공학박사 학위논문

Enhancement of Anti-Fouling Properties of Quorum Quenching Bacteria Entrapping Media for Effective Biofouling Control in Membrane Bioreactors

분리막 생물반응기에서 생물막오염 저감 성능의 향상을 위한

정족수감지 억제 미생물 고정 담체의 제조

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이 상 현

Abstract

Enhancement of Anti-Fouling Properties of Quorum Quenching Bacteria Entrapping Media for Effective Biofouling Control in Membrane Bioreactors

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Quorum quenching (QQ) bacteria entrapping media have been reported as a new method for biofouling control in MBR because, unlike conventional post-biofilm control methods, bacterial QQ can inhibit biofilm formation through its combined effects of physical scouring of the membrane and biological inhibition of quorum sensing (QS). Most of subsequent studies focused on improving the efficiency of QQ media through changing material or method of application. However, they did not account how QS signal molecules and QQ media interact with each other which could lead to a further enhancement in biofouling control efficacy of QQ media. Furthermore, enhancement in physical and biological properties of QQ

media is necessary for making QQ technology more economical and for bringing it closer to practical applications.

In this study, the route by which QS signal molecules come into contact with QQ bacteria entrapped in a hydrogel medium was analyzed. On the basis of this result, a more efficient QQ media design was proposed. Furthermore, enforcement of physical strength as well as extension of lifetime of a QQ-HC was sought by adding a dehydration procedure following the ionic cross-linking of polymeric hydrogel.

Firstly, the interaction between QS signal molecules and QQ media was analyzed by visualizing QS signal molecules in a spherical shaped hydrogel QQ medium (QQ-bead) using a special reporter strain (*Escherichia coli* JB525). On the basis of this result, QQ hollow cylinder (QQ-HC) was developed. QQ activity and physical washing effect of (QQ-HCs) was shown to be higher than those of QQ-beads, by 13% and 140% respectively, due to an increased surface area and more efficient use of media material. The operation of lab-scale MBRs with QQ-HC and QQ-bead confirmed that biofouling was mitigated to a greater degree in the MBR with QQ-HC than in the MBR with QQ-bead.

Secondly, physical strength and lifetime of a QQ-HC was enhanced by adding a dehydration procedure following the cross-linking of polymeric hydrogel by Ca^{2+}, boric acid, and SO_4^{2-}. From the gel fraction determination and SEM analysis, it is speculated that the increase in physical strength (approximately by 100%) was due to the increased degree of physical cross-linking after dehydration procedure. In addition, the lost QQ activity after the dehydration step could be easily restored through re-activation with either a commercial culture medium or real wastewater.

As a result, longer lifetime of QQ-HCs was confirmed which led to an improved biofouling mitigation performance of QQ-HCs in an MBR. Furthermore, QQ-HCs stored under dehydrated condition showed higher QQ activity when the storage time lasted more than 90 days due to better-maintained cell viability. Moreover, the reduced weight of dehydrated media is expected to make handling and transportation of QQ media highly convenient and economical in practice.

Keywords

Membrane bioreactor (MBR), Biofouling control, Quorum sensing, Quorum quenching, Media, cross-linking

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Chapter I

Introduction

I.1. Backgrounds

Membrane bioreactors (MBRs) have been widely accepted as an alternative to the conventional activated sludge (CAS) process due to its compactness and high effluent quality. Since its birth, it's been in practice for over two decades and have evolved into state-of-the-art technology in wastewater treatment and reuse. The global market for MBR reached $838.2 million in 2011 and is projected to rise to $3.44 billion by 2018. These figures translate into a compound annual growth rate (CAGR) of 22% during the period considered above (Hai, 2013). Despite the technical advantages and optimistic market projection, MBR faces a critical issue, the membrane biofouling. Because the process combines CAS and membrane filtration, clogging of membrane by microorganisms is inevitable. The loss of permeability of a membrane leads to frequent chemical washing, which shortens membrane lifetime. In order to alleviate the problem of membrane biofouling, much effort has been made in diverse perspectives including membrane surface modification (Rana and Matsuura, 2010), physical strategies (Rosenberger et al., 2011, Zsirai et al., 2012), and adding specific media (Lee et al., 2006, Yang et al., 2013). However, despite the tremendous efforts to reduce membrane biofouling, the underlying problem still remains because they were limited to physical and chemical strategies, which focus largely on removing already accumulated biomass on membranes. Hence, in order to more effectively reduce biofouling, it was necessary to inhibit the biofilm formation which required understanding the mechanism of biofilm formation.

The formation of microbial biofilm is reported to be caused by the cell to cell communication known as quorum sensing (QS) which mediates other group

behaviors such as expression of virulence, antibiotics resistance, etc. (Greenberg, 2003). With this knoweldge, microbiologists have quenched the quorum sensing (i.e., quorum quenching, QQ) and demonstrated successful inhibition of biofilm formation in a number of bacterial species (Dong et al., 2001).

Motivated by the discovery of the involvement of QS in the bacterial biofilm development, Yeon and his co-workers (Yeon et al., 2009a) found that QS mediated by a signal molecule, *N*-acyl homoserine lactones (AHLs), also plays a key role in biofilm formation on the membrane surface in MBR. The authors proved that biofouling can be mitigated by decomposing signal molecules by QQ enzymes. This opened a new paradigm in biofouling control in MBRs because, unlike conventional post-biofilm control methods, QQ could inhibit the biofilm formation at its developmental stage. In a later study, Oh et al. (2012) isolated a bacterium, *Rhodococcus* sp. BH4, which produces enzymes that decompose QS signal molecules, from a real MBR plant. They encapsulated *Rhodococcus* sp. BH4 inside the lumen of a microporous hollow fiber membrane to protect the bacteria from attack by other microorganisms co-habiting in the mixed liquor while allowing nutrients to pass through. As a result, this "microbial vessel" allowed more sustainable application of QQ than its predecessor (QQ enzyme) in an MBR. Others have extended the concept of the QQ microbial vessel to the ceramic microbial vessel (Cheong et al., 2014) and the rotating microbial carrier frame (Kose-Mutlu et al., 2016) to improve viability of entrapped QQ bacteria and mass transfer.

Recently, Kim et al. (2013b) entrapped QQ bacteria in a porous and spherical hydrogel beads (QQ-beads) to induce smoother mass transfer across the beads. The QQ-beads mitigated biofouling not only through QQ but also physical cleaning of

membrane surface by frequent contacts between the beads and the membrane surface under the fluidized environment in MBR (Kim et al., 2015, Khan et al., 2016).

Although above series of studies have improved performance of QQ media by enhancing cell viability and mass transfer or imbuing the media with physical washing effects, they have not studied how QS signal molecules and QQ media interact with each other which could lead to a further enhancement of biofouling control efficacy of QQ media. Moreover, recent study by Lee et al. (2016c) confirmed biofouling control capability of QQ media in a pilot-scale MBR fed with real wastewater. Although the study has a merit in that it demonstrated a potential of QQ technology to be applied in real MBR plants, in order to bring current QQ technology closer to a practice, however, it would be required not only to enhance physical strength and entrapped cell viability of QQ media, but also to improve methods of storage and transport of QQ media.

I.2. Objectives

The objective of this study was to develop a more efficient QQ medium that has enhanced quorum quenching (QQ) activity and physical washing effect which would allow more effective mitigation of biofouling in MBR for wastewater treatment. Furthermore, enhancement in physicochemical properties and lifespan as well as transportation and storage of developed QQ medium was sought. Therefore, the main objective of this study was to improve physical and biological properties of a QQ medium. The specific objectives of this study are as follows:

(1) Analysis of Interaction between QS Signal Molecule and a Polymeric Hydrogel QQ Medium and Development of a Hollow Cylindrical QQ Medium

The route by which QS signal molecules come into contact with QQ bacteria entrapped in a hydrogel medium was analyzed by visualizing QS signal molecules in a spherical hydrogel QQ medium (QQ-bead) using a special reporter strain (*Escherichia coli* JB525). On the basis of this result, a more efficient QQ medium, a QQ hollow cylinder, was developed. QQ activity and physical washing effect of quorum quenching hollow cylinders (QQ-HCs) was compared to the previously reported QQ-beads in batch experiments. Lastly, the biofouling mitigation performances of QQ-HC versus QQ-bead was tested in lab-scale MBRs.

(2) Enhancement of Physicochemical Properties and Lifespan of Bacterial Quorum Quenching Media through Combination of Ionic Cross-linking and

Dehydration

Enforcement of physical strength as well as extension of lifetime of a QQ-HC was sought by adding a dehydration procedure following the cross-linking of polymeric hydrogel by inorganic compounds like $Ca^{2+}$and boric acid. Physical strength, QQ activity, and shelf life of a dehydrated QQ-HC were analyzed in batch experiments. In addition, enhancement in lifetime of QQ-HC due to addition of dehydration step was confirmed in lab-scale MBR. Lastly, practical implications of our findings on transportation, storage, and application of QQ media were discussed.

Chapter II

Literature Review

II.1. Membrane Bioreactor (MBR)

II.1.1. Definition

The term “membrane bioreactor” (MBR) describes all water and water treatment processes which integrates a membrane with a biological process (Judd and Judd, 2006). Typically, available commercial MBR processes today are said to combine conventional activated sludge (CAS) process and membrane filtration. Figure II- 1 shows schematics of CAS and MBR processes. In CAS process, large particles in the influent are first separated though screening and subsequent primary settling and residual dissolved organic substances, expressed as biochemical oxygen demand (BOD or COD), are removed through aerobic microbial respiration followed by second clarification. On the other hand, MBR employs almost the same primary treatment of influent wastewater as CAS process but effluent from biological treatment is directly filtered with micro- or ultrafiltration. In this configuration, the membrane excludes solid and large dissolved substances produced in biological treatment of water and wastewater to provide clarified and disinfected product water. In other words, while the CAS process incorporates a secondary clarifier to separate solid (sludge) from liquid, an MBR uses a membrane for this function. Figure II- 1b illustrates a membrane filtration unit placed in the exterior of the aeration tank while Figure II- 1c depicts a MBR system with membrane submerged in the bioreactor. Although the treatment performances of external and submerged MBRs are similar, the capital and operating costs for submerged MBRs are less costly compared to those for external MBRs due to additional equipment and spaces. Thus, more than 99% of MBRs around the globe rely on submerged membranes based on the treated

water volume, and this trend is expected to continue in the future (Yoon, 2015). Table II-1 lists details on the differences between side stream and immersed MBR systems.

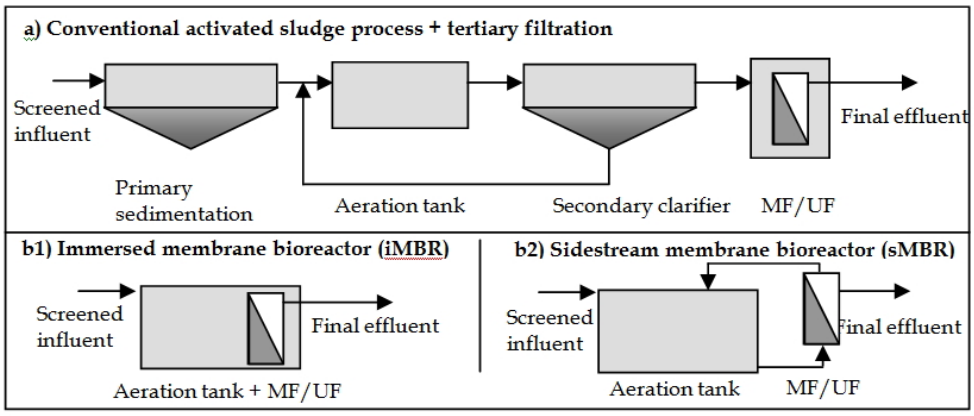


Figure II- 1. Conventional activated sludge process (a) and MBR in both configurations: immersed (b1) and sidestream (b2) (Sebastián Delgado, 2001).

Table II-1. Key facets of the MBR configurations. (Judd, 2004)

Sidestream	Submerged
Longest history (since early 1970s).	Most recent development (since 1990).
Membrane placed external to bioreactor.	Membrane placed in bioreactor.
Pumped system with permeation rate determined by transmembrane pressure and crossflow.	Permeate removed under hydrostatic head, with or without permeate suction, at rate partly determined by aeration
Higher flux and hydraulic resistance; lower aeration and membrane area requirement.	Lower flux and hydraulic resistance; greater aeration and membrane area requirement.
Stabilized flux with periodic chemical cleaning.	Stabilized flux with periodic chemical cleaning for flat plate membrane configuration; short backwash cycle with periodic chemical cleaning for hollow fiber configuration.
Greater overall energy demand; greater process (hydrodynamic) control.	Lower overall energy demand; reduced process (hydrodynamic) control.

II.1.2. Advantages and Disadvantages of MBR

The replacement of secondary clarifier with membrane in MBR offers a number of advantages over CAS process as follows (Judd, 2015):

[1] Uncoupling HRT and SRT

A major advantage of the membrane bioreactor process is that the activated sludge is completely retained by the membrane and thus they remain in the bioreactor. This means the solids retention time (SRT) in the bioreactor can be completely controlled separately from the hydraulic retention time (HRT). In the CAS process, a certain period is required for the flocculant solids ('flocs') that make up the biomass to grow in size to the point at which they can be settled down in the secondary clarifier. Hence, in the CAS, the HRT and SRT are coupled and thereby, larger tank size (or space) is required.

[2] High quality effluent

Membranes typically have small pore size (<0.5 μm) which yields an effluent of very high clarity and significantly reduced pathogen concentration. This allows MBR processes to produce a substantially clarified and disinfected effluent of high enough quality to be discharged. It is especially beneficial in cases where receiving water reservoir is sensitive or end user requires high quality water such as urban irrigation, utilities or toilet flushing.

[3] Small footprint

The uncoupling of SRT and HRT allows long SRT which generates higher biomass solids concentrations. The increased sludge concentrations mean that the same total mass of solids is contained in a smaller volume, such that the footprint is smaller.

[4] Better biotreatment

Lastly, the longer SRT tend to offer better biotreatment efficiency overall. This encourage the development of the slower-growing microorganisms, specifically nitrifiers. MBRs are especially effective at the biological removal of ammonia (‘nitrification’).

On the other hand, compared with conventional activated sludge process, MBR are to some extent constrained primarily by:

[1] Greater process complexity

Membrane separation demands additional operational protocols relating to the maintenance of membrane permeability. Hence, MBR operators should be trained more rigorously.

[2] Higher capital equipment and operating cost

The membrane component of the MBR incurs a significant capital cost over and above that of a CAS and maintaining membrane cleanliness demands further capital equipment (capex) and operating cost (opex) as well as higher energy input.

II.1.3. MBR History

The first commercial MBR was developed by Dorr-Oliver in the late 1960s (Leonard, 1971). The system combined ultrafiltration (UF) with a CAS process for application to ship-board sewage treatment (Bailey, 1971). The system was based on what is known today as ‘sidestream’ configuration (sMBR) as opposed to more commercially significant configuration nowadays which is ‘immersed’ (iMBR). At around late 1980s and early 1990s, Japanese government instigated water recycling program prompted pioneering work by Yamamoto, Hiasa, Mahmood, & Matsuo (Yamamoto et al., 1989) to develop immersed hollow fiber (HF) UF MBR as well as flat sheet (FS) microfiltration (MF) iMBR by the agricultural machinery company, Kubota. Meanwhile, a Zenon Environmental, a company established in 1980 was developing an MBR system. By the early 1990s, Zenon Environmental patent the ZenoGem® immersed HF UF MBR process and the first immersed HF membrane module named ZeeWeed® was introduced to the market in 1993. By the end of 1990s, the total installed treatment capacity of Zenon membrane reached 150 megaliters per day (MLD).

Kubota’s MBR systems were also being popular candidate for water and wastewater treatment worldwide. Kubota conducted successful trials at Kingston Seymour by Wessex water in the mid-90s and installed a municipal wastewater treatment works at Porlock in the United Kingdom, which is the first to be installed outside Japan, in 1997. Zenon’s first international installment took place at the Veolia Biosep® plant at Perthes en Gatinais in France in 1999. Both of these plants had peak flow capacity of about 2 MLD and represented land mark plants in widespread of immersed MBR technology.

Whereas only a few iMBR systems from just two countries (USA ad Japan) were launched I the first half of the 1990s the following years saw the launch of at least 10 products from seven countries. Table II- 2 shows the list of 15 world's largest plants and technology suppliers and Table II- 3 lists the MBRs installed in South Korea. It can be seen that the plant capacity and number of suppliers nowadays has multiplied since the early days of MBR development. Table II- 4 also shows currently available large variety of membrane products which indicates highly competitive market of MBR.

Table II- 2. Largest MBR plants worldwide. (Judd, 2016)

Installations	Location	Technology Provider	(Expected) date of commissioning	PDF (MLD)	ADF (MLD)
Henriksdal, Sweden	Stockholm, Sweden	GEWPT	2016-2019	864	536
Seine Aval	Acheres, France	GEWPT	2016	357	224
Canton WWTP	Ohio, USA	Ovivo USA/Kubota	2015-2017	333	159
Water Affairs Integrative EPC	Xingyi, Guizhou, China	OW		307	
Euclid, OH, USA	Ohio, USA	GEWPT	2018	250	83
9th and 10th WWTP	Kunming, Yunnan, China	OW	2013	250	
Shunyi	Beijing, China	GEWPT	2016	234	180
Wuhan Sanjintang WWTP	Hubei Province, China	OW	2015	200	
Jilin WWTP (Phase 1, upgrade)	Jilin Province, China	OW	2015	200	
Caotan WWTP PPP project	Xian, Shaanxi, China	OW		200	
Brussels Sud	Brussels, Belgium	GEWPT	2017	190	86
Riverside	California, USA	GEWPT	2014	186	124
Brightwater	Washington, USA	GEWPT	2011	175	122
Visalia	California, USA	GEWPT	2014	171	85
Qinghe WRP (Phase 2)	Beijing, China	OW	2011	150	

PDF: Peak daily flow, Megalitres per day
ADF: Average daily flow, Megalitres per day
GEWPT: GE Water and Process Technologies
OW: (Beijing) Origin Water
MRC: Mitsubishi Rayon Corporation

Table II- 3. MBRs in South Korea

Installations	Location	Technology Provider	Commissioned/ Installed date	PDF (MLD)	ADF (MLD)
Suyeong Sewage Treatment Plant	Busan, South Korea	GS Engineering	2012	102	
Unbuk Sewage Treatment Plant	Incheon, South Korea	Pure Envitech	2012	5-20	
Geumchon Sewage Treatment Plant	Paju, South Korea	Pure Envitech	2009-2015	5-20	
Cheongbuk Water Restoration Centre	Pyeongtaek, South Korea	Pure Envitech	2010	5-20	
Tangjung WWTP	Asan, South Korea	GE Water	2009		55

PDF: Peak daily flow, Megalitres per day
ADF: Average daily flow, Megalitres per day
GEWPT: GE Water and Process Technologies
OW: (Beijing) Origin Water
MRC: Mitsubishi Rayon Corporation

Table II- 4. Summary of commercial MBR membrane module products. (Santos and Judd, 2010)

Immersed (iMBR)	
Flat sheet	Hollow fiber
A3-*MaxFlow*[DE]	Asahi Kasei-*Microzoa*®[JP]
Alfa Laval[SE]	Beijing Origin Water Technology Co.[CN]
Brightwater-*Membright*®[IRL]	*Ecologix-EcoFlon*[TM], *EcoFil*[CN]
Colloide-*Sub snake*[NIR]	ENE Co., Ltd.-*SuperMAK*[KR]
Ecologix-*Ecoplate*[TM], *EcoSepro*[CN]	GE Zenon-Zeeweed®[US]
Huber-*VRM*®; *VUM*®/*GreyUse*[DE]	Hangzhou H-Filtration Membrane Technology Engineering Co., Ltd.MR[CN]
Jiangsu Lantian Peier Memb. Co. Ltd.[CN]	Koch Membrane System-*Puron*®[US]
KOReD-*Neofil*[KR]	Korean Membrane System-*KSMBR*®[KR]
Kubota-ES/EK[JP]	Litree-*LH3*[CN]
Microdyn-Nadir-*Biocel*®[DE]	Memstar-SMM[SG]
Pure Envitech Co., Ltd.-*ENVIS*[KR]	Mitsubishi Rayon-*Sterapore SUR*®; *Sterapore SADF*®[JP]
Shanghai Megavision Memb. Tech.[CN]	Philos[KR]
Toray-*TRM*[JP]	Porous Fibers S.L.-Micronet ®[JP]
Vina Filter-*Vinap*[CN]	SENUO Filtration Technology Co., Ltd.-*SENUOFIL*[CN]
Weise-*MicroClear*®[DE]	Shanghai Dehong Biology Medicine Siece & Tech. Development Co., Ltd.[CN]
	Siemens Water Tech.-*Memjet*[TM DE]
	Sumitomo-*PoreFlon*®[JP]
	Superstring MBR Tech. Corp.-*SuperUF*[CN]
	Tianjin Motimo-*FP AIV*[CN]
	Vina Filter-*F08*[CN]
	Zena Membranes-*P5*[CZ]

Table II- 4. *(Continued)*

Sidestream (sMBR)
Multitube/multichannel
A3-*MaxFlow*[DE]
Alfa Laval[SE]
Brightwater-*Membright*®[IRL]
Colloide-*Sub snake*[NIR]
Ecologix-*Ecoplate*[TM], *EcoSepro*[CN]
Huber-*VRM*®; *VUM*®/*GreyUse*[DE]
Jiangsu Lantian Peier Memb. Co. Ltd.[CN]
KOReD-*Neofil*[KR]
Kubota-ES/EK[JP]
Microdyn-Nadir-*Biocel*®[DE]
Pure Envitech Co., Ltd.-*ENVIS*[KR]
Shanghai Megavision Memb. Tech.[CN]
Toray-*TRM*[JP]
Vina Filter-*Vinap*[CN]
Weise-*MicroClear*®[DE]

II.1.4. Trends in MBR: Market and Research

[1] World Market

Since its adaptation in 1990s, the MBR system has continuously been replacing the conventional technologies in wastewater treatment such as CAS system due to lowered cost of MBRs. As a result, the global MBR market was worth $838.2 million in 2011 and is expected to witness a positive growth and revenue sales though 2018 (Frost&Sullivan, 2013). Figure II- 2 shows the trends in global MBR market and treatment volume. The figures translate into a compound annual growth rate (CAGR) of 22.4%, which is faster than the total market for wastewater treatment plant of 7.3% CAGR (Sze Chai Kwok, 2009). This indicates rapidly growing popularity of MBRs in the wastewater treatment industry. In addition, not only plant capacities are enlarging, as evidenced in the previous section, but also the number of MBR plants are rising as more than 4,400 MBRs plants were expected to be installed worldwide by the end of 2009 (Judd and Judd, 2006).

[2] MBR Research: Membrane Fouling

The research trend in MBR technology was analyzed by counting the number of articles published regarding topics of MBR. The search was performed at the Web of Science (https://apps.webofknowledge.com/) and the key words separately used are “MBR” and “Fouling in MBR”. The result was obtained based on the number of articles published for each key word in years from 2000 to 2015 and presented in Figure II- 3.

The result indicates increasing number of publication on MBR in general.

This signifies that MBR has been increasingly gathering attention in the academia possibly due to anticipated increase in market value and plant installment. To look more closely at what specific topic has been highly discussed in MBR research, the search key word of “Fouling in MBR” was used. The number of research regarding fouling in MBR was shown to be negligible in the beginning of the 2000s but it experiences a rapid rise accounting approximately 30% since early 2000s. Since membrane fouling is reported to be the major contributor in energy consumption of an MBR as shown in Figure II- 4, it is speculated that the importance of fouling control in an MBR is critical in reducing the cost of MBR and hence, the number of research is growing to more efficiently mitigate membrane fouling in an MBR.

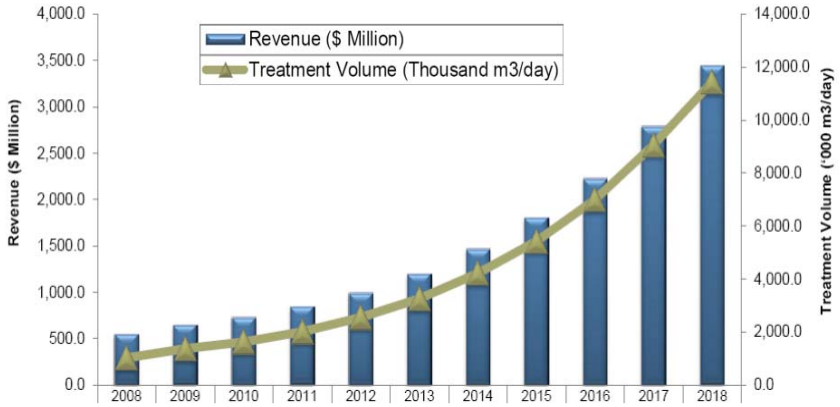


Figure II- 2. Global MBR market value MBR and treatment volume.

(Frost&Sullivan, 2013)

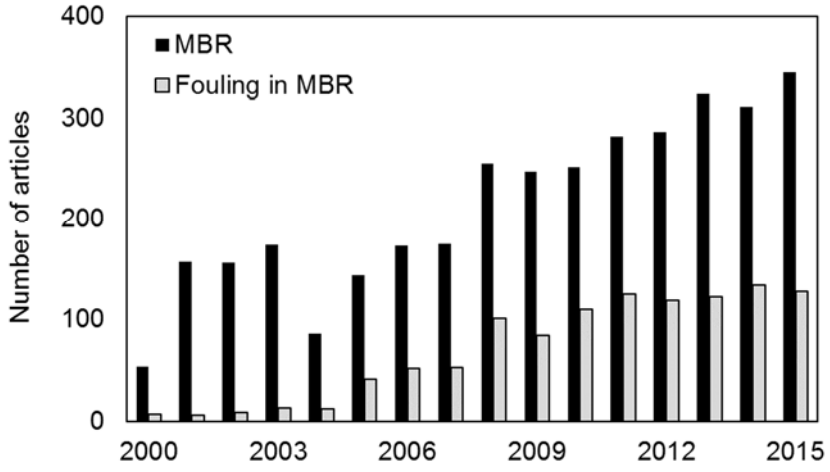


Figure II- 3. The number of research papers since 2000. (Source: Web of science)

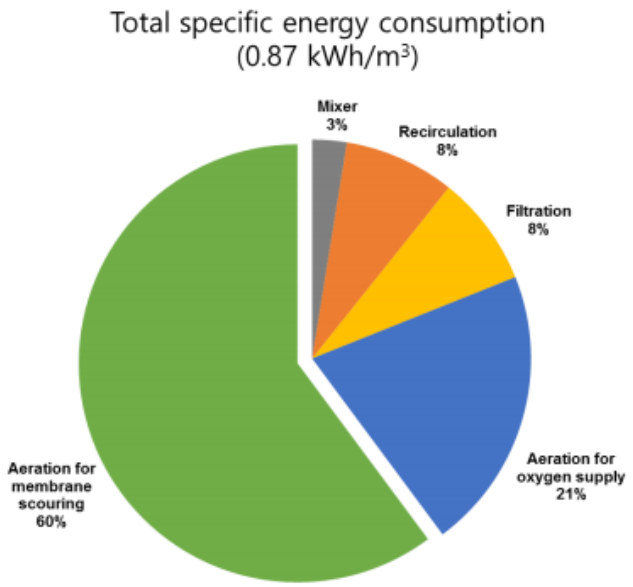


Figure II- 4. Distribution of the energy consumption in the operation of the MBR (17,000 m3 /d) installed at a municipal wastewater treatment plant in Korea. (Lee et al., 2016c)

II.1.5. Membrane Fouling in MBR

Membrane fouling refers to the clogging of membrane by either abiotic or biotic particles or dissolved substances which leads to the loss of water permeability. In practice, most of the filtration process is conducted in constant flux operation mode in which flux is fixed and operating pressure is varied. As the membrane is fouled, resistance to water permeation across the membrane is raised thereby increasing transmembrane pressure or TMP. Often, MBR operators monitor the values of TMP to estimate the degree of membrane fouling in an MBR.

