out at low temperature (4° C).

### 2.3.1. Standard curve for ATP determination

As a linear relationship exists between the intensity of the fluorescent signal and the ATP concentration (Crouch *et al.*, 1993), ATP standard curve was produced to

correlate the microreader measurement as RFU to the ATP molarity concentration. For the fluorometric assay, 1 µL of the ATP standard was diluted with 99 µL of ultrapure water to generate 0.1 mM ATP standard. Different amounts of ATP standard (0, 2, 4, 6, 8, 10 µL) were added into a series of wells and the total volume was adjusted to 50 µL/well with ATP assay buffer to generate different concentrations (0, 0.2, 0.4, 0.6, 0.8, 1 nmol/well) of ATP standard.

The standard curve was prepared for three different temperatures (25, 30, and 35º C) under same spectrophotometer conditions including cutoff wavelength, number of readings and mixture duration before each reading. In this study for ATP measurements, all of the samples were prepared in triplicate with three separate readings. The ATP standard curves are shown in Figure 3-1 to Figure 3-3.

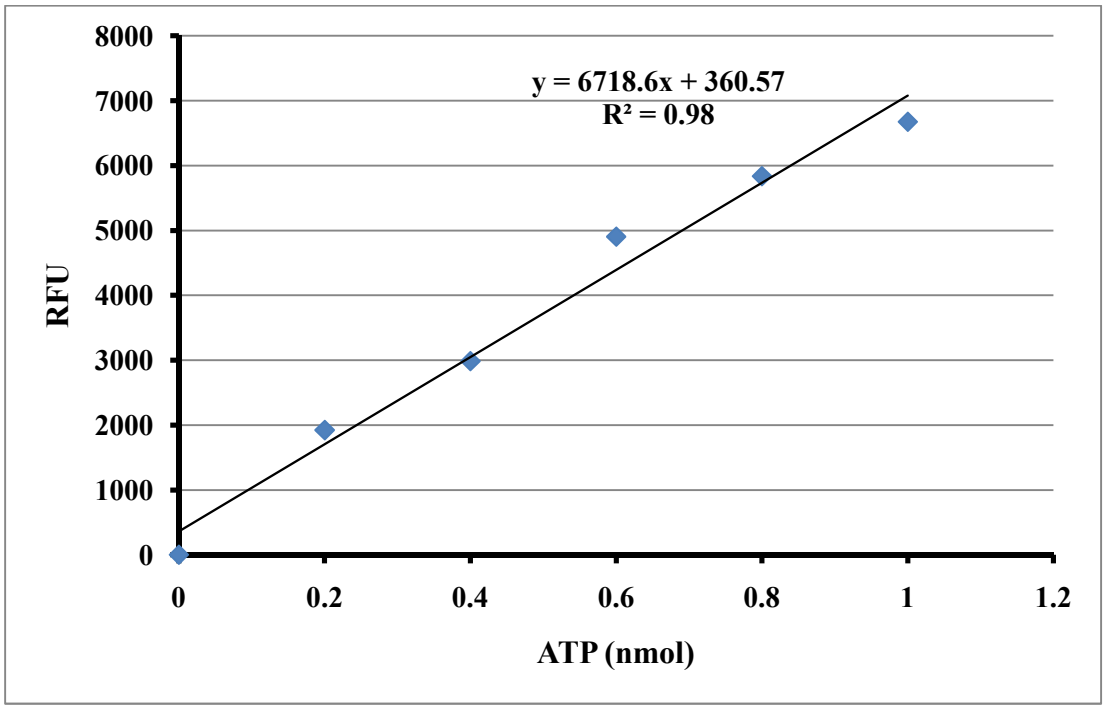



**Figure 2-2: Standard curve of ATP assay resulted at spectrophotometer temperature of 25.3º C**

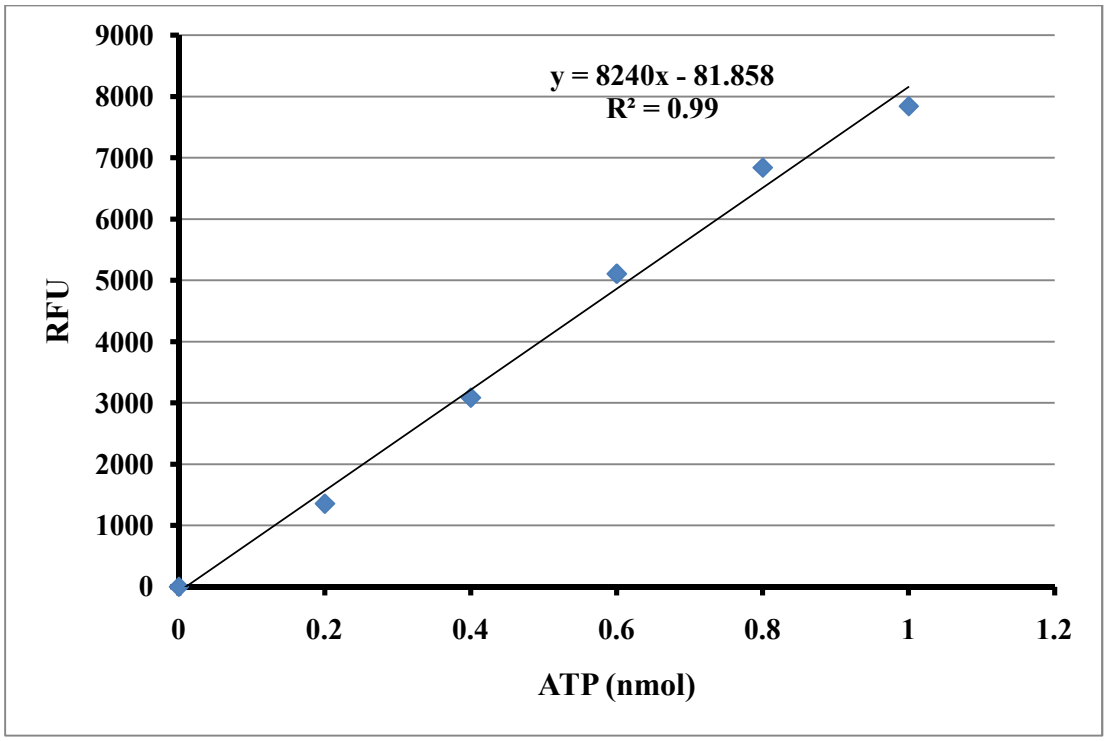



**Figure 2-3: Standard curve of ATP assay resulted at spectrophotometer temperature of 30.5º C**

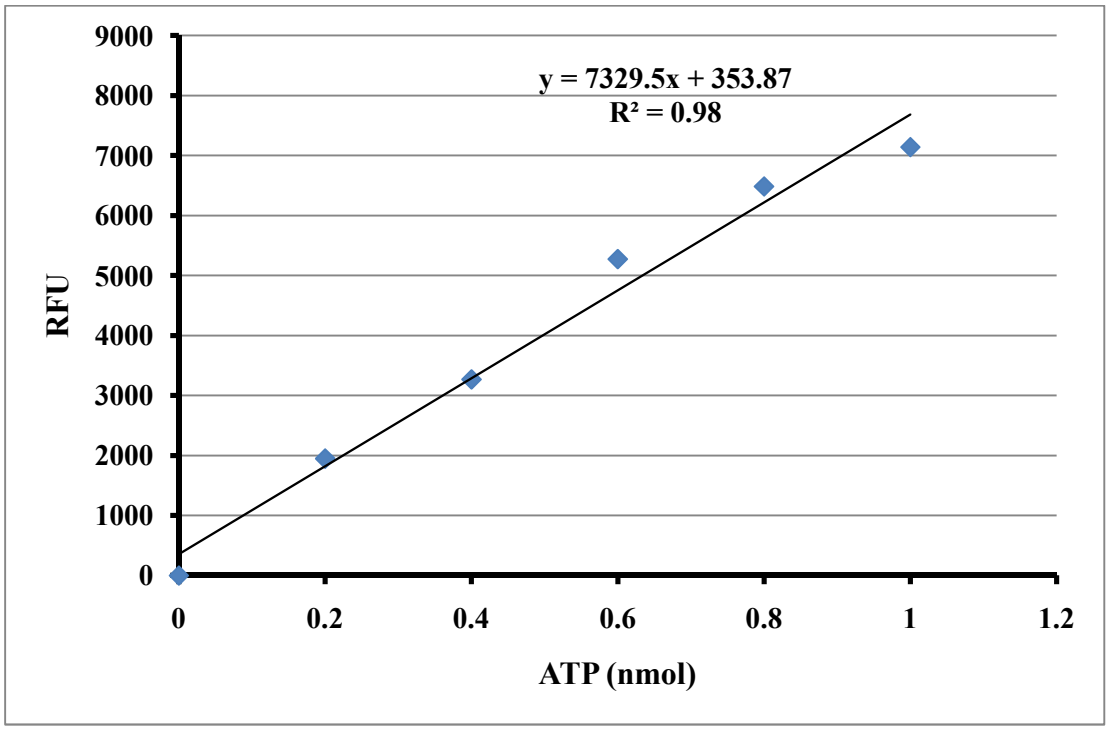



**Figure 2-4: Standard curve of ATP assay resulted at spectrophotometer temperature of 35.1º C**

It was understood that if temperature equals to 30º C, the trend line of the standard curve tends to the standard curve equation provided by the ATP Biovision catalog. Therefore, all of the fluorometric measurements were carried out at 30º C to have consistent readings. The background light emission resulted from both microplate wells and the reagents were subtracted from each sample reading.

## 2.4. Surface biofilm metabolic activity analysis (CTC)

At the end of each filtration experiments, membrane sheets were dried by incubation at 25° C for an hour and two samples were cut from the permeate outlet of the membrane sheet. In order to measure the respiratory activity of the biofilm, fouled membranes were stained without biomass removal since its removal results in the distortion of the natural structure of the biofilm. Biomass staining was carried out using 1 mL of 5-cyano-2, 3-ditolyl tetrazolium chloride (CTC) (Polyscience, Washington, PA) which was prepared in ultrapure water (20.05 mM). As the proportion of detectable CTC-active cells increases over time (Sieracki, 1999), the samples were incubated at 27° C in the absence of light for 12 hours. To measure the total number of the cells in the biofilm, 5 µL of Picogreen (PG) (Invitrogen, Chicago, IL) was added as the counter-stain to each sample and incubated for 10 minutes. The excitation/emission wavelength for the CTC and PG were 475/600 and 502/523 nm, respectively.

In the case of staining feed water or pure culture, the stained samples were added to 0.22-µm pore size polycarbonate membrane filters (Millipore, Billerica, MA) and vacuum pumped to trap the cells on the membrane filters. After air drying, the filters were mounted on a slide with a drop of non-fluorescent immersion oil, and a cover slip

