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BIOLOGICAL CONTROL OF MICROBIAL ATTACHMENT AND MEMBRANE BIOFOULING

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Biological Control of Microbial Attachment and Membrane Biofouling

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ABSTRACT

Microbial attachment to solid surfaces has been widely observed in natural and engineered systems. A number of factors have been considered to contribute to microbial attachment, such as surface charge, hydrophobic interactions, hydrodynamic shear force, bacteria surface properties, extracellular polymeric substances and cell species. Unwanted microbial attachment can cause serious problems, such as membrane biofouling. Increasing global need for water and wastewater treatment has driven the widespread development of membrane processes. It should be realized that the main drawback hindering the wide application and further improvement of membrane systems is membrane biofouling due to microbial attachment onto membrane surface, which leads to increased operation cost due to frequent cleaning and replacement of the clogged membrane. So far, extensive research efforts have been devoted to prevention and cleaning of membrane biofouling. The traditional methods developed for reducing membrane biofouling are mainly based on physicochemical principles, such as modification of membrane surface, optimization of operation conditions, regular physical and chemical cleaning. However, research on biological control of membrane biofouling is limited. Therefore, this study was aimed to investigate novel strategies for biological control of microbial attachment and membrane biofouling through energy dissipation and D-amino acid.

In the first phase of study, biological control of microbial attachment and membrane biofouling through energy dissipation was investigated. For this objective, 2, 4-dinitrophenol (DNP), a typical metabolic uncoupler, which can dissipate the proton motive force and further disrupt adenosine-5'-triphosphate (ATP) synthesis, was employed. It was shown that energy dissipation through energy uncoupling resulted in reduced microbial attachment on various solid surfaces, and subsequently mitigated membrane biofouling on both hydrophobic polytetrafluoroethylene (PTFE) and hydrophilic nylon membranes. Results revealed that higher cell ATP level of suspended microorganisms favored microbial attachment to different solid surfaces, suggesting that energy metabolism was essentially involved in initial

attachment of microorganisms onto a solid surface. In addition, dissipation of ATP synthesis by DNP also led to lowered autoinducer-2 (AI-2) production in suspended microorganisms, and a positive link between the ATP and AI-2 levels in suspended microorganisms was established, i.e., AI-2 synthesis is energy dependent. Moreover, it was found that inhibition of ATP synthesis of suspended microorganisms facilitated prevention and mitigation of membrane biofouling. It appears from this study that energy metabolism is involved in microbial attachment, and inhibition of ATP and ATP-mediated AI-2 of suspended microorganisms would be a promising alternative for control of microbial attachment and membrane biofouling.

Physiology and structure of biofilms is biofilm age-dependent. To explore biological cleaning of membrane biofouling by ATP dissipation, the responses of different-age biofilms developed on membrane surfaces to a metabolic uncoupler 3, 3', 4', 5-tetrachlorosalicylanilide (TCS) were investigated. Results showed that ATP dissipation caused by TCS would promote detachment of different-age biofilms from membrane surfaces. It was observed that chemically inhibited cellular ATP synthesis also suppressed production of AI-2 and extracellular polymeric substances (EPS) of fixed biomass. The extent of biofilm detachment was found to be closely related to AI-2-regulated EPS content of fixed biomass. These results suggest that energy dissipation would lead to a new cleaning strategy of biologically fouled membrane.

Since most amino acids in microorganisms are present in the form of L-isomers, the existence and function of D-amino acids is not well understood yet. In this phase of study, how D-amino acid would affect attachment of mixed-species microorganisms to hydrophilic glass and hydrophobic polypropylene surfaces was investigated, and D-tyrosine was employed as a model D-amino acid. Results showed that D-tyrosine did not influence ATP synthesis, microbial growth and substrate utilization, but significantly inhibited the synthesis of AI-2, extracellular DNA (eDNA) and extracellular polysaccharides and proteins of suspended microorganisms, and subsequently reduced microbial attachment onto glass and polypropylene surfaces. These in turn provide a plausible explanation about how D-tyrosine suppressed

microbial attachment. It appears that D-amino acid would be a non-toxic agent for control of microbial attachment.

In addition, results further revealed that D-tyrosine effectively promoted mixed-species biofilm detachment from membrane surfaces. It was found that D-tyrosine at studied concentration negatively affected AI-2 secretion and extracellular polymeric substances production of fixed biomass on membrane surface. More importantly, the positive correlation between fixed biomass on membrane surface and corresponding AI-2 content of fixed biomass observed in detachment strongly suggest that AI-2 may mediate biofilm detachment from membrane surfaces. These indicate that D-tyrosine not only suppresses microbial attachment, but also promotes biofilm detachment through inhibition of cellular communication and EPS production.

In conclusion, this study clearly showed that energy dissipation and D-amino acid would be possible new alternatives for biological control of microbial attachment and membrane biofouling.

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ABBREVIATIONS

AB	autoinducer bioassay
ABC	ATP-binding cassette
ADP	adenosine diphosphate
AFM	atomic force microscopy
AHL	*N*-acylhomoserine lactones
AIP	autoinducing peptides
AIs	autoinducers
AMC	activated methyl cycle
ATP	adenosine triphosphate
CCCP	carbonyl cyanide chlorophenylhydrazone
CLSM	confocal laser scanning microscopy
COD	chemical oxygen demand
Con A	concanavalin A lectin
DCP	2,4-dichlorophenol
DLVO	Theory of microbial adhesion by Derjaguin, Landau, Vervey and Overbeek
DNP	2,4-dinitrophenol
DO	dissolved oxygen
DPD	4,5-dihydroxy-2,3-pentanedione
eDNA	extracellular DNA
EPS	extracellular polymeric substances
$FADH_2$	flavin adenine dinucleotide (reduced form)
FCCP	fluorocarbonyl-cyanide phenylhydrazone

FISH	fluorescence *in-situ* hybridization
FITC	fluorescein isothiocyanate
HPSEC	high performance size exclusion chromatography
HSL	homoserine lactone
LB	Luria-Bertani broth or agar
LPS	lipopolysaccharides
LuxS	S-ribosylhomocysteinase
MBR	membrane bioreactor
MLSS	mixed liquor suspended solid
MLVSS	mixed liquor volatile suspended solid
NADH	nicotinamide adenine dinucleotide (reduced form)
$NADP^+$	isocitric dehydrogenase
PBS	phosphate buffered saline
PCP	pentachlorophenol
PCR	polymerase chain reaction
PG	peptidoglycan
PI	propidium iodide
PMF	proton-motive force
PNP	para-nitrophenol
PN	extracellular proteins
PP	polypropylene
PS	extracellular polysaccharides
QS	quorum sensing
SAH	S-adenosyl-L-homocysteine
SAM	S-adenosyl-L-methionine

SD	standard deviation
SOUR	specific oxygen utilization rate
TAE	Tris-Acetate-EDTA
TCA	trichloroacetic acid
TCP	2,4,6-trichlorophenol
TCS	3,3’,4’,5-tetrachlorosalicylanilide
TMP	trans-membrane pressure
TOC	total organic carbon

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I: Journal Papers

1. Xu H.J. and Liu Y. (2010) Control of microbial attachment by inhibition of ATP and ATP-mediated autoinducer-2. Biotechnology and Bioengineering 107(1): 31-36.
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3. Xu H.J. and Liu Y. (2011) D-Amino acid mitigated membrane biofouling and promoted biofilm detachment. Journal of Membrane Science 376(1-2): 266-274.
4. Xu H.J. and Liu Y. (2011) Reduced microbial attachment by D-amino acid-inhibited AI-2 and EPS production. Water Research 45(17): 5796-5804.
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II: Conference Papers

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2. Liu Y., Xu H.J., and Jiang B. (2011) Biological control of microbial attachment on membrane surfaces. BIT 1st World Congress of EnviroBio-2011, 19-22 Oct 2011, Dalian, China.

CHAPTER 1
INTRODUCTION

1.1 BACKGROUND

Microbial attachment on natural and abiotic surfaces has been widely observed. Microbial attachment can be either beneficial or potentially destructive. For example, microbial attachment has been widely applied in wastewater treatment. However, in other situations, attached microorganisms are unwanted and cause serious and costly problems, such as contamination of food from processing equipment, causing infection associated with medical implants, enhancement of metal corrosion and formation of marine biofilms on ships' hull. As worldwide demands on water supply intensify, membrane processes are being used increasingly due to its high quality effluent and compact design with smaller footprints, but suffer from chemical and biological fouling. Membrane fouling can cause flux decline, increased filtration pressure and energy demand leading to increased operation cost due to frequent cleaning and replacement of clogged membranes. Among different types of fouling, membrane biofouling due to microbial attachment onto membrane surface is the most problematic. Extensive research efforts have been devoted to the control of membrane biofouling, while attention has been more placed on the physical and chemical interaction between microorganisms and membrane surfaces, such as physical and chemical cleaning, modification of membrane surface and optimization of operation conditions. Recently, some biological methods for controlling membrane biofouling have been proposed, such as inhibition of quorum sensing system and enzymatic disruption of extracellular polymeric substances. However, little has been known about biological control of initial microbial attachment on membrane surface at the level of energy metabolism.

Adenosine triphosphate (ATP) as a universal energy carrier in biological systems plays an important role in various biochemical reactions including active transport,

macromolecules synthesis and cell movement. The physiological behaviors of bacteria are closely related to metabolic energy supply available in the form of ATP. Quorum sensing provides an effective channel for microbial communication and coordination. Bacteria can communicate through signaling molecules, known as autoinducers. Although, quorum sensing has been intensively studied in pure culture, the effect of energy metabolism on the production of autoinducer-2 (AI-2) in mixed-species microorganisms is not yet clear. The fundamental question unanswered is if energy metabolism is involved in microbial attachment and membrane biofouling. Such information is strongly needed for developing new control strategies of membrane biofouling.

The predominant protein-building-blocks are L-amino acids in nature, while D-amino acids have been thought to have relatively minor functions in biological processes. D-amino acids are normally found in the peptidoglycan cell wall of bacteria. Recently, it has been realized that D-amino acids have previously unappreciated regulatory roles, in which D-amino acids are involved in regulating cell wall remodeling in stationary phase and inducing pure culture biofilm dispersion. So far, little information is available about the effect of exogenous D-amino acids on mixed-culture microbial attachment and biofilm detachment. Thus, this study also investigated the biological control of microbial attachment through D-amino acids.

1.2 OBJECTIVES AND SCOPE

The main objectives of this study are:

(1) To investigate feasibility of biological control of microbial attachment, and enhancement of microbial detachment of biofilm through energy inhibition by metabolic uncoupler, further to explore the effect of ATP dissipation on AI-2 production of mixed-culture microorganisms. The contributions of energy metabolism and AI-2 mediated cellular communication to microbial

attachment/detachment and membrane biofouling were also studied.

(2) To examine possible control of microbial attachment and enhancement of mixed-species biofilm detachment by D-amino acid, and to further look into the effect of D-amino acid on AI-2 mediated cellular communication and production of extracellular polymeric substances of suspended microorganisms in microbial attachment as well as in mixed-species biofilm dispersion.

In this study, a series of static microbial attachment assays with different biocarriers was conducted to investigate biological control of microbial attachment and membrane biofouling.

1.3 ORGANIZATION OF THE THESIS

This thesis is divided into the following seven chapters:

(1) Chapter 1 is an overview of the research background and objectives.

(2) A comprehensive literature review is presented in Chapter 2. This chapter covers factors affecting microbial attachment, challenges associated with microbial attachment, i.e., membrane biofouling, and current control strategies of membrane biofouling.

(3) Chapter 3 demonstrates biological control of microbial attachment and membrane biofouling through ATP inhibition. In this chapter, a positive link between ATP and interspecies cellular communication signals AI-2 was established, and the involvement of energy metabolism and AI-2 in microbial attachment and membrane biofouling were clearly demonstrated. This chapter offers insights into the control of biofouling by preventing initial microbial attachment through inhibition of energy metabolism.

(4) Chapter 4 focuses on ATP dissipation-promoted microbial detachment of different-ages biofilms. A possible mechanism of microbial detachment due to energy dissipation was proposed.

(5) Chapter 5 investigates the feasibility of using D-amino acid to control microbial attachment onto both hydrophobic and hydrophilic surfaces. For this purpose, extracellular DNA, extracellular polymeric substances, surface charge and AI-2 of suspended microorganisms were determined.

(6) Chapter 6 shows dispersion of mixed-species biofilms from membrane surfaces induced by D-amino acid. Cellular communication and extracellular polymeric substances of fixed biomass on membrane were found to play important roles in mixed-species biofilm dispersion induced by D-amino acid. It appears from this chapter that D-amino acids would be an effective agent for cleaning of biologically fouled membranes.

(7) Chapter 7 summarizes the major findings of this study and recommendations for future investigation.

CHAPTER 2
LITERATURE REVIEW

2.1 INTRODUCTION

Microbial attachment to a solid surface is a general phenomenon encountered in natural and engineered aquatic environments. Attachment can be found in diverse locations, including human dental plaque, gastrointestinal tract; leaves and root surfaces of plants; biocorrosion and biofouling of industrial materials. Following microbial attachment, growth on solid surfaces results in the formation of biofilm. Biofilm is a sessile microbial community consisting of cells that irreversibly attach to a substratum. The distinctive three-dimensional architecture of biofilm results from towering microcolonies distributed throughout a monolayer of cells, all of which are surrounded by an extracellular matrix (Flemming and Wingender 2010). This pattern of growth creates a series of open water channels, which allow the diffusion of necessary nutrients, including oxygen and carbon source, for cells to grow and the efflux of waste products to and from the cells within the microcolonies (O'Toole et al. 2000; Stoodley et al. 2002). When environment changes not to support this surface-attached lifestyle, the equilibrium of the dynamic process would shift to dissociation from biofilm for better surviving. Detached cells are believed to return to the planktonic mode of growth, thus closing the biofilm development life cycle.

2.2 FACTORS AFFECTING MICROBIAL ATTACHMENT

Microbial attachment involves complex interactions between the cells, solid surface and liquid phase. Microorganisms can be transported to a solid surface by many mechanisms, including sedimentation and Brownian motion, fluid dynamic forces, and interactions between cell surface and the substratum. Studies have shown that physical and chemical properties of cell, solid surface and growth medium

contribute to microbial attachment. Therefore, the extent of bacterial attachment is controlled by various factors, including surface charge, hydrophobic interactions, microorganisms growth phase, hydrodynamic shear force, solid surface roughness, the species and nature of bacteria, and extracellular polymeric substances as summarized in Table 2.1.

Table 2.1 Contributing factors to microbial attachment (Goulter et al. 2009).

Substratum	**Bulk solution**	**Cell**
Hydrophobicity	Presence of antimicrobial chemicals	Cell surface hydrophobicity
Roughness	Nutrient availability	Extracellular appendages
Charge	Ionic strength	EPS
Porosity	pH	Species
Conditioning film	Temperature	Surface charge
Surface chemistry	Shear force	Growth phase

2.2.1 Surface Charge

Bacterial surface carries negative charge under most physiological conditions. As most solid surfaces are also negatively charged, bacteria would experience electric double layer repulsion when approaching to a solid surface. Electrostatic interaction is involved in the initial step of microbial attachment, which is also governed by long-range van der Waals forces. Bacteria in aqueous suspension get electric charged due to the ionization of the surface functional groups, such as carboxyl groups, phosphate groups and amino groups. Most bacteria are always negatively charged as the number of carboxyl and phosphate groups of cell surface exceeds that of amino groups at pH 5-7 (Fletcher 1996). Surface charge density of bacteria may vary according to bacteria species, growth medium, bacteria age and bacteria surface structure (Meinders et al. 1995; Scheuerman et al. 1998; Shi and Zhu 2009).

Cell surface charge is often determined by its zeta-potential or electrophoretic mobility calculated from the bacteria mobility at a defined salt concentration and pH (vanderWal et al. 1997). The bacterial surface charge can also be quantified by colloid titration or electrostatic interaction chromatography (Flint et al. 1997; Araújo et al. 2010; Palmer et al. 2010).

Surface charge is related to ionic strength of bulk solution. A positive correlation between bacterial adhesion and ionic strength had been reported in the literature (van Loosdrecht et al. 1990a; Bolster et al. 2001; Abu-Lail and Camesano 2003). As bacteria and natural solid surfaces in aqueous solution are usually negatively charged, this would result in the repulsive energy. The repulsive energy tends to increase as the ionic strength of an aqueous solution decreases because shielding of the surface charges by the ions in the electrical double layers lessens (Fletcher 1988; Morisaki and Tabuchi 2009). Therefore, at high ionic strengths, the energy barrier disappears and bacterial cells easily and rapidly attain irreversible adhesion.

The role of electrostatic interactions in microbial attachment has been demonstrated. There exists a correlation between cell surface charge and microbial attachment (van Loosdrecht et al. 1987; Dickson and Koohmaraie 1989; van Loosdrecht et al. 1989; Rijnaarts et al. 1996; Ukuku and Fett 2002; Chia et al. 2011; Vilinska and Rao 2011). However, Flint et al. (1997) compared 12 strains of thermophilic *Streptococci* and their attachment to stainless steel with respect to their surface charge and no correlation between cell surface charge and bacterial adhesion to stainless steel was observed. Other studies also observed similar phenomenon (Gilbert et al. 1991; Li and McLandsborough 1999; Palmer et al. 2010), suggesting that microbial attachment is a very complex process, and surface charge could not be the only contribute factor.

The surface charge of solid surface is also highly related to microbial attachment. Vandermei et al. (1992) found initial attachment rate of all *Staphylococcal* strains were 2.5 times higher to positively charged surface than to negatively charged surface. Several studies on bacterial adhesion showed that bacterial attachment

generally increased with increasing cationic charge, while attachment decreased with increasing negative surface charge (Rose et al. 2005). However, different opinion exists in the literature (Dexter et al. 1975; Baker 1984; Baek et al. 2011). For example, Baek et al. (2011) investigated three commercial reverse osmosis (RO) membranes with different surface properties and found that surface charge of RO membrane had no relationship with microbial attachment. These discrepancies could be due to the different organisms used in the tests or the microtopography of the surfaces which may have changed the electrostatic charge densities.

To better evaluate and understand the interactions that control microbial attachment, attempts have been made to explain the phenomenon related to surface charge and bacterial adhesion through theoretical approach. Microbial attachment can be described using the classical Derjaguin-Landau-Verwey-Overbeek (DLVO) model (Derjaguin and Landau 1941; Verwey and Overbeek 1948), derived from colloid and surface chemistry. In the DLVO theory, the interactive energy between cell surface and solid surface of adhesion is the result of attractive van der Waals forces and the repulsive interactions due to electrical double layers (Bos et al. 1999; Hermansson 1999; Rijnaarts et al. 1999; Bayoudh et al. 2009; Goulter et al. 2009; Araújo et al. 2010). Accordingly, the total energy can be illustrated as:

$$V_t = V_R + V_A \qquad (2.1)$$

in which, V_t is the net interaction between bacteria and solid surface, V_R is the repulsion interactions due to negative charge of cell and solid surface, and V_A is the attractive interactions resulting from van der Waals interaction.

As shown in Figure 2.1, when cells approach the surface, attractive or repulsive force occurs depending on the charge and distance between the cell and substratum surfaces. In the first step of cell adhesion, cells have a tendency to accumulate at the secondary minimum, at which the bacteria is weakly held near the surface and easily removed by shear forces. Cells come to this position by their motility or Brownian motion, and adhere to the surface reversibly. A close approach is generally inhibited by electrostatic repulsion. However, if the bacteria overcome this barrier, it may be bound at the closer separation distance, the primary minimum,

where attractive forces are strong and attachment is presumably irreversible (Bos et al. 1999; Rijnaarts et al. 1999). DLVO theory has been widely used to predict bacterial adhesion to different solid surfaces (Gordon and Millero 1984; Simoni et al. 1998; Camesano and Logan 2000; Bayoudh et al. 2009; Vilinska and Rao 2011).

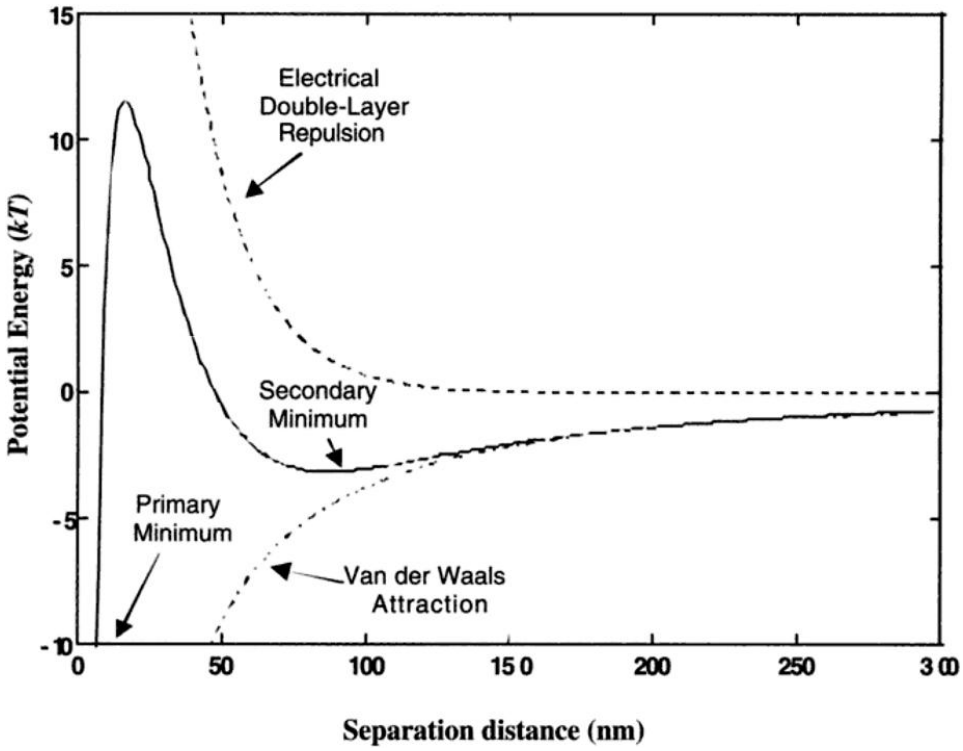

Figure 2.1 Schematic diagram of interaction energy of microbial attachment based on DLVO theory (An and Friedman 2000).

2.2.2 Hydrophobic Interaction

Cell surface hydrophobicity plays an important role in the initial steps of microbial adhesion. Existing evidence shows a correlation between cell surface hydrophobicity and initial adhesion to various solid surfaces (van Loosdrecht et al. 1990a; van Loosdrecht et al. 1990b; Marin et al. 1997; Zita and Hermansson 1997; Bechet and Blondeau 2003; Boks et al. 2008; Tang et al. 2009; Araújo et al. 2010; Palmer et al. 2010; Khan et al. 2011). For example, Rad et al. (1998) reported a significant correlation between cell surface hydrophobicity and attachment ability

of different bacteria strains, such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Streptococcus pyogenes* and *Staphylococcus aureus*.

Wettability is often used to describe surface hydrophobicity. It was found in a number of studies that microbial attachment was directly related to hydrophobicity of substratum surface with respect to numbers of attached cells, rate of attachment and strength of attachment (Rijnaarts et al. 1995; Sinde and Carballo 2000). It had been demonstrated that both hydrophobic *Salmonella* strains and hydrophilic *Listeria monocytogenes* attached in higher numbers to the more hydrophobic materials (Sinde and Carballo 2000). Moreover, microorganisms attached more rapidly to hydrophobic surfaces than to hydrophilic materials (Bendinger et al. 1993) and the binding may be stronger than that on hydrophilic surfaces (Rijnaarts et al. 1993). It has been demonstrated that coating surfaces with non-charged hydrophilic polymers resulted in reducing protein and cell adsorption on a variety of surfaces (Bergstrom et al. 1992; Park et al. 1998; Banerjee et al. 2011; Khan et al. 2011).

Hydrophobic interaction has been included in calculation of the Gibbs adhesion energy $\left(\Delta G_{adh}^{\circ}\right)'$ for microbial attachment (Liu et al. 2004; Hori and Matsumoto 2010) as shown below.

$$\left(\Delta G_{adh}^{\circ}\right)' = \gamma_{\mathrm{sm}} - \gamma_{\mathrm{ml}} - \gamma_{sl} \qquad (2.2)$$

where γ_{sm} , γ_{ml} and γ_{sl} are the solid-microorganism, microorganism-liquid and solid-liquid interfacial free energy respectively.

Evidence shows that γ_{sm} and γ_{ml} are closely related to both microorganism surface charge and hydrophobicity. According to the extended DLVO theory, cell-hydrophobicity represents an attractive force, while cell-hydrophilicity reflects repulsion (van Oss et al. 1986; van Oss 1989; van Oss 1993). It was demonstrated that cell hydrophobicity is inversely correlated to the quantity of surface charge of microorganisms (Briandet et al. 1999; Liao et al. 2001). An increased cell hydrophobicity represents that negative charges on bacterial surface reduce, as a result, repulsive force between particles would become weaker and weaker, which in turn favors the cell-surface interaction and finally lead to the formation of biofilm

(Liu and Tay 2002). Thus, both γ_{sm} and γ_{ml} would be a function of hydrophobic interaction between cell and surface, i.e., cell surface hydrophobicity and surface hydrophobicity.

Liu et al. (2004) proposed the concept of relative hydrophobicity and developed a model relating microbial adhesion to relative hydrophobicity:

$$\left(\Delta G^{\circ}_{adh}\right)' = \Delta G^{\circ}_{adh} - \alpha RT ln H_{o/w} \qquad (2.3)$$

in which ΔG°_{adh} is the change of standard free energy in microorganism-surface attachment, α is positive coefficient, and $H_{o/w}$ is relative hydrophobicity between microorganisms and support surface. $H_{o/w}$ is defined as:

$$H_{o/w} = \frac{overall\ hydrophobicity\ of\ microorganism\ and\ solid\ surface}{overall\ hydrophilicity\ of\ microorganism\ and\ solid\ surface} \qquad (2.4)$$

Equations 2.3 and 2.4 show that $\left(\Delta G^{\circ}_{adh}\right)'$ is negatively correlated to $lnH_{o/w}$. Based on this model, increasing cell surface hydrophobicity would cause a corresponding decrease in the effective Gibbs energy of the microorganism-surface interaction and promote cell-surface interaction. This model provides a direct connection between the hydrophobicity-associated surface thermodynamics and microbial adhesion, and clearly showed that increased cell surface hydrophobicity would favor microbial adhesion on both hydrophilic and hydrophobic solid surface (Liu et al. 2004).

2.2.3 Solid Surface Roughness and Topography

Characteristics of the solid surface are also important in the attachment process. The DLVO theory assumes perfectly smooth surfaces. But in reality, the perfectly smooth surfaces do not exist. In many cases, attachment of bacteria to surface is favored by surface irregularities, such as roughness, crevices and pits. This is because rougher surfaces provide a higher surface area for attachment and protect the adhered bacteria from shear forces (Auerbach et al. 2000; Whitehead and Verran 2006; Lee et al. 2010). Studies on the adhesion of psychrotrophic bacteria on different surfaces showed that maximum attachment occurred on materials with

higher degree of roughness, and significantly fewer cells were attached to glass which has a very smooth surface (Suarez et al. 1992; Shellenberger and Logan 2002; Li and Logan 2004).

Besides surface roughness, surface topography may also play a part in microbial attachment (Kumar and Anand 1998). It was observed in several studies that bacteria were able to attach to the cavities of the steel surface (Zoltai et al. 1981; Verran et al. 2001; Faille et al. 2010), i.e., surface topography may enhance biofilm development by protecting cells from removal and thus allowing biofilm re-growth more rapidly (Verran et al. 2001; Jullien et al. 2003). It was also commented that surface topography around the critical size close to the diameter of the bacterial cells may entrap bacteria on the stainless steel surface, thus providing cells with some degree of protection from cleaning agents (Flint et al. 2000).

2.2.4 Hydrodynamic Shear Force

In a biofilm system, hydrodynamic shear force may result from liquid/air flow or particle-to-particle attrition. Hydrodynamic shear has been regarded as one of the most critical factors in microbial attachment and biofilm formation under hydrodynamic conditions. Hydrodynamic factors can influence biofilms in many ways, including their control of the transport of bacteria to the surface during the initial stages of colonization, the transport of substrate after biofilm formation, metabolite secretion and shear stress, which are related to biofilm erosion and sloughing (Liu and Tay 2002). Biofilm structure under steady state conditions also depends on shear stress, which creates a balance between biofilm growth and cell detachment.

Studies have shown that magnitude of hydrodynamic shear force could influence biofilm density and strength, i.e., a higher shear force results in a thinner and denser biofilm (Ohashi and Harada 1994; Costerton et al. 1995; Chen et al. 1998; Liu and Tay 2002; Rupp et al. 2005). In addition, Liu and Tay (2002) reported that biofilm density was correlated closely with the adhesion strength of attached bacteria,

which depended on the shear force imposed on the biofilm surface. The biofilm formed under low shear stress detached easily, while the adhesion strength of the biofilm formed under high shear stress was much stronger (Ohashi and Harada 1994). Therefore, it appears that a certain shear force in the biofilm system is necessary in order to produce a compact and stable biofilm structure, i.e., higher shear force favours the formation of a smoother and denser biofilm.

The physical hydrodynamic shear forces acting on biofilm could also influence biofilm structure. For biofilm grown under low, laminar flow, the patterns of formed microcolonies are generally isotropic with no direction. However, under high-shear, turbulent flow, biofilm would tend to grow in the downstream direction forming filamentous streamers (Stoodley et al. 1999). The filamentous biofilms were also commonly found in fast-flowing environments, such as marine hydrothermal vents, hot springs (Reysenbach and Cady 2001).

In addition, hydrodynamic shear force would influence mass transfer of substrate. Substrate flux in biofilms would increase with increasing hydrodynamic turbulence (Brito and Melo 1999; Wasche et al. 2000). It had been reported that high effective diffusivities at high substrate concentrations exhibit lower biofilm densities; on the other hand, reduced effective diffusivities at high flow velocities show higher biofilm densities (Tanyolac and Beyenal 1997). Thus, it should be noted that hydrodynamic conditions indeed have a dual effect on the performance of mass transfer in biofilms, i.e. high turbulence would facilitate substrate diffusion in biofilms; however, shear force would enhance biofilm density and this in turn reduces substrate diffusion in biofilms. The observed diffusivity of substrate would be a net result of these two phenomena (Liu and Tay 2002).

Another important shear force-related phenomenon in biofilm is the overproduction of extracellular polymer substances (EPS). In the study of the effect of superficial air upflow velocity (a major hydrodynamic shear force) on the production of EPS in steady state three-phase fluidized bed reactor, it was found that the content of extracellular polysaccharides (PS) is at least 4.5-fold higher than that of

extracellular proteins (PN), and the ratio of PS to PN increase with increasing superficial air upflow velocity (Lopes et al. 2000). Ohashi and Harada (1994) reported a linear relationship between the content of exopolysaccharides in biofilm and biofilm density. These clearly indicate that hydrodynamic shear force-induced EPS in biofilms play a key role in building up and maintaining the architecture of biofilms.

2.2.5 Bacterial Surface Structure

Several studies had shown that cellular appendages, such as flagella, fimbriae, pili, and extracellular polymers as illustrated in Figure 2.2, are also involved in the bacterial adhesion process (Sinde and Carballo 2000).

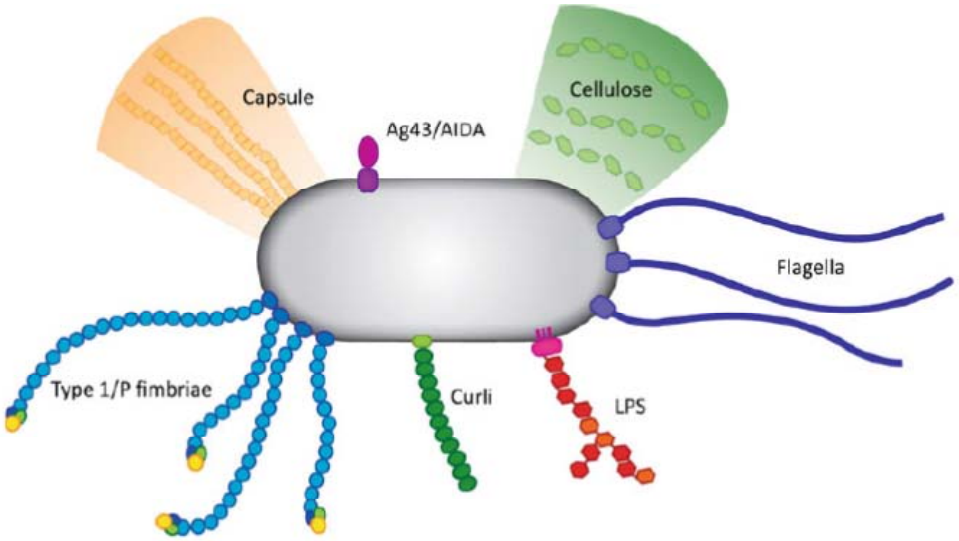

Figure 2.2 Surface structures of *E. coli* involved in microbial attachment (Klemm et al. 2010).

Flagella

Many bacteria have been shown to be motile by using flagella on the cell surface. It has been proposed that the formation of biofilm requires flagellar-dependent association and binding with a surface to form a monolayer. Individual cells on this monolayer then grow and aggregate into a number of microcolonies through

twitching motility (Shirtliff et al. 2002). Several studies have shown that flagella synthesis is down regulated upon adhesion of bacterial cells to a surface and during the development of a biofilm, suggesting that flagella and consequent motility play an important role in the initial attachment of bacterial cells to a surface (Pratt and Kolter 1998). There is no consistent conclusion for the role of flagella in the initial attachment of organisms to solid surfaces. Some studies reported positive role of flagella in attachment (Bouttier et al. 1997; Van Houdt and Michiels 2005), and others found that the presence of flagella had no effect on attachment (Rivas et al. 2005; Rivas et al. 2007). Studies focusing on *E. coli* have shown that motility facilitated the initial microbial attachment between bacterial cells and surfaces submerged in glucose minimal media (Van Houdt and Michiels 2005). Despite this finding, attachment of mutant *E. coli* cells without flagella to solid surfaces was not completely eliminated, suggesting that factors other than flagella also play an important role in the attachment of *E. coli* to surfaces. Without motility, the chances of a bacterial cell coming into contact with a surface are reduced and dependent largely on the shear force of liquid.