[1] Physicochemical Aspects of Membrane Fouling

In the early stage of the MBR research, the fouling layer established on the membrane surface was often considered as an abiotic cake layer. It has been accepted that fouling of membrane proceeds according to a number of widely recognized physicochemical mechanisms, which are described in early stage of filtration studies (Grace, 1953). Figure II- 5 shows the reported fouling mechanisms which are complete blocking, standard blocking, intermediate blocking, and cake filtration. As the diagram illustrates, all models imply a dependence of water permeability loss on the ratio between particle size and membrane pore size. A complete blocking of pore occurs when the size of foulant is almost the same as the size of pores whereas standard blocking take place by the adsorption of small particulates on to the walls of pore thereby reducing the size of pore. When the particle size is larger than the pore size, intermediate blocking plays a role on the surface of membrane. Cake filtration typically occurs in polymer, usually protein, filtration. As polymers are retained, their concentration on membrane surface increases. When the concentration

of polymer is raised high enough, the molecules start to condense forming a gel-like layer on membrane surface.

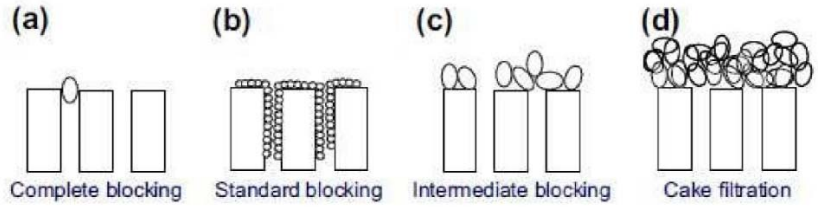


Figure II- 5. Fouling mechanisms of the membrane in an MBR. (Judd and Judd, 2006)

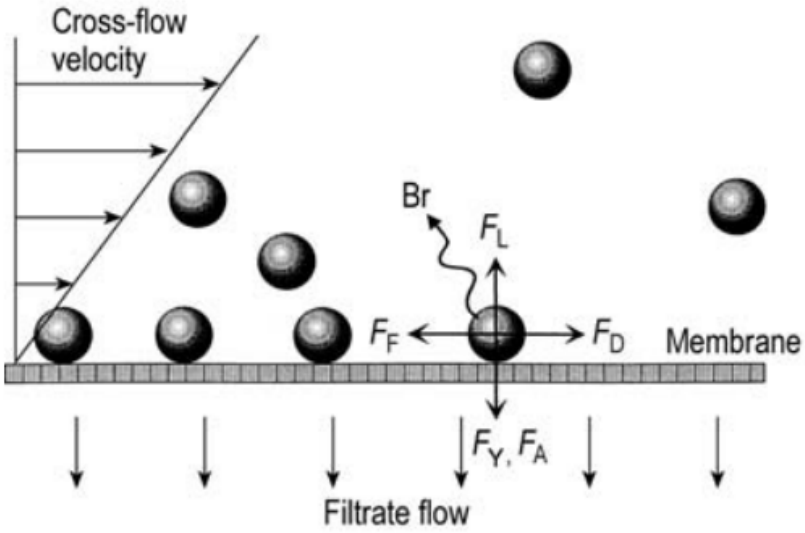


Figure II- 6. Forces and transport effects onto a particle in cross-flow filtration Br = Brownian motion; F_A = adhesion to the membrane; F_D = drag from the cross-flow; F_F = friction force; F_L = hydrodynamic lift force; F_Y = drag from the filtrate flow. (Alt, 2012)

On the fundamental level, attachment of foulant particle on a typical UF membrane is influenced by various forces resulting from physicochemical properties of the filtration system as shown in Figure II- 6. Increasing cross-flow velocity tends to increase the hydrodynamic life force reducing fouling. However, cross-flow velocity is controlled by pumping, consequently energy consumption, thus, its tuning for fouling mitigation is limited. Forces resulting from the Brownian motion (Br), friction (F_F), and drag from the cross-flow (F_D) are either relatively uncontrollable or have negligible impact on membrane fouling. Hence many studies have focused on modifying surface properties of membrane to reduce adhesion force of particles (F_A). The examples of such method are not discussed in this work but one can find detailed reviews on membrane surface modification in following literatures: (Rana and Matsuura, 2010, Lee et al., 2011, Kochkodan and Hilal, 2015).

One way to mitigate fouling is to reduce the permeation drag force (F_Y) on membrane by decreasing flux. Since the fouling rate increases with flux (Le Clech et al., 2003, McAdam et al., 2010a, McAdam et al., 2010b) sustainable operation is achieved through maintaining the flux at a modest value below critical flux. Critical flux is a flux above which an abrupt attachment of foulant takes place due to convective drag force. However, even while operating at a sub-critical flux, fouling occurs likely due to F_A and partially still effective F_Y. The sub-critical flux fouling is reported to occur in a two-stage pattern initiated by a conditioning (Brookes et al., 2006, Pollice et al., 2005): an initial conditioning of membrane via adsorption of soluble compounds (Zhang et al., 2006a, Zhang et al., 2006b), and gradual rise of TMP over a long period due to continued adsorption and attachment of particle by drag force followed by a rapid increase of TMP usually refer to as TMP-jump. The

components involving each fouling stage are described in detail in Figure II- 7.

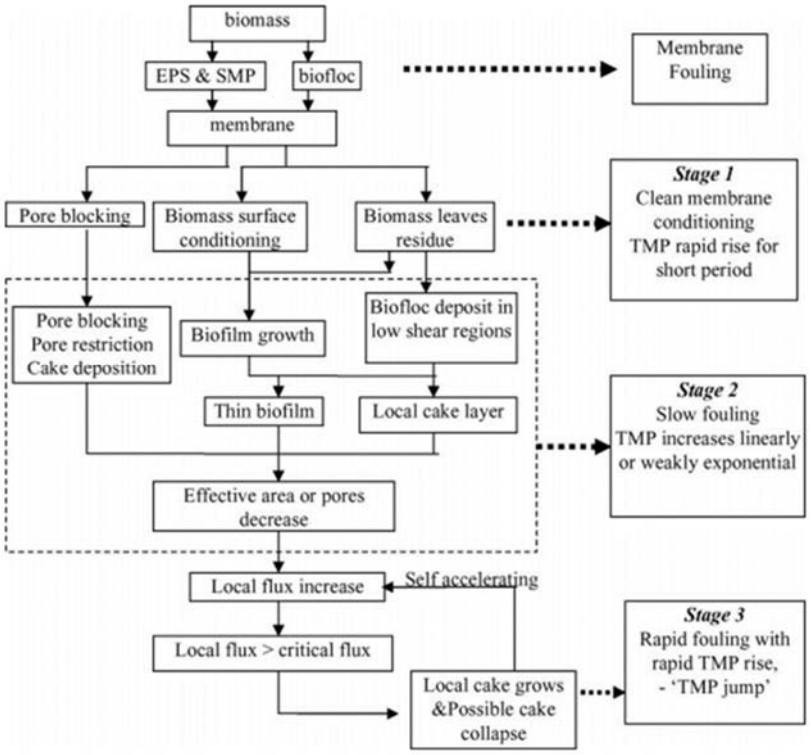


Figure II- 7. MBR fouling mechanism map—the three stages of fouling. (Zhang et al., 2006a)

[2] Biological Aspects of Membrane Fouling: Biofouling

As described in previous section, early stage of the MBR research did consider less on biological aspects of membrane fouling. The researchers at the time regarded the fouling mechanism majorly based on physicochemical phenomena such as adsorption of organic foulants on the membrane surface, accumulation of mixed liquor suspended solids, and formation of cake layers. However, numerous studies have reported the effect of biological activities on reducing water permeability in an MBR which is referred to as membrane biofouling (Hwang et al., 2008, Lee et al., 2008). Since the year of 2000, the concept of growth of microorganisms on the surface of membrane is used to interpret the fouling phenomenon in an MBR and it became one of the main topics in the realm of MBR research. Before looking into studies on biofouling in an MBR, it is necessary to briefly introduce the concept of biofilm.

II.1.6. Biofilm

[1] Biofilm in General

A biofilm is an assemblage of microbial cells that is irreversibly associated (not removed by gentle rinsing) with a surface and enclosed in a matrix of primarily polysaccharide material. Inorganic materials such as salt crystals, clay or silt particle may also be found in the matrix (Donlan, 2002).

Development of a biofilm is reported to follow a cycle of initial attachment, irreversible attachment, early development of biofilm architecture, maturation, and dispersal. as shown in Figure II- 8 (Stoodley et al., 2002). The initial attachment is controlled by both environmental factors such as temperature, pH, hydrodynamics, chemical properties of a substratum and genetic factors including motility, extra-cellular product (EPS) production, etc. The mechanism of attachment is usually species-specific, however above list is known to be common features that mediates cellular attachment to surfaces. Ones attached, cells begin to settle by suppressing expression related to motility while encouraging expression of genes related to EPS production. By doing so, EPS help anchor cells firmly to the substratum forming an irreversible attachment. Thereafter, cells grow and spread as a monolayer on the surface to form microcolonies. During the formation of microcolony, cells undergo developmental changes which induces formation of complex biofilm architecture in a mature biofilm. As the biofilm ages, a number of phenomena is observed. The biofilm may spread by crawling into uncolonized area or cells re-express genes that are accustomed to planktonic mode. This causes dispersal of mature biofilm and allows colonization of a new environment.

The architecture of a mature biofilm resembles that of a mushroom as

shown in Figure II- 9. This mushroom structure illustrates well the concept of 'microscale heterogeneity' in a biofilm (Dirckx, 1997). The microscale heterogeneity of a biofilm gives rise to perturbation of mass and momentum transport processes which in turn affects the interaction of biofilm with its surrounding. Consequently, this illuminates the complex nature of a biofilm and the reason why the biological aspect of fouling was not considered so far was possibly because of its complexity. In addition, recent study revealed that bacterial biofilm formation is mediated by a cellular density dependent cell to cell communication called quorum sensing (Davies et al., 1998). The details of quorum sensing will be discussed in later section.

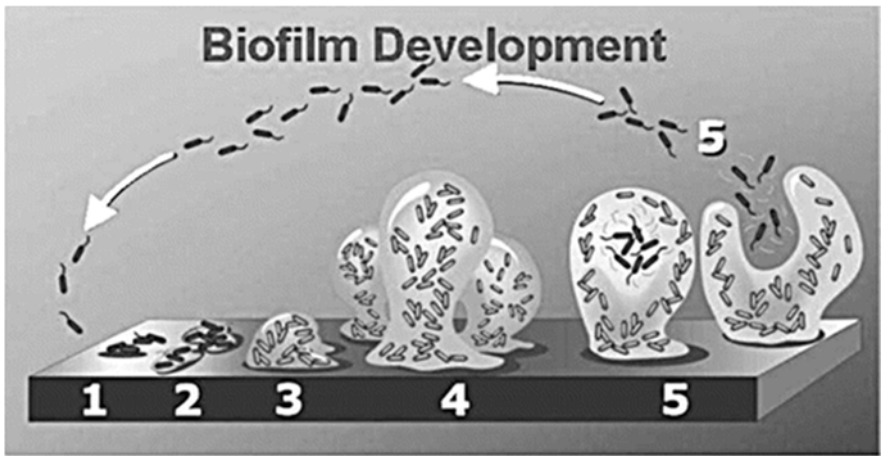


Figure II- 8. Development of a biofilm as a five-stage process. Stage 1: initial attachment of cells to the surface. Stage 2: production of EPS resulting in more firmly adhered “irreversible” attachment. Stage 3: early development of biofilm architecture. Stage 4: maturation of biofilm architecture. Stage 5: dispersion of single cells from the biofilm. (Stoodley et al., 2002)

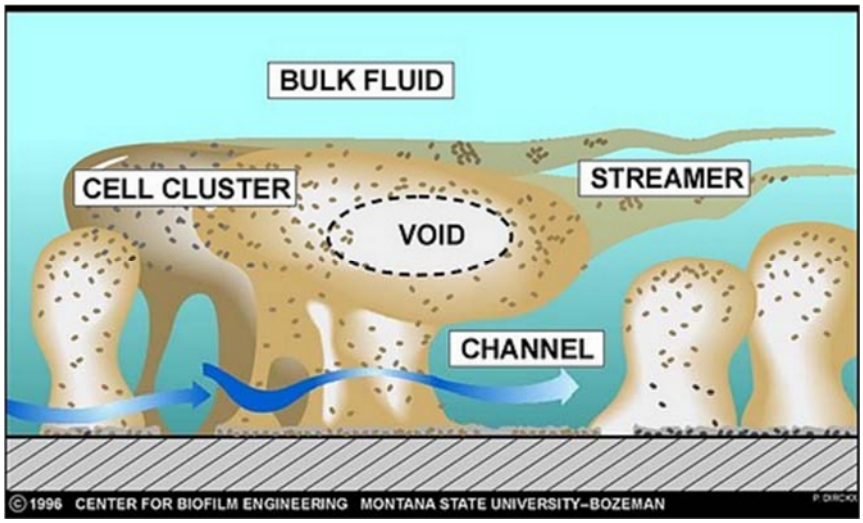


Figure II- 9. A conceptual cartoon to illustrate the structural heterogeneity of biofilms. Depending on the surrounding environment, biofilms can contain bacterial clusters (or microcolonies), channels or pores through which water can flow (carrying nutrients through the biofilm), void areas within biofilms (no longer) populated with bacteria, and streamers created by bulk fluid flow. (Dirckx, 1997)

[2] Biofilm in MBR

A study by Park and Lee (2005) demonstrated foulants accumulated on the surface of the membrane is biologically active. The authors extracted the cake layer formed on the membrane and tested to see if the foulants have biodegradation activity towards soluble chemical oxygen demand (COD). They showed that the foulants were able to degrade a certain percentage of COD and the consumed COD was utilized in the growth of the microorganisms in the cake layer. This suggests that fouling layer in an MBR has characteristic of a biofilm.

As briefly mentioned in the previous section that microscale heterogeneity of a biofilm plays an important role on its surrounding hydrodynamic environment and mass transfer, the structure of the biofilm gained the attention in perspective of analyzing its impact on fouling in an MBR. Yun et al. (2006) analyzed the internal porosity of biofilm formed on membrane surface with respect to different dissolved oxygen (DO) level. For this analysis, a technique consisted of staining components of biofilm such as EPS and bacterial cells and observing it with a confocal laser scanning microscopy (CLSM). They found that high DO concentration resulted in a more porous biofilm than under low DO concentration. The higher biofilm porosity yielded a greater water permeability. Kim et al. (2006) showed that DO affects not only the amount of expressed EPS but also their spatial distribution affects water permeability.

Furthermore, Lee et al. (2009) suggested that position of aerator in an MBR affects the spatial distribution of biocake porosity in a HF iMBR system. A recent, study has revealed that biofouling in an MBR is caused by a cellular density depended cell to cell communication called quorum sensing (Yeon et al., 2009a).

II.1.7. Fouling Control in MBR

As mentioned previously, membrane fouling is the critical challenge in MBR operation. Thus, countless studies have focused on remediating the fouling problem in MBR for decades. The approach to controlling fouling in an MBR can be divided as physical, chemical, and biological depending on the equipment or methods. The pros and cons of each approach are discussed in this section. Fabrication of anti-fouling membrane is also a major area of research but it is not considered in the scope of this study.

But, before getting into the actual discussion, it is worth explaining the classification of fouling. The two classes of membrane fouling are reversible fouling and irreversible fouling (Tsuyuhara et al., 2010) as shown in Figure II- 10. Physically reversible fouling refers to the fouling of a membrane that can be restored through appropriate physical washing procedures such as backwashing or hydrodynamic scouring (surface washing) which will be discussed shortly. Chemically reversible fouling only can be removed by chemical cleaning. On the other hand, iterative use of membrane results in an irreversible fouling. The irreversible fouling occurs by chemisorption and pore plugging mechanisms. The strong adhesion of particles makes recovery of water permeability of a membrane practically impossible either by typical physical or chemical treatment. At this point, the membranes should be treated by extensive chemical cleaning or be replaced.

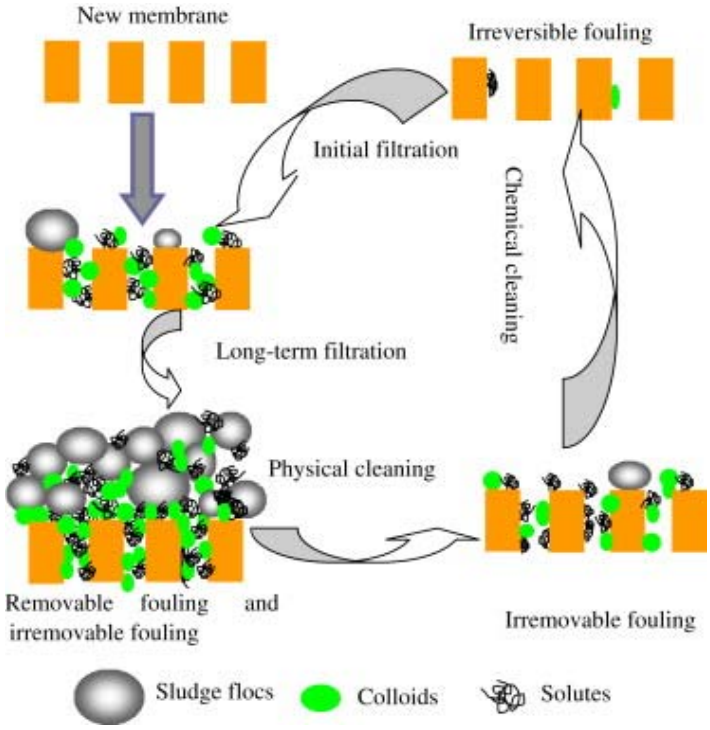


Figure II- 10. Illustration of reversible and irreversible fouling and physical and chemical cleaning cycle. (Meng et al., 2009)

[1] Physical Approach

i) Tuning of Operating Parameters

Physical cleaning of a membrane is often achieved via injecting a pulse of water in reverse direction to filtration through membrane pores (backflushing) or periodic cessations of filtration (relaxation) (Yuniarto et al., 2013, Monclus et al., 2010, Judd and Judd, 2006). Relaxation temporarily eliminates the permeation drag force which draws particles towards membrane surface while backflushing exerts out ward force on accumulated particles so that they are expelled out from the membrane surface and even disintegrate biocake structure. Often, the two techniques are used in combination. In addition, air scouring of membrane through aeration is an indispensable operating factor in an MBR. Along with providing oxygen for microbial respiration, intense aeration is commonly practiced in MBR plants to induce hydrodynamic life force and shear stress on foulants (Hazlett, 1995). However, because aeration rate for membrane scouring exceeds the value required for microbes to respire, energy consumption of an MBR system is significantly raised. Thus intermittent aeration was used (Nagaoka and Nemoto, 2005, Yeom et al., 1999). In addition, cyclic aeration switching between the high and low aeration intensity was used to effectively control membrane fouling in MBRs (Monsalvo et al., 2015, Wu and He, 2012). The physical approach is typically known to reduce only reversible fouling of membrane (Johir et al., 2011).

ii) Adding Particles to Induce Mechanical Cleaning

A number of studies investigated the addition of particles mechanical washing through frequent contact between the particles and membrane to induce detachment of foulants (Rosenberger et al., 2011, Siembida et al., 2010, Johir et al., 2011). Particularly, Rosenberger et al. (2011) inserted the plastic particles into a flat sheet membrane module (BIO-CEL, Microdyn-nadir, Germany) to reduce membrane fouling. As shown in Figure II- 11, an upward flow induced by aeration lifts particles upwards during which collision between membrane sheets occur. Once the plastic particles reach the top, they descend with the activated sludge and re-circulates. They showed that the permeability of MBR with circulating particles was remained higher compared to the one with no particles (Siembida et al., 2010). However, the membrane surface can be damaged by the collision with polypropylene particles (Figure II- 12). Furthermore, Kurita et al. (Kurita et al., 2014) reported that granules were able to reduce membrane fouling, but they could not mitigate the irreversible fouling resulted from a long-term operation (Figure II- 13).

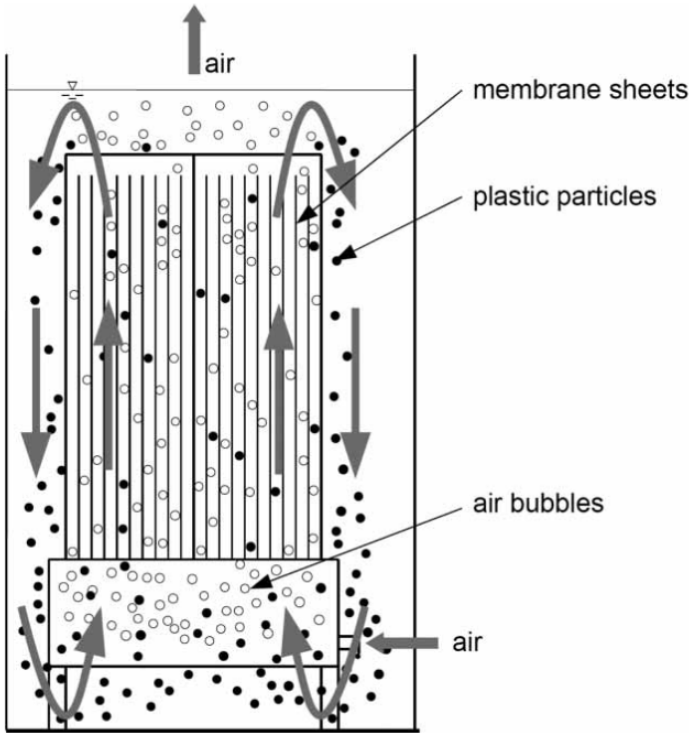


Figure II- 11. Scheme of the MCP flow field in a membrane tank with a submerged flat sheet module. (MCP: mechanical cleaning process). (Rosenberger et al., 2011)

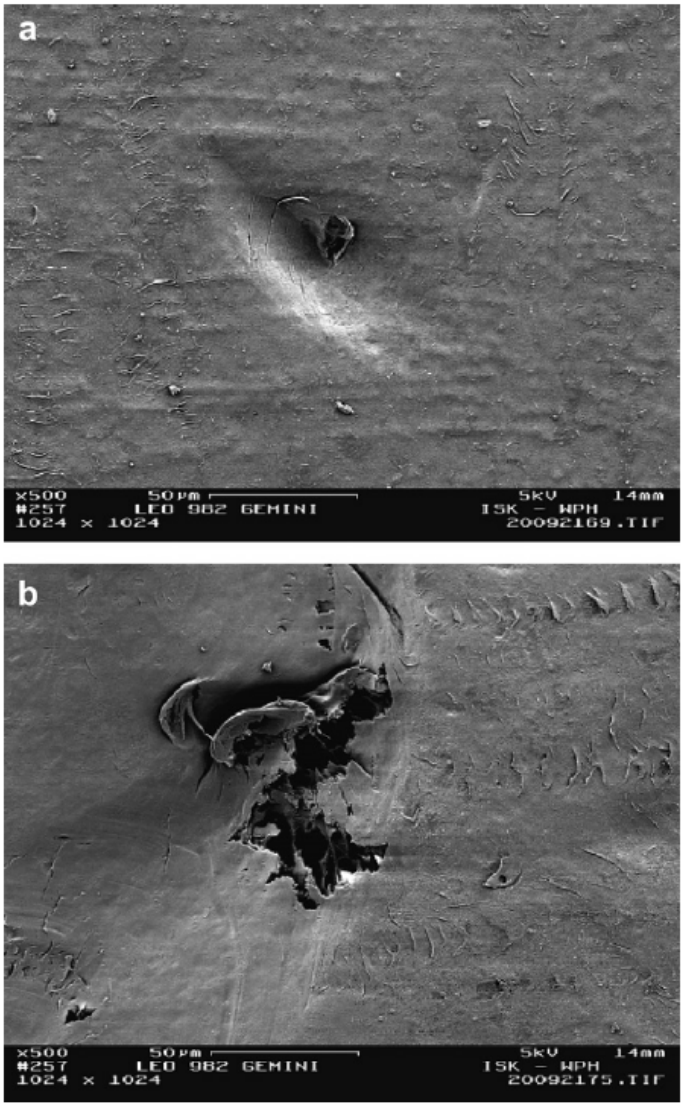


Figure II- 12. SEM images of membrane surface. (a) Membrane operated without plastic particles. (b) Membrane operated with plastic particles. (Siembida et al., 2010)

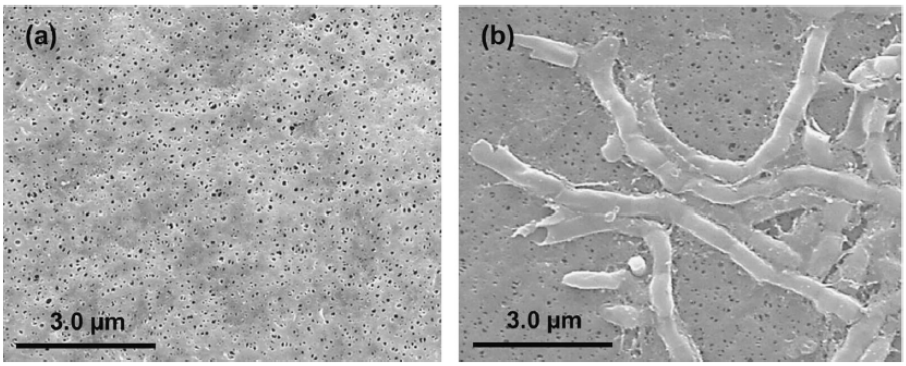


Figure II- 13. SEM images of membrane surface at four months of operation (after physical cleaning): (a) without granules and (b) with granules. (Kurita et al., 2014)

[2] Chemical Cleaning

Chemical cleaning typically involves chemically enhanced backwashing with cleaning agents such as chlorine (or bleach). This method is also known as a maintenance cleaning or clean in place (CIP) since the membrane module stays in the bioreactor during the cleaning. It is typically performed once or twice a week, and 100-1000 mg/L bleach solution is back flushed through the permeate line. Another is strategy is the recovery cleaning. Onsite chemical recovery cleaning involves emptying sludge and filling in the bioreactor with chemicals. In another case, fouled membrane module is transferred to another tank instead for immersion in cleaning solution. Commonly, higher concentration (> 1000 mg/L) of bleach solution and additional chemicals (citric acid, etc.). Such type of cleaning is done on a biannually base to remove the irreversible fouling.