was applied to the filter. The slides were processed by fluorescence microscopy (Olympus BX51) and a minimum of 20 random fields were captured using an integrated digital camera on the microscope (Olymus DP70, Center Valley, PA) at the magnification of 100X.

For the last step in the activity analysis, epifluorescent microscopic images were enumerated using the freeware ImageJ (version 1.41). This software was previously used as a method to detect area of membrane biofilm coverage (Ferrando *et al.*, 2005; Hughes *et al.*, 2006; Kang *et al.*, 2006; Marselina *et al.*, 2009). The intensity of stained cells and area proportional of active cells to total cells were used as indicators of cellular activity. Three growth-related parameters including area of stained cells, intensity of stained cells, and ratio of active cells to total cells of *Pseudomonas* in the CTC/Picogreen stained samples were determined. For each sample, at least 20 microscope field images were taken and the CTC activity was calculated based on the active and total area and intensity (Equation 2-1).

$$\text{CTC Activity} = \frac{\text{Active Area} * \text{Active Intensity}}{\text{Total Area}} \qquad \textbf{Equation 2-1}$$

## 2.5. Standard error calculation

In order to calculate the standard error in the CTC activity calculation the following method was used (Fersht, 1999). In multiplication and division of values with standard deviation, the standard deviations are not simply be added to produce the final standard deviation. Instead, the fractional standard deviation should be squared, be added together and the square root should be taken of the sum. Standard deviation calculation

for multiple values is shown in Equation 2-2 and the standard error is provided with Equation 2-3.

$$SD_r = r * \sqrt{(\frac{SD_X}{X})^2 + (\frac{SD_Y}{Y})^2} \quad \textbf{Equation 2-2}$$

$$\text{Standard Error} = \frac{SD_r}{\sqrt{\text{Total field number}}} \quad \textbf{Equation 2-3}$$

When several small sets have the same sources of indeterminate error (i.e. the same type of measurement but different samples) the standard deviations of the individual data sets may be pooled to have more accurate standard deviation in the analysis method. As there were duplicate runs for each activity and filtration duration, the standard deviation should be pooled with the Equation 2-4 as below.

$$S_{pooled} = \sqrt{\frac{\sum_{i=1}^{N1}(x_i - \overline{x_1})^2 + \sum_{j=1}^{N2}(x_i - \overline{x_2})^2}{N_1 + N_2 - 2}} \quad \textbf{Equation 2-4}$$

$$S_{pooled} = \sqrt{\frac{(N_1 - 1) * {\sigma_1}^2 \ + \ (N_2 - 1) * {\sigma_2}^2)}{N_1 + N_2 - 2}}$$

# Chapter 3

# Results & Discussion

## 3.1. Activity determined by ATP

### 3.1.1. Activity as measured by ATP as a function of growth phase

Figure 3-1 demonstrates the growth curve for *Pseudomonas Fluorescens Migula* at 27° C in LB broth. Data points provide the ability to differentiate between different phases of growth including lag phase, exponential growth phase, and stationary phase. During the experiment, cell concentration increased approximately one order of magnitude from $2x10^8$ during lag phase to $2x10^9$ cells following exponential growth. Since the amount of ATP present in a cell depends on the physiological state of the organism determined by the biomass growth curve (Atlas, 1982) and also on the substrate consumption rate (Namkoong *et al.*, 1989), it was necessary to establish the growth curve for this organism under experimental conditions.