Pili

Pili, or fimbriae, are generally only visible under electron microscopy and are thinner, shorter and more numerous than flagella, usually extending 1-2 μm from the cell surface (Pratt and Kolter 1998). There are several different types of pili, which are classified according to their receptor binding specificities and are different in function, molecular structure, localization on a cell surface and mechanisms of secretion and assembly (Walker et al. 2004; Strauss et al. 2009). A bacterial cell frequently has more than one type of pilus.

Type IV pili in many Gram-negative bacteria such as *P. aeruginosa* have been studied most in terms of the relationship with microbial attachment. This type of pili has been reported to be involved in both nonspecific adhesion to abiotic surfaces for colonization and biofilm formation (Mattick 2002). Bahar et al. (2010) also showed that type IV pili play a critical role in both the surface attachment and the biofilm formation of *Acidovorax citrulli* under a medium flow. Additionally, type IV pili

null mutants were unable to perform twitching movement against the direction of medium flow and subsequent attachment. Type IV pili extend from the poles of a bacterial cell and mediate the movement of bacteria over surfaces without the use of flagella (Craig and Li 2008).

Type I pili and P pili are also well-known to be involved in microbial attachment, which are the most commonly present in Gram-negative bacteria such as *E. coli* and *Salmonella* species (Brunder et al. 2001). The role of type I fimbraie in the attachment of *E. coli* to surfaces has been shown in a number of studies (Prigent-Combaret et al. 2000; Cookson et al. 2002; Chao and Zhang 2011). For example, type I pili significantly enhanced microbial attachment as well as the transformation ratio from reversible attachment to irreversible attachment, however they did not significantly influence the adhesion strength in irreversible attachment (Chao and Zhang 2011).

Lipopolysaccharide

Lipopolysaccharide (LPS) is composed of lipid A, core polysaccharides, and an outer membrane region of O-antigen units (Caroff and Karibian 2003) and is important for initial microbial attachment of bacteria to solid surface (Davey and O'Toole 2000). The LPS has been shown to form hydrogen bonds with mineral surfaces and 1000 or fewer such bonds are sufficient to anchor a cell firmly to a surface (Jucker et al. 1997; Kabanov and Prokhorenko 2010). It was reported that *P. aeruginosa* PAO1 lacking LPS demonstrated reduced adhesion to hydrophilic surfaces (Makin and Beveridge 1996). *E. coli* lacking LPS, as in *P. aeruginosa*, also showed decreased ability to attach to solid surface (Genevaux et al. 1999). Similar phenomenon was also observed for other species (Walker et al. 2004; Strauss et al. 2009; Hori and Matsumoto 2010; King et al. 2010; Boyer et al. 2011; Karunakaran and Biggs 2011).

2.2.6 Extracellular Polymeric Substances

In a biofilm, cells are enmeshed in a bacterial extracellular polymeric substance

(EPS) matrix which defines the morphology, structure and physicochemical properties of the biofilm. It is generally accepted that in a biofilm, EPS forms a highly hydrated gel containing a mixture of polymers like polysaccharides, proteins, lipids, nucleic acids and humic substances that arise as a result of different cellular processes such as active secretion, cell lysis, discarding cellular materials and absorption of matter from the environment (Flemming and Wingender 2000; Flemming and Wingender 2010). The composition and quantity of EPS would vary with environmental conditions, bacterial species and age. For example, less EPS production was observed at the lower level of dissolved oxygen, which would eventually lead to biofilm slough (Applegate and Bryers 1991; Ahimou et al. 2007). Multivalent cations, such as calcium and magnesium, also play a role in biofilm formation by bridging negatively charged EPS to create stable EPS matrix (Mayer et al. 1999; Chen and Stewart 2002). EPS is of importance for biofilm development and structural integrity as summarized in Table 2.2 and the presence of EPS alters the characteristics of the cell surface such as charge density, hydrophobicity and surface morphology (Dufrene et al. 1996; Flemming et al. 2007; Rollefson et al. 2011).

Table 2.2 EPS functionality (Flemming et al. 2007).

Effect of EPS component	Nature of EPS component	Role in attachment and biofilm formation
Constructive	Neutral polysaccharides Structural component	Amyloids Structural component
Sorptive	Charged or hydrophobic polysaccharides	Ion exchange, sorption
Active	Extracellular enzymes	Polymer degradation
Surface-active	Amphiphilic Membrane vesicles	Interface interactions Export from cell, sorption
Informative	Lectins Nucleic acids	Specificity, recognition Genetic information, Structure
Redox active	Bacterial refractory Polymers	Electron donor or Acceptor
Nutritive	Various polymers	Source of C, N, P

Some bacteria complete the transition to irreversible attachment by increasing production of extracellular polymer substance (O'Toole et al. 2000). For example, *E. coli* will increase the secretion of colanic acid to enhance the attachment. Colanic acid is an exopolysaccharide secreted by attached cells, and it is the major constitute of the biofilm extracellular matrix. Danese et al. (2000b) found that a strain of *E. coli* deficient in colanic acid production was able to attach to a surface, but was unable to develop the characteristic three-dimensional architecture of a mature biofilm. The *E. coli* outer membrane protein Ag43 is also required for the initial steps in biofilm formation because it facilitates cell-cell interactions by inducing autoaggregation (Danese et al. 2000a). The resulting aggregates form microcolonies, which are the basic building blocks of a mature biofilm.

Nucleic acids detected in extracted EPS were at first believed to originate from intracellular contamination during the extraction procedure or the presence of dead cells in the matrix. However, Whitchurch et al. (2002) showed that extracellular DNA (eDNA) is required for initial establishment of biofilms by *P. aeruginosa*. Later on, Bockelmann et al. (2006; 2007) demonstrated the importance of eDNA in biofilms structure. Das et al. (2010) also reported that removal of eDNA from several Gram-positive bacteria reduces initial adhesion to and surface aggregation of bacteria on surface.

Several studies have shown that adhered bacteria were significantly detached from surfaces after treated with proteolytic enzymes, and this in turn provides evidence for the role of specific surface proteins in attachment (Danielsson et al. 1977; Bashan and Levanony 1988). A recent study analyzed the protein profiles of *P. aeruginosa* at different biofilm development stages by two-dimentional polyacrylamide gel electrophoresis and showed that the average difference in protein production between each development episode was 35% of detectable proteins (Sauer et al. 2002). Jucker et al. (1997) reported that polysaccharides on bacterial outer surface could significantly affect bacterial adhesion.

In addition to microbial attachment, the contribution of EPS to biofilm formation has been examined through mutation of genes involved in EPS synthesis. Alginate, produced by *P. aeruginosa* strains, is the best-studied component of biofilm EPS and appears to play an important role in determining biofilm structure. The structural complexity of mushroom and mound structures, which developed in wide type biofilms grown from the isolate *P. aeruginosa* FRD1, were suppressed in alginate synthesis mutant strains, which formed flat, patchy biofilms (Nivens et al. 2001). On the contrary, structural complexity was observed in a flat, non-mucoid wide type *P. aeruginosa* PAO1 biofilm in an isogenic mutant where alginate was over expressed (Hentzer et al. 2001).

From the level of gene expression, EPS production may be a strategy for cells to acclimate surface-attached growth mode. It has been reported that microbial attachment could change cells in gene expression (Vandevivere and Kirchman 1993). Davies et al. (1993) demonstrated that the transcription of *algC*, a key gene involved in the biosynthesis of alginate required for the synthesis of EPS, is up-regulated three- to five-fold in adhered cells of *P. aeruginosa* compared with their planktonic counterparts within minutes of adhesion to surfaces. Even more, Garrett et al. (1999) linked the up-regulation of flagellum synthesis with the up-regulation of alginate synthesis. These suggest that cells adjusted to an immobile life on surfaces with increasing production of EPS and decreasing flagellum synthesis. The properties of cell surface play important roles in microbial attachment as summarized in Table 2.3.

Table 2.3 Examples of cell surface properties and EPS involved in microbial attachment.

Bacterial strain	Substratum	Factors involved in adhesion	Reference
Escherichia coli	Abiotic surface	Flagella	(Van Houdt and Michiels 2005)
Escherichia coli	Glass and polystyrene	Curli fimbriae	(Cookson et al. 2002)
Escherichia coli K-12 and its six mutants	glass, two metals (aluminum and stainless steel), and three plastics (polyvinyl chloride, polycarbonate, and polyethylene)	Type I pili	(Chao and Zhang 2011)
Acidovorax citrulli	Stainless steel	Type IV pili	(Bahar et al. 2010)
Escherichia coli K12	Quartz surface	lipopolysaccharide	(Walker et al. 2004)
Pseudomonas aeruginosa	Flow chamber channel	eDNA	(Whitchurch et al. 2002)
Staphylococcus haemolyticus	PVC	Extracellular protein	(Fredheim et al. 2009)
Escherichia coli K12	PVC	Extracellular polysaccharide	(Danese et al. 2000b; Kaplan et al. 2004)

2.2.7 Cell Species

The adhesion process depends on the bacterial species and strains since they have different physicochemical characteristics and cell surface properties. The extent of microbial attachment may vary among bacterial species. It has been shown that Gram-negative species attached more on dairy equipment than Gram-positive organisms due to the differences in structure and chemical composition of their outer layer of cell wall (Lewis and Gilmour 1987; Suarez et al. 1992). Microbial attachment in nature environments often consist of multiple species, and the attachment may be influenced by the interactions among bacterial species. For example, it was found that microbial attachment of *Listeria monocytogenes* was enhanced by the presence of *Pseudomonas fragi* (Sasahara and Zottola 1993). It seems that EPS produced by one species may help to stabilize other species. In addition, the initial colonized species may potentially encourage the colonization of other physiologically compatible species, while inhibiting the attachment of others (Kumar and Anand 1998).

2.3 CHALLENGES ASSOCIATED WITH MICROBIAL ATTACHMENT: MEMBRANE BIOFOULING

2.3.1 Membrane Biofouling

The need for fresh clean water is growing rapidly due to the world population growth that imposes larger demands of water supply for domestic use, agriculture and industry. Hence, there is a strong need to increase fresh-water availability either by recycle of used water or by production of fresh water from seawater (Barnett et al. 2005). The global need to increase fresh water supply and more extensive water treatment has driven the widespread development of large-scale membrane filtration processes due to their unique advantages of high quality effluent and compact design with less space requirement. Membrane processes can be classified as microfiltration (MF), ultrafiltration (UF), nanofiltration (NF), reverse osmosis (RO),

dialysis, and electrodialysis (ED). The general characteristics and the typical operating ranges of each membrane process are summarized in Table 2.4.

Table 2.4 Characteristics of membrane process (Cheryan 1998).

Membrane process	Separation mechanism	Structure (pore size)	Operating range, μm
MF	Sieve	Macropores (>50nm)	0.08-2.0
UF	Sieve	Mesopores (2-50nm)	0.005-0.2
NF	Sieve+solution/ diffusion+exclusion	Micropores (<2nm)	0.001-0.01
RO	Solution /diffusion+exclusion	Dense active layer (<2nm)	0.0001-0.001
Dialysis	Diffusion	Mesopores (2-50nm)	—
ED	Ion exchange with selective membrane	Micropores (<2nm)	—

The main drawback of membrane technology is fouling leading to decreased membrane flux and increased filtration pressure, and subsequently increased operation cost due to frequent cleaning and replacement of the clogged membrane. Mebrane fouling may be due to (a) precipitation of salts, mainly calcium salts (inorganic fouling), (b) suspended solids (particulate and colloidal fouling), (c) dissolved organic matter (organic fouling) and (d) biofilm formation by microorganisms (biofouling) (Le-Clech et al. 2006; Drews 2010; Gao et al. 2011; Tang et al. 2011). Among them, membrane biofouling has been regarded as the most serious problem (Flemming 2002; Mansouri et al. 2010), and was even described as the Achilles heel (Flemming et al. 1997).

Extensive research efforts have been devoted to the prevention or cleaning of membrane biofouling. As membrane biofouling is the complex phenomenon involving membrane surface and microorganisms, present strategies to prevent biofilm formation and membrane biofouling are mainly based on physical-chemical principle, such as optimization of operation condition, new membrane development, pretreatment, and chemical and physical cleaning.

2.3.2 Physical Cleaning

Membrane cleaning is performed when there is a significant drop in permeate flux or salt rejection, or when there is a need to increase the trans-membrane pressure significantly to maintain the desired water flux. Membrane backwashing is normally used as physical cleaning (Chen et al. 2003). Backwashing may be efficient to remove loosely attached sludge on membrane surface, while clogging near membrane surface may only be partially loosened by backwashing. The important operation parameters for backwashing are the duration, frequency and intensity. Although less biofouling is expected when backwashing duration and frequency are increased, optimization of backwashing is needed to balance energy consumption and permeate production. Air scourging can also be used in backwashing. Other mechanical cleaning, such as physical scrubber and the use of UV and pulsed acoustic devices was also used (Brizzolara et al. 2003).

2.3.3 Chemical Cleaning

Many water treatment and membrane filtration processes frequently require exposure to aggressive hydrodynamic/physical and chemical cleaning. These may include aeration of feed solution, backwashing, and highly turbulent flow to remove particles on membrane surface. Once the cause of fouling is identified, various cleaning chemicals could be used to remove foulants from the membrane and help to restore the membrane flux. Chemical cleaning involves intermittent exposure to high concentration of acid/alkali/chlorinated solutions, sometimes continuous addition of hypochlorite and other oxidizing chemical solutions (Al-Amoudi and

Lovitt 2007). Table 2.5 shows typical cleaning chemicals commonly used for membrane cleaning.

Table 2.5 Major chemicals for membrane chemical cleaning (Liu et al. 2001).

Category	Chemicals	Functions
Alkali	NaOH	Hydrolysis, dissolution
Acids	Citric acid, Nitric acid	Dissolution
Chelating Agents	EDTA	Chelation
Oxidants	NaClO, H_2O_2, peroxyacetic acid	Oxidation, disinfection
Surfactants	Detergents, Surfactants	Emulsifying, surface conditioning

Alkali

Alkali is usually used to clean membrane fouled by organic and microbial foulants. The extracellular polymeric substance such as polysaccharide and proteins could be hydrolyzed by alkali. Another important of alkali is to increase negative charges of humic substances (Mallevialle et al. 1989), which have many functional groups that are organic acids. During the high pH of alkali cleaning, the organic acids would dissociate. Subsequently the negative charges on organic compounds and their solubility would increase, easily removed by chemical cleaning.

Acids and Chelating Agents

Acids are used to remove scales and metal dioxide from membrane surface. When membrane is fouled by iron oxide, citric acid is very effective due to its capability to dissolve the iron oxide. Some polysaccharides and proteins may also begin to hydrolyze due to exposure to acids. The chelating agents such as EDTA could be used to remove divalent cations, thus help to improve the cleaning efficiency (Hong and Elimelech 1997).

Oxidants

In industrial processes, including drinking water treatment, the most common means of oxidation for membrane cleaning is chlorination (Le-Clech et al. 2006). Other oxidizing biocides such as ozone, hypochlorite (bleach), hypobromite, chloramine, chlorine dioxide, and hydrogen peroxide can similarly be used. Hypochlorite treatment normally uses sodium hypochloride solutions (0.01 to 0.5%). Sodium hypochloride hydrolyzes the organic molecules, and therefore loosen the particles and biofilm attached on the membrane. The effects of cleaning chemicals like NaClO on microbial community have been studied for MBR process (Lim et al. 2005). Normally, the recovery cleaning would take high concentration of 0.2-0.5 (wt.) % NaClO treatment for two hours.

Surfactants

Surfactants are compounds having both hydrophilic and hydrophobic structure and are semi-soluble in both organic and aqueous solvents. Surfactants can react with proteins, fat and oils and help to clean these materials on membrane. Some surfactants can interfere in the reactions between microorganisms and membrane and can disrupt some functions of cell wall (Paul and Jeffrey 1985). Thus, surfactants affect biofouling by preventing biofilm formation on membrane. These compounds may be combined to clean membrane fouling, such as NaClO coupled with nitric acid in flux recovery cleaning. Commercial cleaning products are also mixtures of these compounds. Cleaning efficiency varies with respect to the conditions applied during cleaning, include cleaning time, type of cleaning agent, cleaning agent dose, cleaning solution pH, flow situation during cleaning, and cleaning solution temperature (Al-Amoudi and Lovitt 2007). A systemic study should be conducted to select cleaning solution for the optimal consideration of both cleaning efficiency and operation cost.

2.3.4 Modifying Membrane Surface Property

Modification of membrane surface properties is another strategy for control

membrane biofouling. Generally, some important properties such as surface zeta potential, surface roughness and hydrophobicity would affect initial microbial attachment on membrane surface. Modification of membrane is to make membrane more fouling-resistant.

Membrane Preparation

From the point view of membrane preparation, the addition of hydrophilic polymers such as polyvinylpyrrolidone (PVP) has become a standard method; commercial UF and MF membranes such as 'hydrophilized' polysulfone (PSF) or polyethersulfone (PES) are mostly produced using this approach. The presence of PVP could to some extent decrease the degree of biofouling of the resulting membranes (Marchese et al. 2003). Polyethylene glycol (PEG) with various molar masses was added during the preparation of PES UF membranes. As the PEG content was increased, the pure water flux increased and the BSA adsorption decreased (Radha et al. 2009). The performance of the resulting membranes was influenced by both molar mass and concentration of PEG. Membranes prepared with higher molar mass of PEG had higher pure water permeation and larger pores.

Grafting

As reviewed above, more severe fouling is expected when hydrophobic membranes are used in the MBR, and efforts have been focused on increasing membrane hydrophilicity through membrane modification. It has been reported that after NH_3 and CO_2 plasma treatment of polypropylene hollow fiber membrane (Yu et al. 2005a; Yu et al. 2005b), membrane hydrophilicity significantly increased and modified membranes presented better filtration performances and flux recovery than those of unmodified membranes (Yu et al. 2005b). UV and plasma grafting provide more permanent surface modification, primarily with poly-2-hydroxyethyl methacrylate (HEMA), polyacrylic acid (PAA), polymethacrylic acid (MAA), and similar families of compounds (Van Der Bruggen 2009; Khulbe et al. 2010).

Coating

In coating, the membrane surface is contacted with a solution/dispersion of

modifying additive and after evaporation a thin layer of coating is formed. Membrane surface properties such as hydrophilicity, roughness and charge are modified to give improved resistance to biofouling. Coating is usually simple to apply and the process can easily optimized to the existing membrane manufacturing processes. Some materials with non-fouling oligoethylene glycol side chains have been used to coat commercial polysulfone membranes (Hyun et al. 2006). Other coating materials are hydrophilic coatings such as polyvinyl alcohol (PVA) and negatively charged polymers such as polyacrylic acid (PAA) on NF membranes etc (Ba et al. 2009; Ba et al. 2010).

Surfactants containing a cationic quaternary ammonium head group and a long alkyl chain tail also have been used as bactericidal agents. Covalently coupled coatings developed based on these surfactants greatly reduce the viability of numerous bacteria and mixed biofilms of bacteria and yeasts (Gottenbos et al. 2002; Oosterhof et al. 2006). Similarly, poly(4-vinyl-N-alkylpyridinium bromide) functionalized surfaces are also effective at killing bacteria on contact (Tiller et al. 2001). These coatings have the common feature of preserving the flexibility and accessibility of the alkyl chain group for penetrating the bacterial cell wall, thereby allowing the cationic nitrogen to disrupt the underlying membrane (Mansouri et al. 2010).

Some coating approaches have focused on preventing the initial adhesion of bacteria to a surface, or lowering the force of adhesion between bacteria and a surface to facilitate removal. Poly (ethylene glycol)-modified surfaces have been shown to greatly reduce bacterial adhesion and protein adsorption by providing a steric repulsive barrier, as well as facilitate the removal of microorganisms (Park et al. 1998; Kingshott et al. 2003; Gudipati et al. 2005).

Others

More diverse approaches have seen the incorporation of biocidal and other functional modifications. Incorporation of nanoparticles into modifying polymeric membrane is another strategy of control of membrane biofouling. These nanoparticles include silica, AL_2O_3 and titanium dioxide (TiO_2) (Lohwacharin and

Takizawa 2009; Maximous et al. 2009; Wang et al. 2011). TiO_2 has been the focus of numerous investigations in recent years, because of its photocatalytic effects that decompose organic chemicals and kill bacteria (Sotto et al. 2011). By creating a reactive membrane surface by the application of TiO_2 nanoparticles, the organic chemicals and bacteria responsible for biofouling may be eliminated. Kim et al. (2003) applied titanium dioxide nanoparticles to a thin-film composite reverse osmosis membrane and found improvement in membrane permeability after incubation in *E. coli* solution, compared to uncoated thin-film composite membranes, contributed to the photo-biocidal effect of the TiO_2. Recently, silver-based materials attracted special interest (Monteiro et al. 2009; Gladis et al. 2010; Gorup et al. 2011; Sawada et al. 2012). For example, the silver-coating was achieved by soaking the membrane in silver nitrate solution, and the membranes showed reduced fouling compared to untreated membranes (Yang et al. 2009). It should be noted that use of these nanoparticles faces potential public concern, need further investigation before their wide application in water and wastewater treatment process.

2.3.5 Biological Control of Membrane Biofouling

In recent years, new control strategies are constantly emerging as reviewed by Xiong and Liu (2010) and illustrated in Fig. 2.4. Biofilms on membrane surface can contain various extracellular substances like extracellular DNA (eDNA), extracellular polysaccharides and proteins such as amyloid fibers. These matrix components might be good targets for enzymes such as DNases, proteases, and extracellular polysaccharide degrading enzymes to prevent biofilms formation or to stimulate dispersal of already formed biofilms (Xiong and Liu 2010). Communication between cells during biofilm formation and dispersal of biofilms is dependent on quorum sensing systems and signaling molecules like AHL, and AI-2, which was also found to be effective to mitigate biofouling.

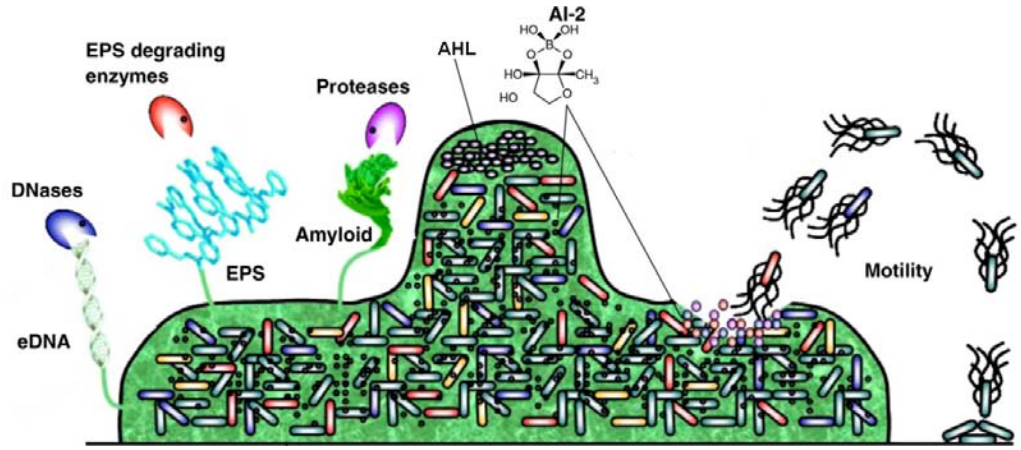

Figure 2.3 Schematic presentation showing mechanisms and components involved in biological control of membrane biofouling (Abee et al. 2011).

2.3.5.1 Phosphate limitation

Phosphate is an essential limiting nutrient for biomass growth. Very low phosphate concentration in feed water of membrane systems would limit biomass growth and subsequent membrane biofouling (Lehtola et al. 2002; Vrouwenvelder et al. 2010a; Vrouwenvelder et al. 2010b). Jacobson et al. (2009) found that phosphate concentrations in RO feed water less than 0.45 µg L^{-1} would be low enough to control microbial growth and subsequent membrane biofouling. In addition, it has been reported that phosphate concentration of feed water in large scale water treatment system could be reduced by ferritin, an enzyme that can remove phosphate, and mitigated membrane biofouling was observed (Jacobs et al. 2010). Therefore, reducing phosphate concentration in feed water may be an effective method to control membrane biofouling (Vrouwenvelder et al. 2010a).

2.3.5.2 Degradation of biofilm matrix

Extracellular polymeric substances (EPS) secreted by microorganisms facilitate microbial attachment and biofouling on membrane surface as reviewed before. Some studies have shown that deprivation of oxygen may lead to reduced EPS production, and subsequently biofilm detachment (Applegate and Bryers 1991; Tay et al. 2001; Ahimou et al. 2007). EPS have been believed to play an important role in the formation of fouling layers and clogging the membrane pores, which can not

be efficiently removed by traditional physical or chemical cleaning methods. However, EPS could be hydrolyzed by some specific enzymes, implying a novel means to control EPS-mediated membrane biofouling. Extracellular proteins (PN), polysaccharides (PS) and extracellular DNA (eDNA) are important and main components of EPS, thus three main EPS-degrading enzymes, i.e., protease for protein degradation, polysaccharases for polysaccharide hydrolysis, and DNase for eDNA degradation have been applied to control membrane biofouling.

Some protease, such as the proteinase K, trypsin, subtilisin, etc., have been employed to remove established biofilms on membranes. Among those protease, proteinase K has been commonly used for both inhibiting biofilm formation and remove biofilm on solid surfaces. For example, proteinase K could lead to 98% detachment of several different clinical biofilm (Fredheim et al. 2009). Protease has been used to remove membrane biofouling on ultrafiltration (UF) membrane for wastewater treatment (te Poele and van der Graaf 2005). In addition, Leroy et al. (2008a) compared the antifouling potential of some commercial enzymes, such as Amano Protease A, papain, umamizyme, and subtilisin, and found the most efficient protease was subtilisin. Subtilisin was also reported to investigate the anti-fouling activity in seawater (Leroy et al. 2008b).

Some polysaccharases are also available for mitigating membrane biofouling by disrupting the matrix structure of extracellular polysaccharides. When dispersin B was applied to biofilm developed, most of the biofilm would be detached from the solid surfaces as dispersin B could efficiently cleave the biofilm matrix (Lee et al. 2008). It was also found that polyurethane and Teflon catheters coated with dispersin B exhibited strong anti-fouling ability (Kaplan et al. 2004).

Extracellular DNA (eDNA) is required for the initial establishment of biofilms, and plays an important role in biofilms structure and stability (Whitchurch et al. 2002; Vilain et al. 2009; Wu and Xi 2009; Kirkpatrick and Viollier 2010). It was shown that DNase I (Deoxyribonuclease I), an enzymatic degrader of DNA, can not only inhibit microbial attachment but also promote biofilm dispersal (Whitchurch et al.

2002). Thomas et al. (2008) reported that eDNA degrading enzyme was able to detach different-age biofilms. Based on the findings, DNase may be a feasible method to control membrane biofouling.

Compared to traditional chemical cleaning, EPS-degradation through enzymes may be highly efficient. A mixture containing several different enzymes seems to be fundamental for a successful biofouling control strategy. However, the use of enzymes in biofouling control is still limited due to the high prices of enzymes compared with the chemicals used today (Johansen et al. 1997).

2.3.5.3 Inhibition of quorum sensing signaling

Biofilm growth cycle is highly complex and regulated. Cell-to-cell signaling or quorum sensing (QS) has been demonstrated to influence biofilm development (Bassler 2002). Bacteria communicate with one another using autoinducers, which are small, chemical signaling molecules that are usually produced as metabolic byproducts. Autoinducer concentrations increase as a function of cell density, allowing populations of bacteria to regulate activities and gene expression in a cell density-dependent manner. At low population density, only small amounts of diffusible signal molecules are produced, provoking no effect. However, when the population density is sufficiently high, signal molecules will bind to a receptor and this signal complex will then induce or repress transcription of QS-regulated genes (Choudhary and Schmidt-Dannert 2010; Galloway et al. 2011). This process, called quorum sensing, enables bacteria to monitor the environment for other bacteria and to react by changing their behavior. This is especially important to cell density-dependent cellular functions such as light production, virulence, sporulation, and biofilm formation.

There are several types quorum sensing regulated systems. According to the different bacteria source, autoinducers are classified into three types: (1) Acyl-homoserine lactone (AHL) in Gram-negative bacteria; (2) autoinducing peptide (AIP) in Gram-positive bacteria; (3) autoinducer-2 (AI-2) for both Gram-positive

and Gram-negative bacteria and involved in inter-species communication (Choudhary and Schmidt-Dannert 2010).

Controlling biofilm formation through regulating QS has proven to be promising for *P. aeruginosa* and *Staphylococcus aureus* biofilm (Wu et al. 2004; Balaban et al. 2005), and could help suppress infections originating from *E. coli* biofilms (Ren et al. 2001; Ren et al. 2004). Some debate exists over whether AI-2 regulates *E. coli* biofilm formation. Reisner et al. (2003) reported no connection of AI-2 to biofilm formation by *E. coli* containing conjugative IncF plasmids. In a study with *E. coli* W3110 and strain MDAI2, it was reported that deletion of luxS had no effect on growth, or biofilm formation (Wang et al. 2005a). However, DeLisa et al. (2001) investigated differential gene expression in wild type *E. coli* W3110 and a luxS deletion mutant (strain MDAI2) after adding AI-2-containing supernatant and found significant transcriptional changes in putative adhesion or fimbrial-like proteins, and carbon storage regulator, which contribute to *E. coli* adhesion or biofilm formation (Genevaux et al. 1999; Torres et al. 2005). Due to the debate of the effect of AI-2 on pure-culture biofilm formation, the study of AI-2 involvement in mixed-species microbial attachment and biofilm development is necessary.

Yeon et al. (2009a) reported that AHL activity was low during the early stages of filtration, intensified around the point at which trans-membrane pressure (TMP) showed the first signs of its significant rise and was fully developed when fouling was severe. They further proposed the addition of acylase, an enzyme can hydrolyze amide bond of AHL, as a novel fouling control strategy. After having proven the feasibility of inhibiting quorum sensing by free enzymes in a lab-scale MBR (Yeon et al. 2009a), the group immobilized the enzymes on magnetic enzyme carriers (Yeon et al. 2009b). Thus, enzymes can be readily retained by the membrane and recovered by magnetic capture, and show a high stability which led to a significant delay in membrane biofouling.

In addition, vanillin (4-hydroxy-3-methoxybenzaldehyde) extracted from vanilla beans was reported to inhibit QS signaling systems by interference with AHL

receptor. This compound was found to inhibit AHL-mediated quorum sensing systems, leading to inhibited biofilm formation on RO membrane (Ponnusamy et al. 2009). There are some furanone-type QS inhibitors in the literature (Zang et al. 2009). Hentzer et al. (2002) have demonstrated that *P. aeruginosa* cultures treated with a synthetic furanone form thinner biofilms. Brominated furanones synthesized by Janssens et al. (2008) were found to repress biofilm formation. These in turn indicate that disruption QS systems would be able to prevent membrane from biofouling.

2.3.5.4 Engineering bacteriophages

Bacteriophages are viruses that infect bacteria and may provide a natural, highly specific, non-toxic, feasible approach for controlling several microorganisms involved in membrane biofouling control. When phages come into contact with biofilms, further interactions occur, depending on the susceptibility of the biofilm cells to the phage and to the availability of receptor sites. For example, Hughes et al. (1998a; 1998b) working in the control of *Enterobacter agglomerans* biofilms by the use of phages found that cells were lysed and the biofilms were degraded by the bacteriophage. The phage then lysed the biofilm cells, the polysaccharide polymerase enzyme expressed by the phage degraded the EPS and caused biofilm slough off. In addition, Lu and Collins (2007) engineered a bacteriophage to express a biofilm degrading enzyme. This enzymatic phage had the ability to attack the bacterial cells in the biofilm and the biofilm matrix, substantially resulting more than 99.9% removal of biofilm. A mixture of multiple phages has been shown to be efficient in removing multispecies biofouling (Richards and Melander 2009).

More recently, bacteriophage was employed by Goldman et al. (2009) to control ultrafiltration membrane biofouling. Results showed that the addition of phages could reduce 40% microbial attachment to membrane surface, and the membrane permeability in the MBR supplemented with phage was nearly 2.5-fold higher than that observed in the control MBR without addition of phage (Goldman et al. 2009). This study sheds lights on the potential application of bacteriophage in mitigating membrane biofouling. The biotechnology for phages has not yet been successfully

developed and relatively little information is available on the application of bacteriophages on biofilms.

2.4 SUMMARY

Microbial attachment is the first step for biofilm development. The adhesion of microorganisms to surfaces is affected by various factors, such as electrostatic interaction, hydrophobic interaction, dynamic shear force, cell structure properties, extracellular polymeric substances (EPS), and cell species etc. (Stoodley et al. 2002; Flemming and Wingender 2010). The inclination for bacteria to colonize surfaces is a double edged sword, i.e., that can prove either beneficial or potentially destructive. For example, bioremediation of wastewater is a beneficial function of microbial attachment, while biofouling in membrane processes is a major economic disadvantage in industrial application.

Membrane biofouling is due to unfavorable microbial attachment on membrane surface. The traditional methods developed for controlling membrane biofouling are mainly based on physicochemical principles, such as modification of membrane surface, optimization of operation conditions, and regular physical and chemical cleaning. However, conventional cleaning may contribute to inefficient biofilm control due to reduced susceptibility of cells in biofilm to cleaning reagents. Consequently, new control strategies are constantly developed with main focus on biological control, such as phosphate limitation, degradation of biofilm matrix, control using phages and inhibiting cellular communications. It can be seen that biological control strategies have the advantage of high antimicrobial activity and specificity, seems to be promising in microbial attachment control.