[3] Biological Approaches

In contrast to physical and chemical approaches, biological strategy offers higher efficiency and lower toxicity. Hence, it is considered more sustainable option and is currently being actively studied. The proposed biological approaches for biofilm control in an MBR include enzymatic disruption (ED), energy uncoupling (EU), nitric oxide treatment, use of bacteriophage, and quorum quenching (QQ).

i) Enzymatic disruption (ED)

ED refers to degradation of foulant by enzymes. Typical disruption targets are protein or polysaccharides. Especially, EPS-degrading enzymes have been successfully applied for biofilm detachment, such as protease (e.g., proteinase K and trypsin), polysaccharases (e.g., Dispersin B, Mutanase and dextranase) and DNases (Petersen et al., 2005, Guezennec et al., 2012, Chaignon et al., 2007).

ii) Energy uncoupling (EU)

EU is the inhibition of adenosine triphosphate (ATP) synthesis by the addition of chemicals [e.g., 2,4- dinitrophenol (DNP), carbonyl cyanide chlorophenylhydrazone (CCCP), and 3,3',4',5-tetrachlorosalicylanilide (TCS)]. Above chemical agents transport protons through the cellular membrane and disrupts the proton gradient required for production of ATP. Hence, cells are stripped of the ability to convert substrates into energy thereby losing their activity. It was reported that DNP suppressed ATP synthesis and interfered with biofilm formation circuit of bacteria on nylon membrane surfaces (Xu and Liu, 2011).

iii) Nitric oxide (NO) Treatment

NO has been identified to play a role of a messenger molecule that regulates biofilm dispersal of *Pseudomonas aeruginosa* (Barraud et al., 2009). In addition, it was found to have an orthodox effect on dispersal of both Gram-positive and –negative bacteria (Charville et al., 2008). However, NO is only soluble in water for small quantity and is easily oxidized in open environment (Bill Cai et al., 2005). Hence, application of NO donor such as e.g., sodium nitroprusside, 3-morpholinosydnonimine, sodium nitrite, S-nitroso-N-acetylpenicillamine and diazeniumdiolate) have been proven to be efficient in the dispersal of biofilms formed on reverse osmosis (RO) membrane as well as in the dispersal of multi-species biofilms from water and treatment systems (Barraud et al., 2009). The NO-based method for biofouling control is still at its nascent stage and restricted to *in vitro* studies, therefore, more effort is needed to further explore its potential in membrane biofouling at pilot scale (Xiong and Liu, 2010).

iv) Use of Bacteriophage

Bacteriophage can be used to inhibit or disrupt biofilm formation on membrane surfaces. The applied bacteriophage infects the host bacteria and causes them to undergo rapid replication of virions which leads to a lysis of the host cells (Mc Grath and Sinderen, 2007). The study by Goldman et al. (2009) demonstrated that the addition of specific bacteriophages to an MBR could lower microbial attachment to the membrane surface and increase the membrane permeability. In presence of multiple species of bacteria, as in most of cases of water treatment process, application of a consortium of several phages is desired to prevent biofilm

development on the membrane surface. However, highly specific parasitic characteristics of bacteriophage may pose a challenge to their application in large-scale wastewater treatment in which bacteria of highly diverse group are involved (Xiong and Liu, 2010).

v) Quenching of Quorum Sensing (Quorum Quenching)

Formation of biofilm in diverse bacterial species is known to be regulated by a cell density dependent cell to cell communication called quorum sensing (QS) (Davies et al., 1998). In 2009, Yeon and his co-workers (Yeon et al., 2009a) have found that QS also plays a key role in biofilm formation on membrane surface in an MBR and biofouling can be inhibited by quenching a type of signal molecule (n-acyl homoserine lactone) mediating QS using enzymes. The inhibition of QS is called quorum quenching (QQ) and the application of QQ in an MBR is often referred to as QQ MBR. QQ MBR technology is deemed revolutionary and emerged as a promising option in fouling control (Drews, 2010).

Details on QS regulated biofilm formation and QQ MBR are discussed in following sections.

II.2. Quorum Sensing (QS)

II.2.1. Definition

Quorum sensing, a density dependent cell to cell communication is the regulation of gene expression related to the group behaviors. Quorum sensing is mediated through synthesis and exchange of signal molecules called autoinducers. They are call autoinducers because of the fact that when their external concentration increases to a certain value (i.e. when cell density reaches a quorum), production of autoinducers are exponentially increased (positive feedback) accelerating its synthesis (Waters and Bassler, 2005). A brief procedure of QS is illustrated in Figure II- 14. Individual cells produce basal level of signal molecules, however, when the cells become an aggregate, the collective synthesis of signal molecules caused its local concentration to increase. When the density of cells (or the concentration of signal molecules) reaches a threshold level, signal molecules binds with a receptor protein, which in turn the receptor-signal molecule complex transcription factor-signal molecule comp modulates expression of QS genes (Fuqua et al., 2001).Gram-positive and gram-negative bacteria use QS communication circuits to regulate a diverse array of physiological group behabiors including symbiosis, virulence, competence, conjugation, antibiotic production, motility, sporulation, and biofilm formation (Miller and Bassler, 2001).

II.2.2. Mechanism

Different bacterial species employs distinctive QS pathway through different QS signal molecules (Figure II- **15**). The mechanism of QS can be divided into three general classes based on the type of signal molecules as follows.

(i) Gram-negative bacteria with *N*-acyl-homoserine lactone (AHL).

(ii) Gram-positive bacteria with oligopeptide signal molecules.

(iii) Interspecies communication with AI-2 molecules.

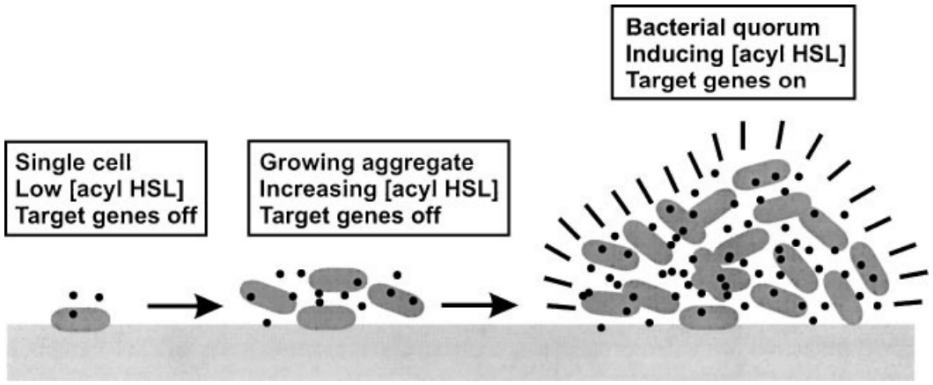


Figure II- 14. Population density-dependent gene regulation. A single population of bacteria accumulating on a surface. Filled dots indicates the intercellular signal molecule. (Fuqua et al., 2001)

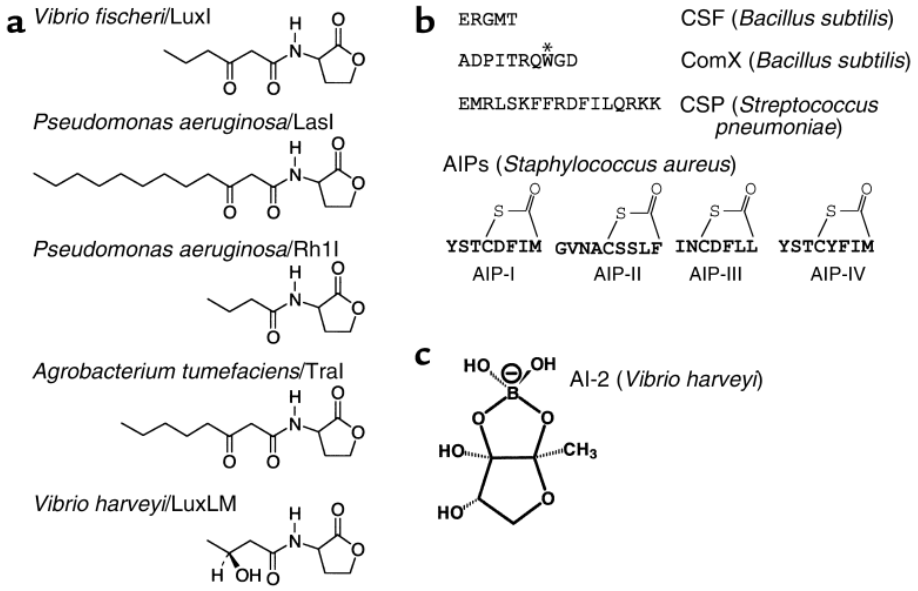


Figure II- 15. Structures of different signal molecules. (a) AHL signal molecules. (b) Oligo peptide signal molecules. (c) AI-2 signal molecules. (Federle and Bassler, 2003)

II.2.3. Gram-Negative Bacteria QS

A graphical illustration of AHL-mediated QS circuit in gram-negative bacteria is shown in Figure II- 16. The stages of such QS consist of 1) signal synthesis, 2) diffusion, 3) formation of receptor-signal molecule complex, 4) binding of the complex to a promoter and lastly, 5) expression of QS regulated genes. For example, in a luxI/luxR AHL QS system Figure II- 17, signal molecule synthesis is dependent on a *luxI* homologue, as well as the production of a transcriptional activator protein, *luxR* homologue, which detects AHL molecules and subsequently, the resulting complex induces gene expression (Waters and Bassler, 2005). As shown in , LuxI (or a homologue) signal molecule synthases (square) produce AHL signal molecules (triangles) which then freely diffuse across the cell membrane. Upon reaching a critical concentration of signal molecules, LuxR (or a homologue), a signal receptor protein (circle) accepts signal molecules forming a complex. The LuxR-AHL complex activates transcription of the target gene related group behavior (Bassler, 2002).

There exists a number of AHL species used for QS of different gram-negative bacteria as shown in Figure II- 18. Different types of AHLs involved in certain species of gram-negative bacteria are listed in Table II-1.

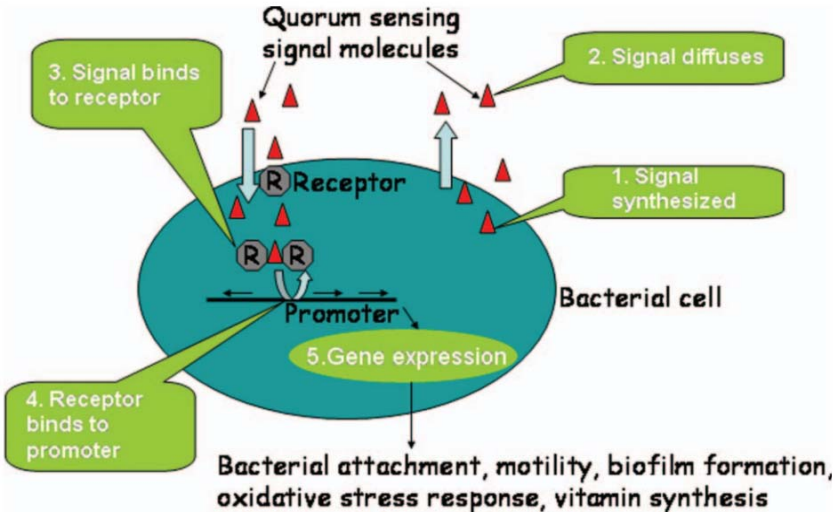


Figure II- 16. Different stages of QS process in generalized model of AHL QS in Gram-negative marine bacteria. Thick arrows show the main direction of signal transport due to diffusion (short chain AHLs or active transport (long chain AHLs). (Dobretsov et al., 2009)

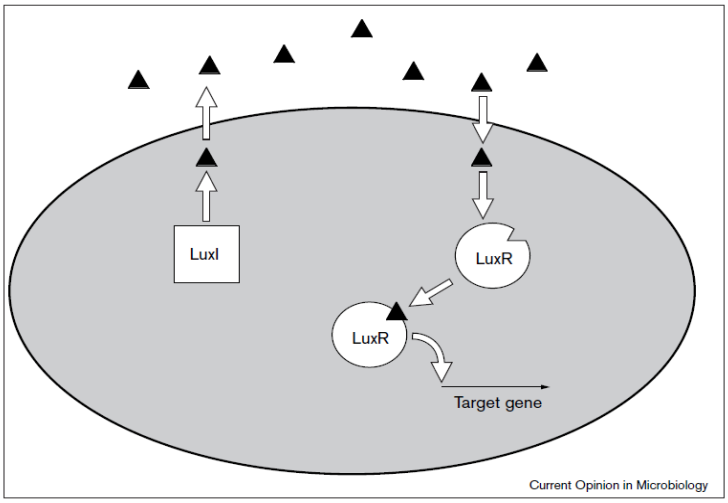


Figure II- 17. LuxI/LuxR quorum sensing. (Bassler, 1999)

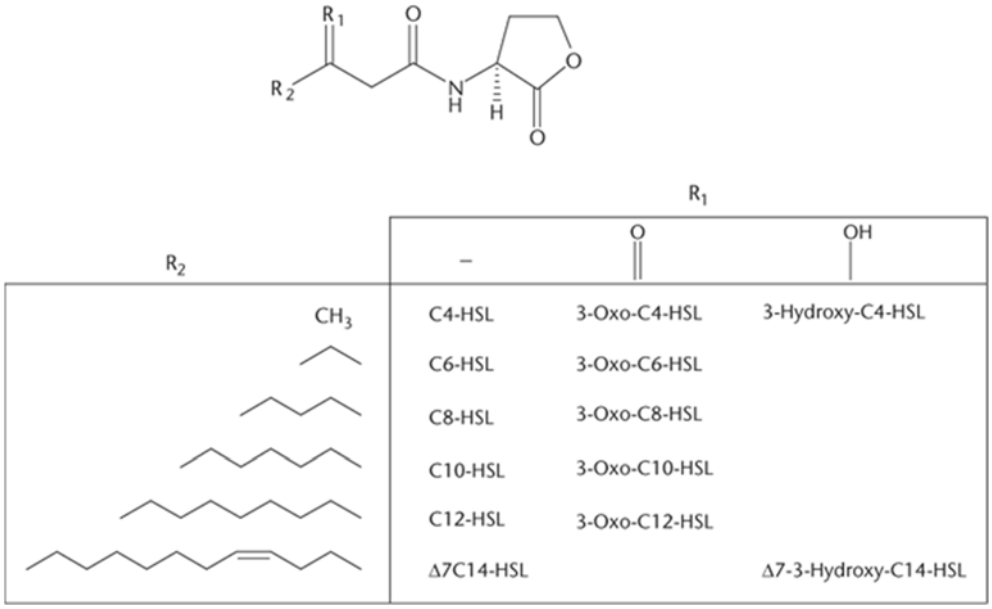


Figure II- 18. Structures of the *N*-acylhomoserine lactone family of molecules. The figure illustrates the structural features of AHL molecules purified from bacterial culture supernatants. AHL molecules differ in substitutions of groups at the C-3 position of the acyl chain (R_1) and also in the composition of the acyl chain itself (R_2). (Holden et al., 2001)

Table II- 5. Some examples of AHL-dependent quorum sensing systems in Gram-negative bacteria. (Holden et al., 2001)

Organism	Phenotype	Major AHLs
Aeromonas hydrophila	Biofilms, exoproteases, virulence	C4-HSL, C6-HSL
Aeromonas salmonicida	Exoproteases	C4-HSL, C6-HSL
Agrobacterium tumefaciens	Plasmid conjugation	3-oxo-C8-HSL
Agrobacterium vitiae	Virulence	C14:1-HSL, 3-oxo-C16:1-HSL
Burkholderia cenocepacia	Exoenzymes, biofilm formation, swarming motility, siderophore, virulence	C6-HSL, C8-HSL
Burkholderia pseudomallei	Virulence, exoproteases	C8-HSL, C10-HSL, 3-hydroxy-C8-HSL, 3-hydroxy-C10-HSL, 3-hydroxy-C14-HSL
Burkholderia mallei	Virulence	C8-HSL, C10-HSL
Chromobacterium violaceum	Exoenzymes, cyanide, pigment	C6-HSL
Erwinia carotovora	Carbapenem, exoenzymes, virulence	3-oxo-C6-HSL
Pantoea (Erwinia) stewartii	Exopolysaccharide	3-oxo-C6-HSL
Pseudomonas aeruginosa	Exoenzymes, exotoxins, protein secretion, biofilms, swarming motility, secondary metabolites, 4-quinolone signalling, virulence	C4-HSL; C6-HSL, 3-oxo-C12-HSL

Organism	Phenotype	Major AHLs
Pseudomonas aureofaciens	Phenazines, protease, colony morphology, aggregation, root colonization	C6-HSL
Pseudomonas chlororaphis	Phenazine-1-carboxamide	C6-HSL
Pseudomonas putida	Biofilm formation	3-oxo-C10-HSL, 3-oxo-C12-HSL
Pseudomonas syringae	Exopolysaccharide, swimming motility, virulence	3-oxo-C6-HSL
Rhizobium leguminosarum bv. *viciae*	Root nodulation/symbiosis, plasmid transfer, growth inhibition, stationary phase adaptation	C14:1-HSL, C6-HSL, C7-HSL, C8-HSL, 3-oxo-C8-HSL, 3-hydroxy-C8-HSL
Rhodobacter sphaeroides	Aggregation	7-cis-C14-HSL
Serratia spp. ATCC 39006	Antibiotic, pigment, exoenzymes	C4-HSL, C6-HSL
Serratia liquefaciens MG1	Swarming motility, exoprotease, biofilm development, biosurfactant	C4-HSL, C6-HSL
Serratia marcescens SS-1	Sliding motility, biosurfactant, pigment, nuclease, transposition frequency	C6-HSL, 3-oxo-C6-HSL, C7-HSL, C8-HSL
*Serratia proteamaculans*B5a	Exoenzymes	3-oxo-C6-HSL
Sinorhizobium meliloti	Nodulation efficiency, symbiosis, exopolysaccharide	C8-HSL, C12-HSL, 3-oxo-C14-HSL, 3-oxo-C16:1-HSL, C16:1-HSL, C18-HSL
Vibrio fischeri	Bioluminescence	3-oxo-C6-HSL

Organism	Phenotype	Major AHLs
Yersinia enterocolitica	Swimming and swarming motility	C6-HSL, 3-oxo-C6-HSL, 3-oxo-C10-HSL, 3-oxo-C12-HSL, 3-oxo-C14-HSL
Yersinia pseudotuberculosis	Motility, aggregation	C6-HSL, 3-oxo-C6-HSL, C8-HSL

II.2.4. Gram-Positive Bacteria QS

Gram-positive bacteria employ a QS system similar to that of gram-negative bacteria but instead of AHLs, use the peptide signal molecules via a dedicated AHL (ATP-binding cassette) exporter protein. Figure II- 19 shows details of QS among gram-positive bacteria. The peptide molecules (extracellular pheromones), represented as three adjacent circles, are synthesized from a pheromone precursor locus and diffused to extracellular environment through cell membrane. Then the peptides are recognized by two-component sensor kinase proteins interacting with cytoplasmic response regulator proteins (Kleerebezem et al., 1997, Novick and Muir, 1999). In detail, the sensors auto-phosphorylate on a conserved histidine residue (H), and subsequently transfer the phosphoryl group to cognate response regulators which are phosphorylated on conserved aspartate residues (D). Following the phosphorelay cascade (indicated by a letter P in a circle), response regulator proteins activate/repress transcription of peptide-mediated QS genes (Bassler, 1999).

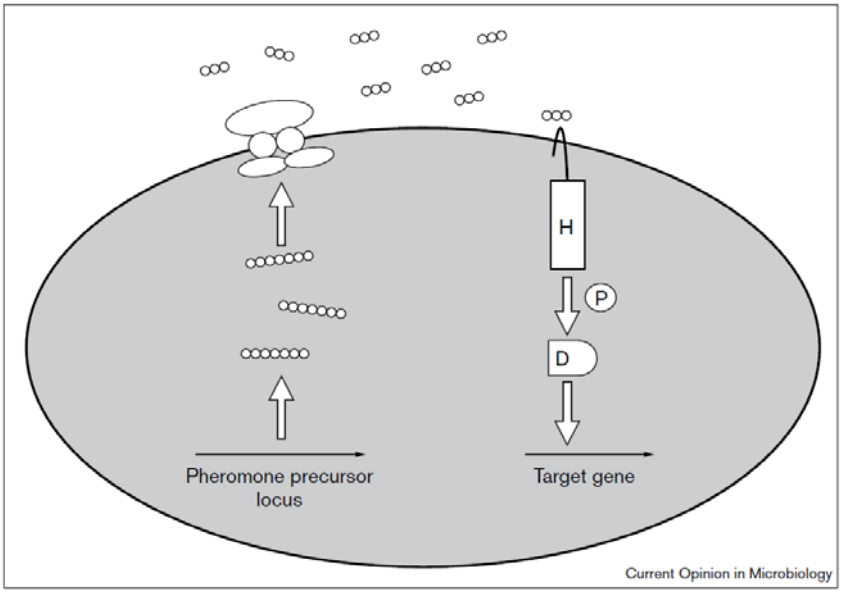


Figure II- 19. Peptide quorum sensing. (Bassler, 1999)

II.2.5. Interspecies QS (AI-2)

Above mentioned QS system are called intra-species QS because they are utilized within only one group of bacteria (either gram-negative or –positve) and thus, cannot be used across the gram-negative and –positive bacterial species. Recently, a family of molecules designated as autoinducer-2 (AI-2) has been discovered (Chen et al., 2002). It has been proposed that AI-2 is an inter-species signal molecule that mediates communication between gram-negative and gram-positive bacteria (Thiel et al., 2009).

The first discovery of AI-2 QS was from the luminous bacterium, *Vibrio harveyi,* which is often found in saline water. This bacterium possesses two QS circuits to control its luminescence through expression of the luciferase structural gene (*luxCDABE*) (Federle and Bassler, 2003). As illustrated in Figure II- 20, *V. harveyi* produces and responds to AHLs (triangles), similar to gram-negative bacteria, however, AHL-mediated QS signal transduction occurs via a two-component circuit, similar to gram-positive bacteria. On the other hand, *V. harveyi* also possesses AI-2 signal molecule (circles) QS transduction circuit which is conducted through the two-component circuit as well. Synthesis of AHLs and AI-2 molecules is mediated by LuxLM and LuxS, respectively. Receptor proteins, LuxN and LuxQ, detects AHL and AI-2, respectively. Signals from both sensors are channeled to the shared integrator protein LuxU and LuxO. Each step is proceeded in phosphorelay cascade. Lastly, LuxO–phosphate controls the expression of the luciferase structural operon *luxCDABE* (Bassler, 1999). It is interesting note that the presence of both AHL- and AI-2 mediated QS system in *V. harveyi* implies even when one of the QS systems is quenched, the bacterium still can produce luciferase

via utilization of the remaining un-quenched QS system.

More surprising discovery was that AI-2 signal molecule and the gene required for its production have recently been identified in a variety of gram-negative and –positive species (Surette and Bassler, 1998, Surette et al., 1999). This suggests that AI-2 could be used in an inter-species communication.

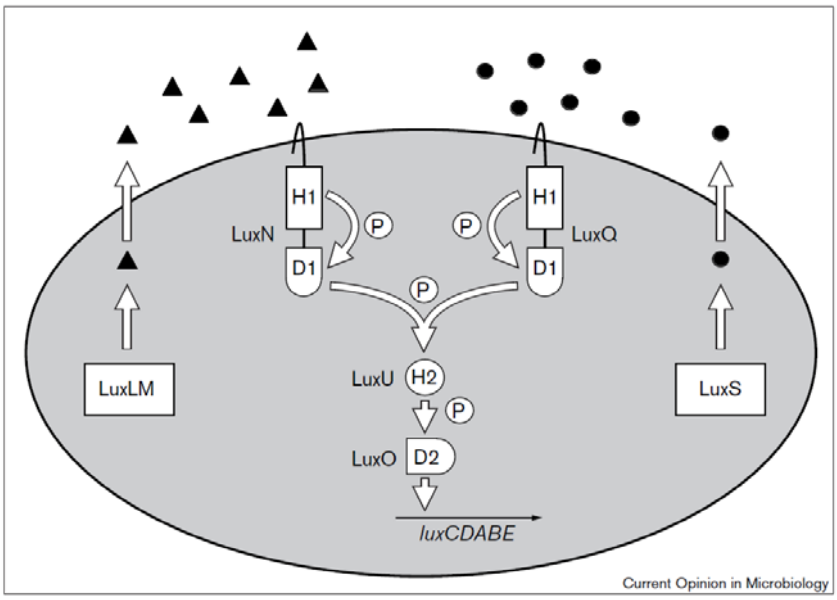


Figure II- 20. Quorum sensing in *V. harveyi* with two QS circuits. (Bassler, 1999)

II.2.6. Role of QS in Biofilm Formation

The cellular density in biofilm is generally accepted to be higher than that in their planktonic state. As such, it would be reasonable to argue that quorum sensing and biofilm are closely related because biofilm includes aggregates of cells which would give rise to the local concentration of QS signal molecules. This hypothesis that biofilm is an ideal site for execution of QS has led numerous studies to investigate the role of QS in biofilm formation of bacteria.

As described in a previous section, development of biofilm from a planktonic cell involves attachment, maturation and dispersal cycle. During the progression of this cycle, several factors have been shown to affect biofilm maturation, including motility, production of extracellular polymeric substance and rhamnolipid production (Hentzer et al., 2001, Davey et al., 2003, Klausen et al., 2003).