The ATP activity was measured at different times during cell growth as follows: in the beginning of lag phase (t=2 hr), beginning of exponential phase (t=9 hr), mid-late of exponential phase which is the time that the cells were used in the membrane filtration experiments (t=21 hr), the transition between the exponential phase and the start of stationary phase (t=27 hr), and in the middle of stationary phase (t=36 hr). The activity as measured by ATP assay and as a function of growth phase (incubation time) is presented in Figure 3-2.

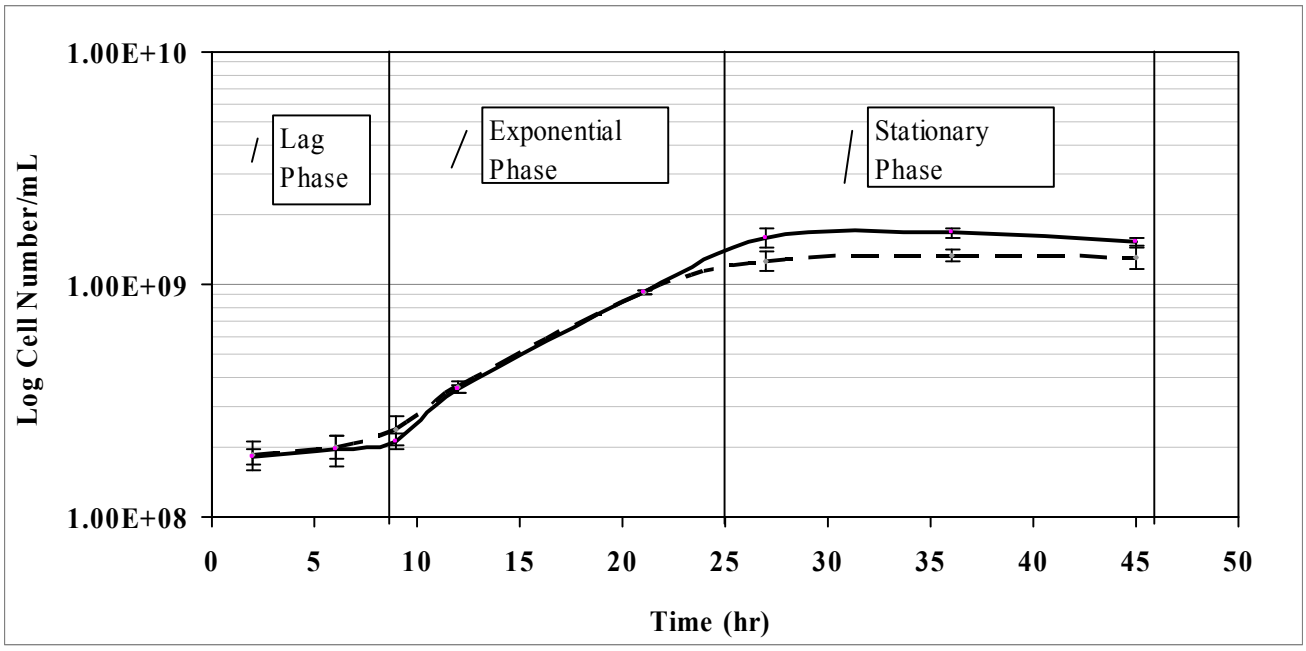



**Figure 3-1: Growth curve for *P. fluorescence* at 27° C in LB broth in the dark. The figure shows the number of cells per mL (log normalized) as a function of time in hours. Error bars represent ± one standard error of the mean.**

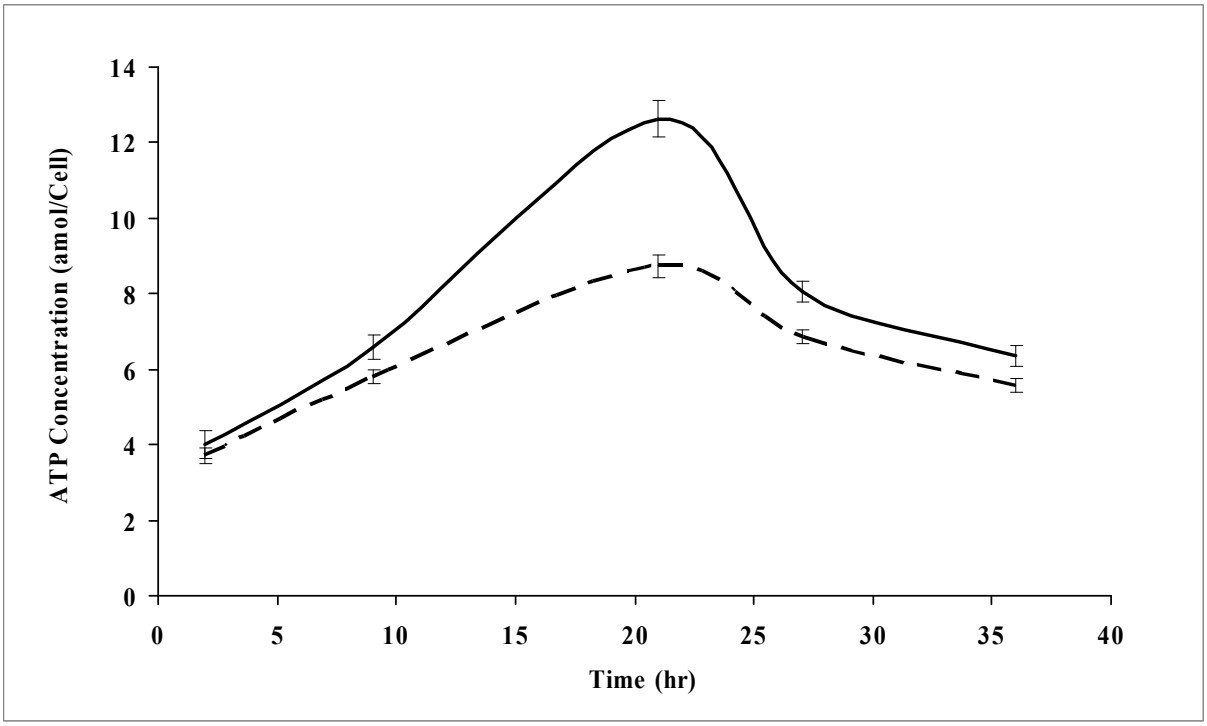



**Figure 3-2: Data shown represents the ATP concentration (amol/cell) as a function of incubation time (hours) for *P. fluorescence* at 27° C in LB broth for two experiments (n=15). ATP measurement was done using a fluorometric based assay. Error bars represent ± one standard error of the mean.**

The ATP content curve of the cell cultures consisted of a linear increase period with the maximum value at late-exponential phase and a rapid decrease with a deceleration period. The two cultures used for these experiments resulted in different ATP amounts which might be attributed to the distinct cell solution that may have had different substrate uptake rates or other physiological differences. However, the patterns between the two experiments were the same. The maximum ATP value (during exponential growth phase) ranged between 9 and 13 amol ATP/cell. The ATP concentrations in the stationary phase were lower than the exponential phase but still significantly higher than in the lag phase. Notably, the ATP concentration decreased in the stationary phase (Figure 3-2) while the number of cells did not measurably decrease (Figure 3-1).

### 3.2. Effect of initial biofoulant activity on biofilm activity and flux decline

In order to investigate the influences of bacterial cell activity on the membrane biofilm activity and membrane performance, the filtration experiments were conducted with active bacterial cells in their late-exponential phase, dead bacterial cells (no EPS), and bacterial cells in different growth conditions. The influence of biofoulant ATP activity on the biofilm activity is shown in Table 3-1.