It appears from this review that biological triggers involved in microbial attachment and membrane biofouling need more focus compared to traditional physicochemical interactions. Information related to biological control of microbial attachment and membrane biofouling is limited and definitely an in-depth and systematic study is

needed. This study is aimed to investigate the feasibility of biological control of microbial attachment and membrane biofouling through energy metabolism inhibition and D-amino acids.

CHAPTER 3
BIOLOGICAL CONTROL OF MICROBIAL ATTACHMENT AND MEMBRANE BIOFOULING BY INHIBITION OF ATP

3.1 INTRODUCTION

Microbial attachment to a solid surface has been widely observed in natural and engineered systems as discussed in Chapter 2. For example, microbial attachment to a membrane surface causes membrane biofouling which is one of the biggest challenges encountered in applications of membrane technology for water and wastewater treatment. Nowadays, membrane bioreactor has emerged as a promising technology for wastewater reclamation, and has advantages of high-quality product water, modular design with less space requirement. However, membrane biofouling would cause increased trans-membrane pressure and lowered water flux (Miura et al. 2007). Prevention and control of biofilm formation would be an ideal way towards mitigation of membrane biofouling. In this regard, several attempts have been made to mitigate membrane biofouling by coating membrane surface with antimicrobial products (Cloete and Jacobs 2001), or by modifying surface physical-chemical properties (Whitehead et al. 2005).

It is well known that the physiological behavior of microorganisms are closely associated with cell energy metabolism with interrelated catabolic and anabolic reactions. According to chemiosmotic theory, uncoupling of electron transport and oxidative phosphorylation would be an effective way to inhibit the energy production, which can be achieved by using various metabolic uncouplers. As presented in Chapter 2, cellular communication has been believed to coordinate the biofilm development of a variety of gram-negative and gram-positive bacteria. Autoinducer-2 (AI-2), as a common signaling molecule, can modulate interspecies communication during biofilm formation (Miller and Bassler 2001; Daniels et al. 2004). The synthesis of AI-2 would be energy-dependent (Chen et al. 2002;

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Stevenson and Babb 2002), while the effect of energy uncoupling on the production of AI-2 in mixed-culture microorganisms is not yet clear. Therefore, this study aimed to investigate the possible roles of energy metabolism and cellular communication in initial microbial attachment. For this purpose, 2,4-dinitrophenol (DNP), a typical metabolic uncoupler (Voet et al. 1998), was employed to inhibit ATP and subsequently AI-2 synthesis. This study would offer new insights into biological control of microbial attachment and membrane biofouling.

3.2 MATERIALS AND METHODS

3.2.1 Biocarriers for Microbial Attachment

Glass slides with the dimension of 24 x 50 mm (CEP, SPD Scientific, Singapore) and polypropylene (PP) coupons (24 x 50 mm, Kinary, Singapore) were used as biocarriers in microbial attachment experiments. In order to investigate biofouling potential of microorganisms with and without exposure to DNP, a series of static attachment assays were also conducted with hydrophilic flat sheet nylon membrane (Osmonics, Minnetonka, USA) with a pore size of 0.2 μm and hydrophobic polytetrafluoroethylene (PTFE) membrane (Sartorius Stedim Biotech, Goettingen, Germany) with a pore size of 0.2 μm at 25ºC. The PP coupons were cleaned with detergent and rinsed thoroughly with distilled water, whereas the glass slides were cleaned by being soaked in 10% nitric acid for 24 hr, and were then thoroughly rinsed with distilled water and dried. The nylon and PTFE membranes were simply rinsed with distilled water before use. The hydrophobicity of the glass slides and PP coupons surface was characterized with contact angle measured by a contact angle goniometer (Dataphysics OCA 20, Filderstadt, Germany). One drop of deionized water (0.8 μL) was placed on each biocarrier surface using a micro-syringe. The contact angle between the water droplet and the surface was measured. Eight measurements were made on triplicate samples. The roughness of biocarrier was measured in triplicate by atomic force microscopy (AFM), and the root-mean-squared roughness (Rq) was recorded. Data of the surface charge were obtained

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from the literature. The properties of the biocarriers were presented in Table 3.1.

Table 3.1 Biocarrier surface contact angles, roughness and surface charge.

	Contact angle (^{o})	Roughness (nm)	Surface charge (mV) at pH 7
Glass slide	16.9 ± 0.5	6.2 ± 0.6	-36.5 ± 2.1 (Li and Logan 2004)
Nylon membrane	44.2 ± 4.5	73.1 ± 2.4	-20.3 ± 0.8 (Pasmore et al. 2001)
PP coupon	99.3 ± 2.2	37.4 ± 0.9	-17.1 ± 1.2 (Meagher and Pashley 1995)
PTFE membrane	142.6 ± 2.3	107.5 ± 1.8	-15.8 ± 1.5 (Pasmore et al. 2001)

3.2.2 Microbial Attachment and Biofouling Development on Biocarriers

Activated sludge used in this study was taken from a local wastewater treatment plant on April 2009 and acclimated with a synthetic wastewater for two months. After 30-min settling, the culture supernatant was replaced with fresh synthetic wastewater on a daily basis. The synthetic wastewater consisted of 690 mg L^{-1} sodium acetate and 240 mg L^{-1} ethanol as carbon sources, 200 mg L^{-1} NH_4Cl, 60 mg L^{-1} K_2HPO_4, 15 mg L^{-1} $CaCl_2 \cdot 2H_2O$, 12.5 mg L^{-1} $MgSO_4 \cdot 7H_2O$ and 20 mg L^{-1} $FeSO_4 \cdot 7H_2O$ (Liu et al. 2003). Two 2-liter beakers were used as batch reactors fed with the above synthetic wastewater at an initial total organic carbon (TOC) concentration of 300 mg L^{-1}, equivalent to 960 mg L^{-1} of theoretical oxygen demand (ThOD), and initial acclimated biomass concentration was fixed at 300 mg L^{-1} in each reactor. The dissolved oxygen concentrations in the two reactors were maintained at quasi-saturation level through air aeration, and all the experiments were carried out at 25ºC. One reactor served as control free of 2, 4-dinitrophenol (DNP), a typical metabolic uncoupler, and another was supplemented with 10 mg L^{-1} DNP. At different exposure times, suspended microorganisms were collected from two reactors for determination of specific oxygen utilization rate (SOUR), cellular ATP and AI-2 content.

A series of static attachment assays were conducted at 25ºC with the

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microorganisms collected from the above two batch reactors with and without supplement of DNP at the exposure time of 1-hr to 4-hr. For each attachment assay, harvested microorganisms resuspended in 30 mL of 10 mM phosphate buffered saline (PBS) solution at 100 mg dry biomass L^{-1} were made contact with the biocarrier for 1-hr in a Petri dish. At the end of contact, biocarrier was gently rinsed three times with distilled water to remove loosely attached microorganisms. Membrane samples were stored in pre-cleaned Petri dishes for further use in microfiltration tests. Fixed biomass on glass slide, PP coupon, nylon membrane or PTFE membrane was harvested by the method suggested by Liu (1997). Briefly, the rinsed biocarrier with fixed biomass was submerged in 20 mL of 1 N NaOH in a pre-cleaned test tube, followed by three minutes of ultrasonic treatment (Sonics & Materials, Newton, CT, USA) to detach fixed biomass from biocarrier surfaces. TOC of the harvested biomass was quantified using a TOC analyzer (ASI-V, TOC-Vcsh, Shimadzu, Japan). Total biomass concentrations and SOUR were measured according to standard method (APHA 2005). Each sample was tested in triplicate otherwise stated. Statistical analysis was conducted by Student's *t*-test with a 95% confidence interval.

3.2.3 Dead-end Microfiltration Test

In order to evaluate the biofouling potential of microorganisms with and without exposure to DNP, standard dead-end microfiltration experiments (Figure 3.1) were carried out at 25ºC with the fouled membranes collected from the microbial attachment tests as described above. The dead-end microfiltration set-up consisted of a feed tank and a 50 mL stainless steel cell (Millipore Corporation, Bedford, MA) housing a 47 mm membrane. The effective membrane surface area was 11.94 cm^2, and trans-membrane pressure was provided by compressed nitrogen gas. Sixty milliliters of Mili-Q water (Milipore, Singapore) was filtered through the fouled membranes at 5 kPa for nylon membrane and 350 kPa for PTFE membrane due to its high surface hydrophobicity. The filtrate was collected in a container placed on an electronic balance, and its weight was recorded at the time interval of 5 s during the microfiltration.

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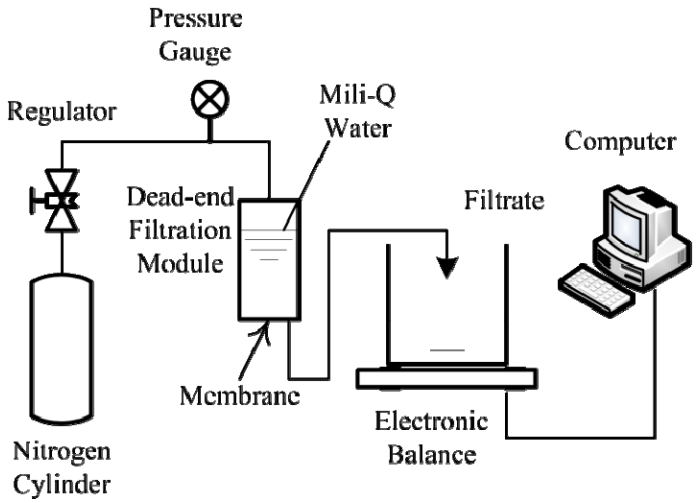

Figure 3.1 Dead-end microfiltration set-up.

In order to analyze the resistance of the fouled nylon membrane, resistance-in-series model (Cheryan 1998) was used for determination of intrinsic membrane resistance, pore blocking resistance and cake layer resistance.

$$J = \frac{\Delta P}{\mu R_t} \tag{3.1}$$

where J is the permeation flux ($m^3\ m^{-2}\ s^{-1}$); R_t is the total filtration resistance (m^{-1}); ΔP is the trans-membrane pressure (Pa); μ is the viscosity of the solution (Pa·s). R_t is the sum of the intrinsic membrane resistance (R_m), the resistance due to pore blocking (R_p) and the resistance caused by the cake layer on membrane surface (R_c).

$$R_t = R_m + R_p + R_c \tag{3.2}$$

The R_m was estimated from Equation 3.1 by measuring the water flux of virgin membrane, while R_t was determined from water flux through fouled membrane. After the fouled membranes were gently wiped with a sponge and rinsed with Mili-Q water to remove the cake layer, the resistance of R_m+R_p was calculated from water flux through wiped membrane, i.e. R_p is known. After knowing R_t, R_m and R_p, R_c can be obtained from Equation 3.2. It is noted that highly hydrophobic PTFE membranes were not used in the above resistance analysis as hydrophilic membrane has been more widely applied for water treatment.

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3.2.4 Measurement of Cellular ATP

The cellular ATP extractions were carried out on freshly collected microorganism samples according to the trichloroacetic acid (TCA) method (Chen and Leung 2000) with some modifications. Briefly, the procedure was as follows: (1) 5 mL of biosample was homogenized with an ultrasonic homogenizer (Sonics & Materials, Newton, CT, USA) for 3 min; (2) 0.1 mL of 5% TCA solution was added to 0.2 mL of the homogenized sample for ATP extraction; (3) the final volume of solution was made up to 4 mL with a pH of about 7.75 by Tris-Acetate-EDTA (TAE) buffer, which was further filtered through a 0.20 μm filter. The collected filtrate was stored at -20°C for use. ATP analysis was performed using firefly luciferin-luciferase bioluminescence method with the FLAA Adenosine 5'-triphosphate (ATP) Bioluminescent Assay Kit (Sigma-Aldrich) and a TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA, USA).

3.2.5 Analysis of Autoinducer-2

The amount of autoinducer-2 (AI-2) was determined by *Vibrio harveyi* bioluminescence assays. *V. harveyi* reporter strain BB170 (ATCC BAA 1117) purchased as freeze-dried cultures from American Type Culture Collection (ATCC, Manassas, VA, USA) were revived overnight according to the provided instructions. The following day, the overnight culture was used to inoculate 20 mL of fresh medium in 100 mL flasks, and cultures were grown to the exponential growth phase (OD_{600} of 1.0) at 150 rpm and at appropriate temperature of 30°C in a shaker. The culture was then aliquoted into cryogenic tubes and a filter-sterilized 50% (v/v) aqueous glycerol solution was added to bring the glycerol concentration up to 25% (v/v). The bacteria *V. harveyi* BB170 with 25% glycerol solution were frozen and stored at -80°C.

Autoinducer bioassay (AB) (Surette and Bassler 1998) medium was prepared by dissolving 17.5 g NaCl, 6.0 g $MgSO_4$, and 2.0 g casein vitamin free per 1 L of deionized water, adjusting pH to around 7.5 with 0.01 M potassium hydroxide

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solution, autoclaving for 30 minutes at 121°C, and allowing to cool. To 1 L of this solution, 10 mL of filter-sterilized 1 M potassium phosphate solution (pH 7.0), 10 mL of filter-sterilized 0.1 M L-arginine, 20 mL of 50% (v/v) autoclaved glycerol, 1 mL of filter-sterilized 10 μg/mL riboflavin, and 1 mL of filter-sterilized 1 mg/mL thiamine were added. Marine broth (BD Difco, Franklin Lakes, NJ, USA) was prepared according to the provided instructions.

For determination of AI-2, cell-free culture fluids were prepared as described below. 5 mL of biomass sample was collected and centrifuged at 13,420 g for 10 min (KUBOTA, Japan). The harvested biomass was resuspended in fresh autoinducer bioassay (AB) medium, and the total volume of the suspension was adjusted to 5 mL. The biomass in the AB medium was chilled and homogenized for 1 min, and subsequently the mixed liquor was centrifuged at 13,420 g for 10 min (KUBOTA, Japan). The supernatant recovered by centrifugation was filtered through a 0.20 μm filter. The filtrate was collected and stored at -20°C. The cell-free culture fluids were thawed before determination of AI-2 activity.

The AI-2 bioassay with *V. harveyi* reporter strain BB170 (ATCC BAA 1117) was done according to the method by Bassler et al. (1997). The *V. harveyi* reporter strain was cultivated in AB medium for 13-16 hr at 30ºC with aeration, and followed by a dilution of 1:5000 into fresh medium. An aliquot 180 μL of the diluted cells was added to the wells (96-well plate) containing 20 μL cell-free culture fluids to be tested for AI-2 activity. The 96-well plates with lid were shaken in a rotary shaker at 150 rpm at 30ºC. For every 30 min, light production was measured using a microplate reader (BioTek, sygnergy 2). The AI-2 activity is reported as the fold induction of the reporter strain over background when AB medium alone was added to the reporter. In the AI-2 bioassay, 4,5-dihydroxy-2,3-pentanedione (DPD) (Omm Scientific, Dallas, TX, USA) were used as the calibration standard. Each sample was analyzed six times in parallel.

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3.2.6 Staining and Visualization of Attached Microorganisms

In order to visualize microbial attachment, the adherent bacteria on glass slides, PP coupons and membrane surfaces were stained with LIVE/DEAD BacLight™ Bacterial Viability kits (Molecular Probes, Eugene, OR, USA), which consisted of two nucleic acid dyes staining on both live and dead cells: SYTO 9 and propidium iodide (PI). SYTO 9 is a green-fluorescent dye which stains both live and dead bacteria with intact and damaged cell membranes while the red-fluorescing PI only stains dead bacteria with damaged cell membranes. The excitation/emission maxima for these dyes are about 480/500 nm for SYTO 9 stain and 490/635 nm for PI. With an appropriate mixture of both dyes, viable bacteria with intact cell membranes are stained green, whereas bacteria with damaged cell membranes fluoresce red. The color assigned to the live and dead cells follows from the color at which the stained cells fluoresce under laser excitation. The sample staining procedure was carried out following the instructions in the manual. First, two hundred microliters of the mixed solution (1000 times diluted SYTO 9 and PI from the stock solution) was added to each attachment sample on glass slides, PP coupons and membranes. The stained sample was then incubated in the dark at room temperature for 15 min. After that, the sample was gently rinsed two times with DI water to remove unbound dyes. Finally, the sample was viewed using an Olympus Fluoview FV300 confocal laser scanning microscopy (CLSM) (Olympus Optical, Tokyo, Japan) with a 100× objective.

3.3 RESULTS

3.3.1 Attachment on Different Biocarriers

Two series of microbial attachment assays with nylon membrane, glass slide, PTFE membrane, and PP coupon were conducted using microorganisms without exposure to DNP (control), and microorganisms exposed to 10 mg L^{-1} DNP as described above. Figure 3.2 shows that attachment of microorganisms exposed to DNP onto the surfaces of nylon membrane, glass slide, PTFE membrane and PP coupon was

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reduced substantially as compared to the control assay free of DNP (Student's *t*-test, $P < 0.05$). After 1-h culture, attachment of microorganisms without exposure to DNP was 9.2 μg TOC cm^{-2} on nylon membrane surface, while attachment of microorganisms with exposure to DNP was 5.0 μg TOC cm^{-2}, indicating a 45.6% reduction in microbial attachment caused by DNP (Figure 3.2a). Similar phenomena were also observed in microbial attachment on hydrophobic PTFE membrane (Figure 3.2c) and PP surface (Figure 3.2d). For instance, attachment of microorganisms exposed to DNP for 1 hr on PTFE membrane was reduced by 41.4%, while a reduction of 42.8% was observed for attachment on PP coupon as compared to the control free of DNP (Figure 3.2c). It appears from Figure 3.2b that the inhibition of DNP to microbial attachment onto glass slide was even more significant than that found in Figure 3.2a for nylon membrane, e.g. microbial attachment onto glass surface was reduced by 54% and 63% for microorganisms exposed to DNP for 1 and 4 hr as compared to the control, respectively. In addition, it should be noted that more significant reduction of microbial attachment after contact with DNP was also observed on nylon membrane than on PTFE membrane. These results were further supported by CLSM microscopic observations (Figure 3.3). All these indicate that DNP could significantly decrease microbial attachment on various solid surfaces.

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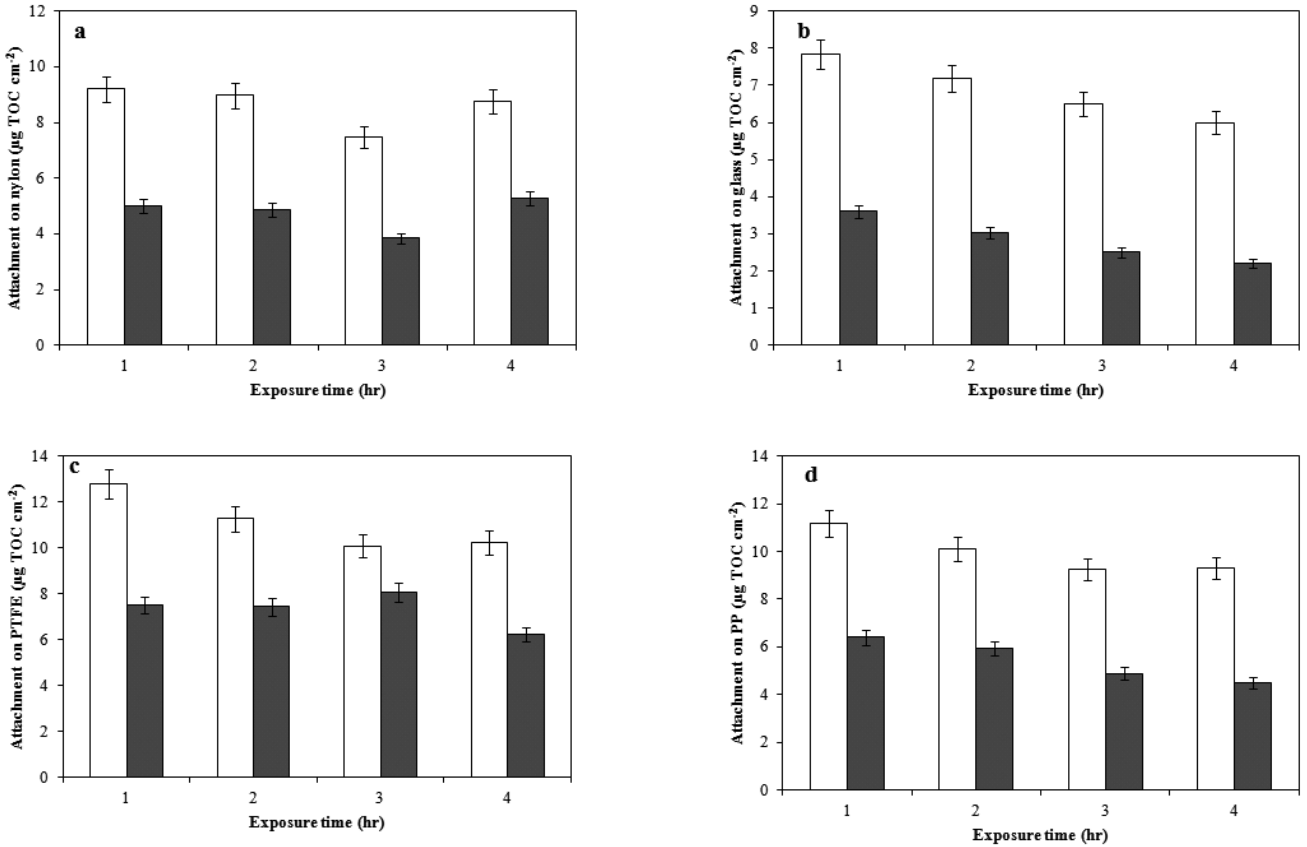

Figure 3.2 Attachment of microorganisms with (■) and without (□) treatment by DNP on nylon membranes (a); on glass slides (b); on PTFE membranes (c); and on PP coupons (d). Each point represents the mean of triplicate measurements, and the error bar is 1 SD from the mean.

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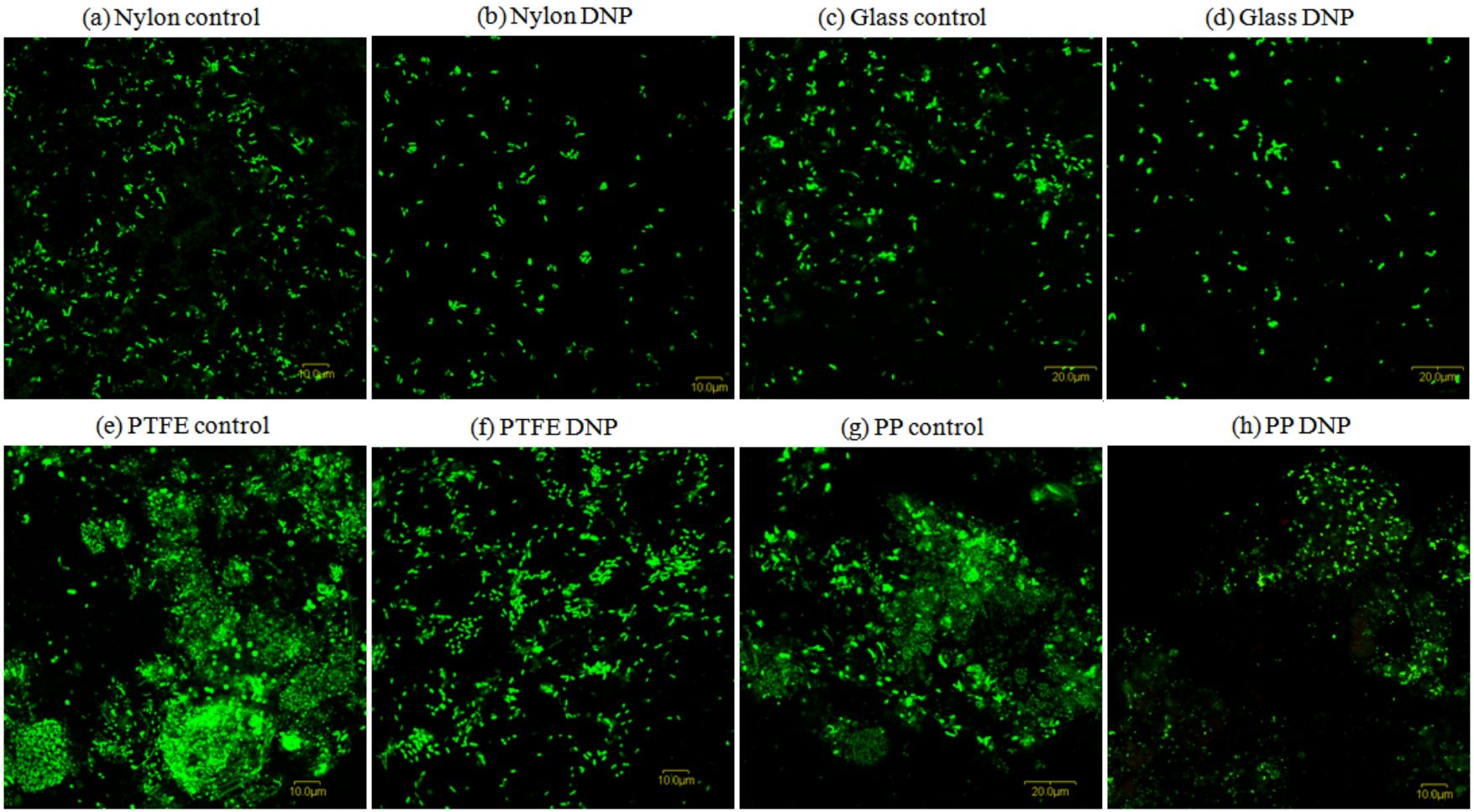

Figure 3.3 CLSM images of attachment of suspended microorganisms with and without exposure to DNP.

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3.3.2 Biofouling Development on Nylon and PTFE Membranes

The dead-end microfiltration experiments were carried out with fouled nylon and PTFE membranes, and the intensity of membrane fouling was characterized by the ratio of water flux (J) of fouled membrane to water flux (J_o) of virgin membrane. Figure 3.4 shows that the water fluxes of both nylon and PTFE membranes fouled by microorganisms exposed to DNP were higher than those obtained from the controls free of DNP. It appears from Table 3.1 that the total resistance of nylon membrane fouled by microorganisms free of DNP was about 20% higher than that of membrane fouled by microorganisms exposed to DNP. In addition, the fraction of cake layer resistance accounted for about 61.8% to 73.1% of the total resistance (Table 3.1), i.e. the main fouling mechanism for the microfiltration of fouled nylon membrane can be attributed to cake layer resistance. These suggest that DNP as a typical metabolic uncoupler is effective in decreasing membrane biofouling.

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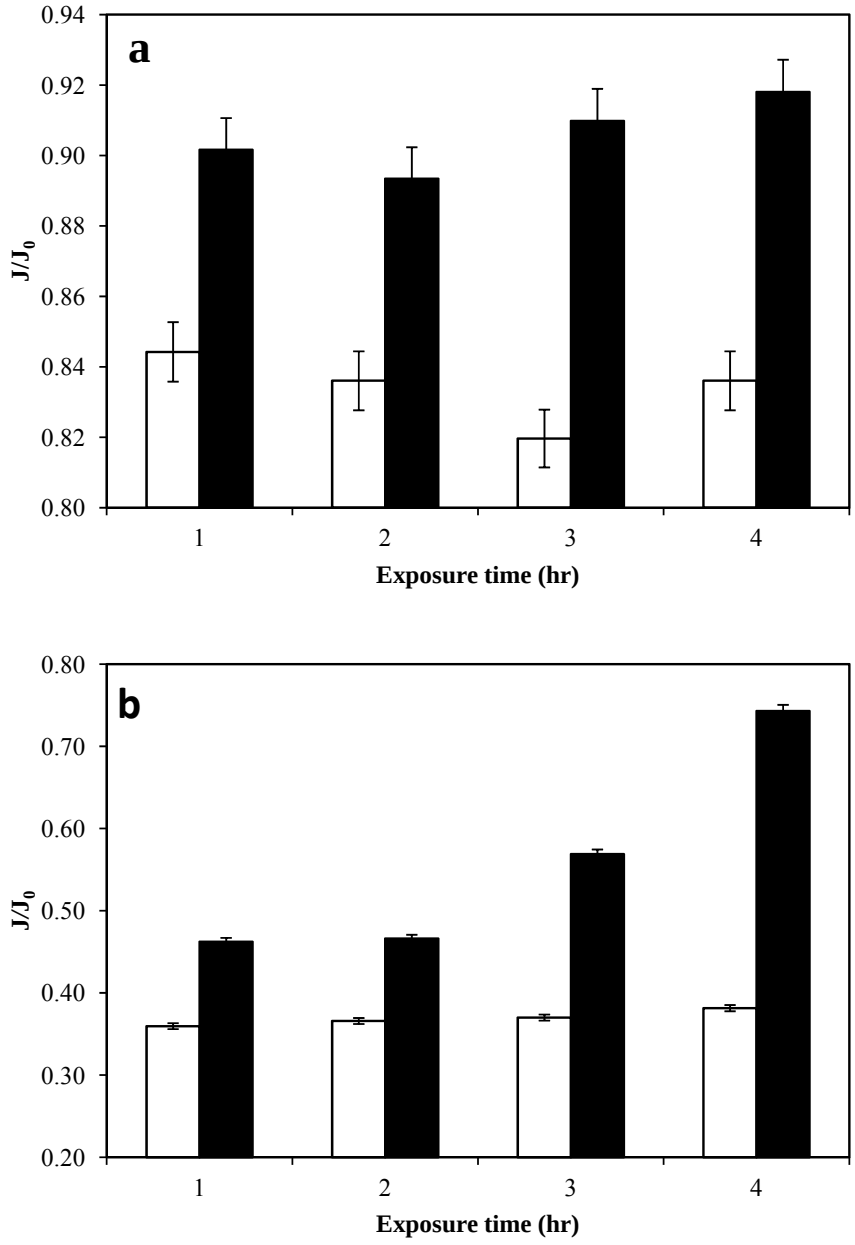

Figure 3.4 Water flux through nylon membrane (a) and PTFE membrane (b) fouled by suspended microorganisms with (■) and without (□) DNP treatment. Each point represents the mean of triplicate experiments, and the error bar is 1 SD from the mean.

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Table 3.2 Filtration resistances of nylon membrane fouled by microorganisms with (DNP-treated) and without (control) exposure to DNP.

		R_t (10^{11} m^{-1})	R_m (10^{11} m^{-1})	R_m/R_t (%)	R_p (10^{11} m^{-1})	R_p/R_t (%)	R_c (10^{11} m^{-1})	R_c/R_t (%)
control	1h	5.89	1.37	23.26	0.26	4.41	4.26	72.33
	2h	5.50	1.37	24.91	0.11	2.00	4.02	73.09
	3h	5.32	1.37	25.75	0.41	7.71	3.54	66.54
	4h	5.45	1.37	25.14	0.49	8.99	3.59	65.87
DNP-treated	1h	4.67	1.37	29.34	0.25	5.35	3.05	65.31
	2h	4.42	1.37	31.00	0.25	5.66	2.80	63.35
	3h	5.19	1.37	26.40	0.54	10.40	3.28	63.20
	4h	4.71	1.37	29.09	0.43	9.13	2.91	61.78

R_t: total resistance; R_m: membrane resistance; R_p: pore blocking resistance; R_c: cake layer resistance

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3.3.3 Activity of Suspended Microorganisms With and Without Exposure to DNP

Since oxygen can serve as the terminal electron acceptor in aerobic respiration, the respiration of microorganism can be evaluated by specific oxygen utilization rate (SOUR). As can be seen in Figure 3.5, the SOUR of microorganisms without exposure to DNP was 32 mg O_2 h^{-1} g^{-1} VSS at the exposure time of 1 h, while SOUR was 56 mg O_2 h^{-1} g^{-1} VSS when exposed to DNP, which was 75% higher than that of microorganisms without exposure to DNP. This indicates that DNP had no inhibitory effect on microbial activity in terms of SOUR.

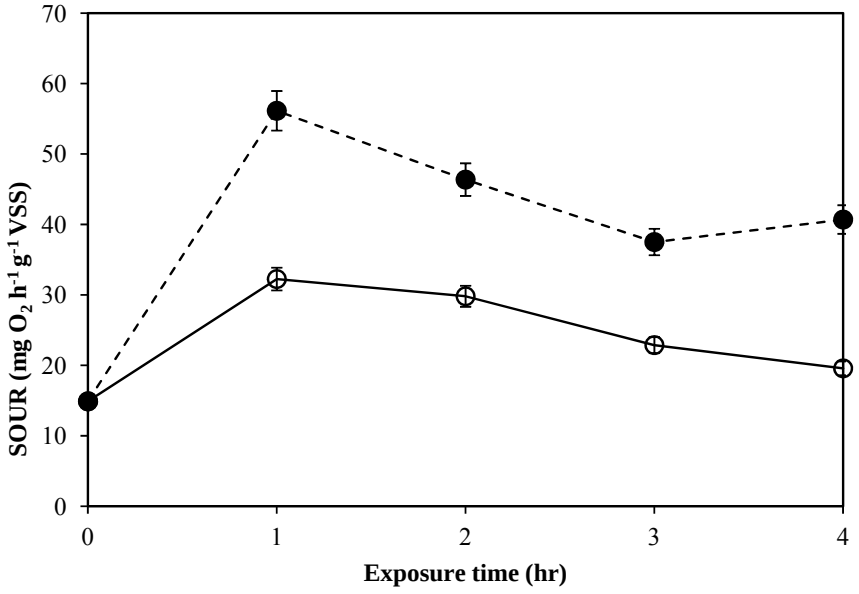

Figure 3.5 SOUR profiles of suspended microorganisms with and without exposure to DNP. ○: without exposure to DNP; ●: with exposure to DNP. Each value represents the mean of triplicate experiments, and the error bar is 1 SD from the mean.