A later study has reported that AHL-based QS has been shown to influence maturation of *Serrtia liquefaciens* (Labbate et al., 2004). Specifically, a mutation in the gene related to AHL synthesis (swri) resulted in immature biofilm of *S. liquefaciens* that lacked aggregates and filaments. In addition, Huber et al. (2001)showed that mutation of either cepI or cepR (QS signal molecule synthase or receptor, respectively) in Burkholderia cepacia H111 resulted in less robust biofilms than the wild-type strain. Moreover, mutation in ahyI (AHL synthase) in *Aeromonas hydrophila* yielded a biofilm that was structurally less differentiated than that of the wild-type. In addition to above studies, several other works suggested involvement of QS in biofilm formation (Eberl et al., 1996, Lynch et al., 2002, Wen and Burne, 2004).

Later studies have revealed that formation of biofilms of *Pseudomonas aeruginosa* (Parsek and Greenberg, 2000), *Burkholeria cepacia*, and *Aeromonas hydrophila* require a functional AHL-mediated QS system (Davies et al., 1998, Huber et al., 2001, Lynch et al., 2002). Especially, Parsek and Greenberg proposed that QS is involved in the evolution of a microcolony into a mature biofilm in P. aeruginosa (Figure II- 21). They also found that the high density microcolonies differential into mature biofilms by a 3-oxo-C12-HSL-dependent QS system. Furthermore, in Figure II- 22, biofilm of wild-type *P. aeruginosa* was thicker than that of lasR, rhlR mutant. Solano et al. (2014)illustrated the relationship between biofilm dispersion and quorum sensing (Figure II- 23). These researches suggest that manupulation of QS could be a highly attractive strategy against unwanted biofilm formation on surfaces.

This idea of involvement of QS in biofilm formation has extended to biofouling in an MBR. Yeon et al. (2009a) revealed that QS plays a key role in biofilm formation on membrane surface in MBRs. Using an AHL reporter strain, they showed that AHL signal molecules was detected (indicated by the blue color) in stages of membrane fouling (Figure II- 24). More specifically, AHLs were not detected at the early stage but the blue color development gradually became stronger as the membrane fouling proceeded. This implied that control of QS in MBR may reduce biofouling.

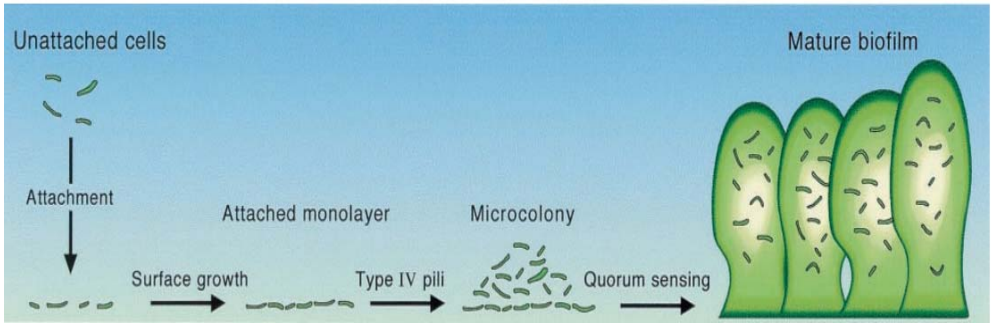


Figure II- 21. Diagram of the *P. aeruginosa* biofilm-maturation pathway. Unattached cells that approach a surface may attach. Attachment involves specific functions. Attached cells will proliferate on a surface and use specific functions to actively move into micro-colonies. The high-density micro-colonies differentiate into mature biofilms by a 3-oxo-C12-HSL-dependent mechanism. (Parsek and Greenberg, 2000)

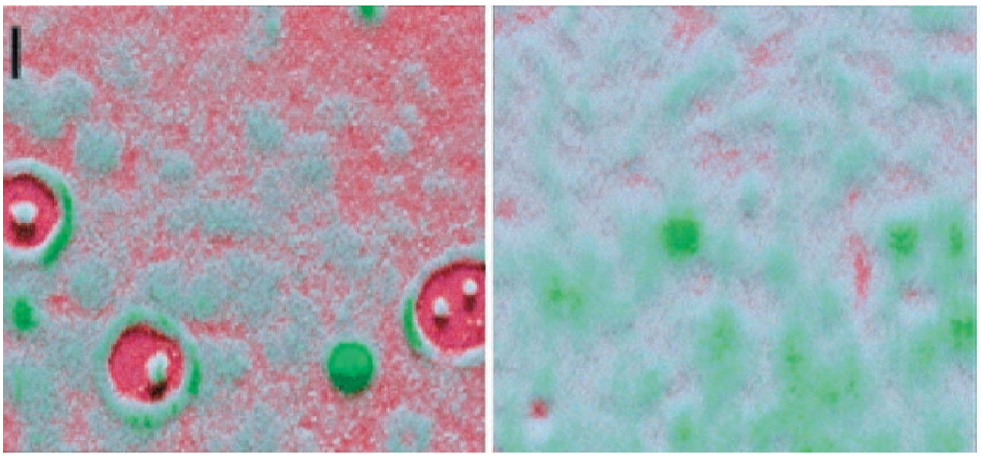


Figure II- 22. Scanning confocal microscope images of a mature *P. aeruginosa* wild-type biofilm (Right) and a quorum-sensing mutant biofilm (Left). In this case the quorum-sensing mutant was a lasR, rhlR double mutant. The glass surface is red, and the green is from the green fluorescent protein encoded by the GFP gene in the recombinant *P. aeruginosa*. The wild-type biofilm consists of thick microcolonies. The immature mutant biofilm appears thinner, and more of the glass surface is exposed. With the lasR, rhlR mutant shown here (but not with lasI, rhlI mutants) zones of clearing around microcolony towers are often observed. The colors were applied to the image by computer enhancement with Adobe PHOTOSHOP 5.0. The black marker bar is 100 mm in length. (Parsek and Greenberg, 2000)

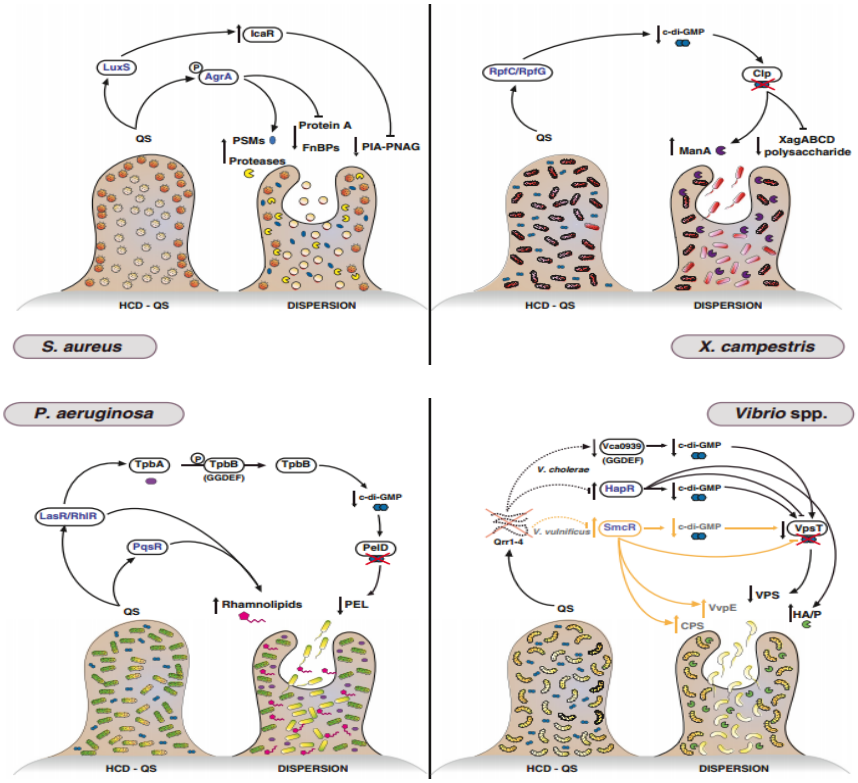


Figure II- 23. Biofilm dispersion mechanisms activated at high cell density by QS in bacteria. Schematic representation of biofilm mushroom-like pillars indicating the mechanisms of biofilm dispersion activated by QS signal accumulation in each bacterial species. In P. aeruginosa, QS positively regulates the expression of the periplasmic tyrosine phosphatase TpbA. TpbA dephosphorylates the membrane-anchored GGDEF protein TpbB deactivating its DGC activity and thus reducing c-di-GMP levels in the cell. As a result, the c-di-GMP receptor PelD is no longer bound to c-di-GMP and PEL polysaccharide production is decreased. QS also promotes the synthesis of rhamnolipids whose overproduction results in biofilm detachment. In Vibrio spp., QS signal accumulation provokes a cessation in qrr1-4 small RNAs transcription. In V. cholerae, qrr1-4 cannot longer base pair with the vca0939 mRNA, which encodes a GGDEF domain protein, and thus its translation is inhibited and c-di-GMP levels decrease. On the other hand, the expression of the HCD master transcriptional regulators HapR and SmcR of V. cholerae and V. vulnificus increases. HapR and SmcR downregulate. (Solano et al., 2014)

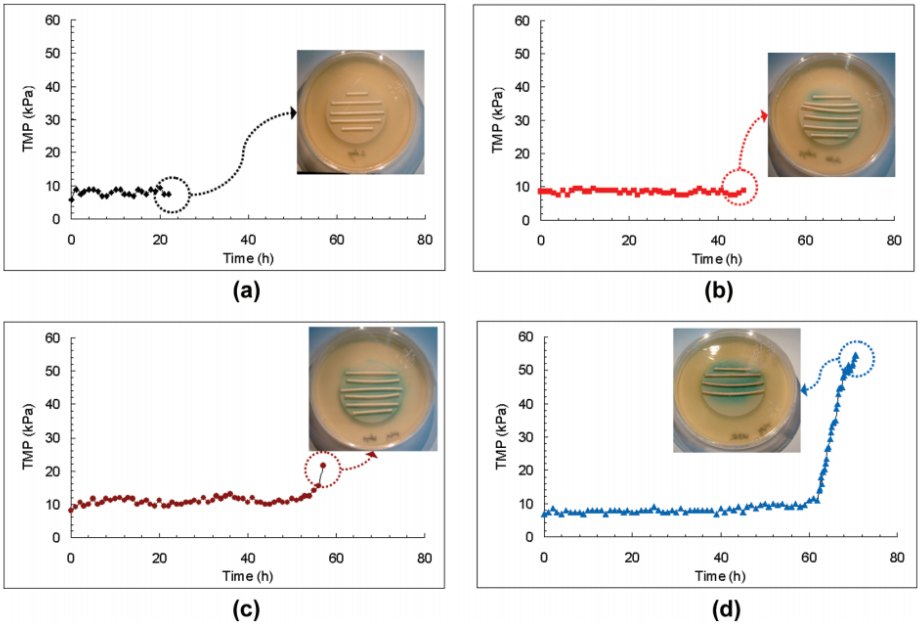


Figure II- 24. Occurrence of AHL signals in biocake during continuous MBR operation: (a) 22 h, (b) 46 h, (c) 58 h, and (d) 72 h. (Yeon et al., 2009a)

II.2.7. Detection of AHL QS Signal Molecules

Since AHLs are known to be present at a pico molar level in natural enviroment, extraction and concentration of AHLs are required for their detection. Commonly used methods of extraction are Liquid-liquid extraction (LLE) and solid-phase extraction (SPE). In a LLE, a sample containing AHLs, usually an aqueous one, is contacted with an organic solvent (e.g., chloroform. ethyl acetate and hexane) to induce diffusion of AHLs to the organic phase. Subsequently the contacted organic solvent is evaporated using appropriate equipment (e.g., rotary evaporator). SPE uses the silica or aluminum columns onto which AHLs can adsorb and be separated.

AHL detection methods can be dividied into two groups 1) biosensors (or reporter strains) and 2) mass spectroscopy. Decriptions of each method are as follows.

[1] Biosensors for detection and visualization of AHLs

Bacterial sensor has been widely used to detect the presence of AHLs for its high sensitivity and convenience. A bacterial strain used for sensing or detecting AHL is called a reporter strain and the detection of AHLs are carried out through the expression of detectable phenotypes such as light emission, expression of β-galactosidase activity or production of pigments upon the presence of exogenous AHLs (Figure II- 25). Two of the commonly used AHL reporter strains are *Agrobacterium tumefaciens* (Farrand et al., 2002) and *Chromobacterium violaceum* CV026 (McClean et al., 1997). When external AHLs were introduced, *Ag. tumefaciens* produce indicating molecules, β-galactosidase which, when reacted with an X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), cleaves the β-

glycosidic bond in D-lactose (Figure II- 26) resulting in a blue color. As a substitute to X-gal, the commercial assay system named Beta-glo can be used. The reagent consists of luciferin-galactoside substrate(6-O-β-galactopyranosyl-luciferin) which is cleaved by β-galactosidase to form luciferin and galactose. Luciferin produces luminescence which can be read through a luminometer. Since the luminescence or blue color intensity proportional to the amount of AHLs present in the sample, these methods are commonly used for quantification of AHLs. Another well-known reporter strain *C. violaceum* CV026 produces a purple pigment upon intake of AHLs.

Other biosensors include those that produce green fluorescence proteins (GFP) in response to the presence of AHLs (Wu et al., 2000). Use of such GFP producing strains allows not only quantification of AHLs in a sample but also visualization of signal molecules in a sample through observation with a fluorescence microscope or a confocal laser scanning microscope (CLSM). For example, Song et al. (2014) have mixed a GFP expressing AHL sensor, *Aeromonas sp.* (pBB–LuxR), with activated sludge from an MBR and observed with a CLSM (Figure II- 27). This method allowed direct observation of AHLs in-situ and it has been proved to be a viable tool in studying spatial distribution of QS signal molecules in a bio-system.

Another GFP producing AHL reporter strain is E. coli JB525. Wu et al. (2000) used this reporter strain to detect AHLs in lung tissues of mice infected with *P. aeruginosa*. The genetic modification of JB525 is as follows: 1) a mutant derivative of a gene *gfpmut3** encoding a 238 amino acid protein the emits fluorescent light at 511 nm when excited by light with wavelength around 488 nm was prepared by insertion of alanine (A), serine (S) and valine (V) at the C-terminus

and denoted as gfp(ASV). 2) gfp(ASV) is fused with a promoter containing luxR (from *P. fischeri*) and PluxI and the resulting *luxR*, *PluxI–gfp*(ASV) fusion was cloned into a stable broad-host-range vector pME6031 giving rise to pJBA132. The plasmid pJBA132 was injected to the E. coli MT102 monitor strain and this AHL biosensor is denoted JB525.

These techniques are convenient, fast and sensitive for detecting the presence of AHLs. However, since a single biosensor can recognize multiple species of AHL and sensitivity towards each AHL is different, differentiating and quantifying each member in AHL family is difficult especially when AHLs present samples are unknown (Wang et al., 2011).

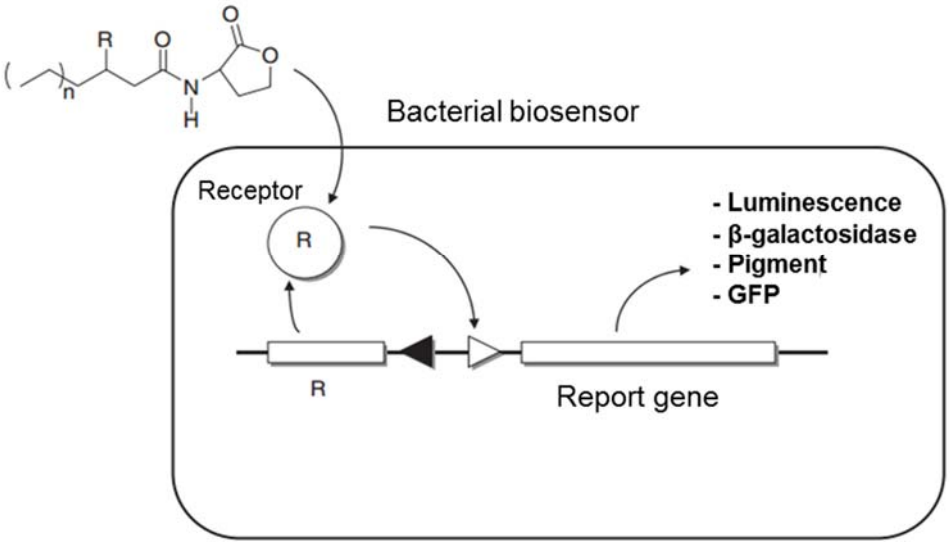


Figure II- 25. Construction and use of a bacterial AHL biosensor. The exogenous AHL interacts with a LuxR family protein inside the bacterial biosensor (non-AHL producer), which results in the transcription of a reporter gene(s) from a LuxR family-AHL regulated promoter as shown by the open triangle. The LuxR family gene is usually expressed from a constitutive promoter as shown with a filled triangle. The properties of biosensor can be dependent on report gene (s) (Modified from (Steindler and Venturi, 2007)).

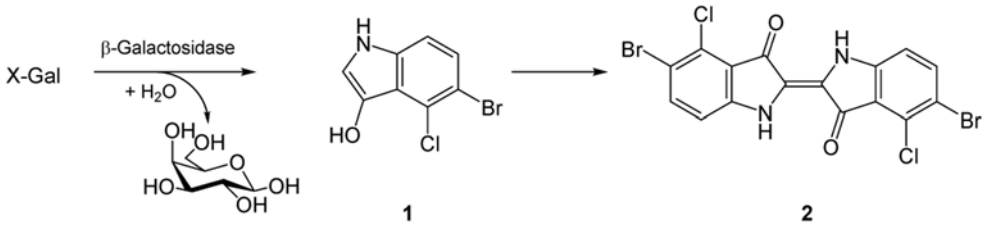


Figure II- 26. Cleavage of X-Gal by β-galactosidase. (Yikrazuul, 2008)

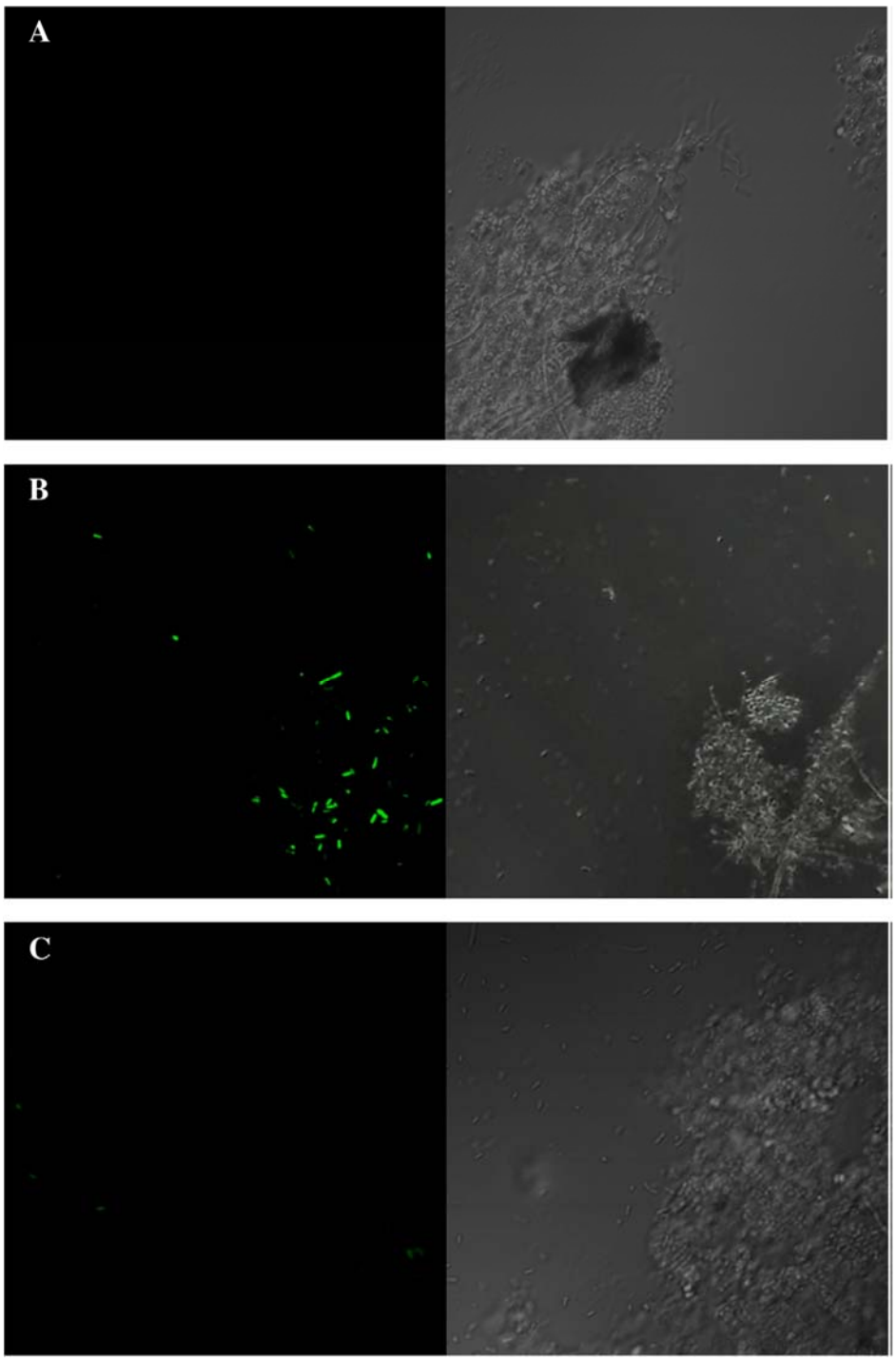


Figure II- 27. CLSM images of the activated sludge sample without the AHL reporter strain (A), with the reporter strain (B), and autoclaved sludge sample with the reporter strain (C). (Song et al., 2014)

Table II- 6. AHL biosensors. (Steindler and Venturi, 2007)

Strain/Plasmid sensor	Host	Based on QS system	Reporter system	Best responds to	Good detection	Commonly used for	Reference
*C. violaceum*CV026	*C. violaceum*	CviI/R (*C. violaceum*)	Violacein pigment	C6-AHL	C6-3-oxo-AHL C8-AHL C8-3-oxo-AHL C4-AHL	T.S., TLC	McClean *et al.*(1997)
pSB401	*E. coli*	LuxI/R (*V. fisheri*)	*luxCDABE*	C6-3-oxo-AHL	C6-AHL C8-3-oxo-AHL C8-AHL	TLC, Q.	Winson *et al.* (1998a)
pHV200I⁻	*E. coli*	LuxI/R (*V. fisheri*)	*luxCDABE*	C6-3-oxo-AHL	C6-AHL C8-3-oxo-AHL C8-AHL	TLC, Q.	Pearson *et al.* (1994)
pSB403	Broad host range	LuxI/R (*V. fisheri*)	*luxCDABE*	C6-3-oxo-AHL	C6-AHL C8-3-oxo-AHL C8-AHL	TLC, Q.	Winson *et al.* (1998a)
pSB536	*E. coli*	AhyI/R (*A. hydrophyla*)	*luxCDABE*	C4-AHL		TLC, Q.	Swift *et al.*(1997)
pAL101	*E. coli* (*sdiA*mutant)	RhlI/R (*P. aeruginosa*)	*luxCDABE*	C4-AHL		TLC, Q.	Lindsay & Ahmer (2005)
pSB1075	*E. coli*	LasI/R (*P. aeruginosa*)	*luxCDABE*	C12-3-oxo-AHL	C10-3-oxo-AHL C12-AHL	TLC, Q.	Winson *et al.* (1998a)
pKDT17	*E. coli*	LasI/R (*P. aeruginosa*)	β-galactosidase	C12-3-oxo-AHL	C12-AHL C10-AHL C10-3-oxo-AHL	TLC, Q.	Pearson *et al.* (1994)
M71LZ	*P. aeruginosalasI*⁻	LasI/R (*P. aeruginosa*)	β-galactosidase	C12-3-oxo-AHL	C10-3-oxo-AHL	Q.	Dong *et al.*(2005)
pZLR4	*A. tumefaciens*NT1	TraI/R (*A. tumefaciens*)	β-galactosidase	C8-3-oxo-AHL	All 3-oxo-AHLs C6-AHL C8-AHL C10-AHL C12-AHL C14-AHL C6-3-hydroxy-AHL C8-3-hydroxy-AHL C10-3-hydroxy-AHL	T.S., TLC, Q.	Farrand *et al.* (2002)

Table II- 6. *(Continued)*

Strain/Plasmid sensor	Host	Based on QS system	Reporter system	Best responds to	Good detection	Commonly used for	Reference
pCF218 + pCF372	*A. tumefaciens*WCF47	TraI/R (*A. tumefaciens*)	β-galactosidase	As above with more sensitivity	As above with more sensitivity	TLC, Q.	Zhu *et al.*(1998)
pJZ384 + pJZ410 + pJZ372	*A. tumefaciens*KYC55	TraI/R (*A. tumefaciens*)	β-galactosidase	As above with more sensitivity	As above with more sensitivity	TLC, Q.	Zhu *et al.*(2003)
pSF105 + pSF107	*P. fluorescens*1855	PhzI/R (*P. fluorescens*2-79)	β-glucuronidase β-galactosidase	C6-3-hydroxy-AHL	C8-3-hydroxy-AHL	TLC, Q.	Khan *et al.*(2005)
S. melilotisinI::lacZ	*S. melilotisinI::lacZ*	SinI/R (*S. meliloti*)	β-galactosidase	C14-3-oxo-AHL	C16:1-3-oxo-AHL C16-AHL C16:1-AHL C14-AHL	T.S., TLC, Q.	Llamas *et al.* (2004)
pJNSinR	*S. melilotisinI::lacZ*	SinI/R (*S. meliloti*)	β-galactosidase	As above with more sensitivity	As above with more sensitivity	T.S., TLC, Q.	Llamas *et al.* (2004)
pAS-C8	Broad host range	CepI/R (*B. cepacia*)	gfp	C8-AHL	C10-AHL	Single cell	Riedel *et al.* (2001)
pKR-C12	Broad host range	LasI/R (*P. aeruginosa*)	gfp	C12-3-oxo-AHL	C10-3-oxo-AHL	Single cell	Riedel *et al.* (2001)
pJBA-132	Broad host range	LuxI/R (*V. fisheri*)	gfp	C6-3-oxo-AHL	C6-AHL C8-AHL C10-AHL	Single cell	Andersen *et al.*(2001)

- T.S. refers to 'T' streak analysis in solid media.
- Note that TLC analysis of unusually long AHLs results in no migration.
- Using epifluorescence microscopy.
- Q refers to quantification.