**Table 3-1: Membrane biofilm ATP activity (amol/cell) and CTC activity (pixels) with respect to initial biofoulant (*P. fluorescence)* ATP activity (amol/cell) as the biofoulant in late-exponential growth phase during different filtration duration (4, 11, 24 hr). Errors represent ± one standard error of the mean.**

| Filtration (hr) | 4 | 11 | 24 |
|---|---|---|---|
| **Biofoulant (ATP in amol/cell)** | 7.38±0.10 | 9.14±0.84 | 5.91±0.12 |
| **Membrane Biomass (ATP in amol/cell)** | 6.68±0.27 | 8.01±0.29 | 8.16±0.29 |
| **Membrane Biofilm (CTC in pixels)** | 98.17±11.61 | 119.22±14.72 | 128.32±15.79 |

During filtration with late-exponential growth phase biofoulant, biofilm activity, as measured by ATP, reached an equilibrium concentration of approximately 8 amol/cell after 10-12 hours of filtration and does not appear to be related to biofoulant ATP levels, which varied between less than 6 amol/cell to more than 9 amol/cell. The consistent level of growth ATP activity in the biofilm formed may be attributed to the limitations to the penetration of nutrients through the biomass. Hence, it seems that there is a certain threshold level called "active thickness" that there will be limiting nutrients above this level (LaMotta, 1976). There is also a possibility that the biofilm cells reached their carrying capacity over time. There is a set amount of nutrients that can support fixed level of activity and biofilm cells gradually lose their capacity to become activated by nutrient supply (Allison et al., 1998).

The inactivated biofoulant cells resulted in approximately 2 amol ATP/cell on the biofilm which was 75% less than that produced by active biofoulant cells while in equilibrium phase. As the bacterial cells were pre-fixed with formaldehyde in the inactive experiments, a stable ATP activity in the inactivated biofoulant was expected, and it was approximately 2 amol/cell in all of the experiments with inactive cells. It is notable that the ATP activity of the membrane biofilm did not change significantly as a result of different filtration durations, and the ATP concentration was similar to that of the initial biofoulant.

In all experiments, there was biomass on the feed side of the membranes and also on the spacer to a lesser extent. Minimal biomass concentration (below detection) was observed on the permeate side of the membranes indicating an efficient removal of microorganisms and growth substrates in the membrane filtration. Figure 3-3(a) and

Figure 3-3(b) illustrates the impact of active and inactive biofilm ATP activity level on the membrane performance in terms of flux loss.

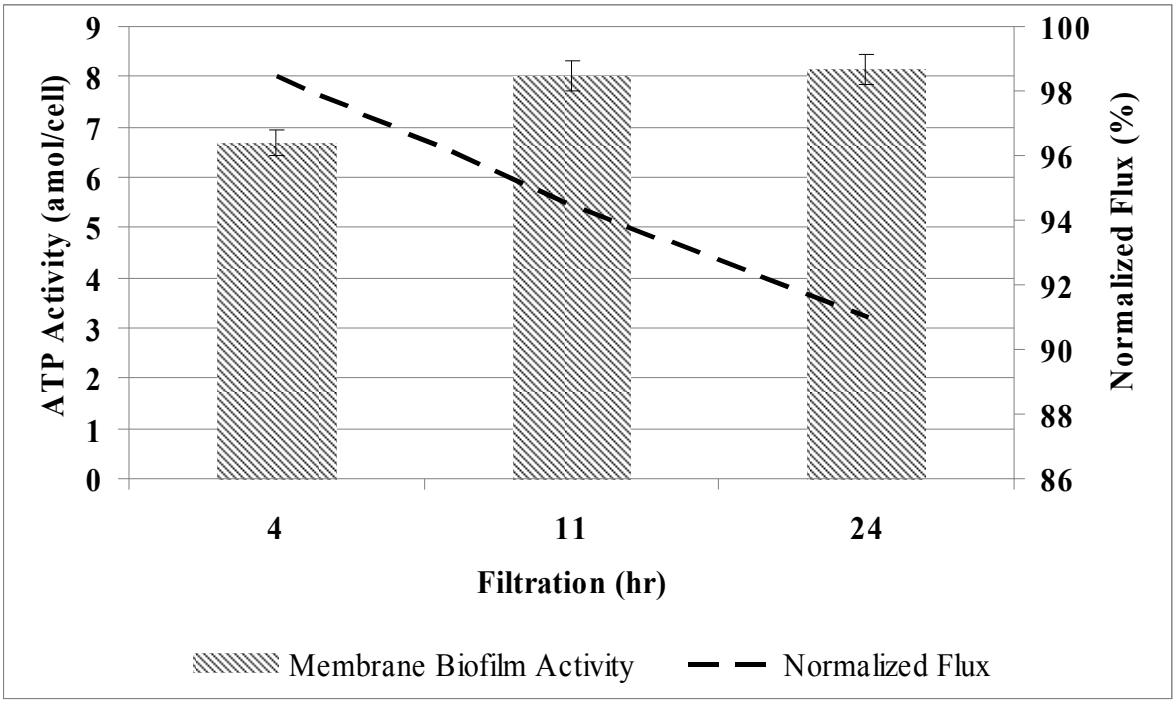



**Figure 3-3(a): Influences of membrane biofilm ATP activity resulted from active *P. fluorescence* biofoulant on the membrane permeate flux during different filtration duration (4, 11, 24 hr). Error bars represent ± one standard error of the mean.**