Figure 3.6 shows TOC profiles in the cultures supplemented with and without DNP. It can be seen that addition of DNP to the culture media had no negative effect on the TOC removal efficiency compared with control. Chen et al. (2006) also observed similar phenomenon in study of sludge reduction by metabolic uncoupler,

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2,4-dichlorophenol. In fact, such an observation can be explained by the unique property of metabolic uncouplers, i.e. they do not block electron transport along the respiratory chain to oxygen.

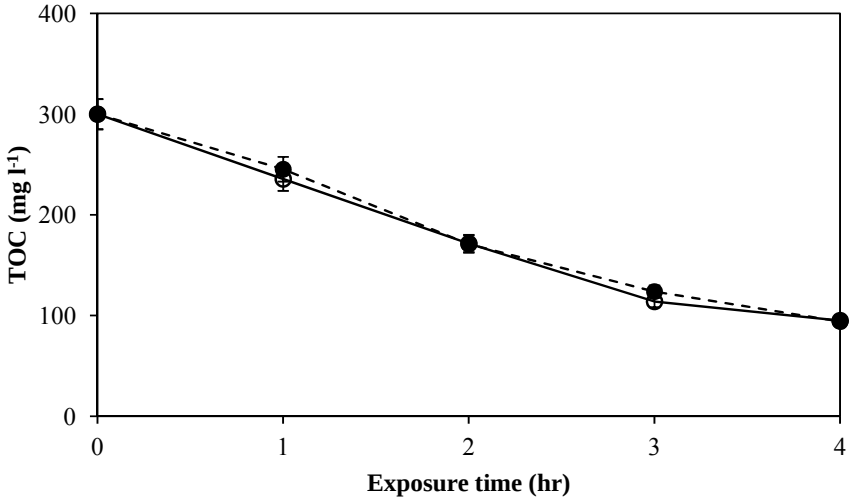

Figure 3.6 TOC profiles in the suspension supplemented with (●) and without (○) DNP. Each value represents the mean of triplicate experiments, and the error bar is 1 SD from the mean.

3.3.4 ATP Content in Suspended Biomass With and Without Exposure to DNP

Figure 3.7 shows that the cellular ATP content of microorganisms without exposure to DNP was significantly higher than that obtained from the culture supplemented with DNP at the same culture times (Student's *t*-test, $P < 0.05$). It should be noted that the inverse V-shape curves for ATP can be explained by consumption of energy sources over culture time. For suspended microorganisms without exposure to DNP, the ATP content increased from 5.4×10^{-7} mol g^{-1} biomass to 10.3×10^{-7} mol g^{-1} biomass, indicating a net ATP synthesis of 4.9×10^{-7} mol g^{-1} biomass. By contrast, for suspended microorganisms exposed to DNP, the net synthesis of cellular ATP was only about 1.72×10^{-7} mol g^{-1} biomass, i.e. a 65% reduction was achieved compared to that of control free of DNP. These results suggest that DNP could effectively decrease cellular ATP synthesis.

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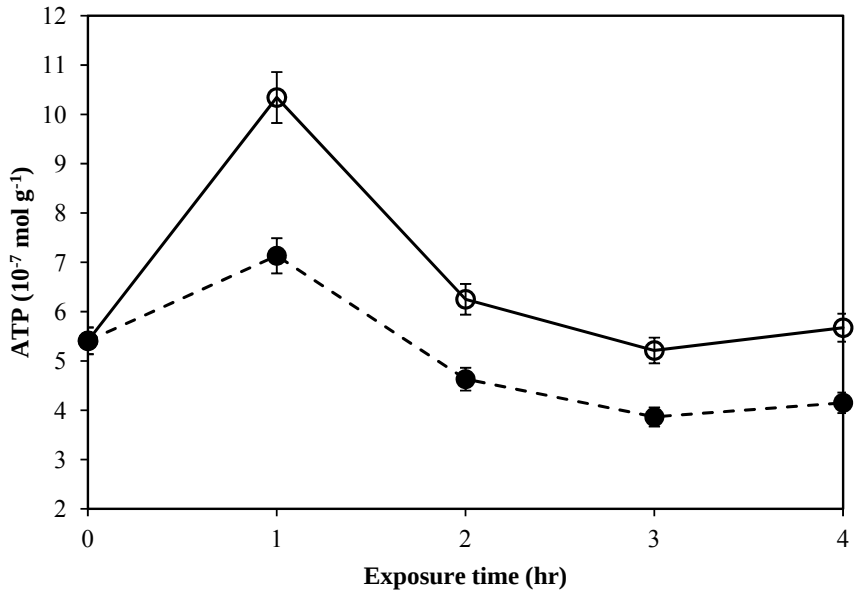

Figure 3.7 Contents of cellular ATP in suspended microorganisms with and without exposure to DNP. ○: without exposure to DNP; ●: with exposure to DNP. Each value represents the mean of triplicate experiments, and the error bar is 1 SD from the mean.

3.3.5 AI-2 Production by Suspended Microorganisms With and Without Exposure to DNP

Figure 3.8 shows the AI-2 content in suspended microorganisms with and without exposure to DNP. It is obvious that the AI-2 content in microorganisms exposed to DNP was reduced substantially as compared to that of the control free of DNP. After 2-hr culture, the AI-2 content of suspended microorganisms without exposure to DNP was about 0.27 μmol g^{-1}, while it decreased to about 0.14 μmol g^{-1} for suspended microorganisms exposed to DNP, i.e. a 48% reduction compared to that of control. These results suggest that DNP would decrease the synthesis or secretion of AI-2 significantly (Student's *t*-test, $P < 0.05$), which is essential for coordinating microbial collective behavior in biofilm formation.

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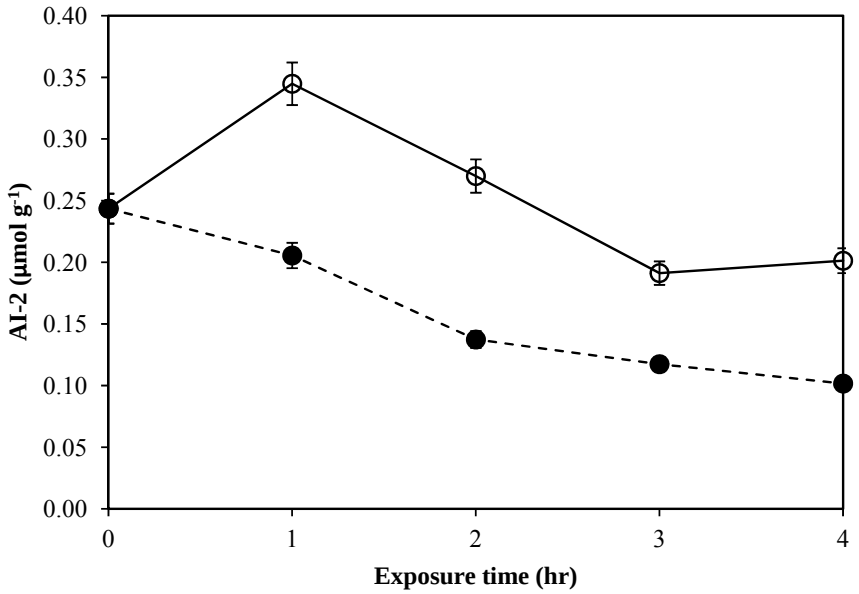

Figure 3.8 AI-2 in suspended microorganisms with and without exposure to DNP. ○: without exposure to DNP; ●: with exposure to DNP. Each value represents the mean of triplicate experiments, and the error bar is 1 SD from the mean.

3.4 DISCUSSION

The results show that DNP can not only hinder microbial attachment on various solid surfaces, but also mitigate membrane biofouling. It is well known that biofilm formation on membrane surface is the major cause of membrane biofouling. As shown in Table 3.1, the main fouling mechanism for nylon membrane was cake layer resistance due to microbial attachment. Miura et al. (2007) also found that biofilm formation resulted in membrane biofouling in a pilot scale membrane bioreactor treating municipal wastewater, and biofilm developed on membrane surfaces had a positive correlation with the increase in trans-membrane pressure.

ATP has been known to be the principal energy carrier of biologically utilizable energy, and is synthesized by coupling two interrelated reactions of electron transport and oxidative phosphorylation (Mathews et al. 2000). The flow of

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electrons from NADH or $FADH_2$ to O_2 through protein complexes located in the inner membrane leads to the pumping of protons out of the matrix. When protons flow back to the matrix through an enzyme complex, ATP is synthesized by the ATP synthase driven by the proton motive force. DNP, a typical uncoupler, binds protons on the acidic side of the membrane, diffuses through the membrane, and releases the protons on the membrane's alkaline side, thereby acting as a proton-transporting ionophore. The net result is proton transport into the cell. In this way the uncouplers dissipate proton motive force (pmf). ATP synthesis will be inhibited due to low pmf. However bacteria respond to a reduction in pmf by increasing respiration in order to maintain a constant ATP/ADP ratio (Brown 1992). As the result, increased SOUR (Figure 3.5) has been often observed in the presence of metabolic uncoupler, such as DNP (Mathews et al. 2000). In the presence of a metabolic uncoupler, electron transport continues (Figure 3.6), but oxidative phosphorylation is inhibited, i.e. the ATP synthesis efficiency would be lowered markedly as shown in Figure 3.7.

For attachment onto nylon membrane, glass slide, PTFE membrane and PP coupon, Figure 3.9 shows the dependence of attached biomass on the ATP content of suspended microorganisms with and without exposure to DNP. For microorganisms exposed to DNP, their ATP contents were reduced substantially due to the uncoupling effect of DNP. As a result, low fixed biomasses on hydrophilic nylon membrane and glass slide (Figure 3.9a) as well as hydrophobic PTFE membrane and PP coupon (Figure 3.9b) were observed. It appears that microbial attachment is favored by high ATP content in suspended microorganisms without exposure to DNP regardless of the biocarriers studied. In study of attachment of *Mycoplasma pneumonia* to glass surface, Feldner et al. (1981) reported that the attachment was strongly reduced by dicyclohexylcarbodiimide, an inhibitor of the membrane-bound Mg^{2+}-adenosine triphosphatase, or carbonyl cyanide chlorophenylhydrazone (CCCP), a metabolic uncoupler. Klebensberger et al. (2006) also found that no macroscopic aggregates could be formed if the energy supply was reduced by inhibiting respiration with KCN or CCCP. This and previous studies suggest that an energized state of suspended microorganisms would favor microbial attachment to a solid surface. This view is indirectly supported by the findings of Fletcher (1977)

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showing that the number of attached cells and the rate of attachment was greatest with log-phase cultures, and progressively decreased with stationary and death-phase cultures. It has been hypothesized that when microorganisms approach a substratum surface, a localized proton motive force (pmf) would be established, and microorganisms can use localized pmf and ATP as the driving force to enable cells attach to a solid surface (Ellwood et al. 1982; Hong and Brown 2009).

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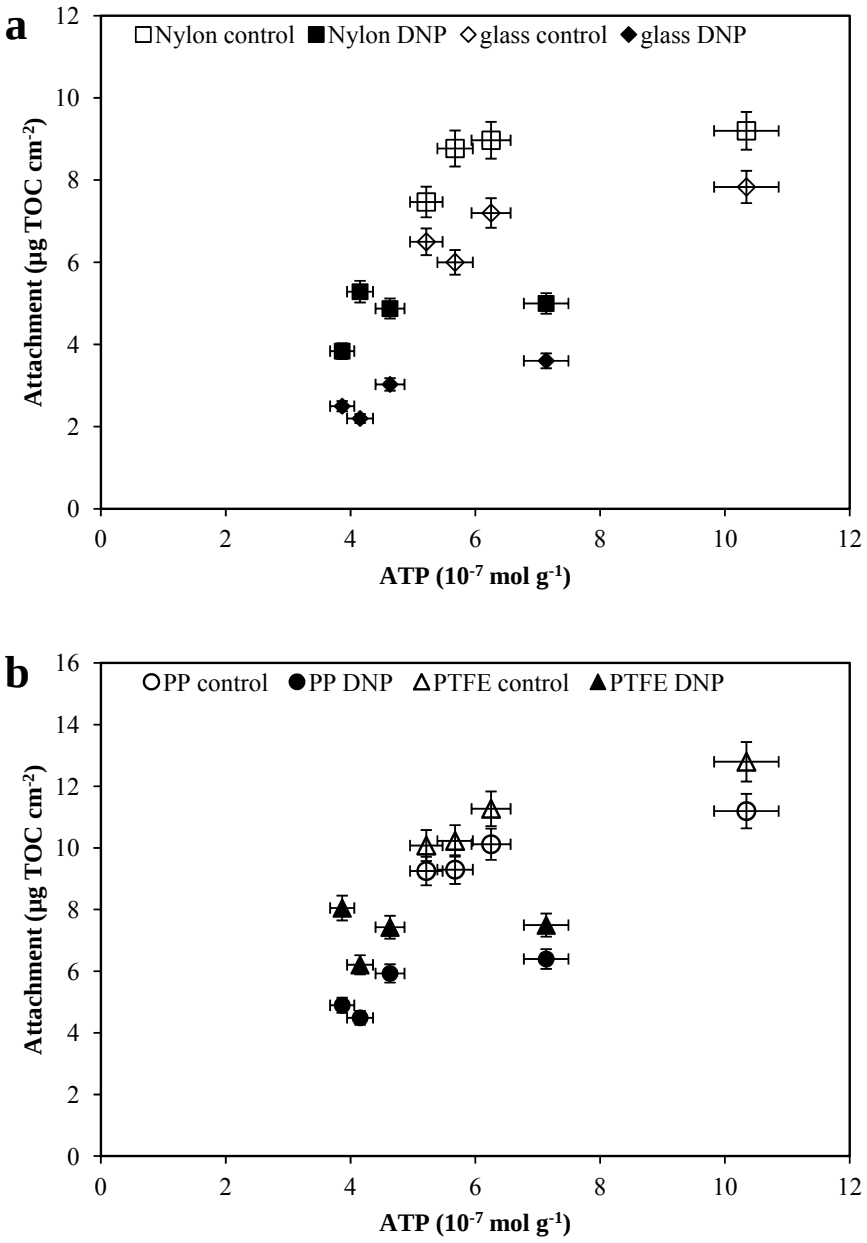

Figure 3.9 Effect of ATP content in suspended microorganisms on microbial attachment onto hydrophilic (a) and hydrophobic surface (b). Attachment of microorganisms without exposure to DNP onto nylon membrane (□), glass slide (◊), PTFE membrane (Δ) and PP coupon (○); attachment of microorganisms with exposure to DNP onto nylon membrane (■), glass slide (♦), PTFE membrane (▲) and PP coupon (●). Each point represents the mean of triplicate experiments, and the error bar is 1 SD from the mean.

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Compared to the control free of DNP, the AI-2 content in microorganisms was reduced by nearly 40% after 1-hr contact with DNP (Figure 3.8). A positive correlation between the AI-2 and ATP contents of biomass was established in Figure 3.10, i.e. the lower AI-2 production was observed at the lower ATP content of biomass in cases where microorganisms were exposed to DNP. Such observation can be explained by the fact that 4,5-dihydroxy-2,3-pentanedione (DPD), an AI-2 precursor, is biosynthesized from S-adenosylmethionine which is made from ATP and methionine by methionine adenosyltransferase (Chen et al. 2002; Stevenson and Babb 2002), meaning that the AI-2 synthesis is energy-dependent. In a study of quorum-sensing systems associated with biofilm formation by *Bacteroides fragilis*, Pumbwe et al. (2008) also reported the similar observation when carbonyl cyanide chlorophenylhydrazone (CCCP) was used as metabolic uncoupler. Therefore, the inhibited ATP synthesis by DNP would be responsible for the observed reduction in the AI-2 content (Figure 3.8). In addition, Pearson et al. (1999) reported that the presence of azide, an inhibitor of pmf, would inhibit the RND efflux pump activities, leading to increased accumulation of autoinducers in bacterial cells, i.e., the pmf-dependent RND pump is responsible for exporting autoinducers. Since DNP is known to have the ability to dissipate the pmf by carrying protons across the cellular membrane. This would result in the less AI-2 exported out of the membrane due to lowered pmf by DNP, which, in part, may explain the observed reduction of the AI-2 content in microorganisms exposed to DNP.

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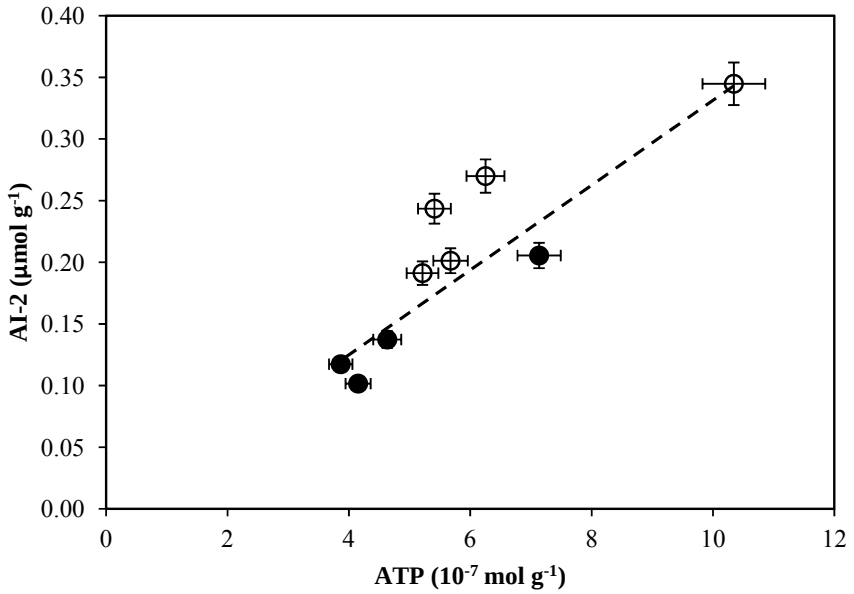

Figure 3.10 Effect of cellular ATP content on AI-2 production of suspended microorganisms with (●) and without (○) DNP treatment. Each point represents the mean of triplicate experiments, and the error bar is 1 SD from the mean.

Cellular communication plays a central role in biofilm development on various supports including membrane (Xiong and Liu 2010). Bacteria can communicate through secreting small chemical signaling molecules, called autoinducers among which AI-2 has been known as an universal signaling molecule for interspecies communication and coordination (Xavier and Bassler 2003; Parsek and Greenberg 2005). Figure 3.11 shows that microbial attachment on both hydrophilic (Figure 3.11a) and hydrophobic (Figure 3.11b) surface was positively correlated to the AI-2 content of suspended microorganisms regardless of DNP treatment. This suggests that AI-2 may play a coordinating role during the microbial attachment on different biocarrier surfaces. It has been known that microorganisms can use AI-2 for interspecies communication for both gram-negative and gram-positive bacteria. For example, AI-2 was required for the formation of mixed species biofilms of *P. gingivalis* and *Streptococcus gordonii*, and production of AI-2 by either bacterium was sufficient for inter-species communication and biofilm formation (McNab et al. 2003). Furthermore, AI-2 has been shown to control *E. coli* biofilm formation through a motility regulatory mechanism. Barrios et al. (2006) suggest that addition

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of synthetic AI-2 would lead to 30-fold biomass increase through improving cell motility, while for isogenic mutant without AI-2 induced motility gene, biofilm biomass was reduced by 8-fold.

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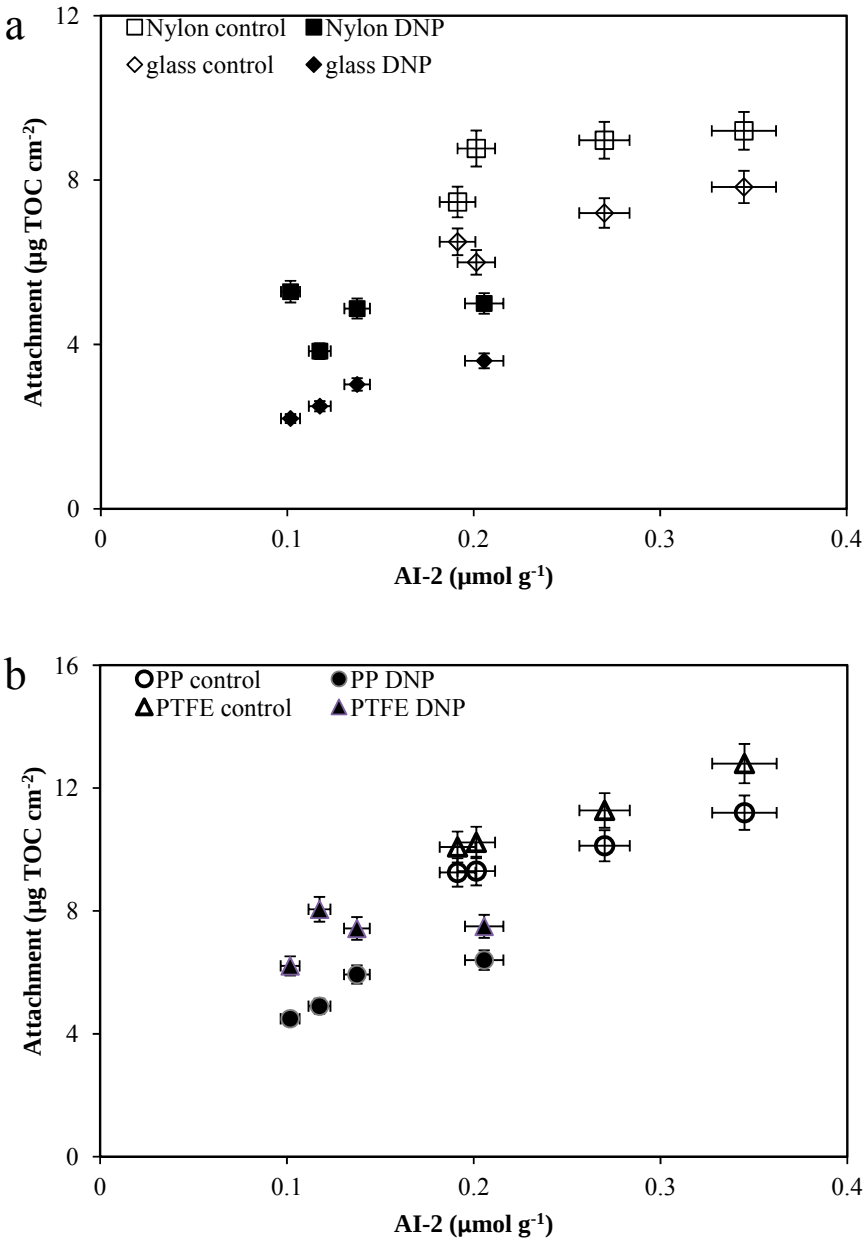

Figure 3.11 Effect of AI-2 content in suspended microorganisms on microbial attachment onto hydrophilic (a) and hydrophobic surface (b). Attachment of microorganisms without exposure to DNP onto nylon membrane (□), glass slide (◊), PTFE membrane (Δ) and PP coupon (○); attachment of microorganisms with exposure to DNP onto nylon membrane (■), glass slide (♦), PTFE membrane (▲) and PP coupon (●). Each point represents the mean of triplicate experiments, and the error bar is 1 SD from the mean.

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In addition, DNP used in this study is a typical metabolic uncoupler known to have the ability to dissipate the pH gradient of cellular membrane (Mathews et al. 2000), leading to dissipation of pmf. It was reported by Jolliffe et al. (1981) that any condition imposed on *B. subtilis* leading to dissipation of the pmf would cause the autolysis of bacteria. Calamita et al. (2001) used nitrogen gas and 1,3-dicyclohexylcarbodiimide to dissipate pmf, and concluded that the method of pmf dissipation is unimportant. Since DNP has been known to be capable of dissipating pmf, it might also cause cell autolysis. Therefore, it is a reasonable consideration that autolysis of suspended microorganisms induced by DNP would not favor microbial attachment to the surfaces of nylon membrane, glass slide, PTFE membrane and PP coupon (Figure 3.2). Additionally, cell motility has been reported to be pmf-dependent (Paul et al. 2008). Existing evidence shows that microbial attachment to a solid surface is largely dependent on cell motility (Kogure et al. 1998; Vatanyoopaisarn et al. 2000; McClaine and Ford 2002; Lemon et al. 2007). As discussed earlier, DNP can dissipate pmf and subsequently affects pmf-dependent motility. Therefore, metabolic uncoupler may compromise microbial attachment to solid surfaces by inhibiting pmf-dependent motility. These in turn provide an explanation for the observed microbial attachment under energy uncoupling conditions (Figure 3.2).

Membrane biofouling has been recognized as one of the biggest challenges in various applications of membrane systems for water and wastewater purification and reuse. As can be seen in Figure 3.2, the amounts of attached microorganisms on nylon and PFTE membranes were reduced significantly through DNP-induced inhibition of cellular ATP and AI-2 synthesis. As a result, the membrane performance in terms of water flux was improved accordingly (Figure 3.4). As can be seen in Figure 3.12, the cake layer resistance on nylon membrane was positively correlated to the AI-2 content of suspended microorganisms. This suggests that AI-2-mediated membrane biofouling is mainly responsible for the observed decline in water flux. Since cellular communication plays a central role in initial microbial attachment (Figure 3.11) and membrane biofouling (Figure 3.12), disruption of

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cellular communication would be a promising alternative of reducing membrane biofouling. For this purpose, the signal synthesis, activation of signal and receptor of the signal should be targeted for disruption of cellular communication (Dobretsov et al. 2009). In addition to AI-2, *N*-acylhomoserine lactones (AHL) is another common autoinducer which regulates quorum sensing system of Gram-negative bacteria, and they have been studied as the target for controlling membrane biofouling (Dobretsov et al. 2009). Paul et al. (2009) used Acylase I, an AHL-degrading enzyme, to hydrolyze AHL and found that the biofilm formation of *Aeromonas hydrophila* and *Pseudomonas putida* on reverse osmosis (RO) membrane was reduced by 20% to 24%. Yeon et al. (2009a) also reported that the presence of AHL in membrane bioreactor (MBR) would be responsible for the membrane biofouling. This and previous studies clearly show that inhibition of cellular communication would be a promising strategy for control of membrane biofouling.

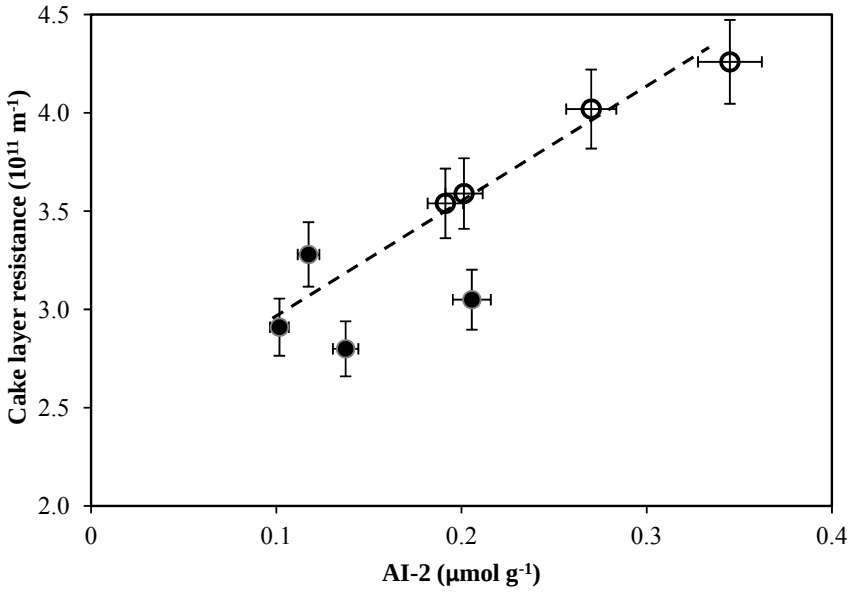

Figure 3.12 Effect of AI-2 content in suspended microorganisms on cake layer resistance of nylon membrane fouled by microorganisms with (●) and without (○) exposure to DNP. Each value represents the mean of triplicate experiments, and the error bar is 1 SD from the mean.

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The PTFE membrane has a more hydrophobic surface than the nylon membrane which is relatively hydrophilic. Under the similar conditions, more microorganisms tended to attach to highly hydrophobic PTFE membrane than to hydrophilic nylon membrane. A similar phenomenon was also observed on the hydrophobic PP coupon and the hydrophilic glass slide. It has been demonstrated that higher hydrophobicity of a solid surface would favor microbial attachment (Fletcher and Loeb 1979; Liu et al. 2004). After exposure to DNP, more significant reduction of attachment was observed on nylon membrane than on PTFE membrane (Fig. 3.2). This suggests that hydrophobic PTFE membrane was able to retain more fixed biomass than less-hydrophobic nylon membrane. In study of membrane biofouling, Fan et al. (2001) reported that the flux decline for a hydrophobic PVDF membrane was considerably quicker than that for its hydrophilic counterpart. Haack et al. (2003) modified the hydrophobic membrane surface by grafting a hydrophilic layer, and found that membrane fouling potential of hydrophobic membrane was significantly higher than that of hydrophilic membrane. These seem to suggest that membrane biofouling would be largely related to surface hydrophobicity of membrane, while hydrophilic membrane would be more beneficial for alleviating membrane biofouling (Figure 3.4).

3.5 CONCLUSIONS

The results showed that attachment of microorganisms treated with metabolic uncoupler, DNP, was significantly reduced on various biocarriers including hydrophilic nylon membrane and hydrophobic PTFE membrane. It was revealed that metabolic uncoupler suppressed ATP synthesis of suspended microorganisms, and subsequently reduced microbial attachment was observed under energy uncoupling conditions, suggesting involvement of energy metabolism in microbial attachment. In addition, DNP-inhibited ATP synthesis in turn lowered AI-2 production of suspended microorganisms, and a positive correlation between the ATP and AI-2 contents was established, i.e., AI-2 synthesis is ATP-dependent. Furthermore, the microfiltration tests confirmed that reduced AI-2 content of

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suspended microorganisms was positively correlated to reduced fouling resistance of nylon membrane. This suggests that AI-2-mediated membrane biofouling was mainly responsible for the observed decline in water flux. Consequently, this chapter provides direct experimental evidence that energy metabolism is involved in microbial attachment, and inhibition of ATP and ATP-mediated AI-2 would be a promising alternative for control of microbial attachment and membrane biofouling.

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CHAPTER 4
EFFECT OF ATP INHIBITION ON DETACHMENT OF DIFFERENT-AGE BIOFILMS FROM MEMBRANE SURFACES

4.1 INTRODUCTION

Prevention of microbial attachment and removal of attached biomass are the two main strategies for effective control and mitigation of membrane biofouling. As shown in Chapter 3, metabolic energetic status plays an important role in biofilm development on various solid surfaces including membranes. However, little has been known about the possibility to disperse or remove attached biomass or biofilm through energy dissipation and the involvement of AI-2 mediated quorum sensing in biofilm dispersion.

It has been known that the physiology and structure of biofilms is biofilm age-dependent, i.e., biofilms at different growth states would respond differently to changes in the culture environment. Thus, this study investigated the responses of different-age mixed-species biofilms precultured on membrane surfaces to energy dissipation induced by a metabolic uncoupler, 3, 3', 4', 5-tetrachlorosalicylanilide (TCS). It should be noted that all metabolic uncouplers have the ability to decouple catabolism and anabolism. In fact, different uncouplers, such as TCS, carbonyl cyanide chlorophenylhydrazone (CCCP), DNP, and others have been employed in various microbial studies (Maier et al. 2004; Klebensberger et al. 2006; Saini and Wood 2008; Aragon et al. 2009). This study offers new insights into the dispersion mechanisms of biofilms, which in turn help to develop more effective cleaning strategies for biologically fouled membranes.

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4.2 MATERIALS AND METHODS

4.2.1 Biofilm Developemnt on and Detachment from Membranes

The methods used to culture biofilms on membranes were similar to those reported in the literature (Steinberger et al. 2002; Priester et al. 2007). Activated sludge microorganisms were taken from a local wastewater treatment plant in June 2010 and acclimated with a synthetic substrate for one month. The synthetic substrate was the same as described in Chapter 3, Section 3.2.2. For biofilm development on nylon membranes, 0.1 mg of activated sludge was filtered on a 47-mm flat sheet nylon membrane (Osmonics, Minnetonka, Minnesota, USA) with a pore size of 0.2 μm. The membranes with uniformly deposited biomass were incubated on the solid Luria-Bertani (LB) agar at 30 ºC (BD Difco, Franklin Lakes, NJ) to let biofilm grow on each membrane. LB medium contained 10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, and 5 g L^{-1} sodium chloride, and was prepared according to the manufacturer's instructions.

To investigate the response of different-growth-phase biofilms to TCS, a typical metabolic uncoupler, different-age biofilms were developed. Based on the preliminary results of biofilm growth curve, biofilms of defined growth phase were developed, i.e. 6-h old biofilm for the initial phase, 12-h old biofilm for the exponential phase followed by 24-h old biofilm for the stationary phase. At the end of a defined biofilm culture time, four membranes were harvested for biofilm with the same age and soaked in TCS-PBS (10 mM phosphate buffered saline) solution under static condition at each TCS concentration. After two hours of exposure to different concentrations of TCS (4, 6, 8 mg L^{-1}), the remained biomass on the membrane was collected to determine the retained biomass quantity and extract cellular ATP, AI-2 and EPS.

Three series of experiments were conducted: one was exposure of 24-h old biofilm to different TCS concentrations for two hours; one was to study the effect of different exposure time on 24-h old biofilm detachment under a given TCS

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concentration (8 mg L^{-1}); the other was to investigate the response of different-age biofilms to 8 mg L^{-1} TCS solution. The experiment was carried out in duplicate, and the significance of data was estimated by Student *t*-tests with a 95% confidence.