[2] Mass spectroscopy for detection of AHLs

Use of mass spectroscopy (MS) offers an effective method of detecting and quantifying individual species of AHLs even at low concentration depending on equipment coupled with the MS. Most analysis follows the organic solvent extraction procedure to purify and concentration AHLs. Thin-layer chromatography is also used for separation of AHLs (Shaw et al., 1997). Techniques, employing MS for detection of AHLs include gas chromatography MS (GC-MS)(Wang et al., 2011), fast atom bombardment MS (FAB-MS) (Eberl et al., 1996, Swift et al., 1997, Winson et al., 1995), electron impact MS (EI-MS) (Bainton et al., 1992), collision-induced dissociation MS (CID-MS) (Kuo et al., 1994), liquid chromatography-MS-MS (LC-MS-MS) (Morin et al., 2003) and HPLC-MS-MS (Tan et al., 2014). These methods are costlier and often requires stricter pretreatment of samples than use of biosensors. Nevertheless, optimization and coupling with other instrument offers accurate quantification of AHLs in the presence of biological matrices.

II.3. QS Control or Quorum Quenching (QQ)

II.3.1. Introduction to QS Control Strategy

As described in the previous section, AHL QS involves three components: the signal generator (LuxI homologue), the signal molecule (LuxR homologue), and the signal receptor (AHL). Therefore, QS can be inhibited by disrupting functionality of above three components. Following strategies are commonly employed for controlling QS which are also depicted in Figure II- 28 (Boyen et al., 2009).

[1] Inhibition of AHL synthesis

[2] Interference with signal receptors

[3] Degradation of AHL signal molecules

Although only control of AHL-mediated QS is dealt in this thesis, quenching of AI-2 QS is conducted in the similar fashion as follows.

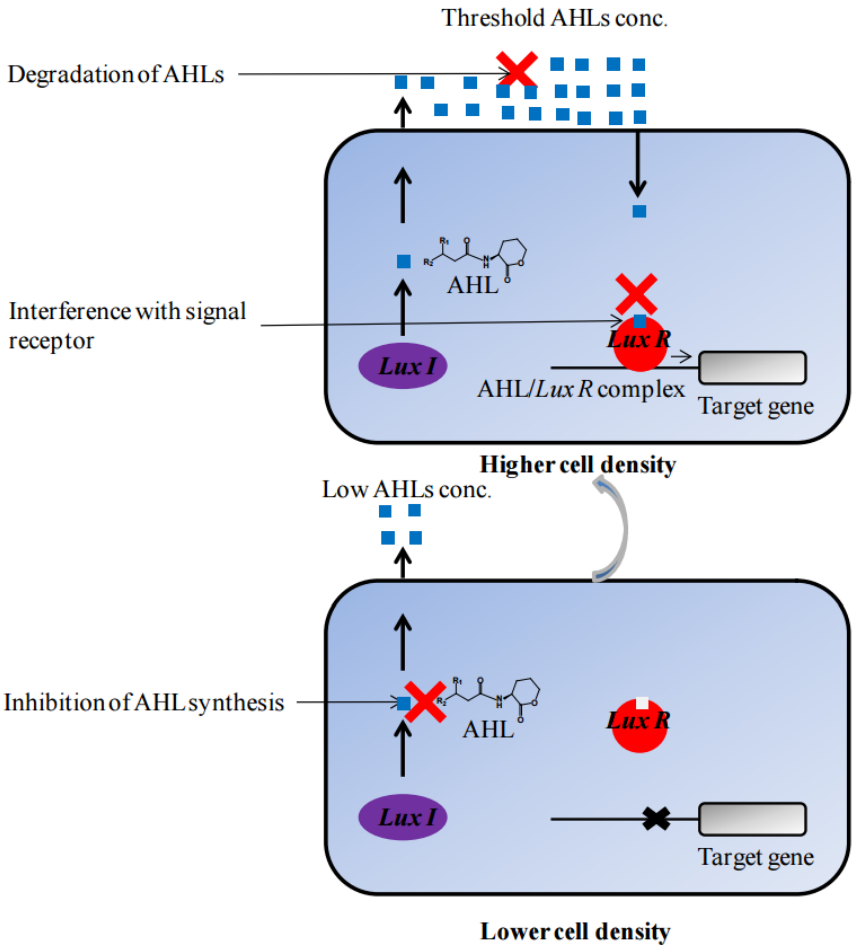


Figure II- 28. Inhibition of quorum sensing in Gram-negative bacteria by various mechanism. Three quorum quenching strategies have been used for attenuating AHL-mediated phenotypes; (i) Inhibition of AHL synthesis (ii) Degradation of AHL signal molecules (ii) Interference with signal molecules. (Lade et al., 2014)

II.3.2. Inhibition of AHL Synthesis

Interference of AHL synthesis is the least investigated strategy among the three listed above due to complexity of the process and preparation of such compounds are often challenging especially when large quantity is to be used. In order to tackle the production of AHLs one needs to understand how the signal molecules are prepared inside of a cell.

Figure II- 29a shows the biochemical reaction pathway for AHL synthesis. AHL signal molecules are synthesized from the acyl-acyl carrier protein (acly-ACP) and S-adenosylmethionine (SAM) by LuxI-type synthases. This catalytic reaction occurs through the two steps as follows: 1) synthesis of intermediate acyl-SAM through transferring an acyl group from acyl-ACP to the amino group of SAM and 2) synthesis of AHL by lactonization of the methionine part, concomitant with the release of methylthioadenosine (MTA) (Parsek et al., 1999, Raychaudhuri et al., 2005). Depending on the acyl side chains, AHLs can have different length and substitution, which confer them with signal specificity (Galloway et al., 2011).

The inhibition of AHL production can be carried out by introducing AHL building block analogs such as L/D-*S*-adenosylhomocystene, sinefungin and butylryl-S-adenosylmethionine (butylryl-SAM) that infere with the AHL synthesis (Parsek et al., 1999). Chemical structure of exemplary AHL precursor analogs are shown in (Figure II- 29b). However, surprisingly, none of these have been test on bacteria *in vivo* and how these analogs would affect cellular function is presently unknown.

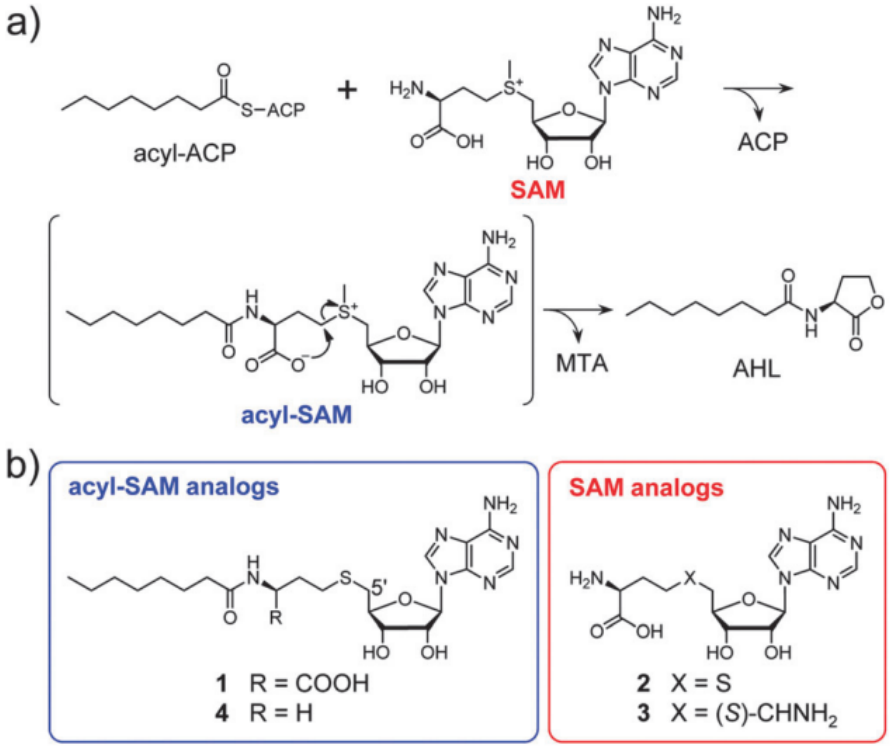


Figure II- 29. (a) Mechanism of AHL production by Lux-type AHL syntheses. (b) Structure of acyl-SAM analogs and SAM analogs. (Kai et al., 2014)

II.3.3. Interference with Signal Receptors

Interference with signal receptors can be done by using an antagonist, a compound having similar structure to signal molecules. The antagonists, due to their structural resemblance to AHLs, outcompete the signal molecules and bind to signal receptors rendering the receptors inactive. (Figure II- 29) The major advantage of this strategy is to control unwanted microbial activity without side effects because the antagonist specifically interferes with expression of specific traits. Many studies have been carried out to identify natural and synthetic analogues of AHLs that function as AHL antagonists. In nature, algae and higher plants have been found to produce substances that inhibit AHL-regulated signaling (Bauer and Robinson, 2002, Givskov et al., 1996). Also, Diketopiperazines (DKPs), which are a family of cylclin dipeptides and found in the supernatant of *P. aeruginosa, Cotrobacter freundii*, and *Enterobacter agglomerans* (Holden et al., 2001) and protein hydrolysates and fermentation broths from yeast, lichen and fungi (Prasad, 1995) are found to act as AHL antagonists in some LuxR based QS systems. In addition, synthetic compounds prepared by adding different substituent in the side chain of 3-oxo-C6 HSL signal molecule is known to displace the native signal from the LuxR receptor. However most of such compounds are found to exhibit agonist effect which limits their use as QS inhibitor (Schaefer et al., 1996). Also, it has been reported that several halogenated furanones interfering AHL based QS by specific interaction with LuxR (Manefield et al., 1999, Manefield et al., 2002).

II.3.4. Degradation of AHL Signal Molecules

Degradation or inactivation of AHL molecules can be achieved through cleavage or modification of the specific sites by quorum quenching enzymes. Five potential cleavage and modification sites in the AHL molecules are presented in (Figure II- 30a) and these leads to either the degradation of homoserine lactone ring or leads to cleavage of acyl chain from AHLs (Figure II- 30b). Currently reported five cleavage/modification mechanisms are carried out by decarboxylase, deaminase, lactonase, acylase, and oxidoreductase having the latter three more investigated. An AHL-lactonase can open the lactone ring (Dong et al., 2001, Dong et al., 2000) while acylase can cleave the acyl side chain from AHL (Leadbetter and Greenberg, 2000, Lin et al., 2003). In addition, oxidoreductase can catalyze the oxidation or reduction of acyl side chain (Chowdhary et al., 2007, Uroz et al., 2005). Meanwhile, an opened ring of AHL molecule by lactonase spontaneously undergoes ring formation at the acidic pH (Camara et al., 2002).

Although these QQ enzymes have been found to interfere with QS, whether AHLs are their primary substrates and the physiological function of these QQ enzymes have not been entirely studied. Some characterized QQ enzymes are shown with their origin and substrate specificity in Table II- 7 (Chen et al., 2013, Fetzner, 2015).

As with other enzymes, it is reported that activity of QQ enzymes are either positively or negatively affected by cofactor or inhibitor, respectively. Chen et al. (2010) reported that Na^{+}, K^{+}, Ca^{2+}, Fe^{3+}, and Mn^{2+} demonstrated positive effect while Cu^{2+}, Cr^{3+}, SDS, Hg^{2+} and Ag^{+} showed significant or complete inhibition of the activity of AHL-lactonase produced from recombinant $AiiA_{B546}$. In addition, the

activity of AHL-lactonase was found to be increased by Mg^{2+}, Zn^{2+}, EDTA at 10 mM but reduced at 1 mM. Li^{+}, Pb^{2+}. Also, *β*-mercaptoethanol improved the enzyme activity at 1 mM but reduced at 10 mM. Moreover, Wang et al. (2004) reported that Mg^{2+}, Ca^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, and Cd^{2+} showed no effect on activity of AHL-lactonase, which was produced from *E. coli* encoding aiiA gene, at 0.2 and 2 mM. Whereas, AHL-lactonase was partially inhibited by Cr^{2+}, Pb^{2+}, and Fe^{2+} at 2 mM and completely inhibited by Cu^{2+} and Ag^{2+} at 0.2 mM. Such dependency of QQ activity on concentration of ionic species should be considered when applying QQ enzymes.

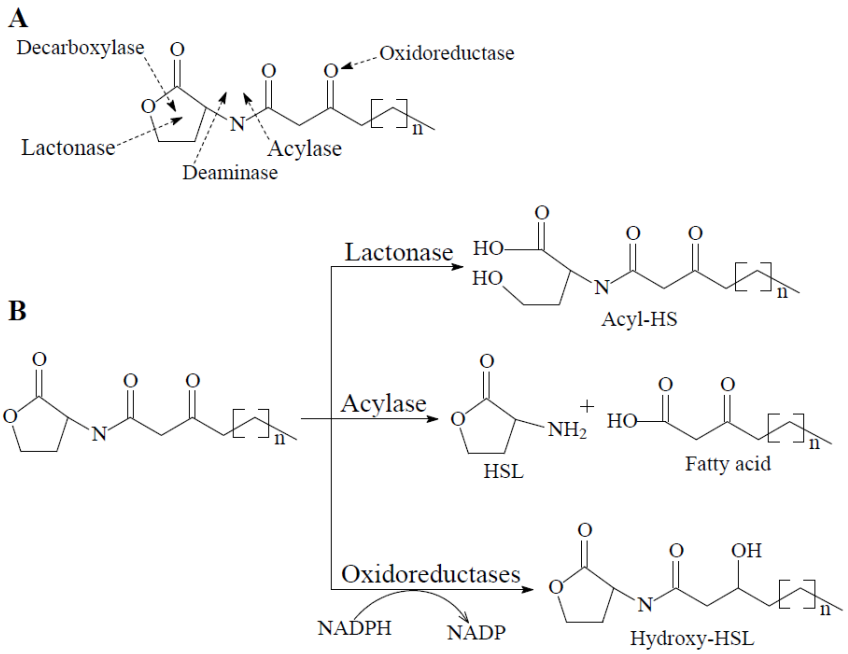


Figure II- 30. Possible linkage degraded by quorum quenching enzymes in quorum sensing molecule *N*-acyl homoserine lactone (A) and corresponding degradation mechanism of quorum quenching enzymes (B). (Dong and Zhang, 2005)

Table II- 7. QQ enzymes involved in the degradation of the QS signal AHLs.(Chen et al., 2013)

Enzyme	Host	Substrate
AHL-lactonase		
AiiA	*Bacillus* sp. 240B1	C6-10-HSL
	Bacillus cereus A24	AHL
	Bacillus mycoides	AHL
	Bacillus thuringiensis	AHL
	Bacillus anthrais	C6, C8, C10-HSL
AttM	*Agrobacterium tumefaciens*	C6-HSL; 3OC8-HSL
AiiB	*Agrobacterium tumefaciens* C58	Broad
AiiS	*Agrobacterium tumefaciens* K84	Broad
AhlD	*Arthrobacter* sp. *IBN110*	Broad
AhlK	*Klebsiella pneumoniae*	C6-8-HSL
QlcA	*Acidobacteria*	C6-8-HSL
AiiM	*Microbacterium testaceum* StLB037	C6-10-HSL
QsdA	*Rhodococcus erythropolis* W2	C6-14-HSL
AhlS	*Solibacillus silvestris* StLB046	C6, C10-HSL
QsdH	*Pseudoalteromonas byunsanensis* 1A01261	C8, C14-HSL; 3OC6-HSL
AidH	*Ochrobactrum* sp. T63	C4, C6, C8, C10-HSL; 3OC6, C8, C10, C12-HSL
PON2	All mammalian tissues	C7, C12, C14-HSL; 3OC6-10,12-HSL
MCP	*M. avium* spp. *Paratuberculosis* K-10	C6, C7, C8, C10, C12-HSL; 3OC8-HSL
BpiB07	Soil metagenome	3OC8-HSL
DlhR	*Rhizobium* sp. NGR234	3OC8-HSL

Table II-6. *(Continued)*

Enzyme	**Host**	**Substrate**
AHL-acylase		
Acylase I	Porcine (Kidney)	C4, C6, C8-HSL; 3OC10, C12-HSL
AibP	*Brucella melitensis 16M*	C12-HSL; COC12-HSL
AhlM	*Streptomyces* sp. M664	C6, C8, C10-HSL; 3OC6, C8, C12- HSL
AiiD	*Ralstonia* sp. Xj12B	3OC6, C8, C10, C12-HSL
PvdQ	*P. Aeruginosa* PAO1	C7, C8, C10, C11, C12, C14-HSL; 3OC10, C12, C14-HSL; 3OH-C14-HSL
HacA	*P. Syringae* B728a	
HacB	*P. Syringae* B728a	C8, C10, C12-HSL; 3OC8-HSL C4, C6, C8, C10, C12-HSL; 3OC6, C8-HSL
AiiC	*Anabaena* sp. PCC7120	C4, C6, C8, C10, C12, C14-HSL; 3OC4, C6, C8, C10, C12, C14-HSL
AiiO	*Ochrobactrum* sp. A44	C4, C6, C8, C10, C12, C14-HSL; 3OC3, C6, C8, C10, C12, C14-HSL
QsdB	Soil metagenome	C6-HSL; 3OC8-HSL
Aac	*Ralstonia solanacearum* GMl1000	C7, C8, C10-HSL; 3OC8-HSL

Table II-6. *(Continued)*

Enzyme	**Host**	**Substrate**
Oxidoreductase		
CYP1041	*Bacillus megaterium*	C12-C16-HSL; 3OC12-HSL
P450BM3	*Bacillus megaterium*	C12, C14, C16 ,C20-HSL; 3OC12, C14-HSL
Not identified	*Rhodococcus erythropolis* W2	3OC8, C10, C12, C14-HSL
Not identified	*Burkholderia* sp. GG4	3OC4, C6, C8-HSL
BpiB09	Soil metagenome	3OC12-HSL

II.3.5. Enzymatic QQ in MBR

[1] Free Enzyme

Yeon et al. (2009a) reported that biofouling in an MBR could be mitigated by adding porcine kidney acylase I (EC-Number 3.5.1.14) into continuous lab-scale MBR. Apart from the reduction in fouling rate the study has found that biofouling in an MBR is proceeded through QS of microorganisms on the surface of the membrane. As shown in Figure II- 30, the QQ enzyme inactivates AHLs by cleaving. However, free enzymes can easily lose their activities due to their low stability under high or low temperature, high shear stress, inactivation by inhibitors and degradation by other microorganisms. Therefore, many researchers applied the QQ enzymes with diverse immobilization into MBR system.

[2] Magnetic Enzyme Carrier (MEC)

In another study by Yeon et al. (2009b), the authors fixed the QQ enzyme, porcine kidney acylase I, on the surface of eveloped magnetic enzyme carrier (MEC) to enhance the stability and recovery of the enzymes. The QQ enzyme fixation procedure is illustrated in Figure II- 31. A magnetic ion-exchange resin (MIEX), which has a net positive surface charge, was used as a magnetic core onto which anionic polyelectrolyte (polystyrene sulfonate, PSS) and a cationic polyelectrolyte (chitosan) were deposited by layer-by-layer (LBL) method. Finally, porcine kidney acylase I was immobilized by cross-linking the enzyme on MIEX-PSS-chitosan substrate using glutaldehyde (GA). This magnetic particle with QQ enzyme immobilized is called MEC. It was shown that fixation of QQ enzymes resulted in a better performance in terms of biofouling control and stability of enzymes than the

application of free enzymes. In addition, magnetic nature of MEC offered convenient method of recovering or separating from the mixed liquor simply by using a magnet. A number of studies followed the explore the advantages imposed by the magnetic carrier. Lee et al. (2014) developed another magnetic carrier based on mesoporous silica. A study by Kim et al. (2013a) anlyzed the effect of MECs on bacterial community and bacterial expression of proteins. They found that population of gram-negative bacteria which utilizes AHL-mediated QS for formation of biofilm was reduced and their EPS production and expression of other biofilm related proteins were decreased (Table II- 8). This suggests that application of MECs successfully inhibit QS and thereby suppress formation of biofilm in an MBR. However, the use of the magnetic particle was regarded economically challenging due to high cost involved in production and purification of enzymes and preparation of the magnetic ion-exchange resin.

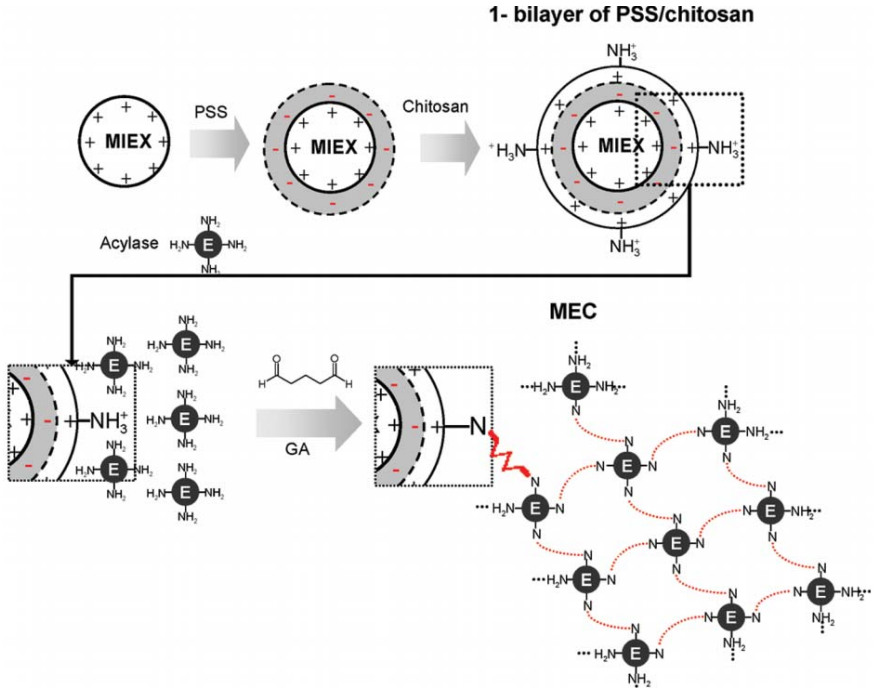


Figure II- 31. Schematic diagram showing the preparation of the MEC through layer-by-layer (LBL) deposition of PSS-chitosan on MIEX resin and enzyme immobilization via glutaraldehyde treatment. (Yeon et al., 2009b)

Table II- 8. Downregulated and upregulated proteins in the biofilm of the quorum quenching channel compared with those of the control channel and their molecular functions. (Kim et al., 2013a)

Downregulated proteins			Upregulated proteins		
Spot number	Protein name	[a]Molecular function	Spot number	Protein name	Molecular function
5	ATP synthase beta subunit (Fragment)	ATPase activity, phosphorylative mechanism, proton-transporting, ATPase activity, rotational mechanism	2	Hemin receptor	Cell outer membrane, plasma membrane, receptor activity, transporter activity
			10	S-(Hydroxymethyl) glutathione dehydrogenase	Oxidoreductase activity, zinc ion binding
	Outer membrane protein TolC	Outer membrane, transporter activity		Acetolactate synthase	Acetolactate synthase activity, flavin adenine dinucleotide binding, magnesium ion binding, thiamine pyrophosphate binding
	Acetolactate synthase	Acetolactate synthase activity, flavin adenine dinucleotide binding, magnesium ion binding, thiamine pyrophosphate binding	12	Acetolactate synthase	Acetolactate synthase activity, flavin adenine dinucleotide binding, magnesium ion binding, thiamine pyrophosphate binding
			23	Glyceraldehyde-3-phosphate dehydrogenase, type I	Oxidoreductase, glucose metabolic process, NAD binding
8	Elongation factor Tu (Fragment)	GTP binding, GTPase activity, translation elongation factor activity			Oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor
9	Putative uncharacterized protein	–	24	Superoxide dismutase	Oxidoreductase, superoxide metabolic process, metal ion binding, superoxide dismutase activity
14	Flagellin	ciliary or flagellar motility, bacterial-type flagellum filament, structural molecule activity			
	Putative uncharacterized protein	–		Acetolactate synthase	Acetolactate synthase activity, flavin adenine dinucleotide binding, magnesium ion binding, thiamine pyrophosphate binding
	Acetolactate synthase	Acetolactate synthase activity, flavin adenine dinucleotide binding, magnesium ion binding, thiamine pyrophosphate binding			
17	Outer membrane protein TolC	Outer membrane, transporter activity			
18	Putative uncharacterized protein	–			
	ATP synthase beta subunit (Fragment)	ATPase activity, phosphorylative mechanism, proton-transporting, ATPase activity, rotational mechanism			
	Outer membrane protein TolC	Outer membrane, transporter activity			
19	Acetolactate synthase	Acetolactate synthase activity, flavin adenine dinucleotide binding, magnesium ion binding, thiamine pyrophosphate binding			
20	Aconitatehydratase 2	Metabolic process, 4 iron and 4 sulfur cluster binding			
	Acetolactate synthase	Acetolactate synthase activity, flavin adenine dinucleotide binding, magnesium ion binding, thiamine pyrophosphate binding			
26	Outer membrane protein OprG	Cell outer membrane, integral to membrane			
	Acetolactate synthase	Acetolactate synthase activity, flavin adenine dinucleotide binding, magnesium ion binding, thiamine pyrophosphate binding			
27	Flagellin	Ciliary or flagellar motility, bacterial-type flagellum filament, structural molecule activity			
	Putative uncharacterized protein	–			
	Sugar fermentation stimulation protein homolog	–			

[a] Molecular function: molecular function of proteins were searched from Protein Knowledge base (UniProtKB), http://www.uniprot.org

[3] QQ Enzyme Immobilization on Alginate Bead

Porcine Kindey acylase I was encapsulated in hydrogel matrix prepared from sodium alginate (Jiang et al., 2013). Cross-linking was performed by extruding the mixture of the acylase and sodium alginate in drop-wise fashion into calcium chloride solution. Na^{+} ions from the sodium alginate was exchanged by Ca^{2+} ions and in doing so, bridges connecting alginate are formed ionically and spherical shaped alginate hydrogel beads were prepared. Subsequently the beads reacted with glutaldehyde (GA) to enhance the mechanical strength and stability They reported that acylase encapsulating beads mitigated the biofouling in MBR and reduced the EPS in both broth and biocake.

II.3.6. Bacterial QQ in MBR

Use of bacteria that continuously produce QQ enzymes are often referred to as QQ bacteria. They are particularly of interest due to greater stability and maintenance of QQ effect. Since MBRs offer a level of nutrients and oxygen required for microbial respiration, these QQ bacteria are able to survive in the bioreactor by feeding off of the feed wastewater. Therefore, application of QQ bacteria is deemed to be more sustainable and economical than that of QQ enzyme.