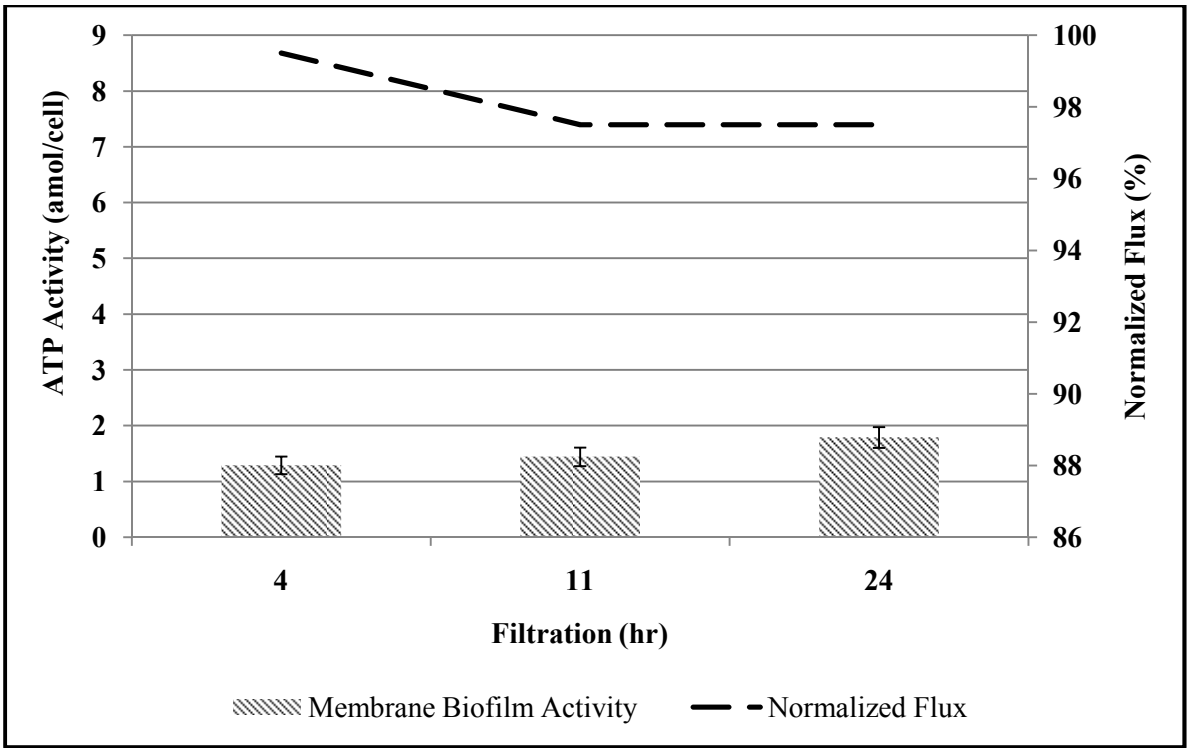



**Figure 3-3(b): Influences of membrane biofilm ATP activity resulted from inactivated *P. fluorescence* biofoulant on the membrane permeate flux during different filtration duration (4, 11, 24 hr). Error bars represent ± one standard error of the mean.**

Notably, there was much less flux decline with inactive biofilm which suggested that the biofilm activity can be an important factor in having flux decline during the membrane filtration. At the end of 24 hours of filtration in the case of inactive biofilm, there was approximately 2% flux decline while much more flux loss was observed in the active biofilm (i.e. approximately 10% flux loss at the end of 24 hours). It's notable that in the case of the active biofilm, flux decline increased with time even though biofilm activity (i.e. ATP concentration) did not considerably increase. Biofilm growth and forming a mature and condense barrier could be a reason for having more flux loss in the filtration process. It is believed that the primary adhesion of microorganisms is an abiotic process and being driven by physicochemical processes (Marshall and Blainey, 1991) and

there might be no physiologically or chemically differences between active or inactive biofilm but as our data indicated, there is a dramatic difference in terms of flux decline. These results are consistent with previous studies on live and dead biofilm (Herzberg and Elimelech, 2007) that showed the effect of pure culture fixing on reduction of flux decline.

In order to represent a variety of possible growth phases that could be present in a real membrane-based water treatment system, the feed water was spiked with cells representing different growth phases. The different growth phases resulted in varied activity, as measured by ATP in pure culture of the biofoulant, the spiked feed water after 24 hours of filtration, and the membrane biofilm (Figure 3-4, Table 3-2).

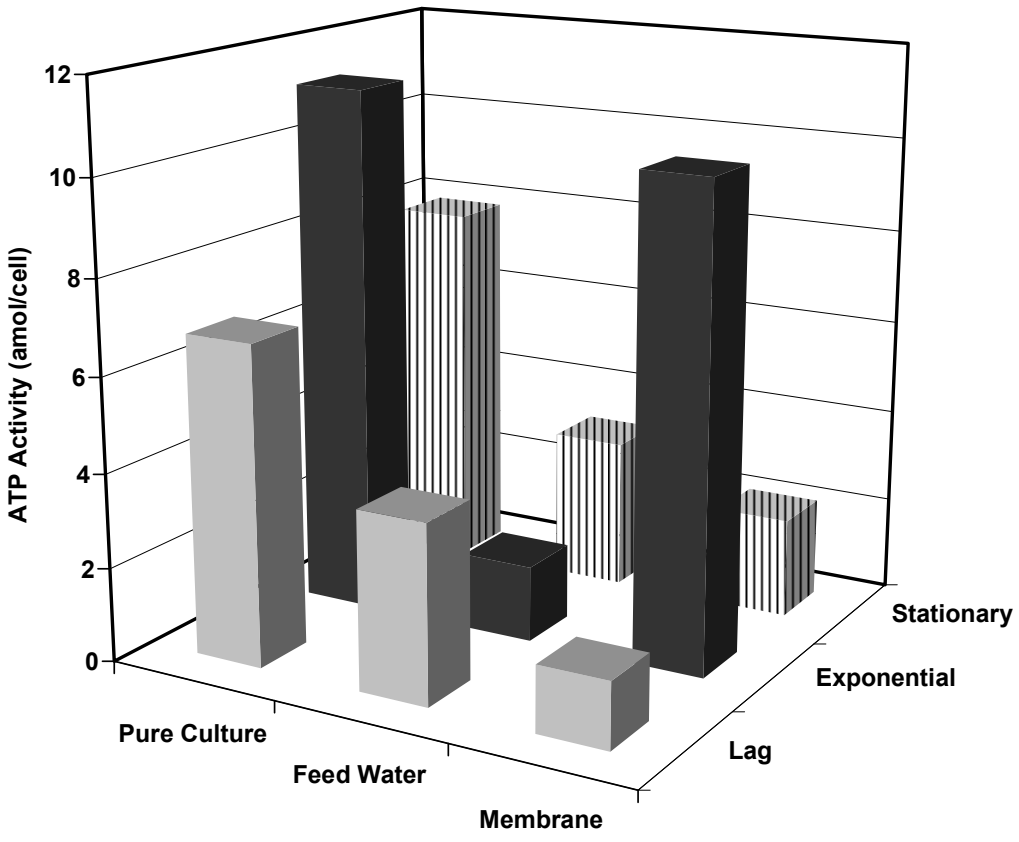


**Figure 3-4: ATP activity changes in different growth phases (lag, late-exponential, stationary) in pure culture (biofoulant), feed water after 24 hours of filtration, and biomass scrapped from the membrane.**

**Table 3-2: ATP activity changes in different growth phases (lag, late-exponential, stationary) in pure culture (biofoulant), feed water after 24 hours of filtration, and biomass scrapped from the membrane. Errors represent ± one standard error of the mean.**

| Growth Phase | Pure Culture | Feed Water | Membrane Biofilm |
|---|---|---|---|
| **Lag** | 6.77±0.23 | 3.79±0.28 | 1.41±0.29 |
| **Exponential** | 11.21±0.26 | 1.61±0.28 | 10.18±0.22 |
| **Stationary** | 7.85±0.27 | 3.19±0.35 | 2.12±0.25 |

The ATP activity in the exponential phase decreased dramatically in the feed water after 24 hours of filtration but the activity on the membrane biofilm is similar to the biofoulant activity that was initially added to the reservoir. This could suggest that the active bacterial cells tend to stay on the membrane due to accumulation of nutrients on the surface which contributes the embedded bacterial growth rate and consequently their metabolic activity (van Loosdrecht, 1990). On the contrary, the lag phase and stationary phase cultures produced similar outcomes which resulted in elevated levels of activity in the feed water as compared to the biofilm. This suggests that these cells are more competitive in planktonic condition rather than in the biofilm during the 24 hours of this experiment.