4.2.2 Measurement of Cellular ATP and Autoinducer-2 of Fixed Biomass

The cellular ATP was extracted from freshly collected biosamples following the trichloroacetic acid (TCA) method (Chen and Leung 2000) as described in Chapter 3 with some modifications. Ten milliliters of 5% TCA solution was directly added into biosample on membrane, and was homogenized with an ultrasonic homogenizer (Sonics & Materials, Newton, CT, USA) for 3 min to release ATP. Aliquots of 0.5 mL homogenized suspension were mixed with 4.5 mL of Tris-Acetate-EDTA (TAE) buffer (Bio-Rad, Singapore) to adjust the pH of about 7.75. The mixture was filtered through 0.2 μm syringe filters to remove residue and the collected filtrate was stored at -20°C for measuring ATP concentration. ATP concentration was determined using the firefly luciferin-luciferase bioluminescence method, as described in Chapter 3.

For determination of AI-2, cell-free culture fluids must be prepared first. Biofilms on membrane surface before or after exposure to TCS were collected and resuspended in 10 mL of fresh autoinducer bioassay (AB) medium (Surette et al. 1999). The extraction and determination of AI-2 concentration were done according to the procedures presented in Chapter 3. Each cell-free sample was assayed six times in parallel and the mean values were reported.

4.2.3 Analysis of Extracellular Polysaccharides and Proteins of Fixed Biomass

Extracellular polysaccharides (PSs) and proteins (PNs) are two major components of EPS secreted by bacteria, which were extracted from fixed biomass on membrane surface by modified cold aqueous technique method (Jia et al. 1996). Ten milliliters of 8.5% NaCl and 0.22% formaldehyde extraction solution was added to biofilm on membrane and the mixture was homogenized for 2 min using

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an ultrasonic homogenizer (Sonics & Materials, CT, USA) in an ice-water bath. The nylon membrane was removed from the suspension and then the mixture was centrifuged at 13,420 g (KUBOTA, 5922, Tokyo, Japan) for 30 min to remove solid residues. The supernatant was harvested to determine PN, PS concentration. PN was analyzed by the modified Lowry method (Lowry et al. 1951) using bovine serum albumin (Sigma-Aldrich) as standard. PS was determined by the phenol-sulphuric acid method (Dubois et al. 1956) in which glucose (Sigma-Aldrich) was used as standard.

4.2.4 Biofilm Staining and Image Acquisition

In order to visualize biofilm on membrane surfaces, the fixed bacteria on the membrane surface were stained with LIVE/DEAD BacLight^{TM} Bacterial Viability kits (Molecular Probes, Carlsbad, CA, USA), as described in Chapter 3. The stained sample was covered with cover slip and viewed using an Olympus Fluoview FV300 confocal laser scanning microscopy (CLSM) (Olympus Optical, Tokyo, Japan) with a 100× objective.

To provide a more detailed picture of biofilm detachment from membrane surfaces, different dyes for specific components of EPS were used. The EPS staining procedure was carried out as follows (Adav et al. 2008). For polysaccharide staining, one hundred microliters of 250 mg L^{-1} fluorescently labelled lectin concanavalin A (Con A) conjugated with tetramethyl rhodamine (Molecular Probes, Carlsbad, CA, USA) was added to the collected sample to bind α-mannopyranosyl and α-glucopyranosyl sugar residues, and incubated at room temperature for 30 min. The stained sample was then washed twice with PBS to remove unbound stain. After that, 100 μL of 300 mg L^{-1} fluorescent brightener 28 (Sigma-Aldrich, St. Louis, MO, USA) was added to mix with the sample for 30 min to stain the β-linked polysaccharides and subsequently washed twice with PBS to remove excess dye. Thus the sample would be ready to visualize extracellular polysaccharide under CLSM.

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To stain extracellular protein, 100 μL of 0.1M sodium bicarbonate buffer was added to the sample to maintain the amino groups in non-protonated status. Then 10 μL of 10 g L^{-1} fluorescein isothiocyanate (Molecular Probes, Carlsbad, CA, USA) solution was used to bind with proteins. The sample was well mixed on a shaker for 1 h at room temperature to let the stain penetrate. After PBS washed twice, the sample was incubated with 60 μL of 10 mg L^{-1} Nile red (Molecular Probes, Carlsbad, CA, USA) solution for 10 min in the dark to stain lipids. After removing the extra dye, the samples were mounted on glass slides to visualize with CLSM.

4.3 RESULTS

4.3.1 Response of 24-h old Biofilm to Different TCS Concentrations

In order to study the dose-response relationship of mixed-species biofilms to TCS, 24-h old biofilms were precultivated on membrane surfaces. Figure 4.1a shows that the fixed biomass on the membrane surface was significantly removed (Student's *t*-test, $P < 0.05$) after being exposed to TCS at different concentrations. Compared to the control, fixed biomass on the membrane surface was reduced by 34%, 42% and 53% after exposure to 4, 6, and 8 mg L^{-1} TCS for two hours, respectively. These results imply that TCS can trigger detachment of mixed-species biofilms at the concentrations studied. In addition, Figures 4.1b and 4.1c present microscopic evidence showing dispersion of 24-h old biofilm after exposure to TCS. Compared to biofilms without exposure to TCS, the architecture of biofilm treated with TCS became loose and more dead cells (red color) were observed. These results indicate that exposure to TCS would help to mitigate membrane biofouling.

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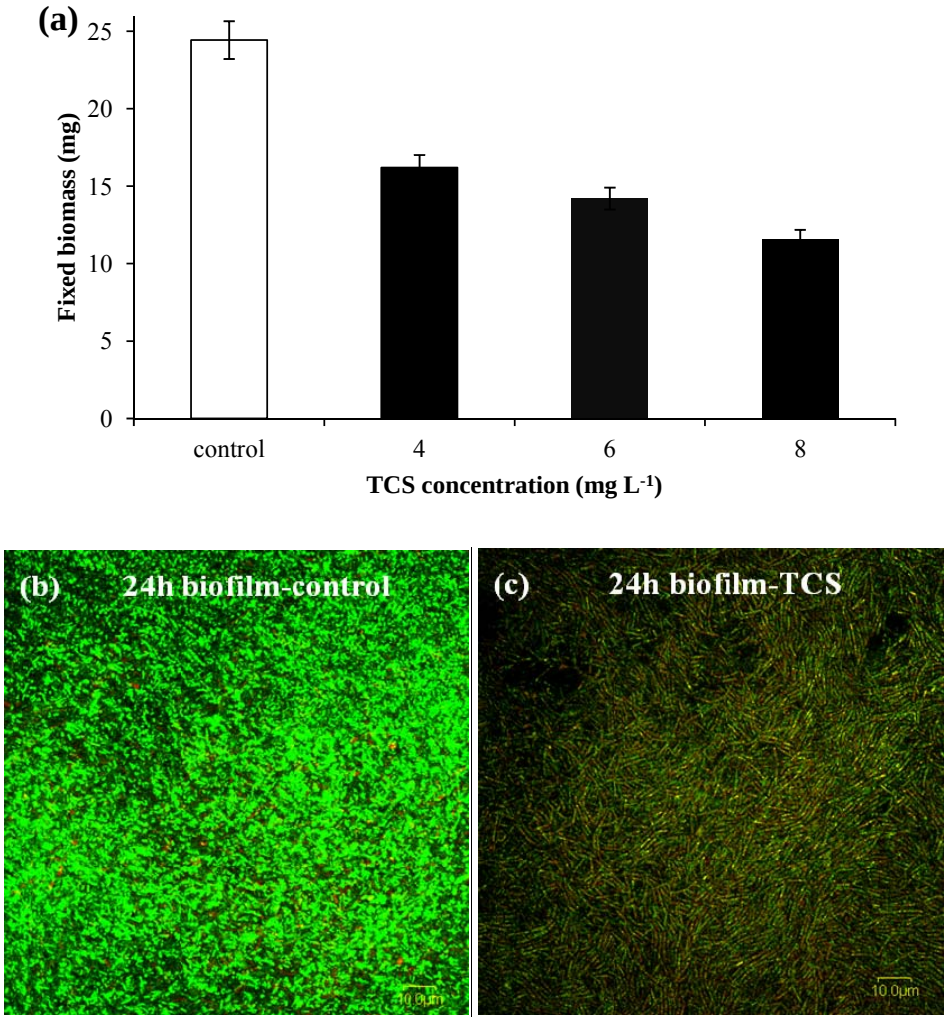

Figure 4.1 Biofilm detachment (a) at different TCS concentrations (□: before TCS treatment; ■: after exposure to TCS), and CLSM images of 24-h old biofilm before (b) and after (c) exposure to 8 mg L^{-1} TCS. Each value represents the mean of duplicate experiments, and the error bar is 1 SD from the mean.

Figure 4.2a shows the ATP contents in 24-h old biofilm exposed to different TCS concentrations. The initial cellular ATP content of 24-h old biofilm before TCS treatment was 4.54×10^{-7} mol ATP g^{-1} MLVSS, while it was reduced by 10-fold after contact with 8 mg L^{-1} TCS for 2 hours. This implies that TCS can significantly inhibit ATP synthesis (Student's *t*-test, $P < 0.05$). Figure 4.2b shows AI-2 contents of 24-h old biofilm exposed to different TCS concentrations. Compared to the biofilm without TCS treatment, it was reduced by 26%, 43% and 57% after contact

with 4, 6 and 8 mg L^{-1} TCS for 2 hours, respectively. Figure 4.3 shows the effect of TCS on extracellular polysaccharides and proteins in biofilms with different ages. It can be seen in Figure 4.3a that the content of extracellular polysaccharides in 24-h old biofilms decreased by 36% after exposure to 8 mg L^{-1} TCS for two hours, while extracellular proteins reduced by 25%. These results indicate that TCS would inhibit production of both extracellular polysaccharides and proteins at the concentration studied.

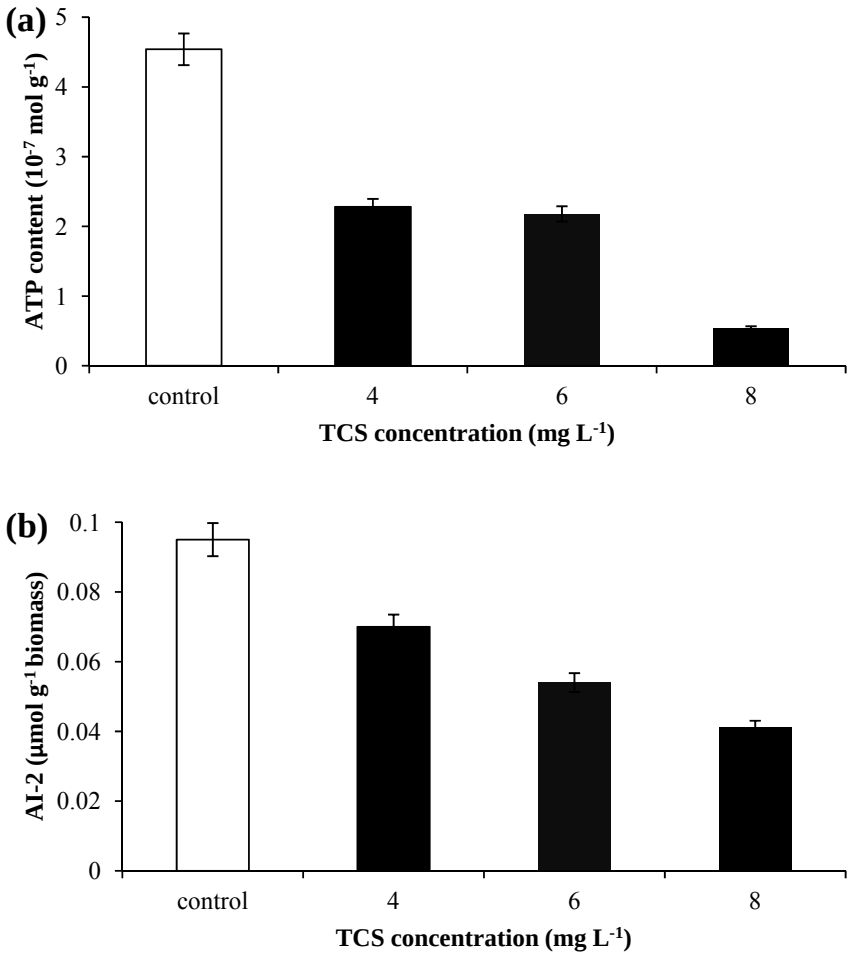

Figure 4.2 Cellular ATP (a) and AI-2 (b) contents of 24-h old biofilm before (□) and after (■) exposure to different TCS concentrations. Each value represents the mean of duplicate experiments, and the error bar is 1 SD from the mean.

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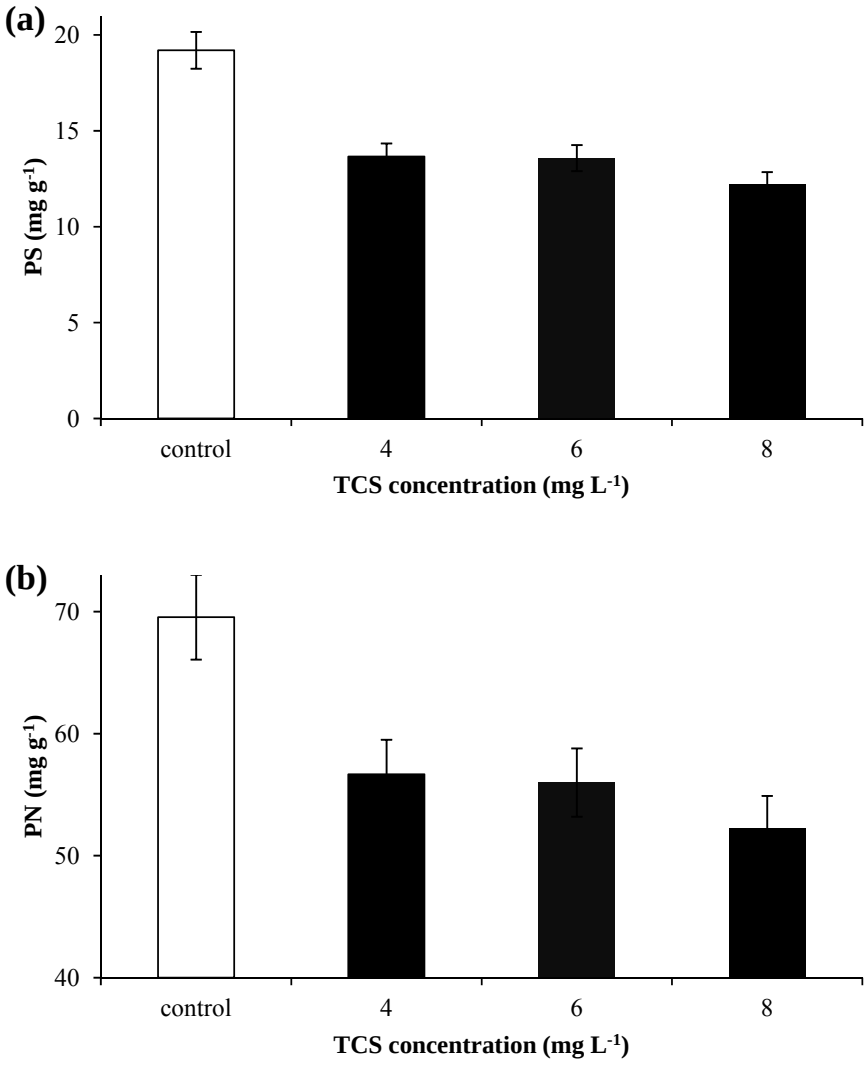

Figure 4.3 Extracellular polysaccharide (a) and protein (b) content of 24-h old biofilm before (□) and after (■) exposure to different TCS concentrations. Each value represents the mean of duplicate experiments and error bar is 1 SD from the mean.

4.3.2 Response of 24-h old Biofilm to Different Exposure Times

To investigate the effect of TCS treatment time on biofilm detachment, the TCS concentration was fixed at 8 mg/L according to the results presented in Section 4.3.1.

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Figure 4.4 shows that fixed biomass decreased with prolonged treatment time from 2 h to 4 h. The most significant reduction in fixed biomass was observed in the first 2-hour treatment. At the end of 2nd hour, 52.5% reduction in fixed biomass was achieved as compared to the control assay free of TCS. These results suggest that a 2-h treatment would be sufficient at the TCS concentration of 8 mg/L.

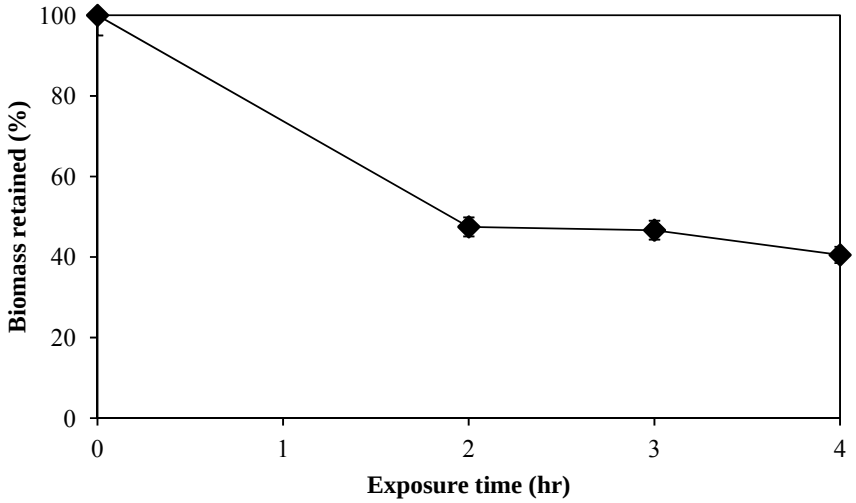

Figure 4.4 Biomass detachment of 24-h old biofilm before and after exposure to 8 mg L^{-1} TCS for different times. Each value represents the mean of duplicate experiments, and the error bar is 1 SD from the mean.

A similar trend has been revealed in cellular ATP content. Figure 4.5a shows that the reduction in ATP was most significant at the 2nd hour, i.e., the ATP content was decreased by 88.0% at the 2nd hour, 88.2% at 3rd, and 90.2% at the 4th hour. This implies that a 2-h treatment by TCS would be optimal for ATP reduction. Figure 4.5b shows the AI-2 content for 24-h old biofilm at different treatment times. It can be seen that the AI-2 content tended to decrease with prolonged TCS treatment, i.e., it was reduced by 56.8%, 64.2 and 77.9% after 2, 3 and 4 hour treatment, respectively. It is known that quorum sensing is biomass density dependent (Bassler 2002; Waters and Bassler 2005). When the biofilm structure was weakened, reduced AI-2 production can be expected.

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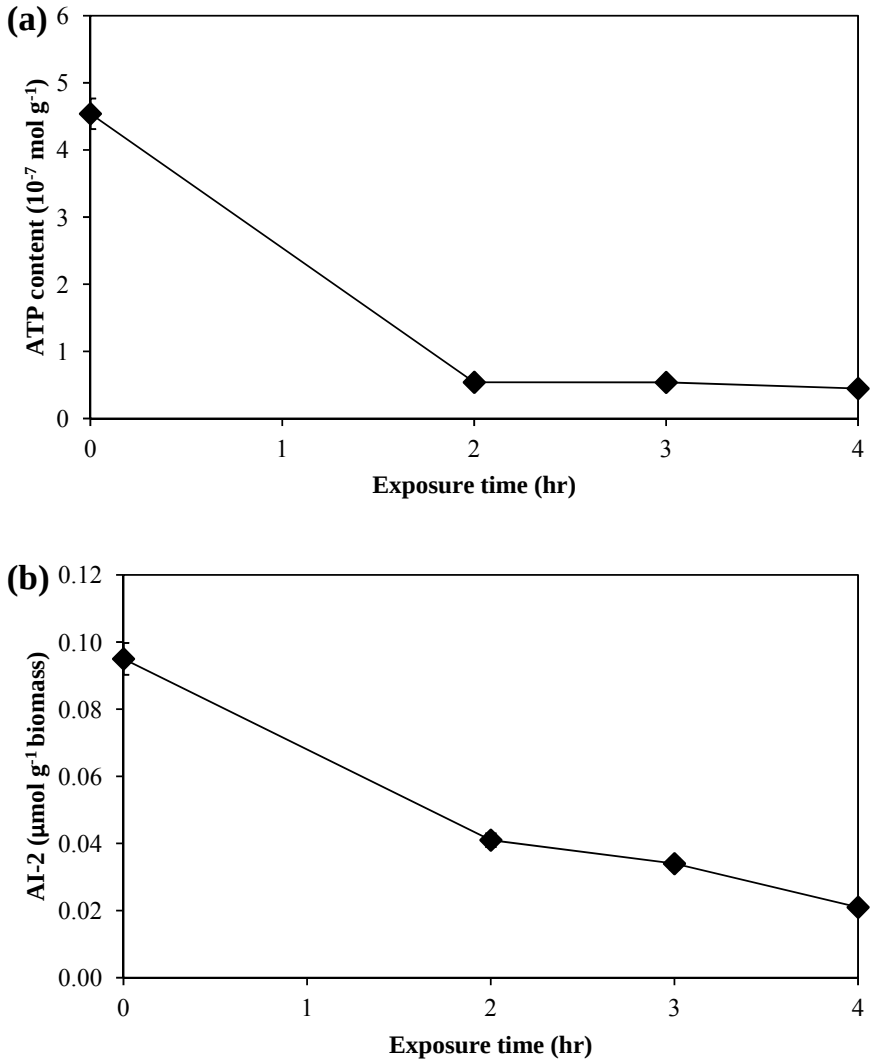

Figure 4.5 Cellular ATP (a) and AI-2 (b) contents of 24-h old biofilm before and after exposure to 8 mg L^{-1} TCS for different times. Each value represents the mean of duplicate experiments, and the error bar is 1 SD from the mean.

Figure 4.6 shows the contents of extracellular polysaccharides and proteins at different treatment times. No significant changes in EPS content were observed after the second hour, indicated by a standard deviation less than 6% for both the polysaccharide and protein contents. These suggest that extended treatment time beyond 2 hours would not result in further reduction in extracellular polysaccharides and proteins.

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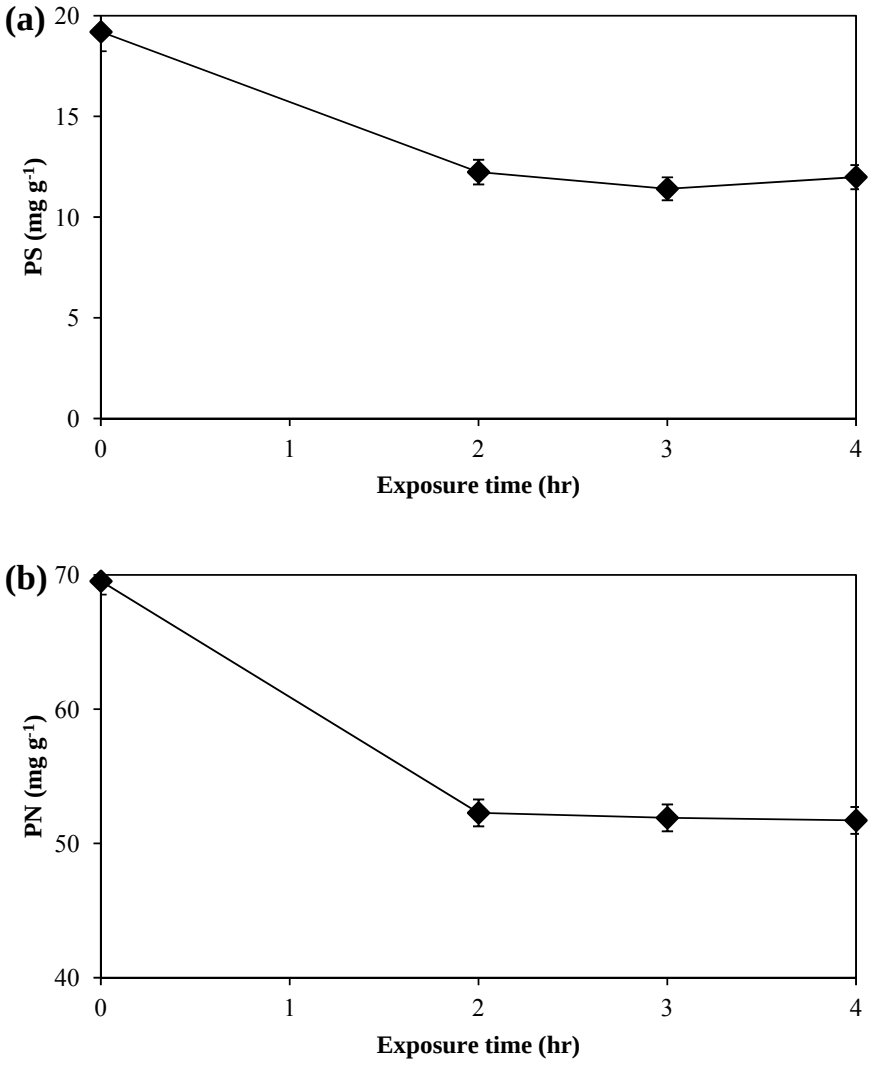

Figure 4.6 Extracellular polysaccharide (a) and protein (b) contents of 24-h old biofilm before and after exposure to 8 mg L^{-1} TCS for different times. Each value represents the mean of duplicate experiments, and the error bar is 1 SD from the mean.

4.3.3 Response of Different-Age Biofilms to Fixed TCS Concentration

As shown in Fig. 4.1, the highest detachment of 24-h old biofilms was observed at the TCS concentration of 8 mg L^{-1}, which was adopted in the study of response of different-age biofilms to TCS. Figure 4.7a shows the detachment of different-age biofilms from membrane surfaces after exposure to 8 mg L^{-1} TCS. Before treatment,

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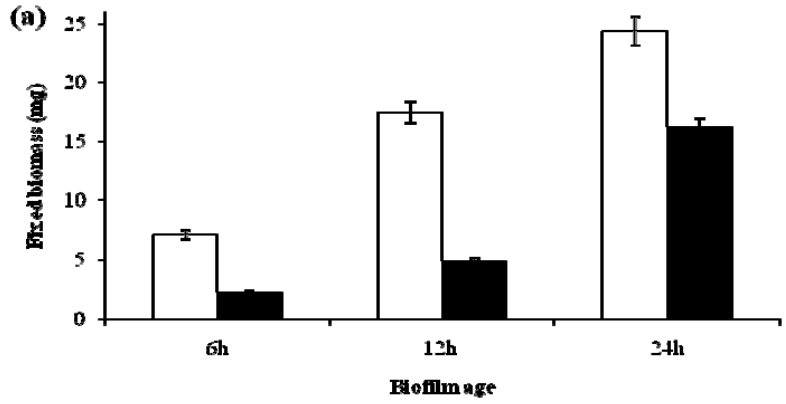
the biomass on membrane for 6, 12 and 24-h old biofilm was 7.13 mg, 17.49 mg and 24.43 mg, respectively. After exposure to 8 mg L^{-1} TCS for 2 hours, the biomass on the membrane surface was reduced by 68%, 72% and 34% for 6-h, 12-h and 24-h old biofilm respectively (Student's *t*-test, $P < 0.05$). Figures 4.7b-c further confirm the changed biofilm structure after TCS treatment, showing that the biofilm on membrane surface became less dense compared to controls for 12-h old biofilms. Similar phenomena were also observed for 6-h old and 24-h old biofilm. These results together with Figure 4.1 indicate that TCS can promote detachment of different-age biofilms from membrane surfaces.



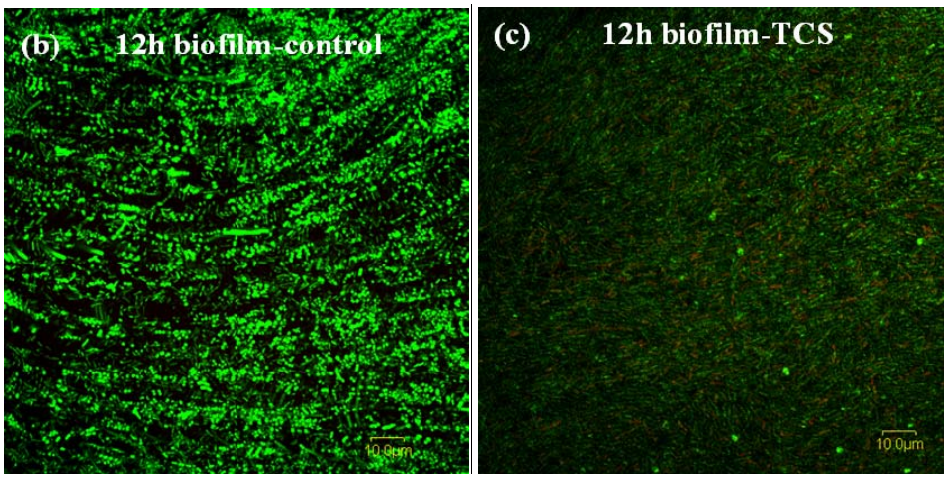


Figure 4.7 Biofilm detachment (a) of different-age biofilms (□: before TCS treatment; ■: after exposure to TCS) and CLSM images of 12-h old biofilm before (b) and after (c) exposure to 8 mg L^{-1} TCS. Each value represents the mean of duplicate experiments, and the error bar is 1 SD from the mean.

The ATP contents of different-age biofilms before and after TCS treatment are shown in Figure 4.8a. After exposed to 8 mg L^{-1} TCS for 2 hours, the ATP content decreased for all the biofilms with different ages. For example, cellular ATP content of 12-h old biofilm decreased from 8.51×10^{-7} mol ATP g^{-1} MLVSS to 1.53×10^{-7} mol ATP g^{-1} MLVSS, indicating an 82% reduction (Student's *t*-test, $P < 0.05$). This suggests that TCS was able to dissipate cellular ATP of biofilms regardless of biofilm age. Figure 4.8b further presents AI-2 contents in different-age biofilms before and after TCS treatment. AI-2 contents of different-age biofilms all decreased, e.g. the decrement was most obvious for 12-h old biofilm where the AI-2 content dropped nearly by 87.1% (Student's *t*-test, $P < 0.05$). Figure 4.9a illustrates polysaccharide contents of different-age biofilms. After treatment by 8 mg L^{-1} TCS, 66% of reduction in biofilm polysaccharides was observed for 6-h old biofilm, and 39% and 36% for 12-h old and 24-h old biofilms, respectively. Figure 4.9b-e further reveals changes in matrix structure of extracellular polysaccharides before and after exposure to TCS. For example, β-polysaccharides, a vital structural component of biofilm, became less in quantity and were loosely distributed in space compared to biofilms without TCS treatment. Similar phenomena were also observed for extracellular proteins in biofilms (Fig. 4.10). Therefore, it is reasonable to consider that TCS can reduce EPS production, but also weaken EPS matrix structure.

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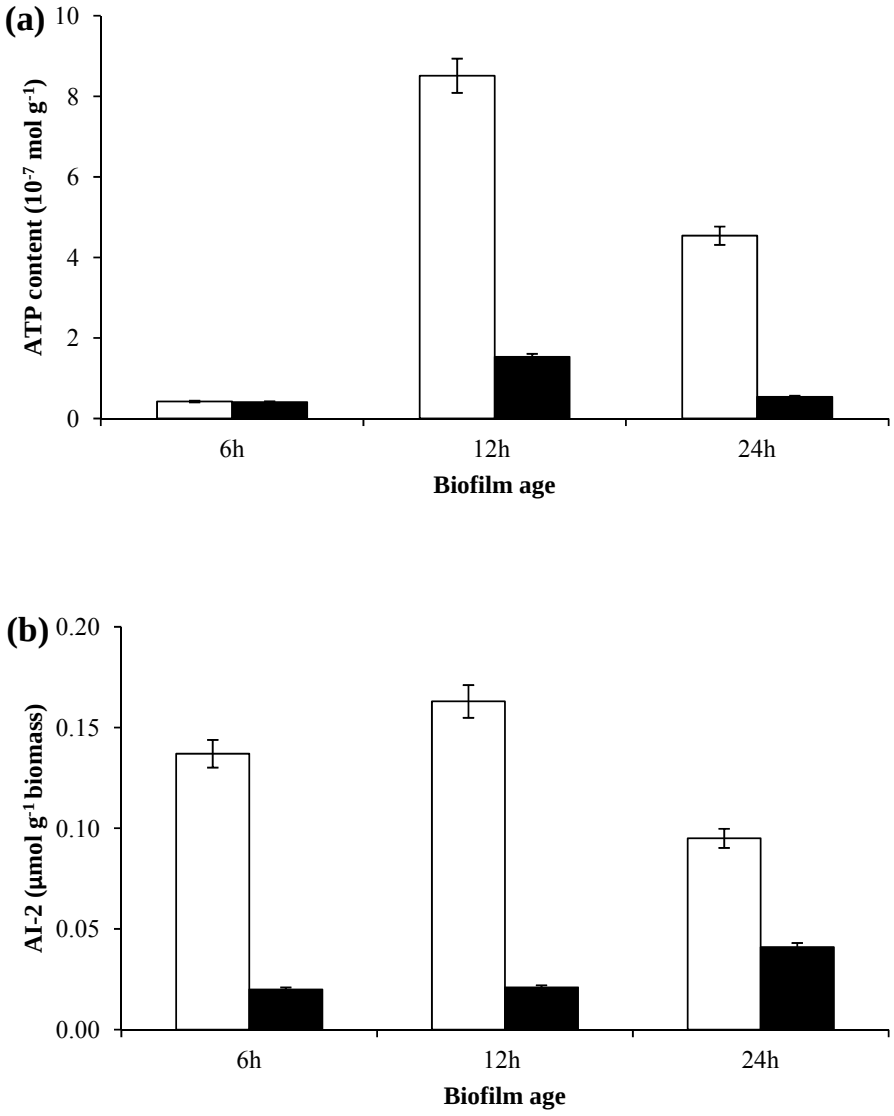

Figure 4.8 Cellular ATP (a) and AI-2 (b) contents of different-age biofilms before (□) and after (■) exposure to 8 mg L^{-1} TCS. Each value represents the mean of duplicate experiments, and the error bar is 1 SD from the mean.