[1] Isolation of QQ Bacteria and Strains

Typical source of QQ bacteria was in the bioreactor because organisms in the bioreactor for wastewater treatment can utilize feed wastewater as their carbon and nutrient sources. Common isolation technique used in separating QQ bacteria is the enrichment culture. The protocol includes samples to be cultivated in minimal medium containing AHLs (e.g., C4-HSL, C6-HSL or oxo-C6-HSL and so on) as the sole carbon source (Christiaen et al., 2011). After three cycles of such cultivation, the cell suspension was spread on LB agar plate, and different types of colony were isolated. By doing so, bacteria able to break down and utilize AHLs as their carbons source are easily isolated. The final candidates are subjected to AHL-degrading activity test and those which exhibit high QQ activity are identified by DNA sequencing (Kim et al., 2014).

Rhodococcus sp. BH4 and *Bacillus methylotrophicus* sp. WY are QQ bacteria isolated through the enrichment culture technique. They are reported to produce lactonase which is capable of degrading a wide variety of AHL molecules (Khan et al., 2016, Oh et al., 2012). In addition, Cheong et al. (2013) isolated *Pseudomonas* sp. 1A1 which produces the acylase. The authors of above studies

found that lactonase and acylase are intracellular and extracellular enzymes, respectively. The topic of which type of enzyme, either intracellular or extracelluar, is better is a still in debate. However, the two certainly manifests distinct pros and cons. Intracellular enzymes are known to be more stable because they are protected by the cell membrane from inhibitors. On the other hand, extracellular QQ enzymes are openly exposed and able to roam outside environment making it less stable. But such mobility of extracellular enzymes makes them more effective due to enhanced mass transfer.

[2] Whole Cell Application of QQ Bacteria

In a study by Kwon (2016), the author applied directly whole cells of QQ bacterium, *Rhodococcus sp.* BH4 to an MBR. In previous studies, 0.5 ~ 1.0% QQ bead loading was applied with respect to the reactor volume and entrapped BH4 concentration in QQ beads was approximately 5 mg BH4 per mL of QQ bead. Considering the reactor volume of 2.5 L, a lump of 125 mg of whole cell BH4 was applied at the beginning of the experiment and 10 mg of BH4 was injected daily to take into account the loss of BH4 through sludge discharge taking place to maintain SRT of 12.5 days. The author assumed that the change of mass of BH4 in the reactor due to growth and death is negligible. Two MBRs were operated in parallel, one as a conventional MBR with no whole cell BH4 injected and another as QQ MBR in which whole cell BH4 was injected. During the two cycles of TMP jump, TMPs in both reactors rose at a similar rate. Rather, TMP rise up in QQ MBR was slightly faster than that of conventional MBR. This suggests that there was no TMP delay or fouling mitigation by QQ. According to a microbial community analysis by Jo (2016) (Table II- **9**), the proportion of *Rhodococcus sp.* In mixed liquor of 10 real

wastewater treatment plant was approximately 0.0116%. This implies that *Rhodococcus sp.* constitute very small portion in the microbial population in activated sludge and hence, they may not be suited for survival in competitive environment such as mixed liquor. With this information, Kwon et al, concluded that BH4s needs protection in order to sustain its QQ activity in MBRs and thus, entrapment of BH4s in a medium will serve as a better method of applying them.

Table II- 9. Relative proportion (%) of Rhodococcus in the activated sludge from 10 full-scale MBRs. The results of microbial community analysis using Miseq sequencing. (Jo, 2016)

genus	**Full-scale MBR**									
	1	2	3	4	5	6	7	8	9	10
Rhodcoccus	0.001	0.000	0.002	0.006	0.014	0.002	0.002	0.002	0.009	0.078

[3] Biostimulation of QQ Bacteria in MBR

Biostimulation refers to a process in which growth of specific bacteria is stimulated through the addition of specific nutrients or electron acceptors (El Fantroussi and Agathos, 2005). Yu et al. applied the technique of biostimulation by using gamma-caprolactone (GCL) to stimulate AHL-degrading bacteria in a membrane bioreactor. The authors exploited the opportunity to magnify the intrinsic QQ activity of sludge resulting from small population of indigenous QQ bacteria in the mixed liquor (Yu et al., 2016). As a result, the QQ activity in the reactor was increased while AHL level was lowered and EPS concentration was found to be reduced.

Most of the following studies employed the QQ bacterium *Rhodococcus* sp. BH4 for its prominent QQ activity.

II.3.7. QQ Bacteria Immobilizing Media

Although QQ bacteria are more stable than QQ enzymes, immobilization of QQ bacteria is also necessary to keep high concentration of QQ bacteria in the reactor by protecting them from the attack of other microorganisms co-habiting in the mixed liquor. In addition, the immobilization kept QQ bacteria from washed out during excess sludge discharge. A number of different types of immobilization techniques are used as follows.

[1] Microbial-vessel

Oh et al. (Oh et al., 2012) isolated a QQ bacterium, *Rhodococcus* sp. BH4 (shortly designated as BH4) from a real MBR plant. Analysis of reaction product through LC-MS revealed that BH4 produces AHL-lactonase which can decompose a wide range of AHL signal molecules. The authors encapsulated BH4 inside the lumen of hollow fiber membrane as shown in Figure II- 32. The assembly consisting of the hollow fiber membrane entrapping QQ bacteria is called 'Microbial-vessel' or MV. The hollow fiber membranes in the MV acted as a barrier protecting BH4s from the attack of other microorganism in mixed liquor in MBR while retaining BH4s within the lumen because the nominal pore size of the hollow fiber membrane was 0.4 μm which was smaller than the size of BH4 bacterium. Such pore size allowed, signal molecules and nutrients to freely diffuse across the porous membrane and thus BH4s were able to survive and produce AHL-lactonase inside membranes. The MVs were fixed at a position inside membrane tank. Hence, in this configuration, QQ was initiated by diffusion of AHL signal molecules into the vessel. As a result, biofouling in an MBR could be mitigated by the MV and authors reported that the QQ activity

of the MV was maintained for 80 days. Furthermore, Oh et al. (2013) reported that QQ activity of microbial-vessel could be affected by materials of membranes, inner volumes of microbial-vessels and encapsulated mass of BH4. In a later study, Jahangir et al. (2012) reported that the position of the MV in an MBR is an important factor and biofouling mitigation can be enhanced by placing the MV close to filtration membrane. Also, they found that when MVs are placed in aeration tank not in the membrane tank, higher recirculation rate between bioreactor and membrane tank could improve performance of MV. There results indicate that mass transfer of microbial-vessel would be one of the important factors because location of microbial-vessel and recirculation rate could be directly related the mass transfer between signal molecules and the MV. Furthermore, Weerasekara et al. (2014) reported that the microbial-vessel could save the energy consumption in MBR fed with synthetic wastewater by reducing the aeration intensity.

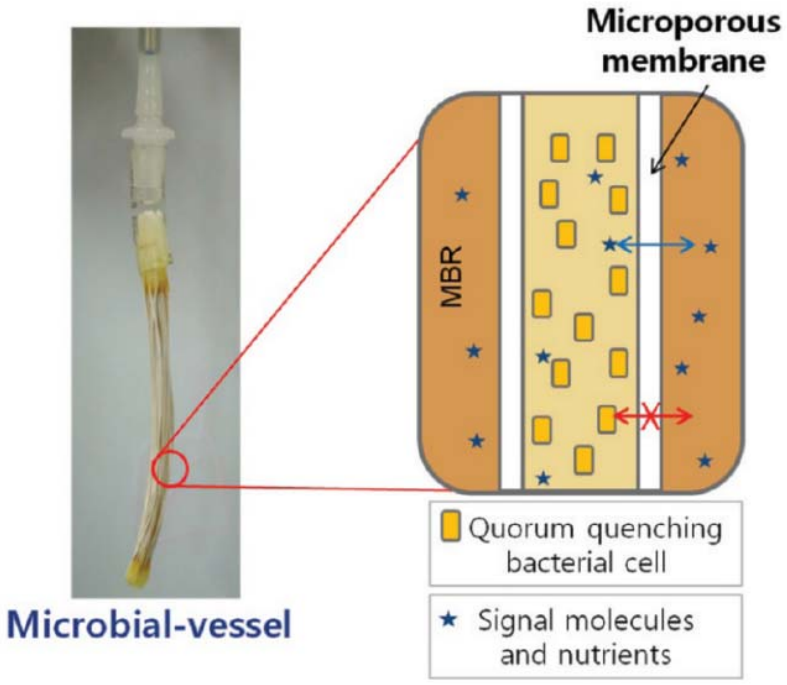


Figure II- 32. Photograph and enlarged diagram of a microbial-vessel. (Oh et al., 2012)

[2] Ceramic-vessel

Cheong et al. (2014) prepared the microbial-vessel with a ceramic membrane, termed as the CMV, to improve viability of encapsulated QQ bacteria inside the lumen of the membranes. The authors considered the food to microorganism (F/M) ratio inside the lumen of MV and pointed that F/M ratio in the MV is quite low and hence the cell viability of encapsulated QQ bacteria could be diminished. They employed a ceramic membrane, instead of previous polymeric hollow fiber membrane, to encapsulate another QQ bacterium, *Pseudomonas* sp. 1A1, and incorporate the 'inner flow feeding mode'. The inner flow feeding directed the piping through which feed wastewater is introduced to the center lumen of the ceramic membrane as shown in Figure II- 33. In this configuration, the concentrated nutrient is firstly accessed and consumed by the encapsulated QQ bacteria in the CMV. As shown in Figure II- 34, the inner flow feeding mode enhanced the viability of QQ bacteria inside the CMV.

[3] Rotating Microbial Carrier Frame

The result from above studies emphasize the importance of mass transfer, either diffusion of nutrient and carbon sources and signal molecules into the microbial-vessels. In an attempt to enhance mass transfer through the vessels, Köse-Mutlu et al. (2016) developed the rotating microbial-vessel (called rotating microbial carrier frame, RMCF). The RMCF consists of a polycarbonate frame with four cubbyholes and a polyvinylidene fluoride (PVDF) flatsheet membrane. The cubbyholes of RMCF were covered with PVDF microfiltration membrane and the interface is sealed with special glue. Concentrated broth of *Rhodococcus* sp. BH4

was infilled into each cubbyholes using a syringe. The assembly was rotated within the reactor by using a rotor and the rotation of RMCF offered improved mass transfer.

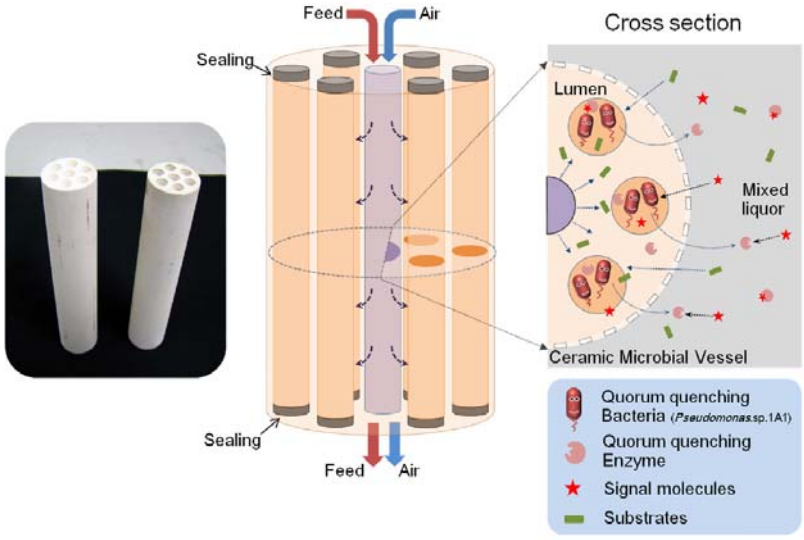


Figure II- 33. Schematic diagram of the ceramic microbial-vessel under the inner flow feeding mode. (Cheong et al., 2014)

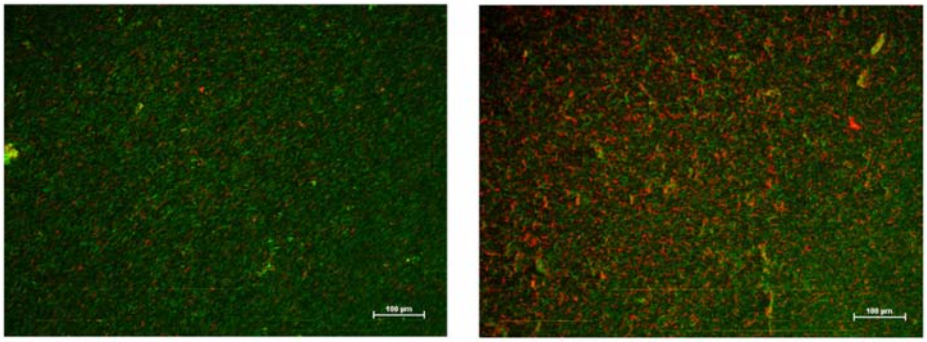


Figure II- 34. Images of live/dead quorum quenching bacteria from the lumens of the used CMVs. (A) MBR-B with the CMV under the inner flow feeding mode, (B) MBR-C with the CMV under the normal feeding mode. Green color: live cell; red color: dead cell. (Cheong et al., 2014)

[4] Alginate bead

Kim et al. (2013b) entrapped the QQ bacteria in a spherical alginate matrix (QQ-bead) which were set mobile in an MBR. This spherical bead had porous microstructure and enough inner space, and then QQ bacteria were entrapped in both inner space and outer surface (Figure II- 35). Apart from the QQ effect coming from entrapment of QQ bacteria, mobility of the QQ-beads facilitated improved mass transfer between QQ media and AHL signal molecules and also physical washing effect was introduced. Physical washing is achieved through collision of beads against the membrane surface and as a result, the biofilm on membrane surface is detached. The combination of QQ and physical washing effects was shown to be highly effective in mitigating biofouling in an MBR as shown in Figure II- 36. Another study by Maqbool et al. (2015) also confirmed the retardation of TMP rise-up in an MBR by QQ-beads. They also found that concentration of AHL molecules and EPS reduced in MBR with QQ-bead compared to those of control MBR.

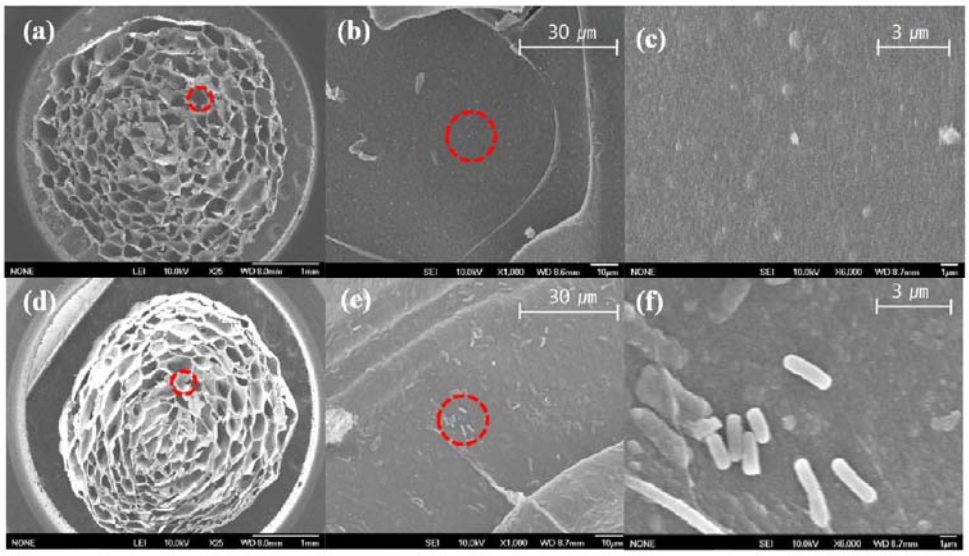


Figure II- 35. SEM microphotographs of the beads: cross section of a vacant bead (a) ×25, (b) ×1000, and (c) ×6000. Cross section of a CEB (d) ×25, (e) ×1000, and (f) ×6000. (Kim et al., 2013b)

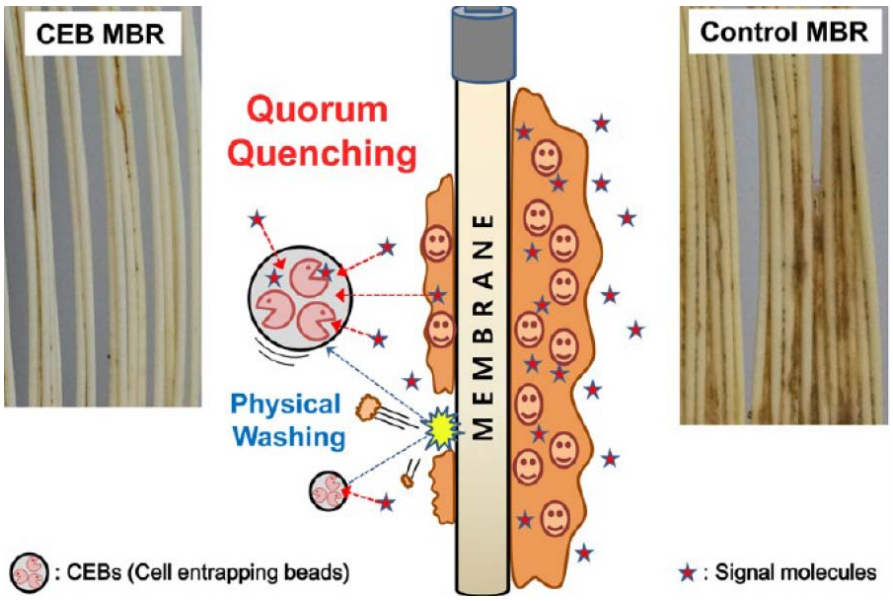


Figure II- 36. Concept of QQ-bead's combined effect of both QQ and physical washing. (Kim et al., 2013b)

In a later study (data not published), it was found that use of alginate based QQ media in real wastewater was hindered by the decomposition of alginate matrix. It was hypothesized that the decomposition of alginate matrix was attributed to the presence of surfactants or non-gel forming ions. In order to increase QQ-beads' lifetime, Kim et al. (2015) reinforced the media by coating the surface with a polymeric layer. The polymeric layer is prepared through the phase inversion method (Figure II- 37) which is the same method used for commercial membrane production. As a result, the coating works as a membrane (Figure II- 38) allowing passage of water, nutrients and dissolved oxygen while retaining the inner material (alginate and QQ bacteria) even when the alginate matrix dissolves. Hence the physical integrity is better-maintained as a consequence. This so-called 'macrocapsule' allowed application of alginate QQ-beads in MBRs fed with real wastewater.

Recently, Lee et al. (2016c) developed a new type of QQ-bead from mixture of alginate and polyvinyl alcohol (PVA). PVA is known to be more resilient to degradation than the alginates. The authors applied such QQ-beads to a pilot-scale real wastewater fed MBR and confirmed maintenance of QQ activity and physical stability up to more than 100 days of operation. Furthermore, reduced biofouling upon application of QQ-bead allowed MBR operation with lowered aeration for membrane scouring. The authors reported that MBR with QQ-beads could save consumption of fouling related energy by 60 %.

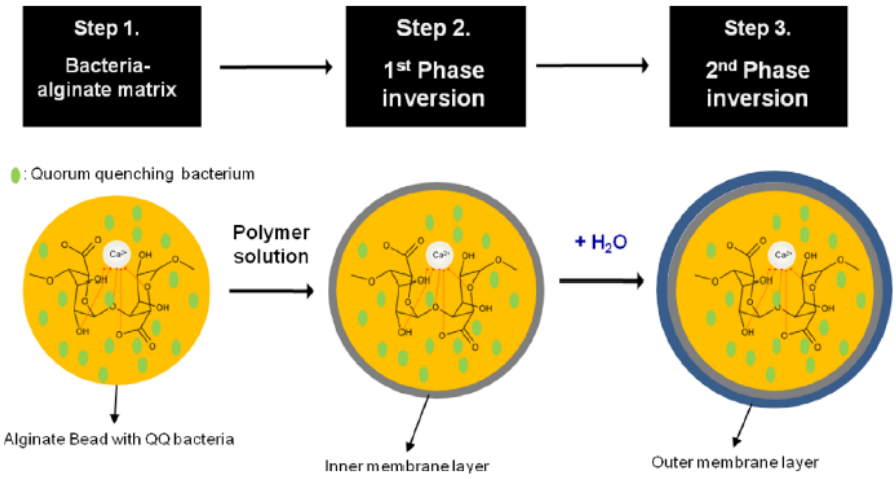


Figure II- 37. Preparation scheme of a macrocapsule coated with a membrane layer through the phase inversion method. (Kim et al., 2015)

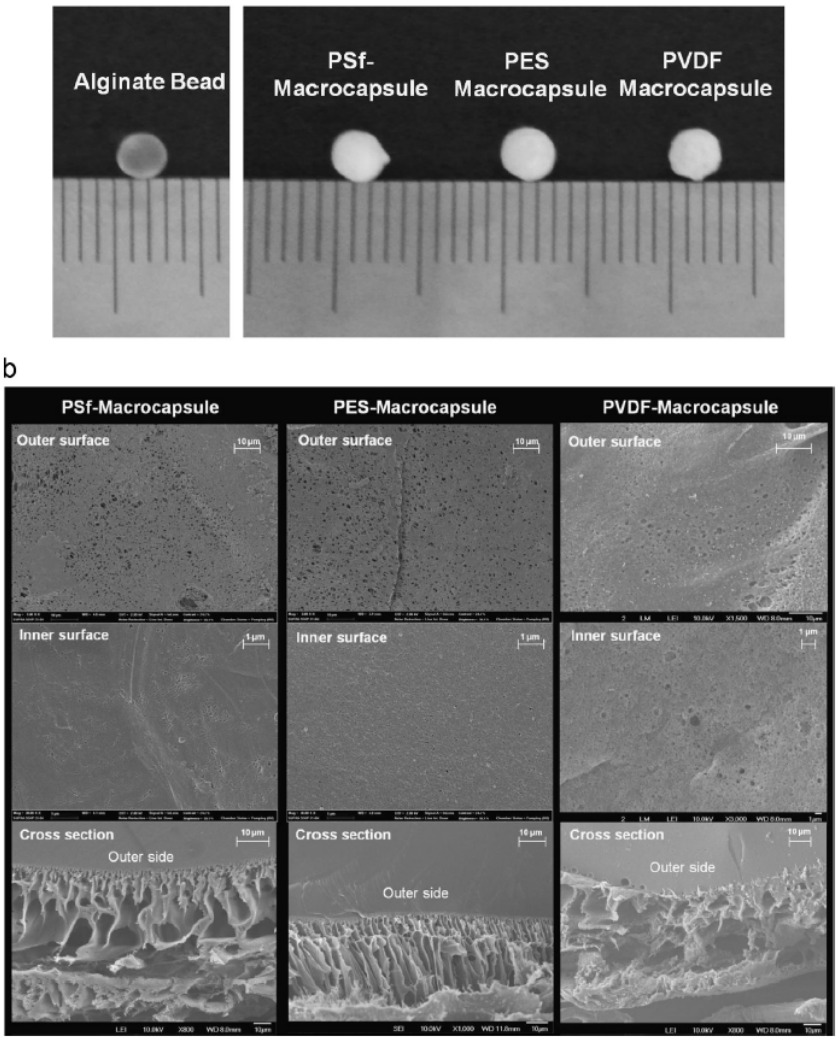


Figure II- 38. (a) Photographs of an alginate bead and PSf, PES, PVDF coated macrocapsules. (b) SEM images of the outer surface, inner surface and cross-section of each macrocapsule coated with PSf, PES, and PVDF, respectively. (Kim et al., 2015)

II.4. Hydrogel for Biotechnology

The terms gels and hydrogels are interchangeably used to describe structures formed with cross-linked polymeric network. Gels are referred to as a substantially dilute cross-linked system, and are categorized principally as weak or strong depending on their flow behavior in steady-state (Ferry, 1961). On the other hand, the term hydrogel describes 3D network structures obtained from a synthetic and/or natural polymers which can absorb and retain significant amount of water (Rosiak and Yoshii, 1999). As such, hydrogels are prepared from hydrophilic polymers.

II.4.1. Classification of Hydrogel

Hydrogels are prepared through a process called gelation. Gelation refers to the linking of polymer chains forming branch structure. At initial stage of gelation, linked polymers slightly lose their solubility forming a structure called ‘sol’. Continuation of gelation further decreases solubility of polymers forming denser network, ‘gel’. This transition from sol to gel is termed ‘sol-gel transition’ (or ‘gelation’) and the critical point at which gel start to appear is call the ‘gel point’ (Rubinstein and Colby, 2003). Gelation can be achieved a number of ways as shown in Figure II- 39. The two types of gelation or cross-linking of polymers are physical and chemical cross-linking. In some instances, combination of the two methods are used.

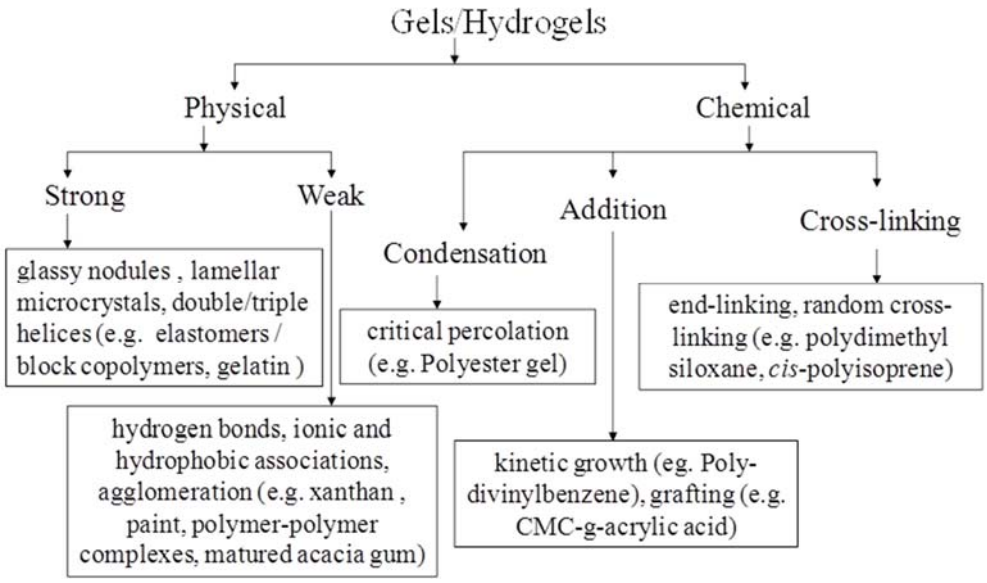


Figure II- 39. Classification of gelation mechanism and relevant examples. (Gulrez and Al-Assaf, 2011)

II.4.2. Physical Cross-linking

Physical cross-linking usually does not involve chemical cross-linkers which could affect the integrity of substances to be entrapped (e.g. microorganisms, enzymes, etc.) as well as removal of them before application. Physical cross-linking can be achieved through heating or cooling a polymer solution, ionically linking polyelectrolytes, formation of H-bonding, and freeze-thawing.