In order to investigate these questions further, the biomass accumulated on the biofilm was compared to that present in the feed water after 24 hours of filtration. Although these numbers cannot be directly compared (planktonic vs. biofilm cells), the

trends are evident (Figure 3-5). This figure also demonstrates the flux decline during the filtration using biofoulant cells from various growth phases.

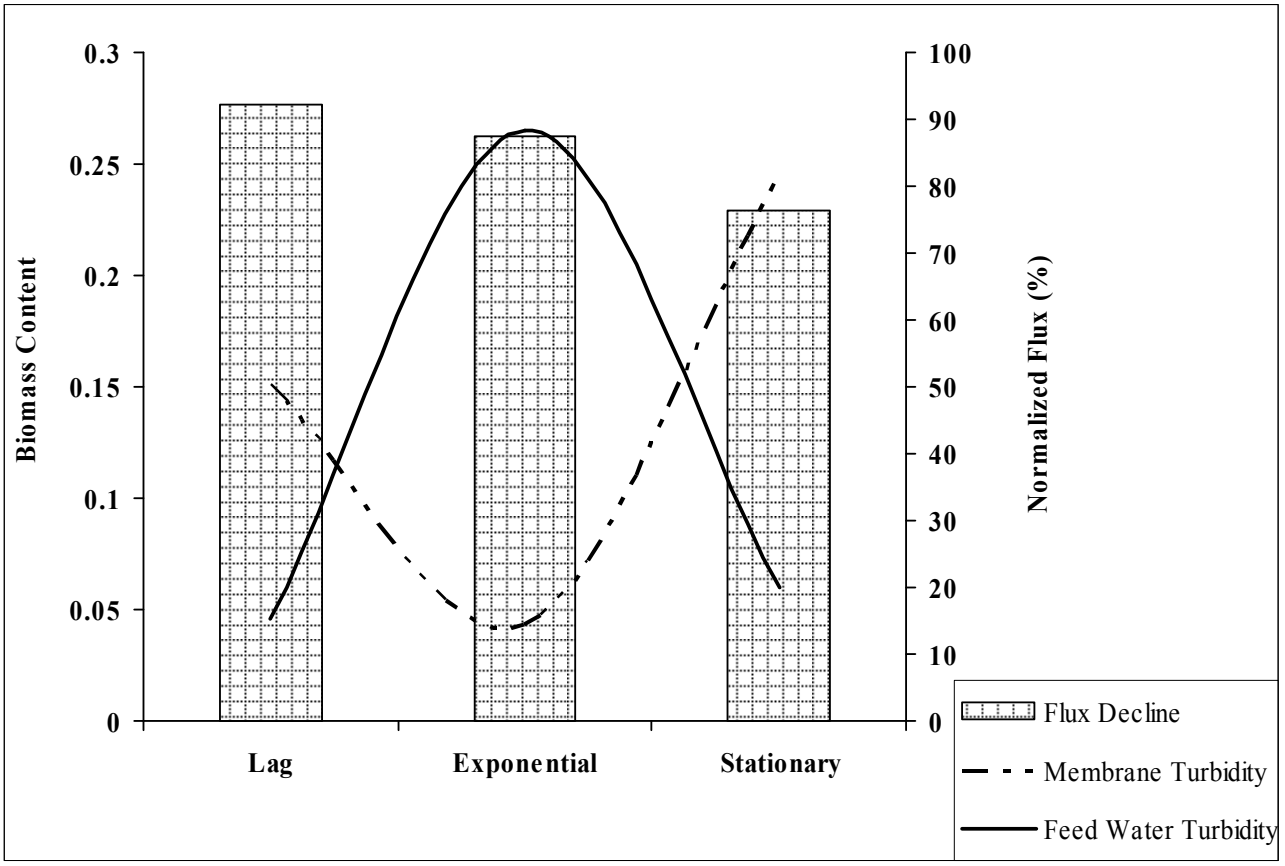



**Figure 3-5: Feed water and membrane biomass content in different growth phases after 24 hours filtration with respective flux decline.**

The data illustrates that despite the lowest amount of biofilm formed on the membrane during 24 hours filtration with the exponential phase biofoulant, the respective biofilm activity is in the highest value compared to other phases. On the other hand, the feed water biomass content is the highest value among other growth phases (e.g., lag and stationary) showing the affinity of low active biomass to detach in the feed water rather attaching to active biofilm on the membrane. In addition, Figure 3-5 demonstrates that the percentage of flux decline is increasing as the pure culture used as the biofoulant

grow. In other words, the most flux decline (24% normalized flux) occurred with pure culture in the stationary phase and the least flux decline happened with biofoulant in the lag phase (8% normalized flux).

It was observed that the biomass embedded in the biofilm was increased when bacterial cells were in stationary phase. This could suggest the presence of a signal substance, present within stationary phase cultures, which promotes attachment to membrane surface and biofilm formation (Decho, 2000). In addition, the difference in biofilm formation could be originated due to different types of EPS produced. It has been found that *Pseudomonas* strain produces two very dissimilar polysaccharides that compose the EPS. The first type that is produced only in exponential phase of growth contains sugars glucose, galactose, glucuronic acid, and galacturonic acid. The second type of EPS that is produced only in stationary phase contains N-acetylglucosamine, 2-keto-3-deoxyoctulosonic acid, and an unidentified 6-deoxyhexose (Christensen *et al.*, 1985). This difference in the polysaccharides can result in the dramatic variation observed in the membrane biomass content. For instance, amino sugar N-acetylglucosamine forms several hundred to several thousand repeating units to form the final EPS (Degeest *et al.*, 2001) which might cause significant flux decline compared to other growth phases.

### 3.3. Activity determined by CTC reduction

In order to provide an alternate measure of activity for comparison, CTC was used to measure the respiratory activity of the cells. As intact membrane biofilms were stained, CTC analyses clarified the activity of cells on the surface of the membrane biofilm. Three

different growth phases (i.e. lag, late-exponential, and stationary phase) which results in varied activity was measured using CTC in pure culture of the biofoulant, the feed water after 24 hours of filtration, and the membrane surface biofilm (Figure 3-6, Table 3-3).