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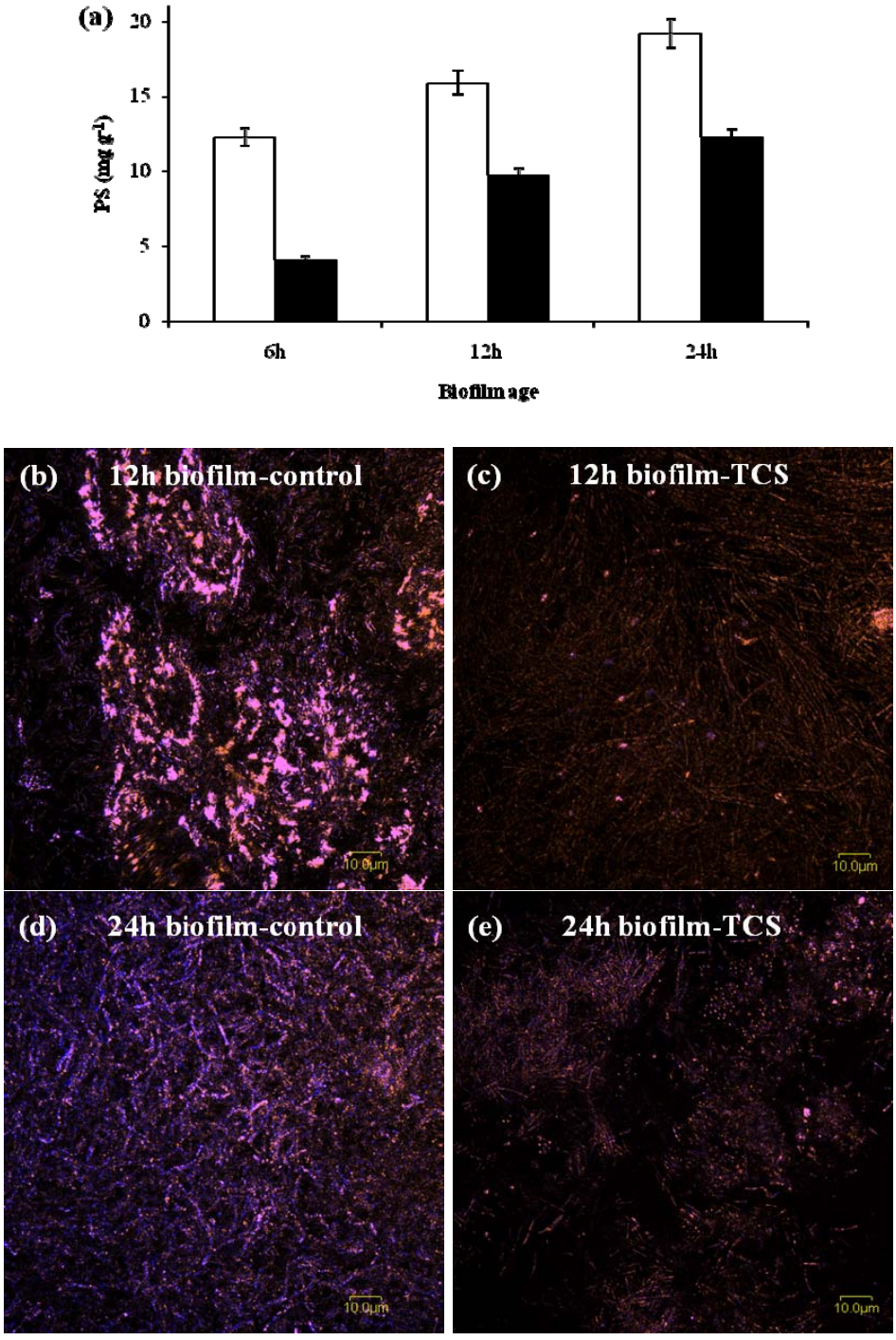

Figure 4.9 Extracellular polysaccharide production (a) (□: before TCS treatment; ■: after exposure to TCS) of different-age biofilms and stained PS before (b, d) and after (c, e) exposure to 8 mg L^{-1} TCS (β-polysaccharide, blue; α-polysaccharide, orange). Each value represents the mean of duplicate experiments, and the error bar is 1 SD from the mean.

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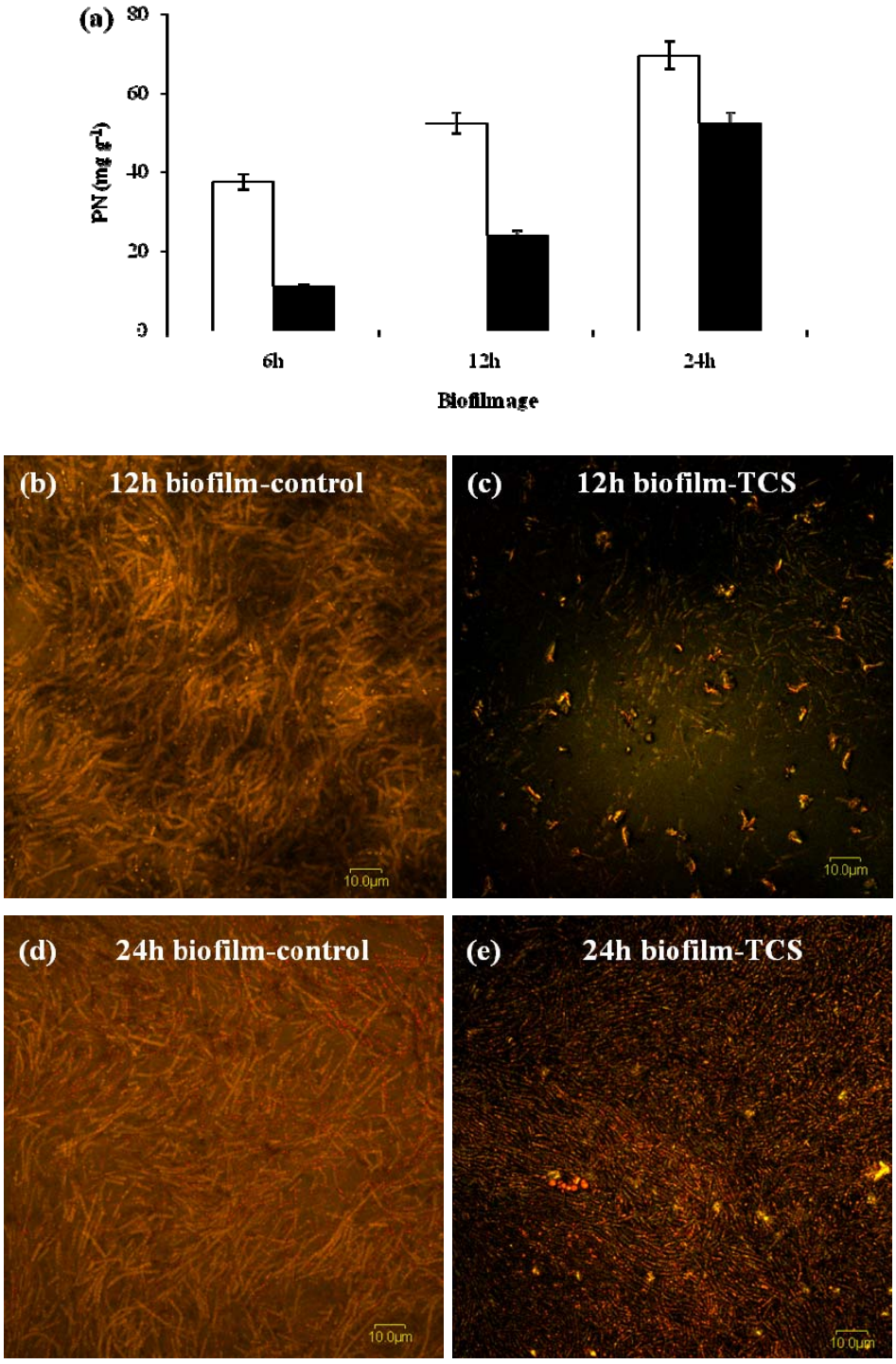

Figure 4.10 Extracellular protein production (a) (□: before TCS treatment; ■: after exposure to TCS) of different-age biofilms and stained PN before (b, d) and after (c, e) exposure to 8 mg L^{-1} TCS (protein, yellow; lipid, red). Each value represents the mean of duplicate experiments, and the error bar is 1 SD from the mean.

4.4 DISCUSSION

It appears from Figs. 4.1 and 4.7 that TCS can promote detachment of biofilms with different ages from membrane surface. As an uncoupler of oxidative phosphorylation, TCS has the ability to disrupt the proton motive force; therefore, it inhibits ATP synthesis. As such, ATP contents in biofilms exposed to TCS were reduced significantly (Fig. 4.2a and 4.8a). Meanwhile, it can be seen in Fig. 4.1 that biofilm detachment would be closely related to chemically dissipated ATP, indicating that maintaining the structure and integrity of biofilm is energy-dependent. It has been known that AI-2 *in vitro* production uses *S*-adenosyl-methionine as substrate, while *S*-adenosylmethionine is made from ATP and methionine by methionine adenosyltransferase (Schauder et al. 2001), suggesting that AI-2 synthesis requires ATP. As observed in Figs. 4.2 and 4.8, reduced ATP synthesis of biofilms by TCS resulted in a significant drop in AI-2 content in biofilms, subsequently a positive correlation between AI-2 and ATP contents in biofilms was observed in Fig. 4.11. Furthermore, reduction of extracellular proteins and polysaccharides was found to be positively correlated to reduction of AI-2 content in biofilms (Fig. 4.12), implying that the AI-2 may be involved in or regulate EPS synthesis. In fact, in comparison of biofilm formation of wild type *Klebsiella pneumoniae* and the AI-2 synthesis gene LuxS mutants, De Araujo et al. (2010) found that exopolysaccharide production was regulated by AI-2 mediated quorum sensing in the formation of *K. pneumoniae* biofilm, whereas quorum sensing had been found to positively regulate *pel*, a major biofilm-related exopolysaccharide operon (Sakuragi and Kolter 2007).

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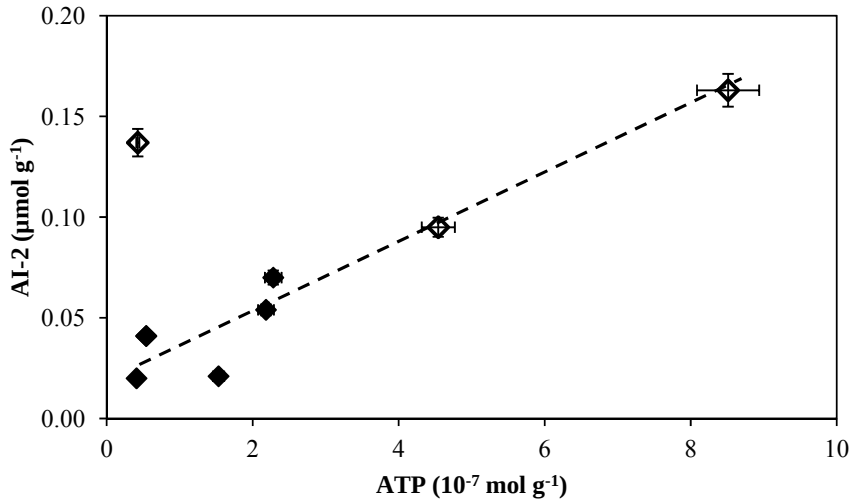

Figure 4.11 Dependence of AI-2 production on ATP synthesis before (◊) and after (♦) TCS treatment. Each point represents the mean of duplicate experiments, and the error bar is 1 SD from the mean.

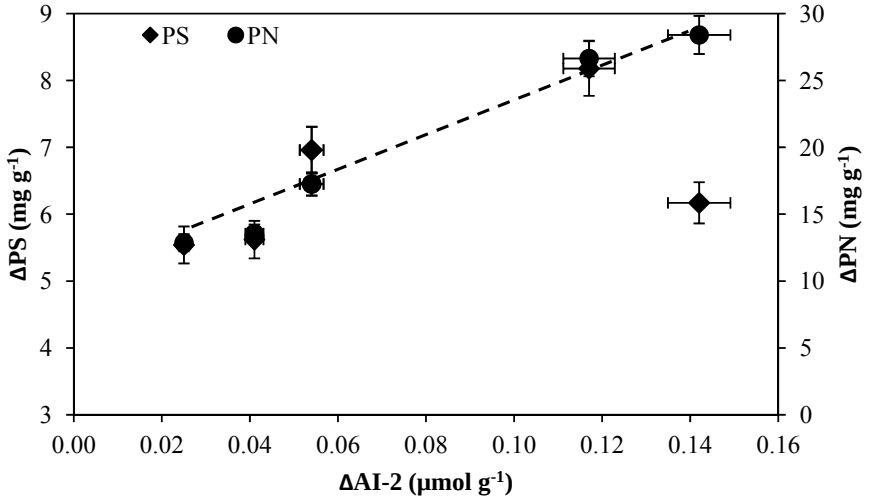

Figure 4.12 Effect of reduction of AI-2 contents in biofilms on decreased PS (♦) and PN (●) production. Each point represents the mean of duplicate experiments, and the error bar is 1 SD from the mean.

Figure 4.13 shows the correlation of fixed biomass and EPS in biofilms, indicating that reduced EPS secretion would induce biofilm detachment. For example, when extracellular protein was hydrolyzed by specific enzyme proteinase K, 98%

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biomass detachment of 72 h *Staphylococcus haemolyticus* biofilm was reported (Fredheim et al. 2009). In addition to EPS quantity in biofilms, the spatial distribution and specific function of vital extracellular polysaccharide and protein also play important role in biofilm detachment. EPS have been believed to facilitate biofilm formation and can further strengthen microbial structure through forming a polymeric matrix. Thus, EPS act like glue and shelter to protect bacteria in the EPS matrix. EPS deficiency would result in a weak structure of biofilms. As shown in Figures 4.9b-e and 4.10b-e, the EPS structure was clearly disrupted after exposure to TCS regardless of biofilm age, especially less secretion of β-linked polysaccharides was observed. It has been reported that β-linked polysaccharides serve as backbone of biofilm structure (Sutherland 2001). These suggest that reduced β-linked polysaccharides, in part, would result in collapse of the EPS matrix structure and subsequent biofilm dispersion.

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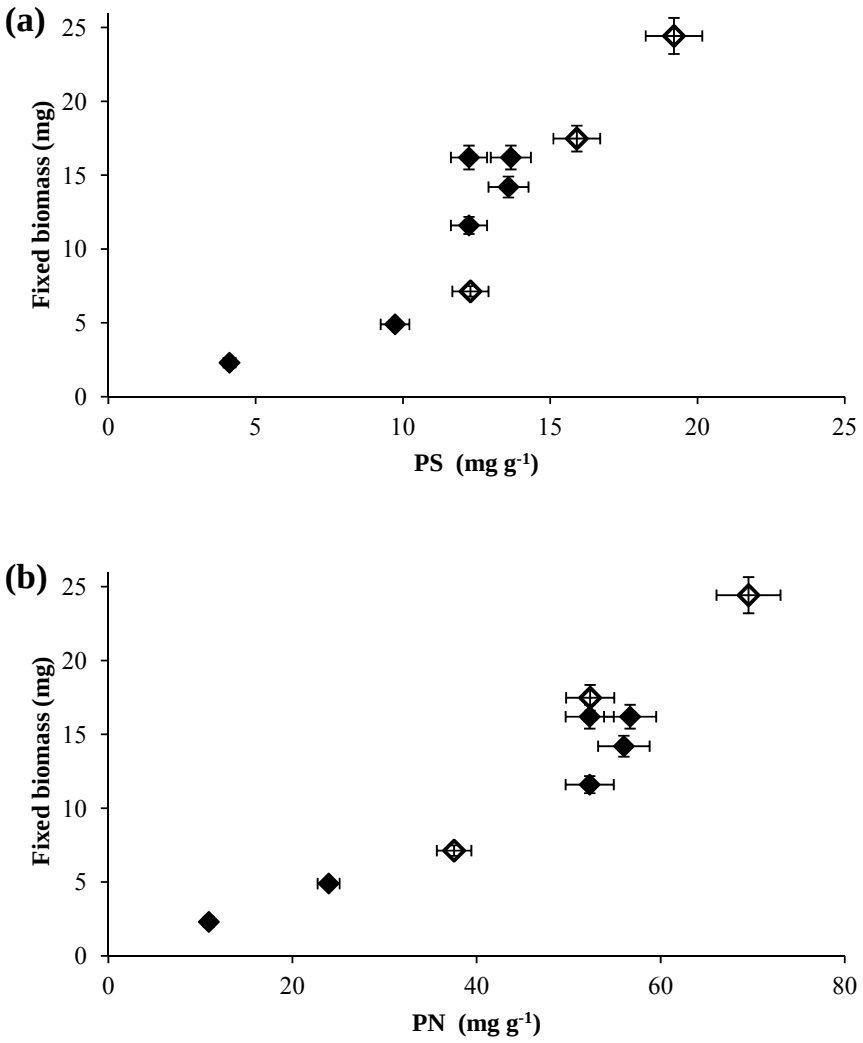

Figure 4.13 Dependence of biofilm detachment on PS (a) and PN (b) production before (◊) and after (♦) TCS treatment. Each point represents the mean of duplicate experiments and error bar is 1 SD from the mean.

It appears from Figures 4.12 and 4.13 that EPS production was mediated by inter-species signaling molecules AI-2 and positively correlated to different-age biofilms detachment, implying that signaling molecules AI-2 may be involved in biofilm detachment through AI-2-regulated EPS production. It had been shown that cells can use different signaling molecules to break biofilm (Karatan and Watnick 2009), and quorum sensing system was involved in biofilm dispersion (Purevdorj-Gage et al. 2005; Rice et al. 2005). AI-2 mediated QS system has been known to facilitate

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biofilm formation (Petersen et al. 2006; Rickard et al. 2006; Ahmed et al. 2008), thus the reduced AI-2 content (Figures 4.2b and 4.8b) may reversely induce the detachment of different-age biofilms as shown in Figures 4.1 and 4.7. In the study of the effect of quorum sensing on *Vibrio cholera* biofilm dispersal, Liu et al. (2007) reported that the AI-2 deficient mutants of *V. cholera* would experience difficulty in detaching from biofilm structure, i.e. AI-2 regulated cellular communication was involved in controlling biofilm thickness and biofilm detachment rate. Thus, this and previous study suggests that AI-2-mediated quorum sensing may be involved in biofilm dispersal through AI-2-regulated EPS production.

Results presented in this study suggest that TCS has the potential to clean biofouled membrane regardless of biofilm age, providing a new alternative for mitigation membrane biofouling.

4.5 CONCLUSIONS

This chapter shows that TCS-dissipated ATP synthesis promoted microbial detachment of different-age biofilms from membrane surfaces. This together with Chapter 3 revealed that reduced cellular ATP content inhibited AI-2 synthesis, whereas reduced production of extracellular proteins and polysaccharides of fixed biomass was found to be positively related to reduction of corresponding AI-2 content in detachment experiments. It seems that reduced cellular ATP content by TCS would lead to inhibited AI-2 and subsequent AI-2-regulated EPS production, which in turn promoted dispersion of different-age biofilms from membrane surfaces. Consequently, energy dissipation through metabolic uncoupling appears to be a new alternative for promoting microbial detachment.

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CHAPTER 5
REDUCED MICROBIAL ATTACHMENT BY D-AMINO ACID

5.1 INTRODUCTION

D-amino acids have been excluded from living systems except in the cell wall of microorganisms. Both the thick cell wall of Gram-positive bacteria and much thinner cell wall of Gram-negative bacteria consist of peptidoglycan which contain D-amino acids. Besides being components of bacterial cell wall, D-amino acids have been known to regulate bacterial germination and to be incorporated into peptides (Wood et al. 2011). Specific D-amino acids have been discovered in a variety of foods (e.g. alcoholic beverages and dairy products) as well as in living organisms from bacteria to mammals (Friedman 2010). In fact, many bacteria would produce various D-amino acids. For example, Lam et al. (2009) found that *Vibrio cholerae* produces D-methionine and D-leucine at the concentration of milimoles, while *Bacillus subtilis* secretes D-tyrosine and D-phenylalanine. The bacteria begin to synthesize those D-amino acids in the stationary phase, which may govern the chemistry of the cell wall since D-amino acids would change the cell wall-building proteins, leading to slow production of peptidoglycan that is crucial for cell wall (Lam et al. 2009).

Kolodkin-Gal et al. (2010) probably for the first time reported the role of D-amino acids in pure culture biofilm dispersal. But little has been known about the effects of exogenous D-amino acids on the formation of mixed-culture biofilm and synthesis of cellular ATP, AI-2, eDNA and EPS, which are all essential for biofilm development on a solid surface as shown in Chapters 3 and 4. Therefore, this study aimed to investigate how D-tyrosine would affect attachment of mixed-culture microorganisms onto hydrophobic PP and hydrophilic glass surfaces through determination of cell surface charge, ATP, AI-2, eDNA and EPS.

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5.2 MATERIALS AND METHODS

5.2.1 Microbial Attachment Assay

Activated sludge microorganisms were taken from a local wastewater treatment plant in May 2010 and acclimated with a synthetic substrate for one month. The synthetic substrate was the same as described in Section 3.2.2, Chapter 3. Experiments were designed to investigate the effect of D-tyrosine on microbial growth and attachment potentials. Thus, microorganisms with and without exposure to D-tyrosine for different times were used in 1-hr static microbial attachment experiments conducted under the same conditions, as detailed below: (i) two series of batch experiments were conducted: one served as control, free of D-tyrosine, while the other was added with 6 mg L^{-1} of D-tyrosine (Sigma-Aldrich, St. Louis, MO, USA); (ii) suspended biomass cultivated with and without exposure to D-tyrosine was collected at different exposure times of 1 to 4 hr for 1-hr microbial attachment assay and determination of surface charge, cellular ATP, AI-2, eDNA and EPS. Glass slides and PP coupons were used as biocarriers in microbial attachment. The static microbial attachment was conducted as illustrated in Chapter 3 Section 3.2.2. Fixed biomass was quantified in terms of TOC by a TOC analyzer (ASI-V, TOC-Vcsh, Shimadzu, Japan).

5.2.2 Surface Charge

The colloid titration method was used to determine surface charge of suspended microorganisms with and without exposure to D-tyrosine (Wilen et al. 2003). Polybrene (Sigma-Aldrich) was used as positive colloidal reagent and polyvinyl sulphate potassium salt (PVSK) (Sigma-Aldrich) as negative reagent. For titrating negatively charged suspended microorganisms, 5 mL of 0.001 N polybrene was added to the sample. The excess polybrene was back titrated with 0.0005 N PVSK using 100 μL of 0.1% toluidine blue (Sigma-Aldrich) as the end-point indicator. Titration was terminated when the color changed from blue to pink, indicating that electrical neutrality was reached. Equal volumes of polybrene in distilled water

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were used as blanks. The surface charge, expressed as milliequivalents per gram of dry biomass can be determined from the equation given below.

$$\text{Charge (meq g}^{-1}\text{ SS)} = \frac{1000(A-B)N}{XV} \quad (5.1)$$

Where A is the volume of PVSK added to the sample (mL), B is the volume of PVSK added to the blank (mL), N is the normality of PVSK solution used (0.0005 N), V is the volume of the sample (mL), X is the biomass concentration of the sample (g L^{-1}).

5.2.3 Determination of Cellular ATP and AI-2

The cellular ATP were extracted from freshly collected biosamples according to the trichloroacetic acid (TCA) method (Chen and Leung 2000) with some modifications. Cellular ATP contents were extracted by 5% TCA solution and determined as described in Chapter 3 Section 3.2.4.

The cell-free culture supernatant was thawed before determination of AI-2 concentration as described in Chapter 3 Section 3.2.5. The amount of AI-2 was measured by *V. harveyi* BB170 (ATCC BAA 1117) bioluminescence reporter assay (Rickard et al. 2008). The results were presented by the molar concentration of AI-2, as DPD (Omm Scientific, Dallas, USA) were used as the calibration standard. Each filtrated sample was assayed six times in parallel and the mean values reported.

5.2.4. Response of AI-2 Reporter Strain to D-tyrosine

In order to further investigate the effect of D-tyrosine on AI-2 repression, an AI-2 bioluminescence assay in presence and absence of D-tyrosine was conducted. In this assay, five thousand times diluted reporter strain *V. harveyi* BB170 as described before was grown in fresh AB medium supplemented with 0.1-0.7 μM of DPD in the wells of a 96-well plate. Two series of experiments were conducted: for control, the wells were free of D-tyrosine; 6 mg L^{-1} D-tyrosine was added to the other wells. The 96-well plate was shaken in a rotary shaker at 30 ºC. The light intensity was

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assayed over time using a microplate reader until get the maximum fold induction of bioluminescence.

5.2.5 Extraction and Quantification of eDNA

Extracellular DNA was extracted from suspended microorganisms sample according to (Steinberger and Holden 2005) with modification. Five milliliter of bacterial suspension was collected and resuspended in 0.9% NaCl solution. The resuspended sample was well mixed with a homogenizer (Sonics & Materials, CT, USA). The treated cell solution was filtered through 0.2 μm syring filter and the collected filtrate was stored at -20°C for determining eDNA concentration. The concentration of DNA was measured by using PicoGreen dsDNA Quantification Kit (Molecular Probes, Invitrogen, Eugene, OR, USA) following the protocol provided by the kit. The calf thymus DNA was used as the standard. The fluorescence intensity was recorded by a microplate reader (BioTek, sygnergy 2, VT, USA).

5.2.6 Determination of Extracellular Polysaccharides and Proteins

Extracellular polysaccharides (PS) and proteins (PN) were extracted from biosample as illustrated in Chapter 4 Section 4.2.3. The extracted EPS solution was stored at -20°C for PS, PN measurement and high performance size exclusion chromatography analysis.

High performance size exclusion chromatography (HPSEC) analysis was carried out with Series 200 HPLC system (Perkin Elmer, Waltham, MA, USA) equipped with a Series 200LC quaternary pump, Series 200 autosampler, a Perkin Elmer 600 interface and a UV/Vis detector (785A). A 300 x 7.8mm size exclusion chromatography column BioSep SEC S2000 (Phenomenex, Torrance, CA) was used. The mobile phase consisted of 9.0 mM NaCl and 0.9 mM Na_2HPO_4 at pH 7.0 (Comte et al. 2007). Extracellular polymeric substances were extracted from suspended microorganisms as mentioned above and all samples were filtered through 0.20 μm filters prior to injection. All measurements were conducted at 25

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ºC, mobile phase flow 1.0 mL min^{-1}, the sample injection 100 µL. The detection was carried out with a UV detector at 280 nm.

5.2.7 Staining and Visualization

In order to visualize microbial attachment, the adherent bacteria on glass slides and PP coupons surfaces were stained with LIVE/DEAD BacLight^{TM} Bacterial Viability kits (Molecular Probes, Eugene, OR, USA) as explained in Chapter 3 Section 3.2.6. The sample was viewed using an Olympus Fluoview FV300 confocal laser scanning microscopy (CLSM) (Olympus Optical, Tokyo, Japan) with a 100× objective.

5.2.8 Statistical Analysis

All tests were performed in triplicate otherwise stated. Results were expressed as mean value ± absolute deviation. Student *t*-tests were employed for analyzing the significance of results at the level of $P < 0.05$.

5.3 RESULTS

5.3.1 Microbial Attachment on Glass and PP

Figure 5.1 shows that attachment of microorganisms exposed to 6 mg L^{-1} D-tyrosine was reduced significantly on glass slides compared to the control free of D-tyrosine (Student's *t*-test, $P < 0.05$). After 2-h culture, attachment of microorganisms without exposure to D-tyrosine was 10.8 µg TOC cm^{-2} on the glass surface, while attachment of microorganisms with exposure to D-tyrosine was 8.5 µg TOC cm^{-2}, indicating 22% reduction in microbial attachment caused by D-tyrosine. A similar phenomenon was also observed in microbial attachment on PP surface (Figure 5.1b). These results indeed are supported by the microscopic observations (Figure 5.2). It should be noted that mixed-culture microorganisms with and without exposure to D-

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tyrosine were collected at different culture (exposure) times of 1 to 4 hr, and used for 1-hr microbial attachment assays, as shown in Fig. 5.1. According to substrate availability over 4-hr culture, 1 hr- and 2-hr old microorganisms were basically in earlier exponential and post exponential growth phases, while 3 hr- and 4 hr-old microorganisms already entered into earlier stationary and post-stationary growth phases, respectively. It is reasonable to consider that microorganisms at different growth states would have different attachment abilities as observed in the control assays (Fig. 5.1). This view is strongly supported by the findings of Fletcher (1977), showing that the number of attached cells harvested from exponential growth phase was greatest, followed by those from stationary and decay phases.

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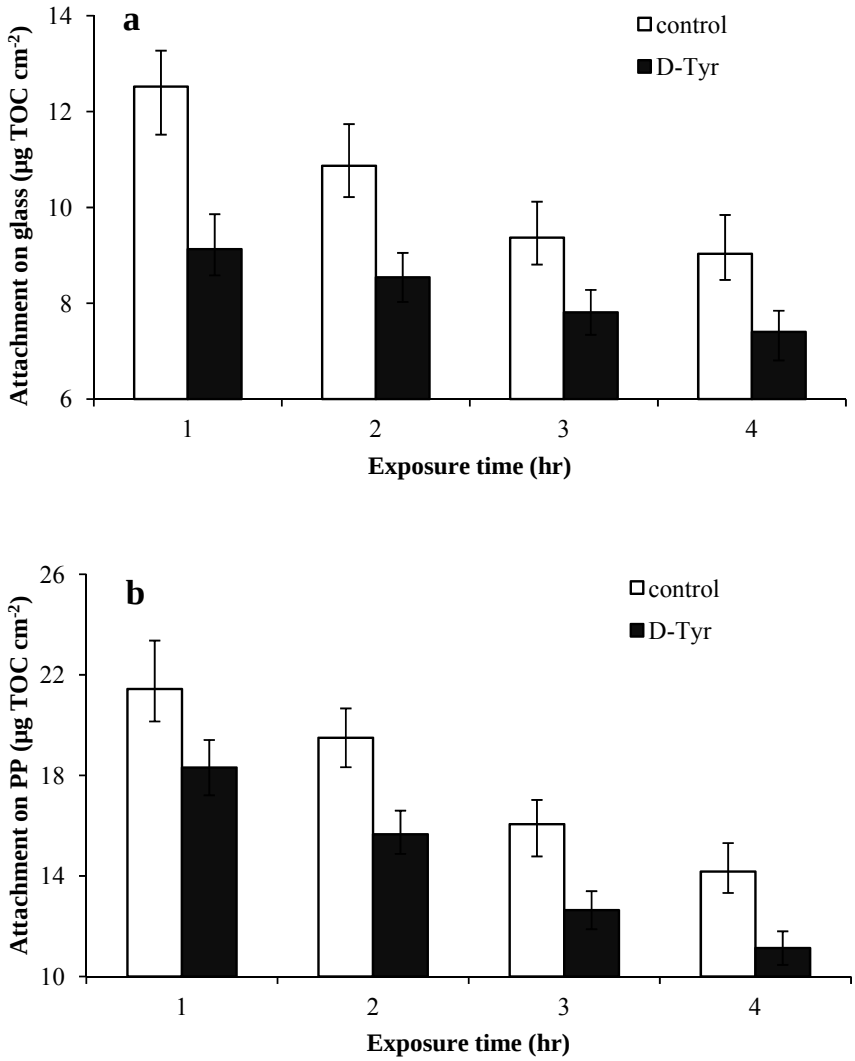

Figure 5.1 Attachment of microorganisms with (■) and without (□) treatment by D-tyrosine on glass slides (a); on PP coupons (b). Each point represents the mean of triplicate measurements, and the error bar is absolute deviation from the mean.

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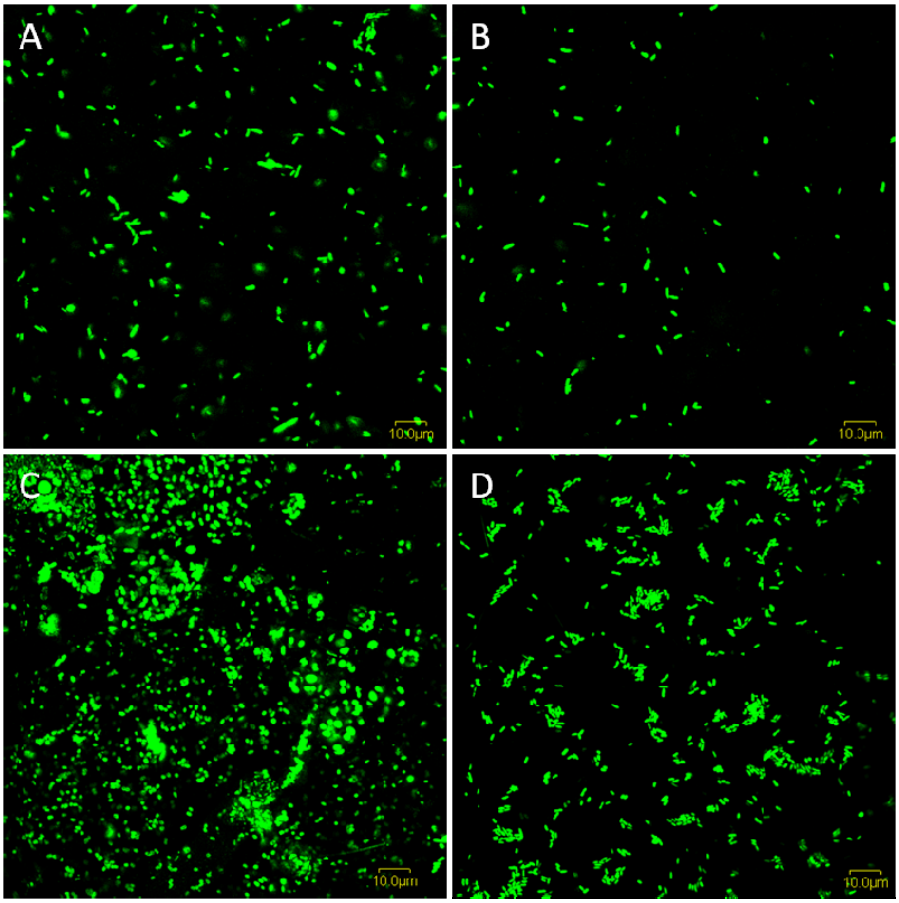

Figure 5.2 CLSM images of attachment of microorganisms without D-tyrosine treatment on glass slides (A) and PP coupons (C) at the exposure time of 1 hr; with D-tyrosine treatment on glass slides (B) and PP coupons (D) at the exposure time of 1 hr.