[1] Heating/cooling

Mechanism of heating or cooling to prepare a hydrogel from carrageenan is shown in Figure II- 40. When carrageenan is heated above the melting transition temperature, they take a random coil conformation. However, upon cooling, they transform to a rigid helical rods of which can be cross-linked with ions such as K^+, Na^+, etc. due to screening of repulsive force exerted by sulphonic group in carrageenan(Funami et al., 2007).

Another method of inducing physical cross-linking is heat treatment resulting in the formation of aggregates. For example, gum arabic, a carbohydrate compound contains 2~3% protein, aggregates when heated (Aoki et al., 2007). It is due to the collapse of proteinaceous components forming aggregation transforming the compound to a hydrogel. The process requires a maturation period for the molecules to arrange in a specific conformation to induce intermolecular force to bring together the molecules.

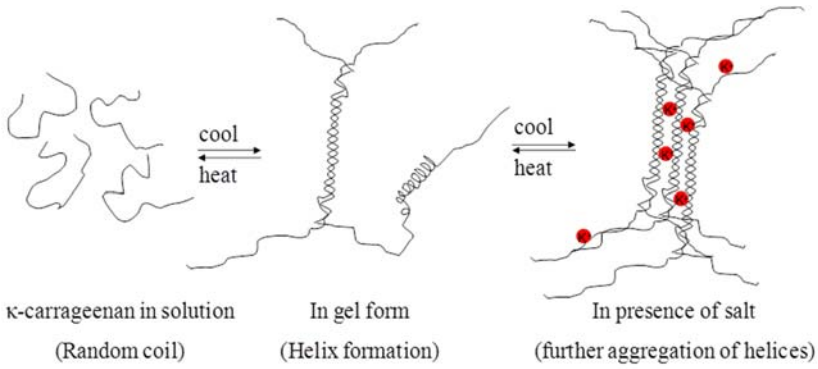


Figure II- 40. Gel formation due to aggregation of helix upon cooling a hot solution of carrageenan. (Gulrez and Al-Assaf, 2011)

[2] Ionic Interaction

Polymers with charged groups can be cross-linked by the addition of di- or tri-valent counter ions. Schematics of ionic cross-linking of alginate, a commonly used building block for hydrogel preparation, is illustrated in Figure II- 41. The linking of polymer chains is done by exchange of monovalent counter ion with multivalent counter ions which act as a bridge between charged sites on polymer chains. Also, complex coaervation methods utilizes electrostatic interaction between oppositely charged polymers (Magnin et al., 2004). Mix of polyanion and a polycation creates complementary charge screening causing them to for an insoluble complex.

[3] H-bonding

H-bonded hydrogel can be obtained by lowering the pH of aqueous solution of polymers carrying carboxyl groups. This methods is typically employed in cross-linking carboxymethyl (CMC) cellulose by dispersing CMC into 0.1M HCl (Takigami, 2007). At a low pH, the sodium in CMC is replaced with hydrogen which forms hydrogen bonding between CMC chains (Figure II- 42). This reduces CMC's solubility in water and result in the formation of an elastic hydrogel.

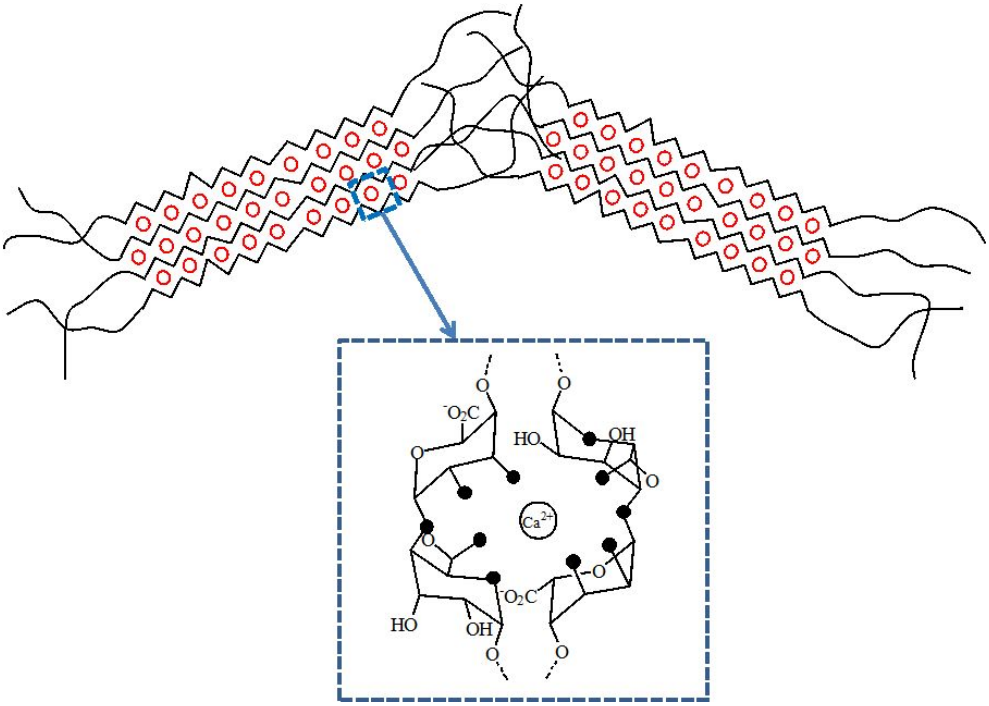


Figure II- 41. Ionotropic gelation by interaction between anionic groups on alginate (COO^-) with divalent metal ions (Ca^{2+}). (Gulrez and Al-Assaf, 2011)

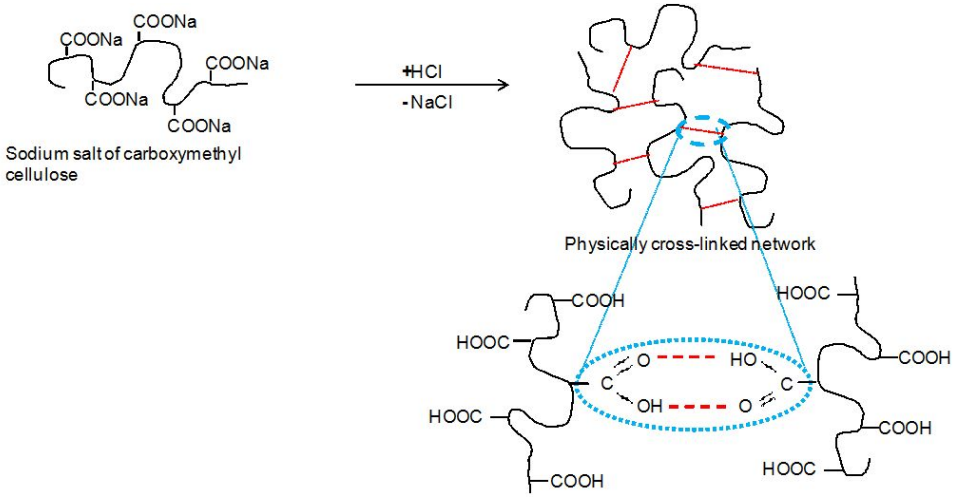


Figure II- 42. Hydrogel network formation due to intermolecular H-bonding in CMC at low pH. (Gulrez and Al-Assaf, 2011)

[4] Free-thawing

A polymer solution gone through a number of freeze and thawing cycle is physically cross-linked. The mechanism involves the formation of microcrystals in the structure due to freeze-thawing. Examples of this type of gelation are freeze-thawed gels of polyvinyl alcohol and xanthan (Giannouli and Morris, 2003, Hoffman, 2002)

II.4.3. Chemical Cross-linking

Unlike physical cross-linking which are typically reversible, chemical cross-linking involves formation of chemical bonds through addition of cross-linkers forming irreversible linkage between polymers. Commonly used cross-linkers include glutaraldehyde (Wu et al., 2007), epichlorohydrin (Chang et al., 2009), etc.. Figure II- 43 shows cross-linking mechanisms of above mentioned cross-linkers. The technique mainly involves the introduction of new molecules between the polymeric chains to produce cross-linked chains. One example of hydrogel preparation via chemical cross-linking is polyvinyl alcohol cross-linked by glutaraldehyde under an acidic condition.

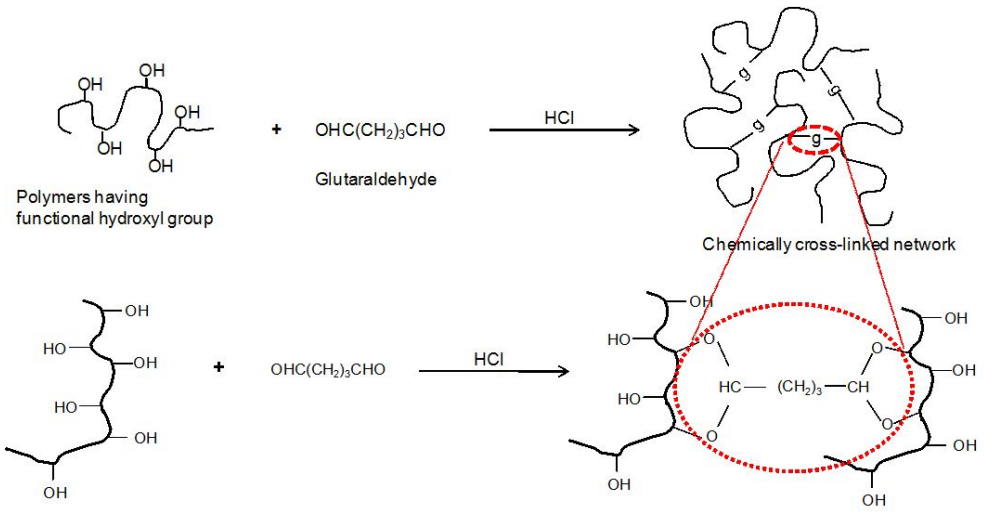


Figure II- 43. Schematic illustration of using chemical cross-linker to obtain cross-linked hydrogel network.(Gulrez and Al-Assaf, 2011)

II.4.4. Application of Hydrogel

Hydrogel prepared from synthetic and natural polymers have been widely used in tissue engineering, pharmaceutical, and biomedical fields (Hoare and Kohane, 2008). Due to their high water absorption capacity and biocompatibility, they have been used in wound dressing, drug delivery, agriculture, sanitary pads as well as trans-dermal systems, dental materials, injectable polymeric systems, ophthalmic applications, hybrid-type organs (encapsulated living cells) (Benamer et al., 2006, Nho et al., 2005, Rosiak et al., 1995, Rosiak and Yoshii, 1999). Table II-10 shows a list of hydrogels and their application.

Table II- 10. List of hydrogel applications and polymers used for synthesis.

Application	Polymers	References
Wound care	polyurethane, poly(ethylene glycol), poly(propylene glycol),	(Rosiak and Yoshii, 1999)
	poly(vinylpyrrolidone), polyethylene glycol and agar	(Benamer et al., 2006, Lugao and Malmonge, 2001, Rosiak et al., 1995)
	carboxymethyl cellulose, alginate, hyaluronan and other hydrocolloids	(Kim et al., 2005, Rosiak et al., 1995, Rosiak and Yoshii, 1999, Walker et al., 2003)
Drug delivery, pharmaceutical	poly(vinylpyrrolidone)	(Benamer et al., 2006, Rosiak et al., 1995)(Benamer et al., 2006; Rosiak et al., 1995)
	starch, poly(vinylpyrrolidone), poly(acrylic acid)	(Kumar et al., 2008, Spinelli et al., 2008)
	carboxymethyl cellulose, hydroxypropyl methyl cellulose	(Barbucci et al., 2004, Porsch and Wittgren, 2005)
	polyvinyl alcohol, acrylic acid, methacrylic acid	(Nho et al., 2005)
	chitosan, αβ-glycerophosphate	(Zhou et al., 2008)
	κ-carrageenan, acrylic acid, 2-acrylamido-2-methylpropanesulfonic acid	(Campo et al., 2009, Pourjavadi and Zohuriaan-Mehr, 2002)
	acrylic acid, carboxymethyl cellulose	(El-Naggar et al., 2006, Said et al., 2004)
Dental Materials	Hydrocolloids (Ghatti, Karaya, Kerensis gum)	(Al-Assaf et al., 2009)

Table II- 10. *(Continued)*

Application	Polymers	References
Tissue engineering, implants	poly(vinylalcohol), poly(acrylic acid)	(Rosiak et al., 1995)
	hyaluronan	(Kim et al., 2005, Shu et al., 2004)
	collagen	(Drury and Mooney, 2003)
Injectable polymeric system	β-hairpin peptide	(Yan et al., 2010)
Technical products (cosmetic, pharmaceutical)	Starch	(Trksak and Ford, 2005)
	gum arabic	(Al-Assaf et al., 2006, Al-Assaf et al., 2007, Katayama et al., 2008)
	xanthan, pectin, carrageenan, gellan, welan, guar gum, locust bean gum, alginate, starch, heparin, chitin and chitosan	(Phillips et al., 2005)
Others (agriculture, waste treatment, separation, etc.)	Starch	(Jeremic et al., 1999, Trksak and Ford, 2005, Yoshii and Kume, 2003, Zhao et al., 2003)
	alginate, polyvinyl alcohol	(Kim et al., 2013b, Lee et al., 2016c, Hashimoto and Furukawa, 1987)

II.4.5. Application of Hydrogel in Wastewater Treatment

There have been many studies which utilized hydrogels for wastewater treatment. Hashimoto and Furukawa (1987) immobilized activated sludge by entrapping them in a PVA hydrogel cross-linked with boric acid. The authors demonstrated degradation of organic carbon and nitrogen in wastewater. Immobilized activated sludge is not only used for degradation of organic matters, but also biosorption of chemicals such as phenol (Aksu and Gonen, 2004).

Application of hydrogel immobilizing microorganism in wastewater treatment is particularly beneficial for nitrogen removal because nitrifiers typically require long SRTs. Immobilization of nitrifiers has shown to improve removal of ammonia in the wastewater (Furukawa et al., 1993, Qiao et al., 2008). Moreover, encapsulation of slow-growing anammox bacteria in PVA cryogel has shown to be effective for deammonification treatment of wastewater (Magri et al., 2012). Apart from cell immobilizing hydrogels, abiotic hydrogels were also used in treating certain species in wastewater. Jin and Bai (Jin and Bai, 2002) have investigated the mechanism of lead adsorption on chitosan/PVA hydrogels. The authors reported that complexation, ion exchange, and electrostatic interaction played roles in adsorption of lead onto the beads.

Recently, quorum quenching (QQ) bacteria entrapping hydrogel media are used in MBRs to mitigate biofouling of filtration membranes. Kim et al. (2013b) prepared QQ-bead from alginate cross-linked with Ca^{2+} for QQ and physical scouring of membrane for inhibition of biofouling. In addition, PVA/alginate composite hydrogel has been used as a carrier for QQ bacteria (Lee et al., 2016c). The addition of PVA enhanced physical stability of the beads which allowed them to

maintain physical and biological stability for over 100 days in pilot-scale MBR fed with real wastewater.

Chapter III

More Efficient Media Design for Enhanced Biofouling Control in a Membrane Bioreactor: Quorum Quenching Bacteria Entrapping Hollow Cylinder

III.1. Introduction

As described in the chapter of literature review, it was shown that QS plays a key role in biofilm formation on the membrane surface in an MBR and it could be inhibited by bacterial QQ typically through immobilizing QQ bacteria in various types of media. This QQ MBR technology opened a new paradigm in biofouling control in MBRs because, unlike conventional post-biofilm control methods, QQ could inhibit the biofilm formation at its developmental stage.

Series of recent studies have immobilized QQ bacteria in hollow fiber membranes (microbial vessel, MV) (Oh et al., 2012), ceramic membranes (ceramic microbial vessel, CMV)(Cheong et al., 2014), rectangular frame prepared from flat sheet membranes (rotating microbial carrier frame, RMCF)(Kose-Mutlu et al., 2016), and spherical hydrogel beads (QQ-bead)(Kim et al., 2013b) which were set mobile in the membrane tank. These studies highlighted the importance of mass transfer between QQ bacteria and QS signal molecules and maintenance of cell viability. In addition, incorporation of physical washing of membrane through contact between mobile QQ-beads and the membrane could further mitigate fouling in an MBR.

However, all of the above-mentioned studies have neglected the interaction between QS signal molecules and QQ media, i.e., the mass transfer and reactions of QS signal molecules across and within the QQ media. Such information could be highly valuable in developing more efficient QQ bacteria entrapping media. Especially, QQ bacteria entrapped at the core of QQ-beads may exert negligible QQ activity towards overall AHL degradation of the media. In addition, physical washing effects of mobile media could have more rooms for enhancement.

Hence, in this study, the route by which QS signal molecules come into

contact with QQ bacteria entrapped in a hydrogel medium was monitored using a special reporter strain (E. coli JB525), and based on this result, a new geometry of QQ media, i.e., a QQ hollow cylinder, was designed. A quorum quenching hollow cylinder (QQ-HC) was proposed as a more efficient QQ medium than a QQ-bead in MBRs because it was expected to have higher QQ activity and a greater physical washing effect (i.e., detachment of accumulated biomass from membrane surface through scouring upon contact) due to its shape. To confirm this, biological and physical aspects of a QQ-HC in biofouling control were assessed in batch scale in comparison with QQ spherical beads and among HCs having the same outer, but different inner, diameters. Lastly, the anti-biofouling ability of QQ-HCs was evaluated further in continuous MBRs.

III.2. Materials and Methods

III.2.1. Preparation of QQ-Beads and QQ-HCs.

For preparation of both QQ-beads and QQ-HCs, *Rhodococcus* sp. BH4 was used as the QQ bacterium because it has been reported to produce N-Acyl homoserine lactone (AHL)-lactonase, which is capable of decomposing a wide variety of signal molecules of AHLs(Oh et al., 2012). Consequently, its QQ ability in an MBR for wastewater treatment has been demonstrated in a number of previous studies(Oh et al., 2012, Kose-Mutlu et al., 2016, Weerasekara et al., 2014, Kim et al., 2013b, Kim et al., 2015, Oh et al., 2013, Maqbool et al., 2015). Spherical media (QQ-beads) were prepared as described in previous work12. Hollow cylindrical media with (i.e., QQ-HC) and without (i.e., vacant-HC) QQ bacteria were prepared from a mixture of polyvinyl alcohol (Wako, Japan) and sodium alginate (Junsei, Japan). BH4 grown in Luria-Bertani (LB) broth was centrifuged (6500 g, 15 minutes, 4 °C) and resuspended with deionized (DI) water. The concentration of BH4 was measured by analyzing its dry mass in the resuspended solution (Michael L. Shuler, 2001). In detail, 0.10 g of BH4 resuspended solution was dropped onto a disk filter (GF/C, Whatman, US), which was then dried in an oven at 105 °C for 1 hour. For a QQ-HC, the aliquot amount of BH4 was mixed with the polyvinyl alcohol-sodium alginate mixture to yield final a concentration of 7 mg BH4 (by dry weight) per g of QQ-HC (by dry weight) whereas, for a vacant-HC, an equivalent volume of DI water was added to the polymeric mixture instead of BH4. Cross-linking of the polymer mixture was done by extruding it through a nozzle into a CaCl2 (Daejung, Republic of Korea) and boric acid (Samchun, Republic of Korea) solution as shown in Figure

III- 1. Using various sizes of spinneret outlets (2.0 - 2.5 mm for outer flow and 0.8 - 2.0 mm for inner flow), cylinders and HCs, with similar outer diameters but different inner diameters, were prepared as shown in Figure III- 2. The flow rate of the polymer and cross-linking solutions were adjusted by syringe pumps (KDS Legato 100, KD Scientific, US) to yield linear velocities of fluids of 30 - 35 mm/s. The densities of all kinds of media were approximately 1.01 (± 0.01) g/mL. Also, the unit mass of each medium was approximately 0.035 (± 0.001) g, 0.163 (± 0.007) g, 0.153 (± 0.002) g, 0.123 (± 0.003) g, and 0.109 (± 0.001) g for bead, cylinder, HC (Thick), HC (Med), and HC (Thin), respectively. Furthermore, their surface was observed through a scanning electron microscope (SEM, JSM-6701F, Jeol, Japan). Prior to observation, all media were freeze-cut in liquefied nitrogen followed by freeze-drying at – 50 °C and - 6.7 kPa.

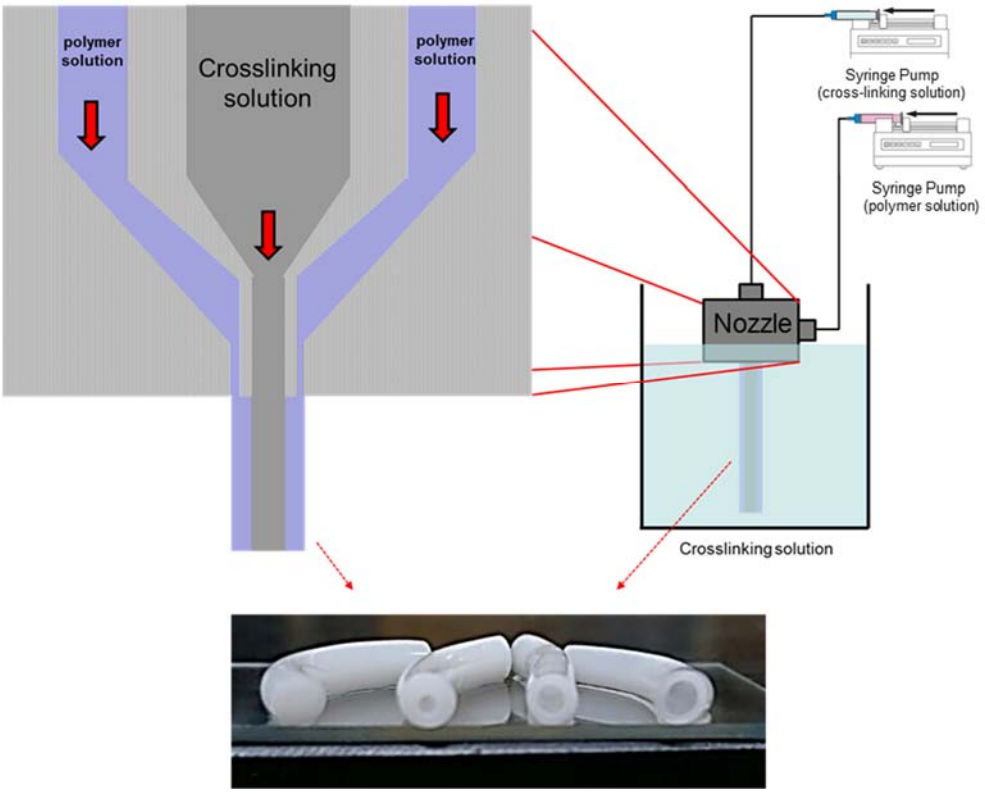


Figure III- 1. Schematics of QQ-HC preparation.

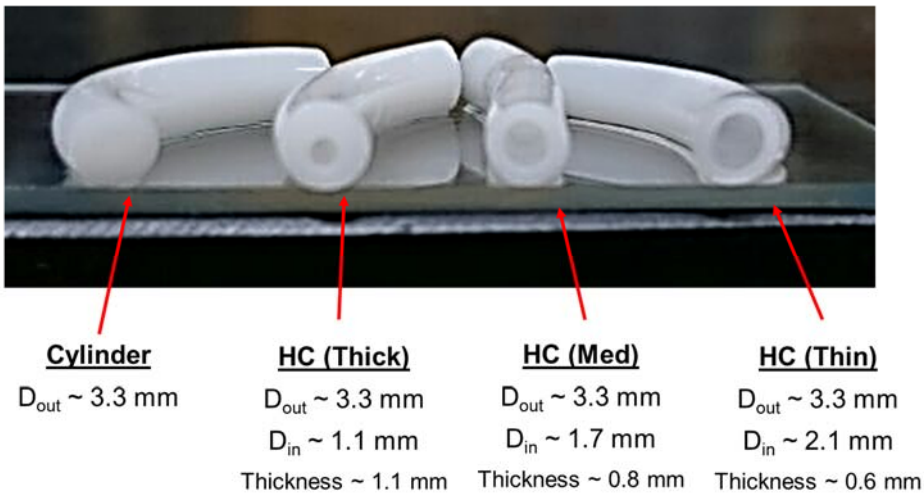


Figure III- 2. Prepared cylindrical media with different inner diameters and thicknesses but fixed outer diameter.

III.2.2. Visualization of QS Signal Molecules in QQ-beads

To visualize the signal molecule within the media, *E. coli* JB525, which produces green fluorescence protein (GFP) upon intake of a range of AHLs (Wu et al., 2000), was used. Two types of beads were prepared, one with only JB525 entrapped and another with both BH4 and JB525 entrapped, following the procedure described in the previous section. The beads were then exposed to 1 μM N-Octanoyl-DL-homoserine lactone (C8-HSL) in 20 mM phosphate buffer (PB) at pH 7 with 1/10 diluted Luria-Bertani (LB) broth for 2 hours and their cross sections were observed with a fluorescence microscope (Eclipse E600, Nikon, Japan). Similarly, the JB525 only and JB525 and BH4 together entrapping HCs were reacted with 1 μM C8-HSL and then their cross sections were observed with a confocal laser scanning microscope (CLSM, SP8 X, Leica, Germany). The control experiment with whole cells of BH4 and JB525 was conducted as follows. An overnight culture of JB525 or BH4 was centrifuged (6500 g, 15 minutes, 4 °C) and resuspended to one tenth its original volume with 1/5 diluted LB broth. To yield a final concentration of C8-HSL of 0 or 1 μM, 0.5 mL BH4 or JB525 resuspended solution or the mixture of 0.25 mL BH4 and 0.25 mL JB525 resuspension solutions was mixed with 0.5 mL of 0 or 2 μM C8-HSL dissolved in 20 mM PB at pH 7. The mixture was left to react on an orbital shaker at 70 RPM and 25 °C for 2 hours and observed with a fluorescence microscope.