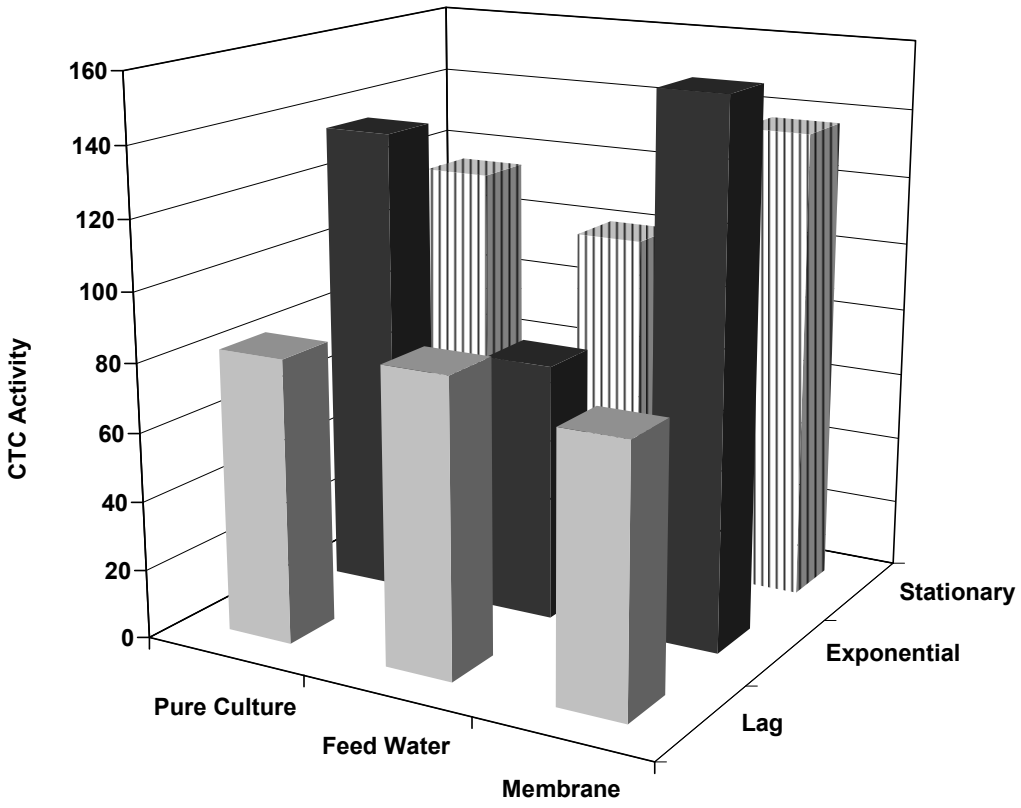



**Figure 3-6: CTC activity changes in different growth phases (lag, late-exponential, stationary) in pure culture (biofoulant), feed water after 24 hours of filtration, and biomass on the membrane.**

**Table 3-3: CTC activity changes in different growth phases (lag, late-exponential, stationary) in pure culture (biofoulant), feed water after 24 hours of filtration, and biomass on the membrane. Errors represent ± one standard error of the mean.**

| Growth Phase | Pure Culture | Feed Water | Membrane Biofilm |
|---|---|---|---|
| **Lag** | 82.36±8.85 | 85.53±7.21 | 76.69±4.35 |
| **Exponential** | 135.73±9.55 | 74.78±6.31 | 155.72±7.32 |
| **Stationary** | 114.88±8.07 | 100.21±17.90 | 137.06±19.70 |

Notably in the exponential phase, the membrane surface biofilm had a remarkable respiratory activity even more than the activity of the pure culture of biofoulant. This may be because *Pseudomonas fluorescens* has a strong tendency to aggregate and form colonies that may result in underestimation of total number of viable cells (Lazarova and Manem, 1995). Based on the Equation 2-1 in previous chapter, this underestimation will result in larger CTC activity. As the *Pseudomonas* has an affinity in biofilm production and aggregation even in other growth phases, the same problem but in less extent was observed in other experiments. Moreover, surface organisms in biofilms are more active due to more nutrients accessibility than the layers below (Watnick and Kolter, 2000).

There is a hypothesis that starvation in the biofilm may lead the bacteria to detach from the membrane surface to locate a nutrient available habitat (O'Toole *et al.*, 2000). This hypothesis can explain the detached dispersing bacterial pattern in the feed water as shown in Figure 3-7. In addition, when examined with epifluorescence microscopy, CTC stained biofilms tended to fade more rapidly in the lag phase samples representing extracellular activity present in the samples (Gruden *et al.*, 2003).

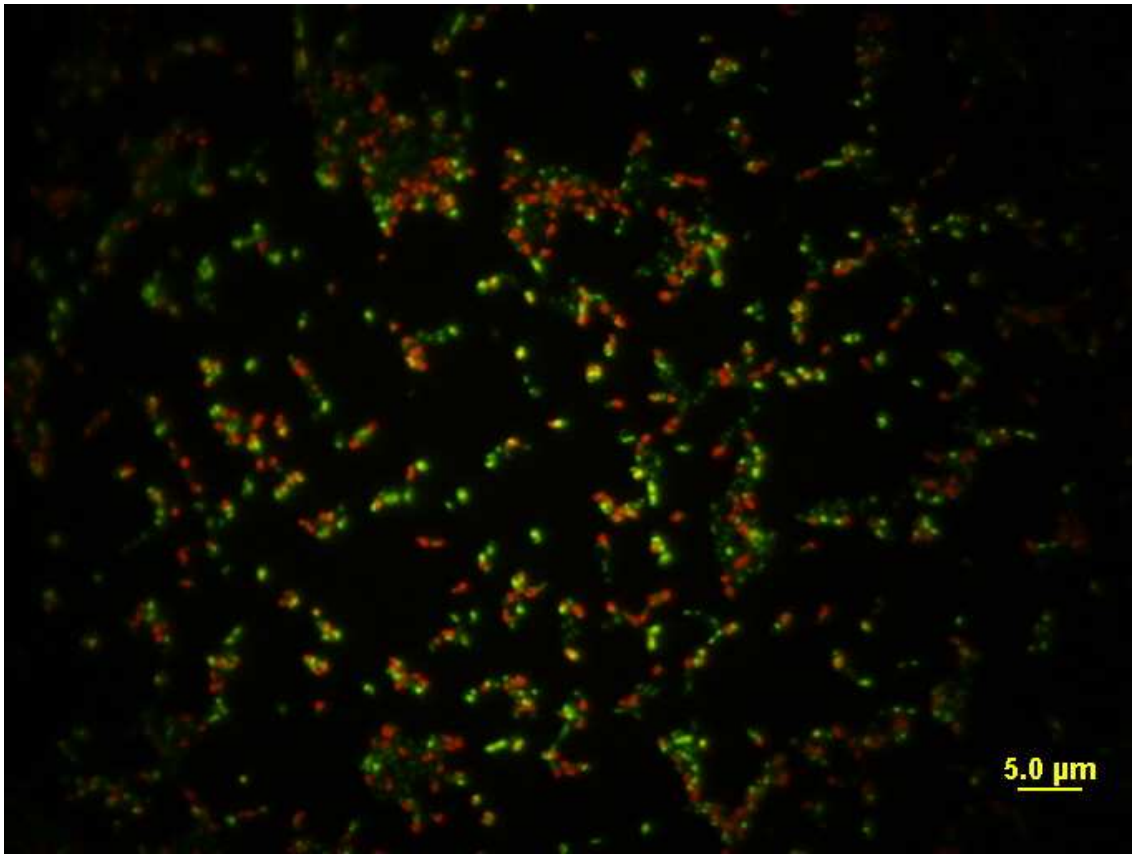



**Figure 3-7: CTC-stained and Picogreen-counterstained sample feed water resulted from 24 hours filtration of *P. fluorescence* in the lag phase (100X).**

### 3.4. ATP-CTC correlation

Kim *et al*. obtained a correlation between ATP activity content and INT-dehydrogenase respiratory activity but as far as we are aware, no studies were done to correlate ATP to CTC activity so far. Although INT and CTC measure the same aspect which is dehydrogenase respiratory activity, but it has been shown that INT has a good performance in anaerobic systems, while CTC has been set up to perform aerobically (Bhupathiraju et al., 1999). Although biofilm intracellular ATP activity can be used to measure the activity in the whole biomass composing the biofilm, and CTC provides a

direct way of measuring the respiratory activity of surface cells on the intact biofilm, an attempt was made to correlate these two factors (Figure 3-8).