In addition, Figure 5.3a shows that addition of D-tyrosine to the culture media had no negative effect on the TOC removal efficiency. It was shown in Figure 5.3b that the suspended biomass concentration increased from 450 mg L^{-1} to 630 mg L^{-1} in the cultures supplemented with and without D-tyrosine. These suggest that D-tyrosine is not inhibitory to substrate utilization and microbial growth at the concentration studied. Figure 5.4 shows that suspended microorganisms with and without exposure to D-tyrosine both carried negative surface charge, but microorganisms exposed to D-tyrosine carried more negative surface charge compared to that of control free of D-tyrosine.

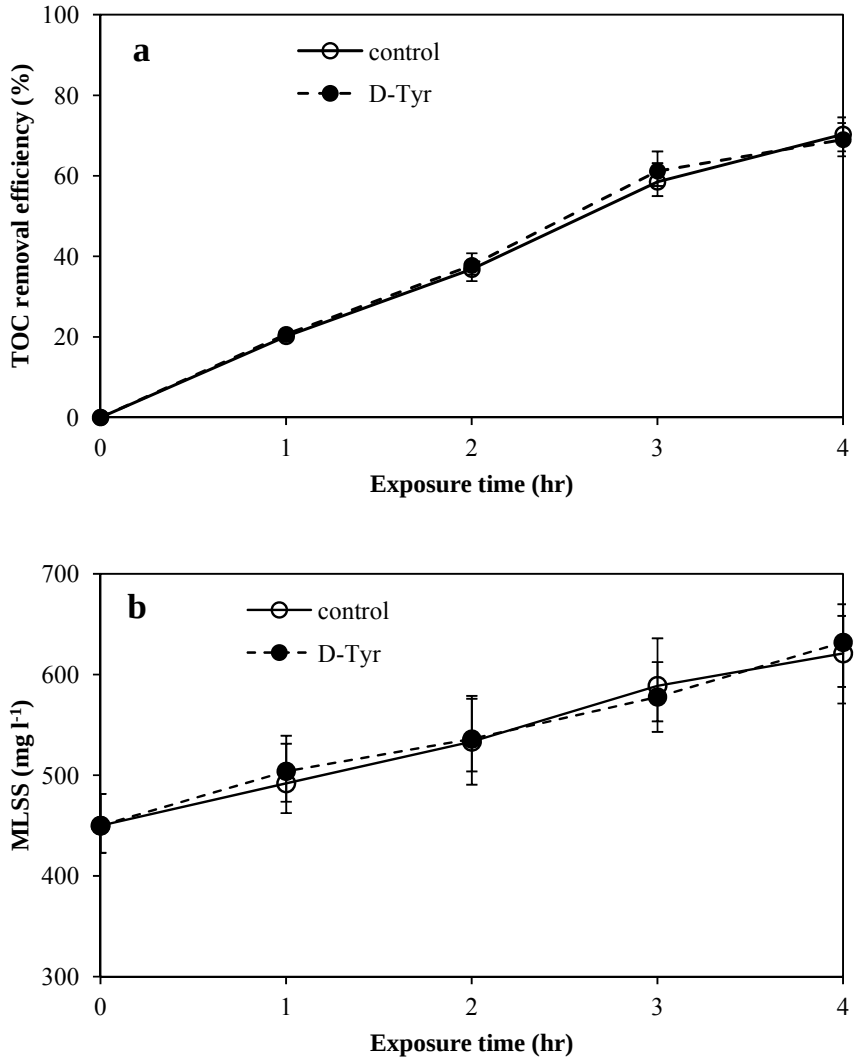

Figure 5.3 Profiles of TOC removal efficiency (a) and microbial growth (b) in the cultures supplemented with (●) and without (○) D-tyrosine. Each point represents the mean of triplicate measurements, and the error bar is absolute deviation from the mean.

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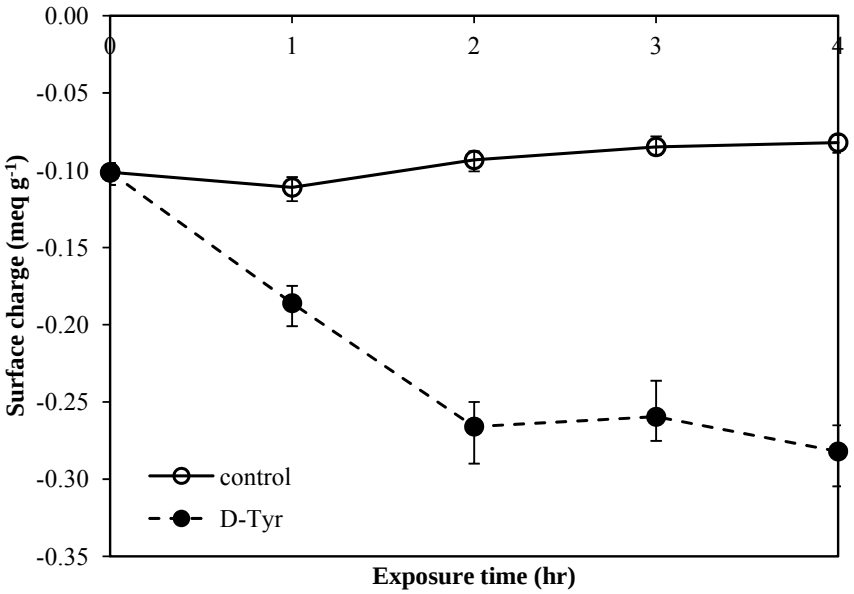

Figure 5.4 Surface charge of suspended microorganisms with (●) and without (○) exposure to D-tyrosine. Each point represents the mean of triplicate measurements, and the error bar is absolute deviation from the mean.

5.3.2 Cellular ATP and AI-2 Contents of Suspended Microorganisms

Figure 5.5a showed the possible effect of D-tyrosine on energy metabolism of suspended microorganisms. It can be seen in Figure 5.5a that the cellular ATP content did not change significantly in the presence of D-tyrosine compared to that of control. D-tyrosine thus does not appear to inhibit the ATP synthesis. AI-2 as inter-species signaling molecules coordinates the formation of biofilm by various species (Rickard et al. 2008). To investigate the effect of D-tyrosine on cellular communication, Figure 5.5b showed the respective AI-2 content of suspended microorganisms with and without D-tyrosine addition. After one hour culture, the AI-2 content of suspended microorganisms without exposure to D-tyrosine was about 0.27 nmol mg^{-1} in the control, while it decreased to about 0.19 nmol mg^{-1} for suspended microorganisms with exposure to D-tyrosine, i.e. D-tyrosine could hinder the synthesis or secretion of AI-2. The response of reporter strain *V. harveyi* BB170 to D-tyrosine was further studied, and results were presented in Fig. 5.6, showing fold induction of luminescence in presence and absence of D-tyrosine, and

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obviously luminescence decreased significantly when the culture was supplemented with D-tyrosine (Student's *t*-test, $P < 0.05$). For example, for 0.4 μM DPD, the fold induction of luminescence was 23.6 in the control free of D-tyrosine, whereas it decreased to 12.4 in the media with addition of D-tyrosine, indicating 48% reduction as compared to that of control. These imply that D-tyrosine at the concentration studied had an inhibitory effect on AI-2 expression.

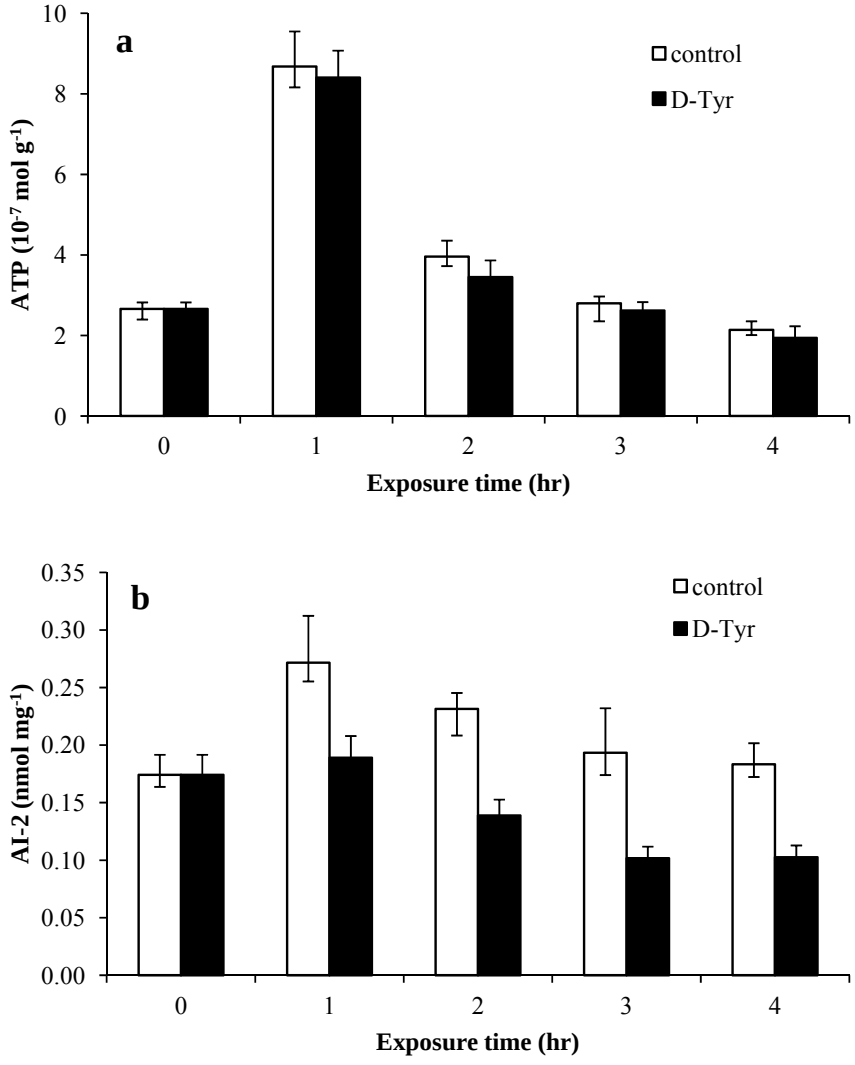

Figure 5.5 Cellular ATP content (a) and AI-2 concentration (b) in the cultures supplemented with (■) and without (□) D-tyrosine. Each point represents the mean of triplicate measurements, and the error bar is absolute deviation from the mean.

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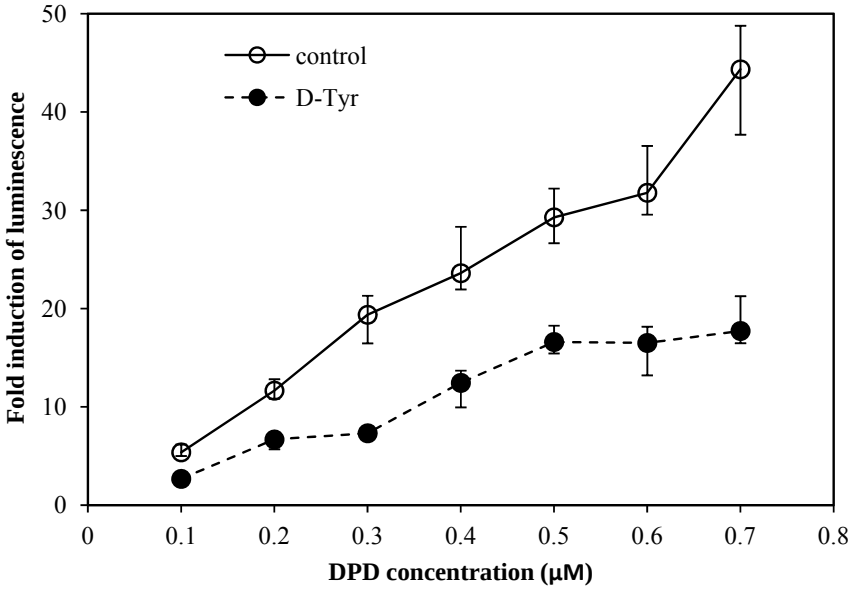

Figure 5.6 Response of AI-2 reporter strain in the cultures supplemented with (●) and without (○) D-tyrosine. Each point represents the mean of six measurements in parallel, and the error bar is absolute deviation from the mean.

5.3.3 EPS Production of Suspended Microorganisms

EPS are composed of a variety of organic substances, in which polysaccharides and proteins are two major components, and play an important role in microbial attachment onto a solid surface (Flemming and Wingender 2010). Figure 5.7 shows the respective contents of extracellular polysaccharide (PS) and protein (PN) in microorganisms with and without exposure to D-tyrosine. As compared to the control, a 31% reduction in PN and 17% decrease in PS were observed in microorganisms after one hour exposure to D-tyrosine, leading to a lowered PN/PS ratio. eDNA is a unique component of the organic substances in the matrix of suspended microorganisms. Figure 5.8 shows eDNA content of suspended microorganisms with and without exposure to D-tyrosine. After one hour exposure to D-tyrosine, eDNA was reduced to 0.006 mg g^{-1} biomass, i.e. a 68% reduction compared to the control. These results suggest that D-tyrosine would significantly hinder eDNA secretion.

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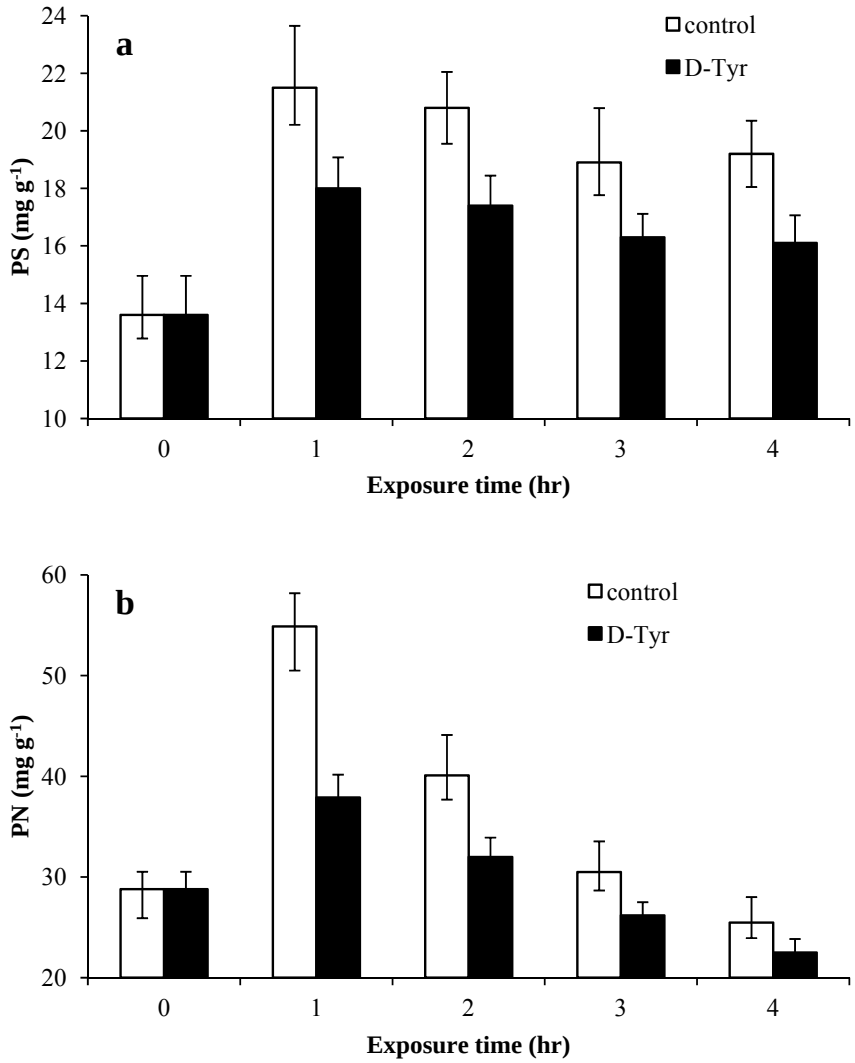

Figure 5.7 PS (a) and PN (b) contents of suspended microorganisms with (■) and without (□) exposure to D-tyrosine. Each point represents the mean of triplicate measurements, and the error bar is absolute deviation from the mean.

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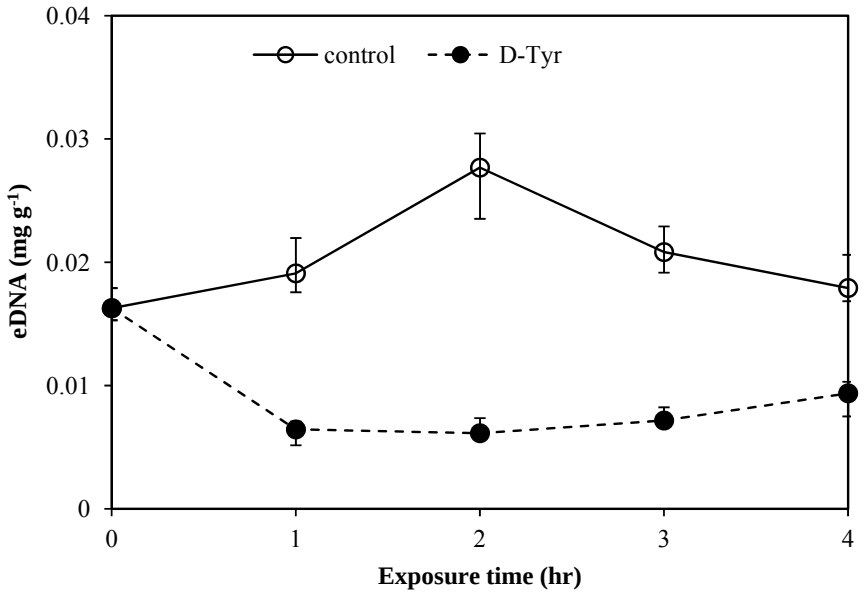

Figure 5.8 eDNA of suspended microorganisms with (●) and without (○) exposure to D-tyrosine. Each point represents the mean of triplicate measurements and error bar is absolute deviation from the mean.

Fig. 5.9 shows the HPSEC spectra of the EPS extracted from microorganisms with and without exposure to D-tyrosine. In general, retention time in HPSEC spectrum reflects molecular weight of a target chemical, i.e. the peak of a chemical with higher molecular weight appears quicker. For EPS extracted from microorganisms exposed to D-tyrosine, the peaks showed a significant decrease in area compared with those of the control, indicating a lowered EPS production that is consistent with the results in Figure 5.7. Two peaks were observed for the EPS extracted from microorganisms without exposure to D-tyrosine at the retention time of 10 to 13 min, while only one peak appeared for the EPS from microorganisms exposed to D-tyrosine. This implies that EPS with higher molecular weight was reduced due to exposure to D-tyrosine. These suggest that D-tyrosine would not only inhibit the EPS production, but also can alter the EPS composition.

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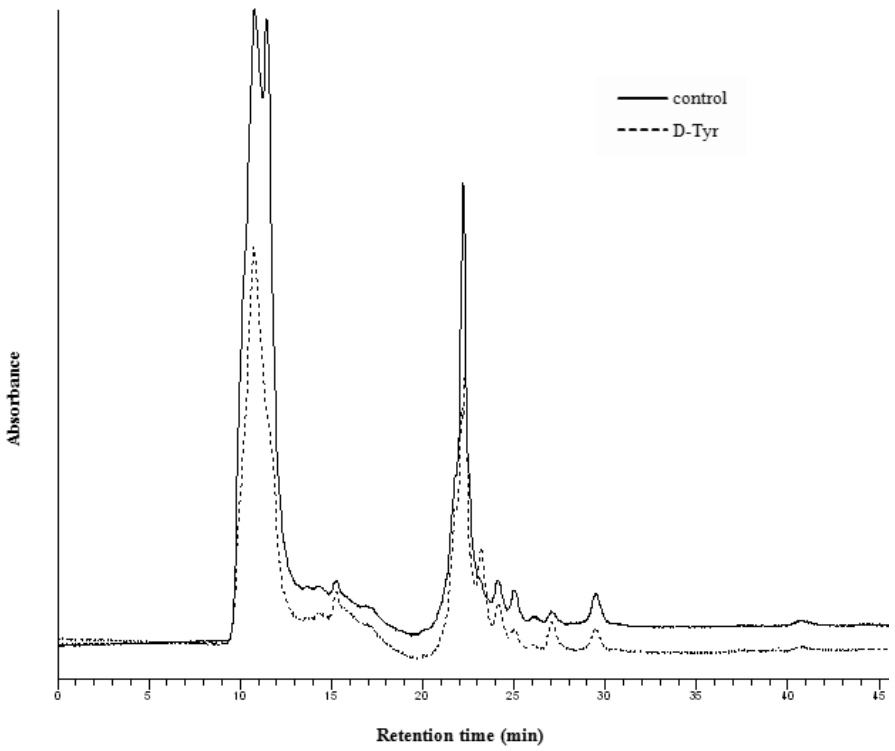

Figure 5.9 HPSEC chromatograms of EPS extracted from suspended microorganisms with (---) and without (—) exposure to D-tyrosine at the exposure time of 1 hr.

5.4 DISCUSSION

Figs. 5.1 and 5.2 show that microbial attachments onto hydrophobic PP and hydrophilic glass surfaces were inhibited by D-tyrosine. So far, little information is available for the role of D-tyrosine in control of mixed-culture biofilm development. Kolodkin-Gal et al. (2010) reported that D-tyrosine triggered release of amyloid fibers from cell surface would suppress development of a pure culture biofilm. It has been known that the long amyloid fiber facilitates the anchoring of cells to various surfaces, which is essential for microbial attachment and biofilm formation. In addition, the PP coupons used in this study have a contact angle of 99.3 ± 2.2°, while 16.9 ± 0.5^{o} for glass slides. As observed in Figures 5.1 and 5.2, more microorganisms attached to hydrophobic PP than to hydrophilic glass slide. In fact,

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it has been well documented that higher hydrophobicity of a solid surface would favor microbial attachment (Liu et al. 2004); on the contrary, coating surfaces with non-charged hydrophilic polymers resulted in reduced cell adsorption on a variety of surfaces (Park et al. 1998).

Figure 5.3 showed that D-tyrosine did not appear to affect the biomass growth and substrate removal efficiency. Such observation is consistent with the results obtained from pure culture experiments (Kolodkin-Gal et al. 2010). It had been reported that D-amino acids at a concentration higher than 20 mg L^{-1} would inhibit bacterial growth (Teeri and Josselyn 1953). Peptidoglycan is an important mesh-like polymer component of cell wall. In the peptidoglycan polymer, there are two unique amino acids at the terminal of a peptide side chain of peptidoglycan: D-alanine as opposed to its isomer L-alanine. D-tyrosine can replace D-alanine in the peptide side chain of cell wall (Lam et al. 2009), and further alter the cell wall-building protein so that the peptidoglycan production would be slowed down, i.e. D-amino acid negatively regulate the amount of peptidoglycan production. In the presence of D-methionine, peptidoglycan synthesis could be severely inhibited, whereas biomass continued to grow (Caparros et al. 1992). The amount of peptidoglycan per cell decreased significantly due to the increased biomass and decreased peptidoglycan synthesis. However, it had been reported that *E. coli* would be able to grow properly with 60% decrease of the normal peptidoglycan content (Prats and De Pedro 1989). The unchanged cellular ATP synthesis in microorganisms with and without exposure to D-tyrosine (Figure 5.5a) strongly supports this.

It appears from Figure 5.5b that AI-2 content of suspended microorganisms decreased due to D-tyrosine in the culture. To exclude other factors affecting AI-2 quorum sensing, the response of reporter strain *V. harveyi* BB170 to D-tyrosine showed that the inhibition of AI-2 regulated bioluminescence was only observed in presence of D-tyrosine (Figure 5.6), which further confirmed that AI-2 expression was suppressed due to the presence of D-tyrosine. In study of D-amino acids regulated cell wall remodeling, Lam et al. (2009) found that exogenous D-methionine produced by *Vibrio cholera* were incorporated into *E. coli* at the same

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position in the peptide even though *E. coli* bacterium did not produce or release D-amino acids. Thus, these rapid diffused small D-amino acids molecules could regulate cells releasing them and the neighbouring cells of different species. In study of biofilm inhibition by D-amino acids, Kolodkin-Gal et al. (2010) hypothesized that D-amino acid may play an important role of chemical signal, but opposite to quorum sensing signal molecule, to mediate interspecies communication for facilitating cell dispersion from biofilm. In addition, AI-2 is known to be a cellular communication signal molecule both for Gram-negative and Gram-positive bacteria and has a positive effect on biofilm formation (Federle and Bassler 2003). Due to the opposite effects of these two signal molecules on biofilm formation, D-amino acid may co-coordinate AI-2 regulated quorum sensing as shown in Figure 5.5. However, how these two signals may co-regulate each other needs further investigation. Furthermore, it is speculated that D-amino acids may play a coordinating role through regulating the gene expression to control biofilm community structure or induce biofilm dispersion.

Figure 5.7 shows that the PS and PN contents of suspended microorganisms tended to decrease with exposure to D-tyrosine. Tsuruoka et al. (1984) also observed that D-amino acid caused reduction of lipoprotein in study of D-amino acid incorporation into peptidoglycan. It has been reported that an incorporation of D-tyrosine into the cellular proteins of *B. subtilis* (Champney and Jensen 1970) and *E. coli* (Miyamoto et al. 2010). As the D-isomer has a similar shape and size to the L-isomer molecule, the D-analogue incorporated into proteins in the place of the natural amino acid would modify the structure of the proteins and the enzymic activity (Richmond 1962). These would eventually lead to the reduced production of PS and PN. As can be seen in Fig. 5.9, high molecular-weight EPS at the retention time of 10 to 13 min disappeared in microorganisms exposed to D-tyrosine. In fact, in study of the effect D-amino acid on structure and synthesis of peptidoglycan, Caparros et al. (1992) also found a direct inhibition of D-methionine on the production of high molecular-weight proteins. In addition, EPS have been believed to play an important role in microbial attachment. The reduced production of PS and PN would result in inhibited microbial attachment (Figure 5.1). Oliveira et al. (1994) reported that

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extracellular polysaccharides could promote a preconditioning of the surface, making attachment more favorable, whereas Flint et al. (1997) found that treatment the cells with trypsin or sodium dodecyl sulphate to remove cell surface proteins resulted in a 100-fold reduction in the attachment of *Thermophilic streptococci* onto stainless steel.

It had been shown that eDNA would play an important role in initial microbial adhesion to hydrophobic and hydrophilic surfaces (Das et al. 2010). Many studies have shown that eDNA is an important component of extracellular network that mediates cell-cell and cell-surface interactions (Bockelmann et al. 2006; Das et al. 2010). As can be seen in Figure 5.8, the presence of D-tyrosine in the culture media caused reduction of DNA in the extracellular network, as the result, less attachment was observed both on glass and PP surface (Figure 5.1). These suggest that eDNA may facilitate microbial attachment onto both hydrophilic glass and hydrophobic PP surfaces. Further study is needed to elucidate how D-amino acid would regulate eDNA production. In study of the role of eDNA in *Listeria monocytogenes* attachment, Harmsen et al. (2010) observed that peptidoglycan, specifically *N*-acetylglucosamine, together with eDNA could induce adhesion. Inhibited production of peptidoglycan and subsequently eDNA by D-tyrosine would also be responsible for reduced microbial attachment (Fig. 5.1). It appears from Figure 5.4 that microorganisms carried more negative surface charge in presence of D-tyrosine. Since EPS often have charged functional groups, the higher negative charge density would be associated with changes in the composition and quantity of EPS induced by D-tyrosine. According to DLVO theory, increased negative charge would lead to strong electrostatic repulsion between cell and approaching surface (Zita and Hermansson 1994). Hence, the extent of attachment would be less (Figure 5.1) as a result of increased surface charge or a greater level of electrostatic repulsion.

5.5 CONCLUSIONS

This study showed that D-tyrosine as a typical D-amino acid could decrease

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microbial attachment on both hydrophilic glass and hydrophobic PP surfaces, while no inhibitory effects on microbial growth, ATP synthesis and substrate utilization were observed at the concentration studied. In addition, suspended microorganisms were found to carry more negative charges after exposure to D-tyrosine, leading to strong electrostatic repulsion between cell and approaching surface. The synthesis of eDNA, EPS and AI-2 were all reduced in the presence of D-tyrosine in the culture media. These in turn imply that that D-amino acid may suppress microbial attachment through inhibiting eDNA, EPS and AI-2 production. Moreover, the response of AI-2 reporter strain to D-tyrosine also confirmed that AI-2 expression was suppressed by D-tyrosine. This study demonstrates the potential of D-amino acid as a non-toxic agent for control of microbial attachment.

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CHAPTER 6
D-AMINO ACID PROMOTED BIOFILM DETACHMENT FROM MEMBRANE SURFACES

6.1 INTRODUCTION

Biofilm detachment is the least investigated strategies for removing unwanted biofilm from membrane surfaces. Use of biocides and disinfectants may not be ideal due to reduced susceptibility of biofilm cells to cleaning reagents (Stewart and Costerton 2001). So far, some chemicals have been used to promote biofilm detachment, including enzymes, chelating agents etc, which can help to reduce biofilm cohesive strength through different mechanisms. As discussed in Chapter 5, D-amino acid can reduce microbial attachment onto hydrophobic PP coupon and hydrophilic glass surfaces, thus this chapter further looked into the effect of D-amino acid on dispersal of mixed-species biofilm from membrane surfaces.

6.2 MATERIALS AND METHODS

6.2.1 Adherence Assay

Activated sludge microorganisms taken from a local wastewater treatment plant on May 2010 were acclimated with a synthetic substrate for one month. The synthetic substrate has the same composites as illustrated in Chapter 3 Section 3.2.2. Two series of batch experiments were conducted: one served as control free of D-tyrosine, while the other was added with 6 mg L^{-1} of D-tyrosine (Sigma-Aldrich, St. Louis, MO, USA) according to minimum concentration of D-tyrosine for biofilm inhibition (Kolodkin-Gal et al. 2010). At different exposure times, suspended microorganisms were collected from two reactors for microbial attachment experiments as well as for determination of EPS, cellular ATP and AI-2 contents. In order to investigate the effect of D-tyrosine on biofouling inhibition onto nylon membrane surface, static

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adherence assay using microorganisms with and without exposure to D-tyrosine was conducted in Petri dishes mounted with hydrophilic flat sheet nylon membrane (Osmonics, Minnetonka, USA) with a pore size of 0.2 μm. The attachment experiment was conducted according to the procedure in Chapter 3 Section 3.2.2. Fixed biomass was quantified in terms of TOC by a TOC analyzer (ASI-V, TOC-Vcsh, Shimadzu, Japan). Each sample was analyzed in triplicate otherwise stated.

6.2.2 Biofilm Development on Membrane and Dispersion

To investigate the effect of D-tyrosine on biofilm dispersion on membrane surface, biofilm were precultured on membrane surfaces for 24 hours. The membranes fouled with biofilm were treated by different concentration of D-tyrosine. The retained biomass on membrane was collected to measure ATP, AI-2 and EPS respectively. The procedure of biofilm development on nylon membranes was according to Chapter 4, Section 4.2.1. For each tyrosine concentration, six membranes were harvested at the end of 24 hours and soaked in D-tyrosine (in PBS) solution. After two hours of treatment, the remaining biomass on the membrane was collected to determine biomass quantity (APHA 2005), ATP, AI-2, EPS and staining for visualization, respectively.

6.2.3 Determination of Cellular ATP and AI-2

The cellular ATP was extracted from freshly collected microorganism samples according to Chapter 3 Section 3.2.4. ATP analysis was performed using firefly luciferin-luciferase bioluminescence method with the FLAA Adenosine 5'-triphosphate (ATP) Bioluminescent Assay Kit (Sigma-Aldrich, St. Louis, MO, USA) as recommended by the manufacture.

The amount of autoinducer-2 (AI-2) of microorganisms was measured by *V. harveyi* BB170 (ATCC BAA 1117) bioluminescence reporter assay as illustrated in Chapter 3 Section 3.2.5.

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6.2.4 Determination of Extracellular Polysaccharides and Proteins

Extracellular polysaccharides (PS) and proteins (PN) were extracted according to the method in Chapter 4 Section 4.2.3. PS was determined by the phenol-sulphuric acid method (Dubois et al. 1956), whereas PN was analyzed by the modified Lowry method (Lowry et al. 1951). Glucose and bovine serum albumin bought from Sigma-Aldrich were used as the standards for PS and PN, respectively.

6.2.5 Staining and Microscopic Observation

Bacteria on membrane surfaces were stained with LIVE/DEAD BacLight^{TM} Bacterial Viability kits (Molecular Probes, Carlsbad, CA, USA) as described in Chapter 3 Section 3.2.6. The color assigned to the live and dead cells follows from the color at which the stained cells fluoresce under laser excitation: live cells were shown in green while dead cells were shown in red.

The EPS staining procedure was carried out as illustrated in Chapter 4 Section 4.2.4: Con A to bind α-mannopyranosyl and α-glucopyranosyl sugar residues; fluorescent brightener 28 to stain the β-linked polysaccharides; fluorescein isothiocyanate solution to bind with proteins, Nile red to stain lipids. The stained samples were mounted on glass slides to visualize with CLSM.