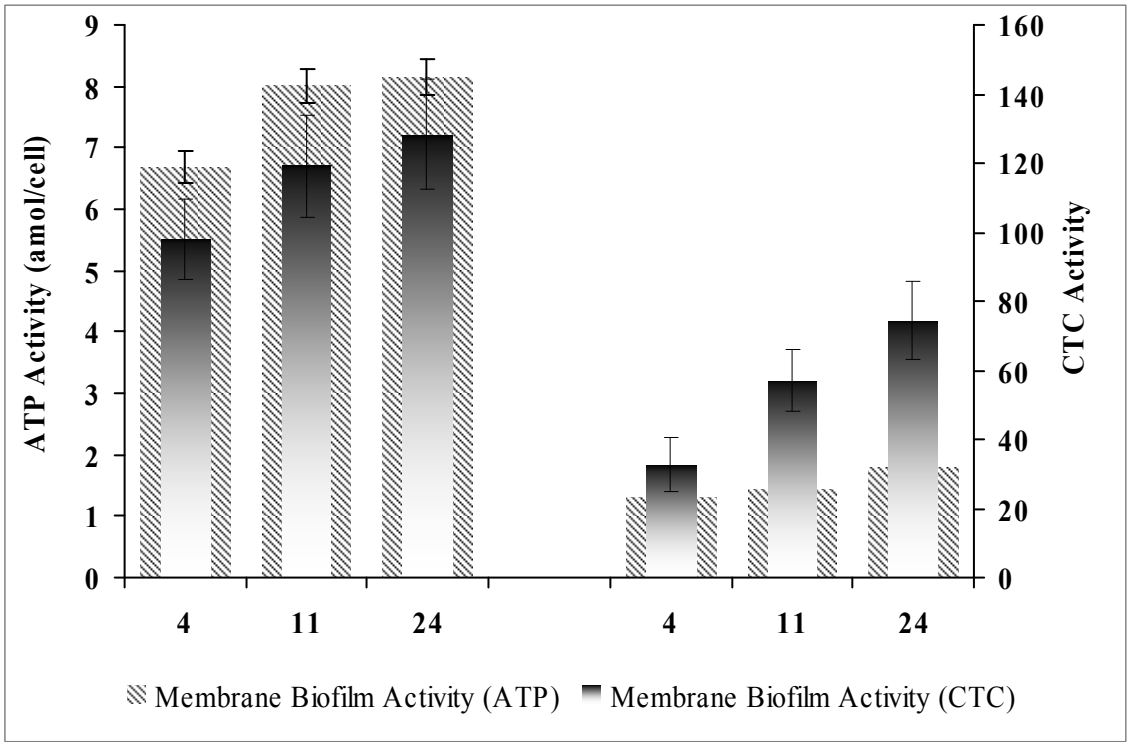



**Figure 3-8: ATP-CTC correlation on the membrane biofilm after different filtration duration with active and inactive biofilm. Error bars represent ± one standard error of the mean.**

Although there wasn't an excellent correlation between the biomass ATP and the surface CTC activity ($R^2 = 0.69$), it is obvious from Figure 3-8 that these two factors are related and as ATP increased by time, there was more CTC activity as well. Differences are expected since the methods we used for collecting biomass between CTC and ATP assays vary. We processed all of the biomass for ATP analysis and only visualized surface cells for CTC assay. It is possible that bacteria embedded in the membrane biofilm are less CTC-active under the stressful and turbulent conditions of cross-flow filtration. It has been also suggested that CTC has some toxic effects on bacteria resulting

in underestimation of the fraction of viable cells (Ullrich *et al.*, 1996; Bartscht *et al.*, 1999). In addition, cell aggregation and colonies forming underestimated the total number of cells and consequently overestimates CTC activity because it only accounts for measurements on the membrane surface.

# Chapter 4

# Conclusions & Future Work

## 4.1. Conclusions

This research was conducted to investigate effects of different growth phase conditions and metabolic activity of a pure culture biofoulant on biofilm formed on the membrane through short term (up to 24 hours) cross-flow filtration. The results indicate that the bacterial growth phase affects metabolic activity as determined by ATP concentration, and the maximum value occurs during late exponential growth phase. Results indicated that the initial biofoulant activity has no measurable impact on activity of the membrane biofilm as measured by ATP and CTC.

It is concluded that fixing and killing the biofoulants can be a great help in a filtration system to reduce the chance of biofilm formation. There is much less flux decline with fixed (inactive) cells as compared to an active biofilm, as measured by ATP. However, the biofilm appears to reach a steady-state activity level (after approximately 12 hours) while the flux continues to decrease as incubation time increases (up to 24 hours).

The growth phase of the initial biofoulant determines the ATP activity in the feed water and the biofilm. Lag phase and stationary phase had similar and low levels of ATP activity in the biofilm (<2.5 amol/cell). However, the exponential phase cells resulted in

significant activity (>10 amol/cell) in the biofilm formed. The lag phase resulted in the least amount of flux decline. Although the activity level in the biofilm created via stationary phase cells was lower, this experiment resulted in the most flux decline and most biomass formed on the membrane, likely due to EPS formation. The growth phase of the biofoulant also impacted the CTC activity of the biofilm formed. In this case, the CTC activity levels of stationary and exponential biofilms were similar and relatively high (135-155 pixels) as compared to the lag phase biofilm activity (77 pixels). This indicates that the surface activity (CTC) of the stationary biofilm is quite high as compared to the overall stationary biofilm activity measured by ATP. This might explain the increased flux decline for stationary phase biofilms as the surface may provide more resistance due to a higher concentration of active microorganisms present there.

It can be concluded that sole measure of activity is not enough, and that quantifying the cell activity is not adequate to predict flux decline. More information is needed as demonstrated by variable flux declines with similar levels of activity in the growth phase experiment. Lastly, a positive correlation between ATP and CTC activity was observed in comparing membrane biofilm activity levels. CTC values (74-155 pixels) provide a broader range and allow activity assessment on an intact biofilm, however these are limited to surface measurements only. On the other hand, ATP measurements represent a composite activity level of the whole biomass, but the main drawback with ATP assays is its high cost (USD 3-4 per sample).

**This research demonstrated that microorganisms in the lag phase or fixed microorganisms result in much less flux decline. Also, it indicated that stationary phase organisms create the worst case scenario. Activity assays are comparable but may not be useful in quantifying or predicting flux declines.**

## 4.2. Future Work

There is a strong probability that EPS matrix covered is a significant contributor to microbial activity as well as flux decline. Future studies should include a focus on the EPS matrix by measuring proteins and polysaccharides functional group through FTIR and investigating EPS structure and metabolic activity by some higher resolution microscopy such as CSLM to elucidate more information. This study showed that the biofoulant activity had no significant effect on the biofilm activity formed on the membrane. For having better knowledge about the EPS structure, effects of biofoulant activity on formation and activity of EPS can be studied.

Nutrients have an important effect on the bacterial cell growth and metabolic activity. They can either accumulate on the membrane surface or become entrapped into biofilm matrix and influence the biofilm activity. Study of the biofilm activity in both absence and presence of different concentrations of substrates (nutrients) will help in providing better understanding of biofilm growth.

# Chapter 5

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