6.3 RESULTS

6.3.1 Reduced Attachment onto Membrane by D-tyrosine

Figure 6.1 shows attachment of microorganisms with and without exposure to 6 mg L^{-1} D-tyrosine. It can be seen from Figure 6.1 that attachment of microorganisms exposed to D-tyrosine was reduced significantly on nylon membrane compared to the control without the contact with D-tyrosine (Student's *t*-test, $P < 0.05$). For example, after 2-h culture, attachment of microorganisms without D-tyrosine was

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12.5 µg TOC cm^{-2} on the membrane surface, while attachment of microorganisms exposed to D-tyrosine was 8.8 µg TOC cm^{-2}, indicating about 30% reduction in microbial attachment caused by D-tyrosine. These results indeed are supported by microscopic observations (Figure 6.2). Thus, D-tyrosine can effectively inhibit microbial attachment on membrane surface.

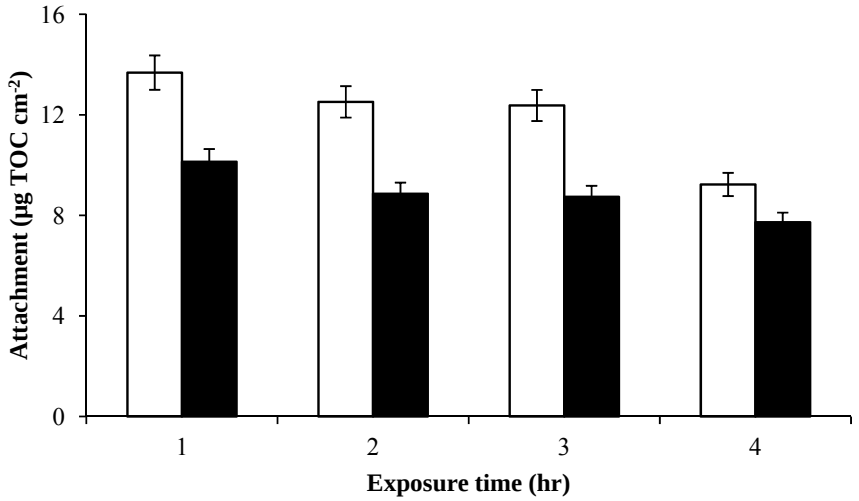

Figure 6.1 Attachment of microorganisms with (■) and without (□) exposure to D-tyrosine. Each point represents the mean of triplicate measurements and error bar is 1 SD from the mean.

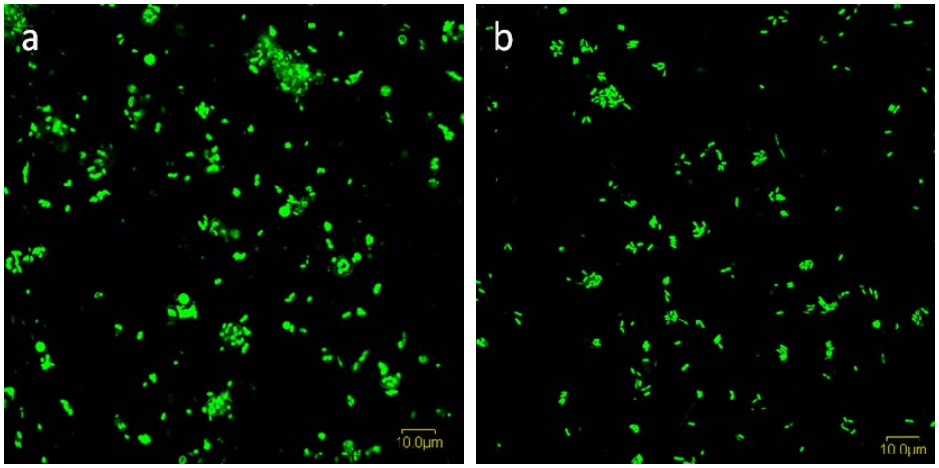

Figure 6.2 CLSM images of attachment of microorganisms without (a) and with (b) exposure to D-tyrosine.

AI-2 as an universal inter-species signaling molecule coordinates the formation of biofilm by various species (Surette et al. 1999). To look into the possible D-tyrosine-induced changes in cellular communication, AI-2 contents of suspended microorganisms with and without exposure to D-tyrosine were determined (Figure 5.5b). After 2-hour cultures, the AI-2 content of microorganisms was about 0.23 nmol mg^{-1} biomass in the control free of D-tyrosine, while it decreased to about 0.14 nmol mg^{-1} biomass in the culture with addition of D-tyrosine, indicating 40% reduction in AI-2. These suggest that D-tyrosine could suppress the synthesis or secretion of AI-2. Meanwhile, it was observed in Figure 5.5a that the cellular ATP content of microorganisms with and without exposure to D-tyrosine did not change significantly. Figures 5.5a clearly indicate that D-tyrosine in the concentration range studied has no inhibitory effect on energy metabolism.

EPS exist in a complex and heterogeneous polymeric matrix in which polysaccharides and proteins are two major components. EPS has been believed to play an important role in microbial attachment onto a solid surface (Flemming and Wingender 2010). Figure 5.7 in Chapter 5 shows respective contents of extracellular proteins (PN) and polysaccharides (PS) in suspended microorganisms with and without exposure to D-tyrosine. As compared to the control, the PS and PN contents were reduced significantly in microorganisms exposed to D-tyrosine, i.e. D-tyrosine would inhibit EPS production.

6.3.2 Cleaning of Biofouled Membrane by D-tyrosine

In order to study feasibility of using D-tyrosine to clean fouled membrane, 24-hr-old biofilms were precultured on membrane surfaces. Figure 6.3 shows the biomass reduction before and after D-tyrosine treatment. For example, the amount of fixed biomass on nylon membrane was reduced by 25% after treatment by 10 mg L^{-1} D-tyrosine, while further decreased to 60% at 500 mg L^{-1} D-tyrosine. These imply that fixed biomass on nylon membrane was significantly removed after treatment with D-tyrosine (Student's *t*-test, $P < 0.05$). In addition, reduced fixed biomass after D-

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tyrosine treatment is consistent with CLSM observation (Figure 6.4). These indicate that D-tyrosine could induce biofilm dispersion from membrane surface.

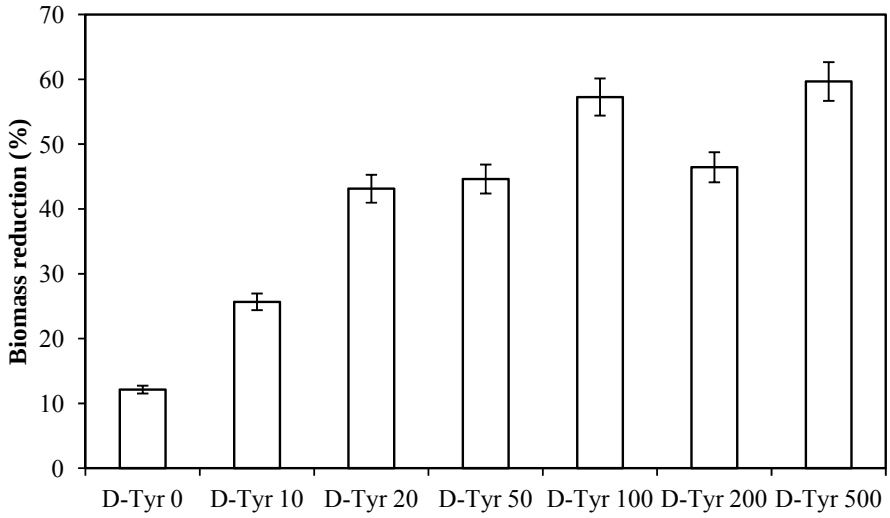

Figure 6.3 Biomass reduction on membrane preculturing with biofilm before and after treatment by D-tyrosine. Each point represents the mean of triplicate experiments and error bar is 1 SD from the mean.

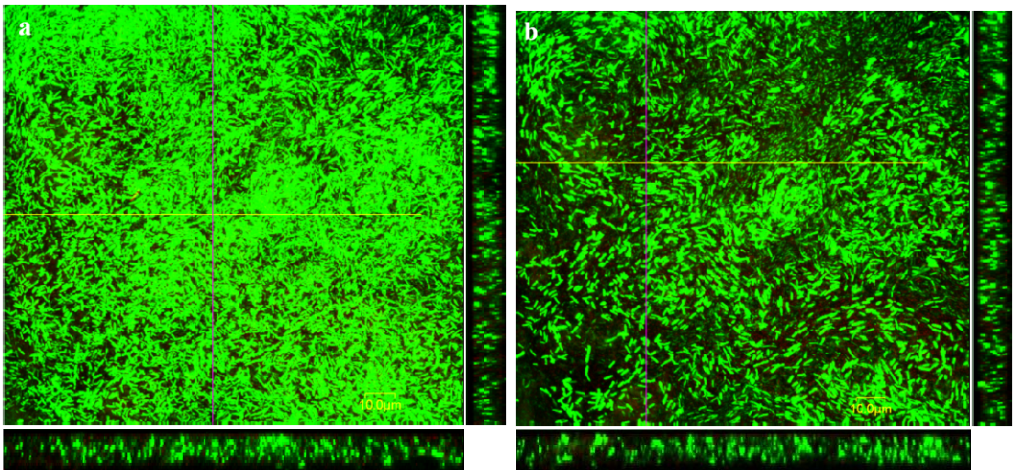

Figure 6.4 CLSM images of retained biofilm precultured on membrane before (a) treatment; after (b) treatment by 100 mg L^{-1} D-tyrosine solution.

Figure 6.5a shows AI-2 content of biofilms on membrane before and after D-tyrosine treatment at different concentrations. The AI-2 content of biofilms before

treatment by D-tyrosine was 0.77 nmol mg^{-1} biomass, but decreased to 0.34 nmol mg^{-1} biomass after two hour treatment with D-tyrosine at the concentration of 10 mg L^{-1}. Moreover, the AI-2 content of biofilms was further reduced to 0.21 nmol mg^{-1} biomass after treatment at 200 mg L^{-1} D-tyrosine. Such observation is consistent with Figure 5.5b. However, it was found in Figure 6.5b that D-tyrosine would not inhibit ATP synthesis, revealed by the fact that there was not significant differences in ATP contents after treatment by PBS solution (D-Tyr 0) and D-tyrosine solutions. This observation indeed is in good agreement with the results obtained in microbial attachment experiments as shown in Figure 5.5a.

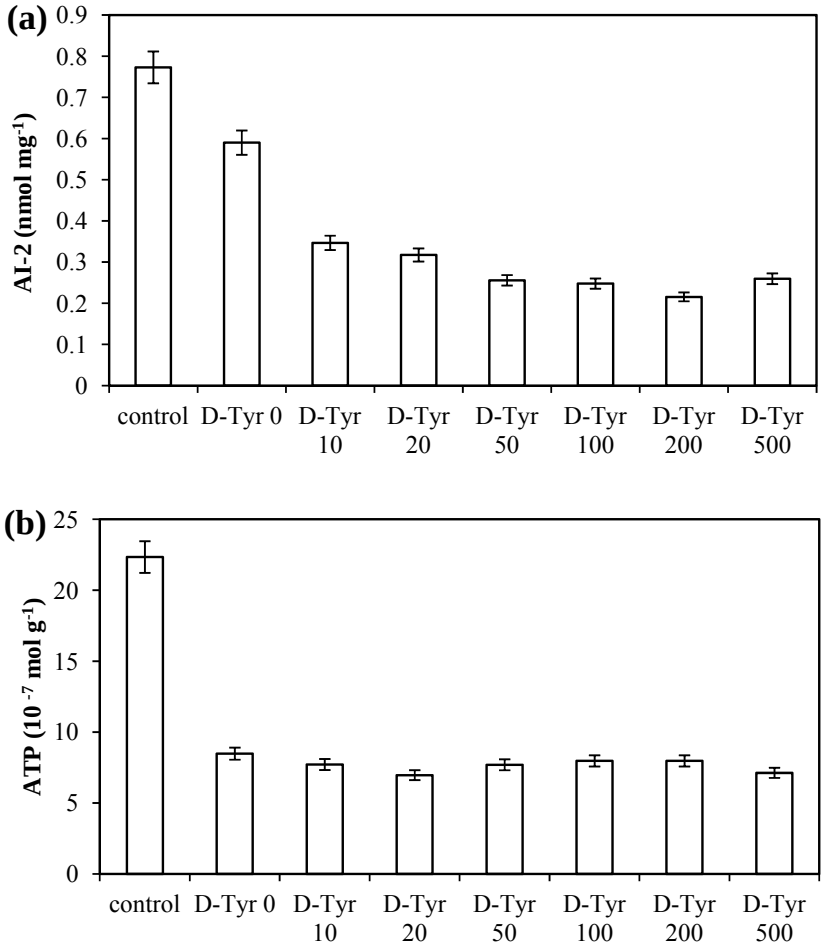

Figure 6.5 AI-2 concentration (a) and cellular ATP content (b) of retained biomass precultured on membrane before and after treatment by D-tyrosine. Each point represents the mean of triplicate measurements and error bar is 1 SD from the mean.

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Fig. 6.6 shows the amount of EPS extracted from biofilms precultured on membrane surfaces after D-tyrosine treatment at different concentrations. It seems that higher D-tyrosine concentrations would result in less PN and PS in biofilms after treatment. This is supported by CLSM images (Figure 6.7). Figures 6.7a, c show spatial distribution of proteins, lipids, β-polysaccharides and α-polysaccharides in biofilm before treatment by D-tyrosine, which form a highly complex, inter-connected EPS matrix structure. The maximal EPS density was observed near the bottom of the EPS layer. It was found that the EPS matrix collapsed after treatment by D-tyrosine. For example, the fluorescence of proteins in biofilm after treatment (Figure 6.7b) was much weaker than that in the control counterpart (Figure 6.7a). For polysaccharides, a much thinner and looser layer of β-polysaccharides was observed (Figure 6.7d) compared with the control (Figure 6.7c). These suggest that β-polysaccharides would be an important component for biofilm structure and stability. Such observation is in good agreement with that found in aerobic granular biofilm (Wang et al. 2005b). Consequently, D-tyrosine would not only decrease the EPS production, but also can alter the EPS matrix structure in biofilms.

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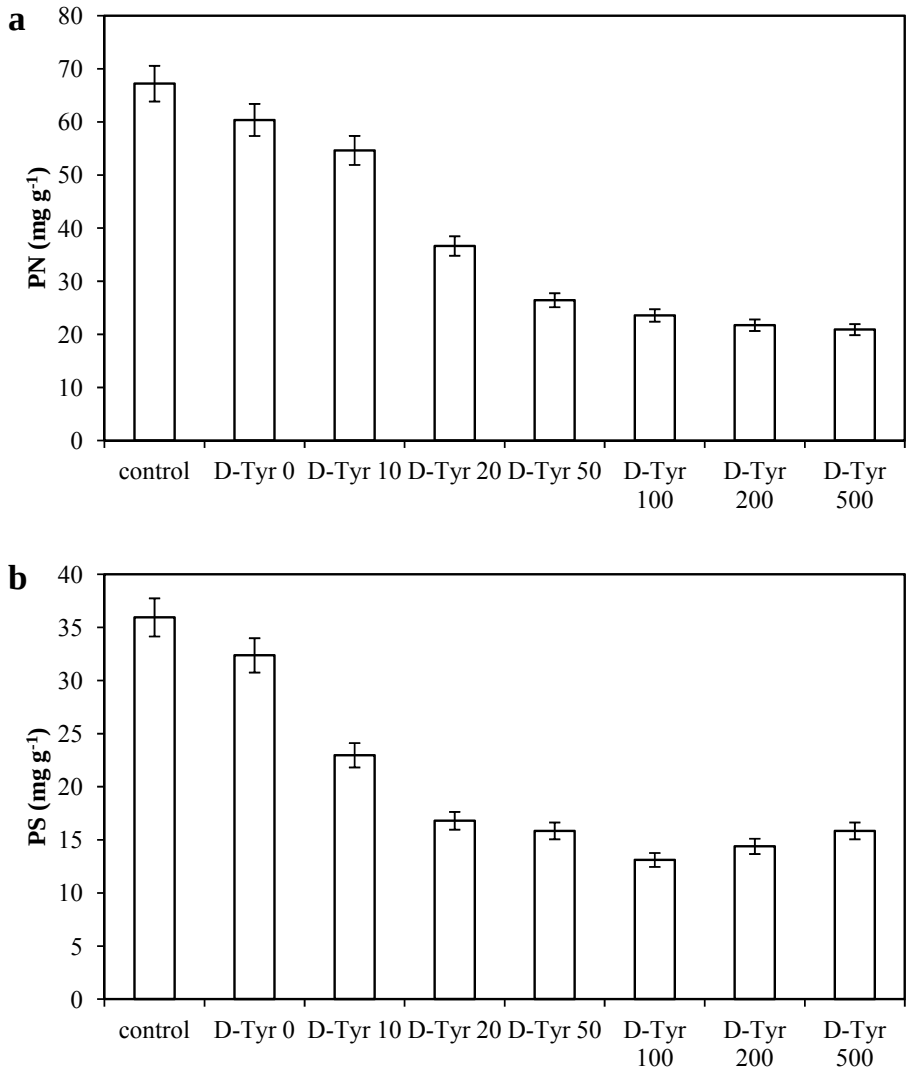

Figure 6.6 PN (a) and PS (b) contents of retained biomass precultured on membrane before and after treatment by D-tyrosine. Each point represents the mean of triplicate measurements and error bar is 1 SD from the mean.

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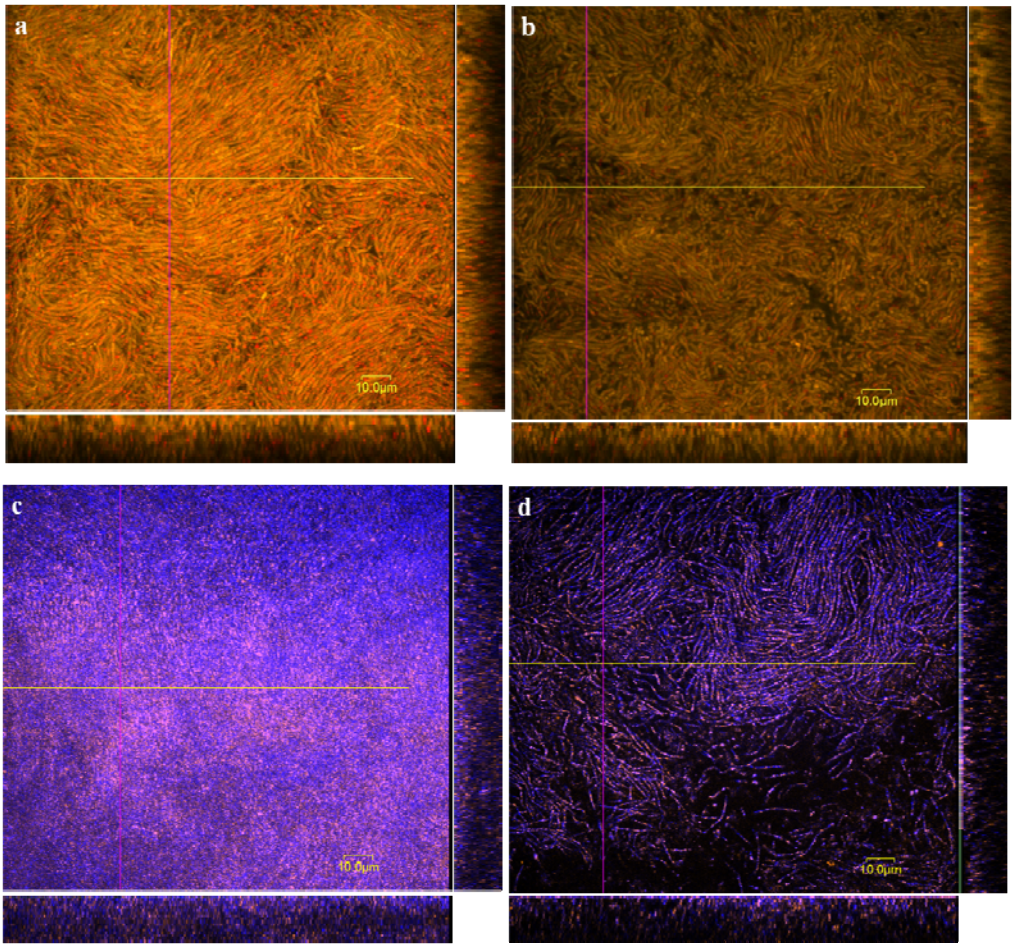

Figure 6.7 CLSM images of PN in retained biomass precultured on membrane before (a) and after (b) treatment by 100 mg L^{-1} D-tyrosine (protein, yellow; lipid, red); CLSM images of PS in retained biomass precultured on membrane before (c) and after (d) treatment by 100 mg L^{-1} D-tyrosine (β-polysaccharide, blue; α-polysaccharide, orange).

6.4 DISCUSSION

The results show that D-tyrosine can not only help prevent microbial attachment on membrane surfaces, but also enhance biofilm detachment from membrane surface. As observed in Figs. 6.1 and 6.2, microbial attachment or membrane biofouling onto nylon membrane surfaces was inhibited by D-tyrosine significantly. Bacteria

are protected by a cell wall made of a strong, elastic, mesh-like layer of polymers, called peptidoglycan outside the plasma membrane of bacteria. Peptidoglycan determines a bacterium shape, structural strength and counteracts the osmotic pressure (Voet et al. 1998). In the peptidoglycan polymer, the terminal two amino acids of a peptide side chain of peptidoglycan are unique amino acids: D-alanine as opposed to its isomer L-alanine (Voet et al. 1998). D-tyrosine can replace D-alanine in the peptide side chain of cell wall (Lark et al. 1963; Lam et al. 2009), and further alter the cell wall-building protein so that the peptidoglycan production would be slowed down, i.e. D-amino acids can block cell wall synthesis through changing peptidoglycan composition and structure (Caparros et al. 1992). Moreover, it has been reported that the incorporation of D-amino acids into the cell wall trigger bacterial amyloid fibers to disassemble, which bridge cells together in the biofilms (Kolodkin-Gal et al. 2010). As the result, less microbial attachment can be expected. In addition, inhibited synthesis of peptidoglycan in bacteria may result in a weak cell wall and lysis of bacterium due to osmotic pressure (Waks and Tomasz 1978), leading to less microbial attachment. Compared to the attachment on glass slide (Chapter 5), it was found that more microorganisms attached to nylon membrane due to higher degree of hydrophobicity and roughness of nylon membrane. After exposure to D-amino acid, the reduced attachment on nylon membrane and glass slide seems to have little difference. This in turn suggests that the effect of D-amino acid on microbial attachment was more profound than the physical properties of biocarriers. Such information is insightful for better understanding the observed mitigation of D-tyrosine to membrane biofouling onto membrane surfaces.

It appears from Figures 5.7 and 6.6 that the PS and PN contents tended to decrease in the culture supplemented with D-tyrosine. In study of D-amino acid incorporation into peptidoglycan, Tsuruoka et al. (1984) also observed that D-amino acid caused reduction of lipoprotein. As the D-isomer has a similar shape and size to the L-isomer molecule, the D-analogue incorporated into proteins in the place of the natural amino acid would modify the structure of the proteins and the enzymatic activity (Richmond 1962). These would eventually lead to the reduced production of PS and PN. In fact, in study of the effect D-amino acid on structure and synthesis

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of peptidoglycan, Caparros et al. (1992) also found a direct inhibition of D-methionine on the production of proteins. More recently, Kolodkin-Gal et al. (2010) observed that the specific extracellular TasA protein disappeared from cell surface remarkably due to the presence of D-tyrosine in the culture media. The long-fiber TasA protein would help the anchoring of the cell to various surfaces, which is essential for microbial attachment and biofilm development. These may partially explain the reduced microbial attachment onto membrane surfaces (Figure 6.1) and biofilm dispersion from membrane surfaces (Figure 6.3). EPS may play a role of bioglue in microbial attachment on a solid surface. The reduced production of PS and PN would result in inhibited microbial attachment (Figure 6.1). It had been reported that the deposition of bacteria on surfaces can be enhanced by the presence of a larger amount of EPS (Long et al. 2009). In addition, EPS provide architectural structure and mechanical stability to maintain biofilm integrity and stability and enzymatic disruption of the extracellular polymeric substance matrix could cause collapse of biofilm structure, leading to detachment (Xavier et al. 2005). Figure 6.7 clearly shows that reduced EPS content would result in collapse of the EPS matrix structure and subsequent biofilm dispersion.

Cells can use different signaling molecules to build and break biofilm (Karatan and Watnick 2009). Biofilm dispersal is a dynamic process involving multiple determinants and regulatory processes. It has been reported that the D-amino acids were secreted by various bacteria into the environment just before biofilm disassembly and both released D-amino acids and externally added D-amino acids were effective against biofilm of *P. aeruginosa* and *Staphylococcus aureus*, suggesting D-amino acids may be a common signal for biofilm dispersion (Kolodkin-Gal et al. 2010). As small molecules like D-amino acids could rapidly diffuse in aqueous environment, bacterial may act a quick and synchronized response to D-amino acids, such as transition from biofilm to planktonic lifestyle, similar to quorum sensing molecules regulation. Furthermore, released D-amino acids signaling could act on not only cells that released D-amino acids, but also neighboring cells, which may be of different species (Cava et al. 2010). This may explain D-tyrosine induced mixed biofilm detachment from nylon membrane

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surface shown in Figure 6.3. As a chemical signal, D-tyrosine at studied concentration seems to negatively affect AI-2 synthesis or secretion (Figure 5.5b and 6.5a). As D-tyrosine could co-regulate biofilm dispersal, while AI-2 coordinates biofilm formation, the relationship between quorum sensing molecule AI-2 and D-amino acid needs further study, for example how distinct signals in mixed biofilm would mediate different processes and coregulate with each other.

Figure 6.8 shows a positive correlation between biomass on membrane surface and corresponding AI-2 content observed in the attachment and detachment experiments. These strongly support the view of that AI-2 can mediate both microbial attachment onto and detachment from membrane surfaces. AI-2 is well known as an inter-species cellular communication molecule (Federle and Bassler 2003), and has been shown to control biofilm formation in *E. coli* through quorum sensing regulated motility (Barrios et al. 2006). As AI-2 co-ordinates cell-cell adhesion and biofilm formation (Buck et al. 2009), reduced AI-2 content (Figure 6.5a) in turn may trigger cell dispersion, and further promote biofilm detachment from membrane surface (Figure 6.3).

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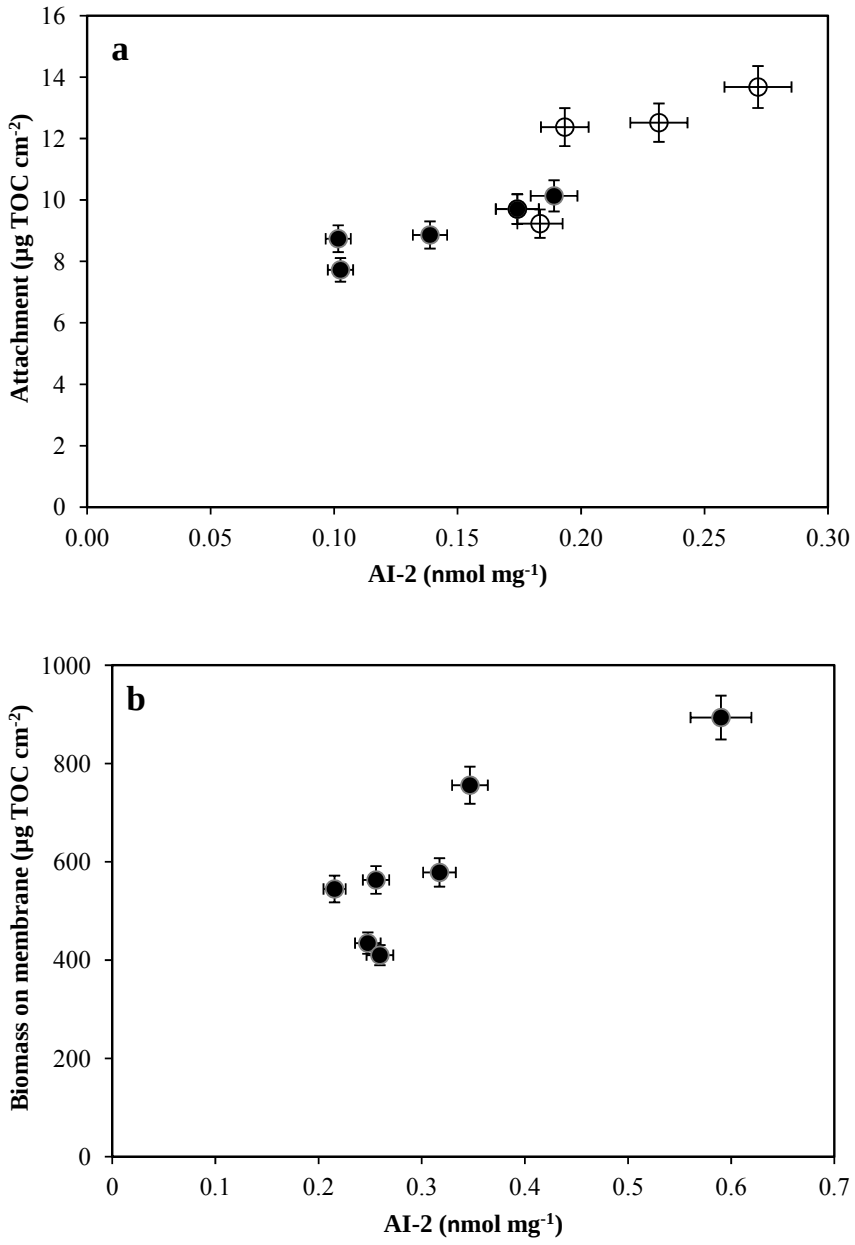

Figure 6.8 Effect of AI-2 content in suspended microorganisms on attachment (a) of microorganisms with (●) and without (○) exposure to D-tyrosine; effect of AI-2 content on retained biomass precultured onto membrane after treatment (b) by D-tyrosine (●). Each value represents the mean of triplicate experiments and error bars are 1 SD from the mean.

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6.5 CONCLUSIONS

D-tyrosine as a typical D-amino acid significantly promoted mixed-species biofilm detachment from membrane surfaces. This study and Chapter 5 found that D-amino acid at the studied concentration had no inhibitory effect on ATP synthesis, while extracellular polymeric substances and AI-2 tended to decrease after exposure to D-amino acid. More importantly, the positive correlation between fixed biomass on membrane surface and corresponding AI-2 content observed in both attachment and detachment experiments strongly support the idea of that AI-2 can mediate both microbial attachment onto and detachment from membrane surfaces. These provide a reasonable explanation for the D-tyrosine-triggered reduction in microbial attachment (Chapter 5) and promotion in biofilm detachment (this chapter). Consequently, previous Chapter 5 and this chapter together demonstrates the potential of D-amino acids as an effective agent for control of microbial attachment on solid surface as well as cleaning of biologically fouled membranes.

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CHAPTER 7
CONCLUSIONS AND RECOMMENDATIONS

7.1 CONCLUSIONS

This study investigated different biological strategies for control of microbial attachment and membrane biofouling, and the following conclusions can be drawn.

ATP dissipation by metabolic uncoupler can inhibit microbial attachment and promote microbial detachment. ATP appeared to be involved in microbial attachment as needed to overcome the energy barriers between microorganisms and solid surfaces, meanwhile it was also required to maintain the structure integrity and stability of biofilms. In addition, inhibited ATP synthesis by metabolic uncoupler subsequently decreased secretion of AI-2, an interspecies cellular communication signalling molecule. The correlation between AI-2 and extent of fixed biomass indicated that ATP-dependent AI-2 was responsible for the collective interactions of microorganisms attached to and detached from solid surfaces. This study clearly illustrates the feasibility of biological control of microbial attachment, membrane biofouling and cleaning of biologically fouled membranes through ATP inhibition.

D-amino acid effectively reduced microbial attachment onto various solid surfaces, as well as promoted detachment of mixed-species biofilm from membrane surfaces. It was found that D-amino acid at the studied concentration had no negative impact on ATP synthesis. In contrast, reduced extracellular polymeric substances (EPS) were observed after exposure to D-amino acid in both microbial attachment and biofilm detachment. It provided evidence of that EPS could facilitate adhesion in initial microbial attachment, and maintain biofilm architectural structure and stability. Furthermore, it was shown that the synthesis or secretion of AI-2 was inhibited after exposure to D-amino acid; implying D-amino acid had a negative effect on AI-2 synthesis. The positive correlation between fixed biomass on membrane surfaces and corresponding AI-2 content observed in both attachment

and detachment experiments strongly supported that D-amino acid-suppressed AI-2 production could mediate both microbial attachment onto and biofilm detachment from surface. Consequently, this study demonstrates the potential of D-amino acids as an effective agent for biological control of microbial attachment on different solid surface as well as enhancement of biofilm detachment from membrane surfaces.

Biological methods targeting inhibition of ATP synthesis and D-amino acid would have great potential in controlling microbial attachment and promoting microbial detachment. These in turn can be applied in mitigating membrane biofouling as well as cleaning biologically fouled membrane respectively. This novel insight targeting ATP dissipation and D-amino acid may widen the vision of biological control strategies. In addition, this study may provide clues and stimulate development for better control of membrane biofouling in industrial application.

7.2 RECOMMENDATIONS

Future work should be needed in the following aspects:

(i) Microbial community composition and dynamics would be analyzed at the different phases of membrane biofouling for identifying the key players responsible for membrane biofouling. For this, the advanced molecular biology techniques, such as DGGE and 16S rRNA gene sequencing, would be employed.

(ii) In this study, AI-2 was chosen as inter-species signal molecule in mixed-species cellular communication. However, there are different types of signal molecules for bacteria, such as acyl-homoserine lactones (AHLs) for Gram-negative bacteria. Thus, it is necessary to investigate the effect of energy inhibition and D-amino acids on production of AHLs and their roles in initial biofouling development and biofilm dispersion.

(iii) The potential of D-amino acids for control biofilm formation was clearly demonstrated in this study. The future study will investigate what kind of EPS will preferably be inhibited in the presence of D-amino acids and how this may affect biofilm formation on membrane surface. In addition, the combined methods for control of membrane biofouling will need to be further investigated and may be optimized for more effectively preventing and cleaning membrane biofouling.

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