III.2.3. Assessment of QQ Activity

QQ activity of the media was analyzed in terms of the degradation rate of commercially available C8-HSL (Sigma-Aldrich, US), a QS signal molecule. The concentration of this molecule was measured by using the reporter strain *A. tumefaciens* A136 as described in previous studies (Kim et al., 2013b, Lee et al., 2016c). C8-HSL was used as the model QS signal molecule in this experiment because it was previously reported to be one of the major QS signal molecules (Yeon et al., 2009a). In detail, QQ media, prepared by fixing either their volume at 1.0 mL or their number at 10 pieces, were added to 20 mL of 1 μM C8-HSL dissolved in 20 mM PB (pH 7) and left to react on an orbital shaker at 70 RPM and 25 °C. After 60 minutes, 0.5 mL of the solution was sampled. The sample was filtered using a 0.45 μm syringe filter and then immediately immersed in a 100 °C water bath for 15 minutes. The C8-HSL concentration was measured through an A136 bioassay as follows. The reporter strain *A. tumefaciens* A136 and samples were mixed and loaded into a 96-well plate, which then was incubated at 30 °C for 90 minutes. The Beta-Glo® Assay System (Promega, US) was added to the wells, and the plate was stored at 25 °C for 30 minutes. The luminescence intensity was measured by a luminometer (Synergy 2, Bio-Tek, US) and the AHL concentration was determined by plotting the calibration curve of standard C8-HSL. The QQ activity was presented as the number of nmoles of C8-HSL degraded per min, which was measured over 60 minutes.

III.2.4. Assessment of Physical Washing Effect

The physical washing effect of a prepared medium was assessed in a batch reactor (working volume of 3 L) with concentrated synthetic wastewater and 5 mL

activated sludge inoculum (Figure III- 3). Instead of using an actual membrane module, a polycarbonate (PC) plate module of identical physical dimensions was used. Four PC coupons were placed on the PC plate. The batch reactors were operated with or without vacant media at an aeration rate of 1.5 L/min. After 24 hours, the biofouled PC plate was taken out of the batch reactor and stained with 0.1% wt/vol crystal violet and dried for 24 hours. Crystal violet on the coupons was dissolved in ethanol by separately immersing the coupons in 10 mL ethanol solution for 30 minutes. The crystal violet concentration in the ethanol solutions was measured using a spectrophotometer set at 570 nm. The optical density values of the four coupons from one PC plate were averaged and their standard deviation was calculated and plotted as an error bar.

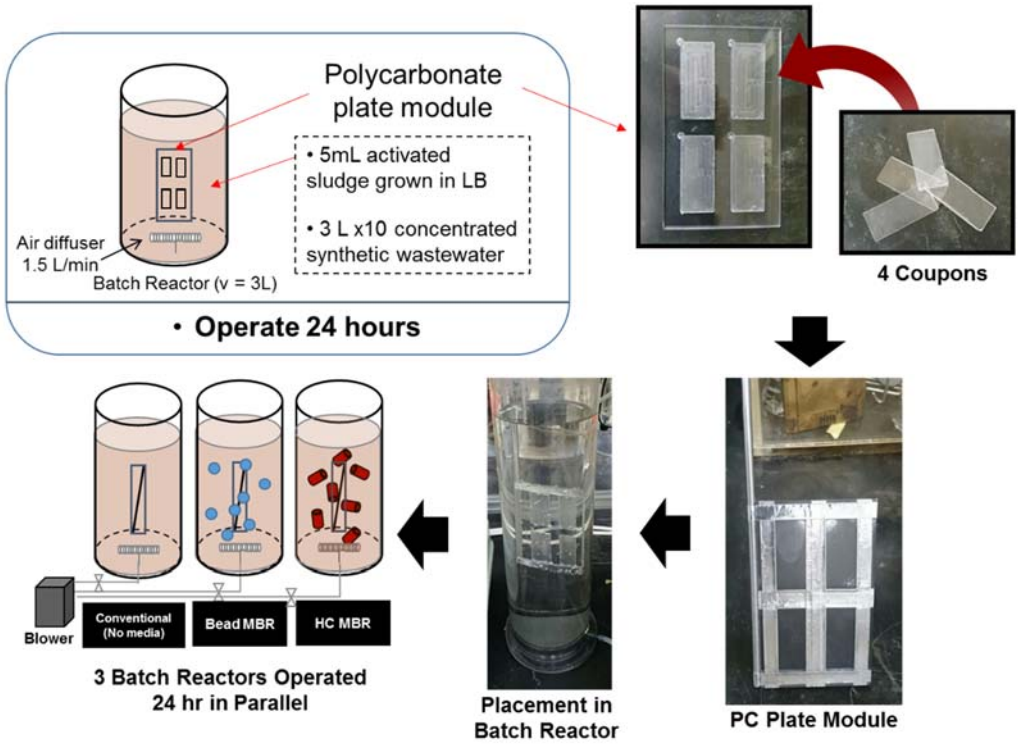
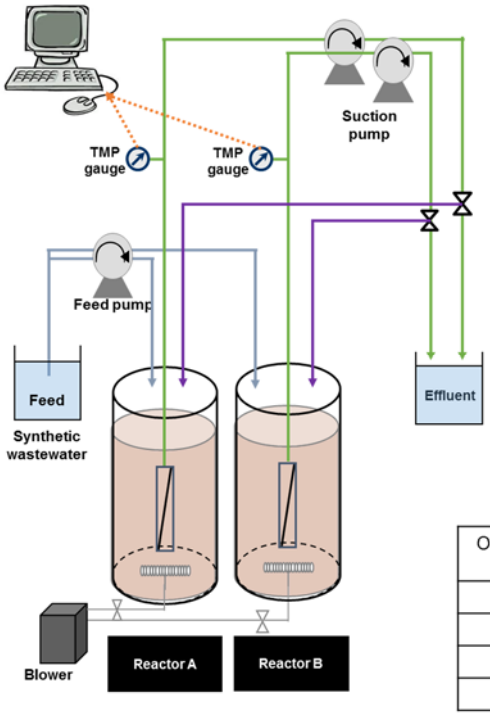


Figure III- 3. Illustration of experimental procedure in assessing physical washing effect of media.

III.2.5. MBR Operation

Two MBRs, Reactor A and Reactor B, were operated continuously in parallel, as shown in Figure III- 4. Activated sludge was taken from a wastewater treatment plant (Tancheon, Korea) and acclimatized until the MBR operating parameters stabilized. The concentration of media applied to each reactor was 1% (v/v – volume of media / volume of reactor) unless stated otherwise. Operation of the two MBRs was performed in four different phases, as listed in the lower table in Figure III- 4. The composition of the synthetic wastewater in this experiment was as follows (all in mg/L)(Oh et al., 2012): glucose, 400; yeast extract, 14; bactopeptone, 115; $(NH_4)_2SO_4$, 104.8; KH_2PO_4, 21.75; $MgSO_4$, 15.63; $FeCl_3$, 0.075; $CaCl_2$, 2.45; $MnSO_4$, 1.8; and $NaHCO_3$, 255.5. A hollow fiber membrane (ZeeWeed 500, GE-Zenon, U.S.) with an effective filtration area of 186 cm^2 was used for filtration. The MBRs were operated at constant flux of 20 liter per m^2 per hour (LMH) with an aeration rate of 2.0 L/min. Membranes that became fouled during operation were taken out of the reactor and cleaned by soaking them in 1000 ppm NaOCl for 3 hours. Prior to Phase 1 (Figure III- 4), the mixed liquors of the two reactors (A and B) were mixed and then divided equally back into Reactors A and B. The trans-membrane pressure (TMP) in each reactor was monitored to estimate and compare the extent of biofouling in the MBRs. For comparison of anti-biofouling performance of QQ-HCs in an MBR at each operation phase, the average number of days for one TMP-Jump to occur at 40 kPa (T_{TMP40}), was calculated by dividing the operation period in days by the number of TMP-Jumps during the operation period.



Operating conditions

Working volume (L)	3
SRT (day)	30
HRT (hour)	8
Membrane area (cm^2)	186
Flux ($L/m^2/h$)	21
Aeration (L/min) - disk type	2.0
Feed COD (mg/L)	480 - 527
COD removal efficiency (%)	Broth: 95 - 96 Permeate: 97 - 98
MLSS (mg/L)	4720 - 6240

Operation Phase	Reactor A	Reactor B
1	Conventional-MBR	Conventional-MBR
2	QQ-HC-MBR	Conventional-MBR
3	QQ-HC-MBR	Vacant-HC-MBR
4	QQ-HC-MBR	QQ-Bead-MBR

Figure III- 4. Schematic, operating conditions, and operation phases of the two of MBRs.

III.2.6. Detection of AHLs in MBR

Detection of AHLs in an MBR was performed by bioassays using reporter strains *A. tumefaciens* A136 and *E. coli* JB525. AHLs were extracted from mixed liquor and biofilm samples containing 1.6 g of biomass by dry weight. For the extraction of AHL from mixed liquor, mixed liquor was directly contacted with ethyl acetate (EA) in a volumetric ratio of 1: 1/3 (mixed liquor : EA) for 2 min. The EA phase was retrieved. This process was repeated 2 more times and the collected EA phase was evaporated by using a rotary evaporator. The residue was re-suspended in a 20 mM PB at pH 7 and AHL content was measured using A136 bioassay as described in earlier section. For the extraction of AHL from biofilm, biofilm was suspended in 300 mL of 20 mM PB at pH 7 and the biofilm suspension was subjected to AHL extraction as described above.

For detection of AHLs in-situ, mixed liquor and overnight grown culture of JB525 (OD_{600} = 0.5) were mixed (one to one by volume) and incubated at 25 °C for two hours. A small drop of sample was placed on a slide glass, covered with a cover glass, and observed with a fluorescence microscope.

III.2.7. Analytical and Statistical Methods

Mixed liquor suspended solids (MLSS) and chemical oxygen demand (COD) were measured according to standard methods (Keith, 1998). The QQ activity and the physical washing effect of different media were compared by a one-way analysis of variance (ANOVA) test or a one-tailed t-test assuming unequal variance.

III.3. Results and Discussion

III.3.1. Interaction between QQ-beads and QS Signal Molecules

To design a more efficient QQ medium, the interaction mechanism between QS signal molecules and QQ media when they came into contact with each other needed to be studied. In other words, it was necessary to look deeply at how QS signal molecules cross the medium, diffuse toward the medium's core, and react with QQ bacteria entrapped in such a hydrogel medium. For this analysis, two types of bacteria entrapping beads were prepared: 1) beads entrapped with only the AHL reporter strain, *E. coli* JB525 and 2) beads entrapped with both JB525 and the AHL decomposing QQ bacteria BH4. Because the bacteria and polymer solution were mixed thoroughly before being shaped into beads, BH4s were likely to be well distributed throughout the entire area of a bead. This was confirmed by observing a cross section of the media using a CLSM (Figure III- 5). When JB525 reacts with AHL, it produces GFP, which can be visualized using a fluorescence microscope. On the other hand, GFP expression was not observed when BH4 was exposed to C8-HSL (Figure III- 6). Thus, by observing the cross section of a JB525 entrapping bead exposed to AHLs, diffusion of QS signal molecules through pure bead material without the QQ bacteria, BH4, can be monitored. In other words, whether AHLs can diffuse freely within the bead can be verified visually. Furthermore, by observing the cross section of JB525 and BH4 entrapping beads exposed to AHLs, diffusion of the AHLs to be decomposed by BH4 can be monitored.

The two types of beads were exposed to 1 μM C8-HSL in 20mM PB (pH 7) with 1/10 diluted LB for 2 hours and then cross sections of the beads were observed with a fluorescence microscope. Figure III- 7 shows the cross-sectional images of the near surface (Figure III- 7a) and center (Figure III- 7a') regions of JB525 only entrapping beads. In Figure III- 7a & a', the bright green spots represent expression of GFP by JB525, indicating the presence of QS signal molecules (C8-HSL). The near surface (Figure III- 7a) and center (Figure III- 7a') regions are expressing GFP, indicating free diffusion of signal molecules within the bead. However, in beads entrapped with both JB525 and BH4, a thin bright band of fluorescence is observed along the surface only (Figure. 3b), which rapidly fades towards the center, leaving only a few bacteria expressing GFP (Figure III- 7b'). For quantitative comparison, we quantified the area of GFP expression from images in Figure 3 using ImageJ software. All color images in Figure 3 were first converted to 8 bit image (black and white) and then threshold values were set to 50 and 255 for all images. % area occupied by GFP, expressed by JB525 in each image, was calculated using the Analyze Particles tool. The results were 16% and 3.8% for Figures 3a and 3b, respectively, and 2.2% and 0.18% for Figures 3a' and 3b', respectively. This indicates that GFP was expressed much more in the JB525 only entrapping bead (Figure III- 7a & a') than in the JB525 and BH4 together entrapping bead (Figure III- 7b & b'). Consequently, we could conclude that C8-HSL concentration was higher in the JB525 only entrapping bead than in the JB525 and BH4 together entrapping bead.

Analyzing the results from observing the two types of beads, the presence of BH4 in a bead seems to limit the diffusion of QS signal molecules into the bead, not by exerting resistance to diffusion but by rapidly decomposing the signal molecules

at the outer surface of the bead. In other words, even though AHLs can diffuse freely to the center of a bead, BH4 located near the surface decomposes most of the QS signal molecules, leaving only a few AHLs available for degradation at the center, and thereby depriving the QQ bacteria located deep in the medium of opportunities to encounter and degrade QS signal molecules. Therefore, QQ bacteria located in the inner part of a medium may appear to have no QQ activity. This result also implies that the surface area of a QQ medium is a more dominant parameter in QQ activity over total mass of entrapped QQ bacteria. Based on this, a QQ-HC was designed and prepared as a new QQ medium. Preparation of two types of HCs, one entrapping JB525 only and the other entrapping JB525 and BH4 together and exposure of them to 1 μM C8-HSL HC yielded the same result: most AHLs were degraded near the surface (Figure III- 8). As such, a QQ-HC's high surface area per unit medium volume was expected not only to enhance QQ activity but also to enable the efficient use of QQ bacteria in the medium. Furthermore, a QQ-HC could be separated more easily from activated sludge microbial flocs than a QQ-bead could, due to its relatively longer length. A QQ-HC was also expected to have a greater physical washing effect because the contact site between a QQ-HC and the membrane surface would be a line, whereas that between a QQ-bead and the membrane surface is a point.

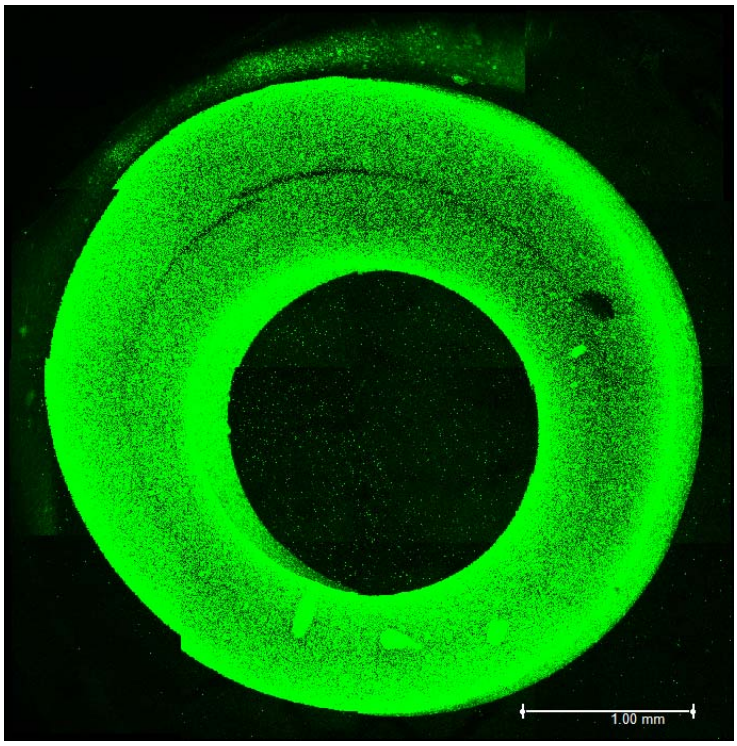


Figure III- 5. CLSM image of BH4s in a cross-section of a QQ-HC stained with a LIVE/DEAD *Bac*Light Bacterial Viability Kit.

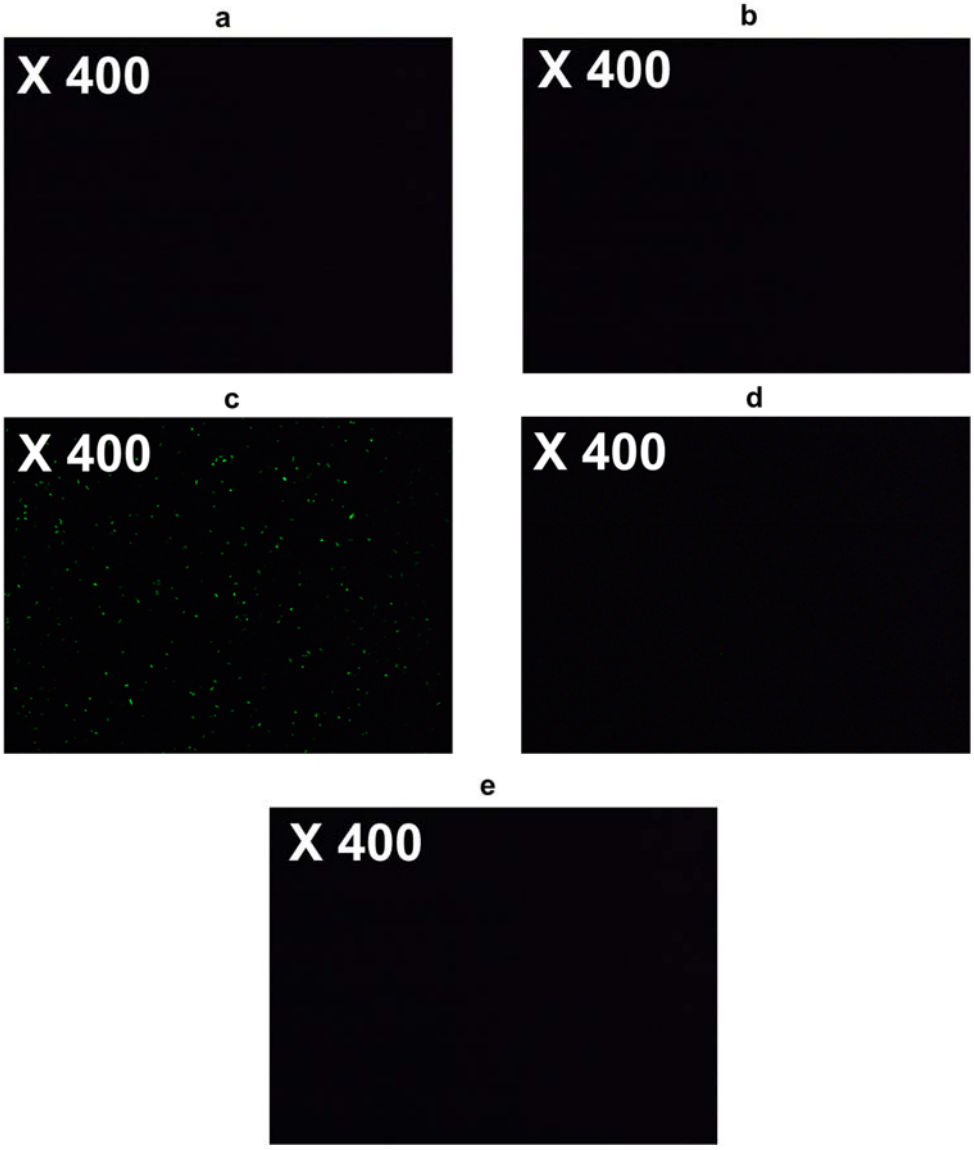


Figure III- 6. Test of fluorescence expression by BH4 and JB525. a: whole cell BH4 with 1 μM C8-HSL; b: whole cell BH4 with 20 mM PB (0 μM C8-HSL); c: whole cell JB525 with 1 μM C8-HSL; d: whole cell JB525 with 0 μM C8-HSL; and e: mixture of whole cells of BH4 and JB525 with 1 μM C8-HSL.All samples were observed with a fluorescence microscope.

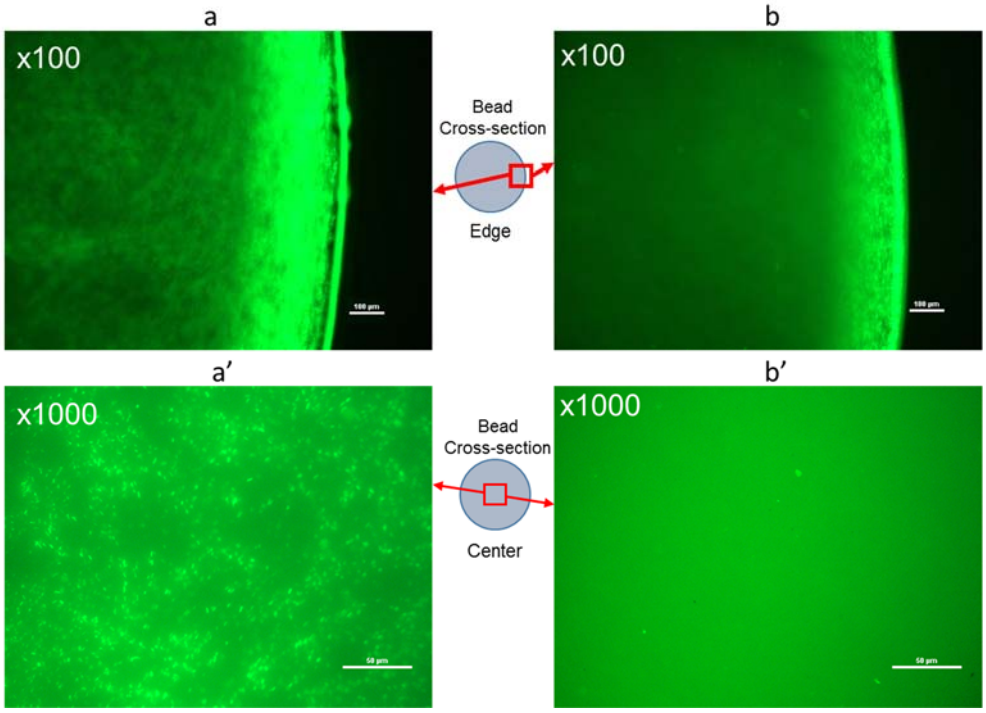


Figure III- 7. Fluorescence images of bead cross sections entrapped with JB525 only (a: near surface and a': center) and with both JB525 and BH4 (b: near surface and b': center).

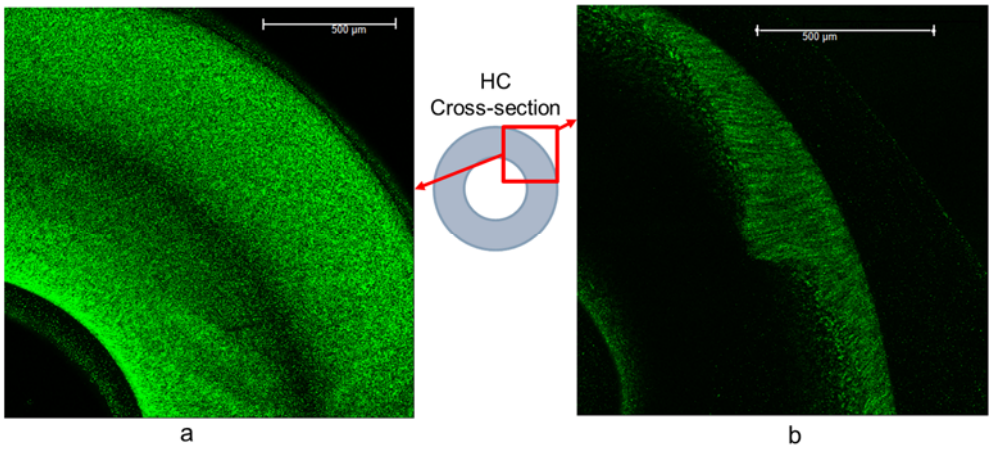
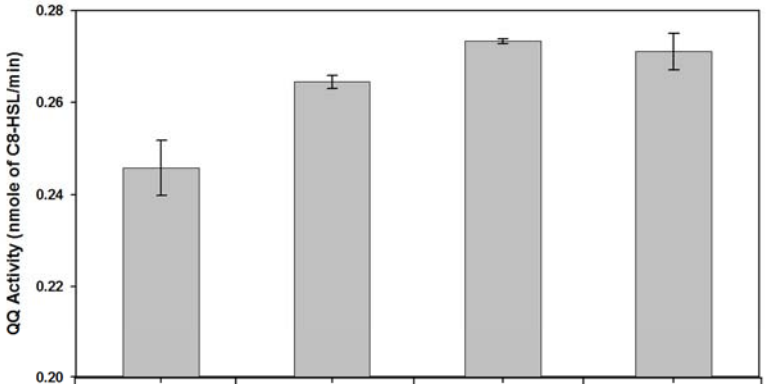


Figure III- 8. CLSM images of the cross-section of a: JB525 entrapping HC and b: JB525 and BH4 entrapping HC obtained from 2-hour after exposure to 1 µM C8-HSL.

III.3.2. QQ Activity of Inner Part of QQ Media

To confirm how those expectations based on the fluorescence image analysis may turn out, four different QQ cylindrical media with various inner diameters but fixed outer diameters were prepared as follows: 1) Cylinder: outer diameter(D_{out}) ~ 3.3 mm, inner diameter (D_{in}) 0 mm; 2) HC (Thick): D_{out} ~ 3.3 mm, D_{in} ~ 1.1 mm; 3) HC (Med): D_{out} ~ 3.3 mm, D_{in} ~ 1.7 mm; and 4) HC (Thin): D_{out} ~ 3.3 mm, D_{in} ~ 2.1 mm. The QQ activities of each medium were compared to one another (Figure III- 9). Because the media were prepared with the homogeneous mixture of BH4 and polymer solution, BH4 was considered to be distributed well throughout the medium. As the inner diameter increases, the proportion of inner material (i.e., BH4 plus polymer) decreases, because the volume of material used for media preparation decreases as surface area increases. Thus, as the inner diameter increases, the amount of entrapped BH4 decreases. Nevertheless, the QQ activity shows an increasing trend as the inner diameter of the cylinder increases as shown in Figure III- 9. More specifically, as the inner diameter increases from that of a QQ-HC (Thick) to that of a QQ-HC (Med), the QQ activity was enhanced ($p < 0.05$, one-tailed t-test with unequal variance), whereas the QQ activity of a QQ-HC (Thin) was not greater than that of a QQ-HC (Med) ($p > 0.05$, one-tailed t-test with unequal variance). It can be hypothesized that an increase in the total surface area due to an increase in inner diameter up to that of a QQ-HC (Med) enhanced QQ activity of the medium to a greater degree than the loss of QQ activity resulting from the reduction in the total amount of entrapped QQ bacteria. In other words, in the range of an inner diameter increase from that of a QQ-cylinder to that of a QQ-HC (Med), the surface area of the QQ medium might be a more dominant parameter for QQ activity over

total mass of entrapped QQ bacteria, which coincides with the result from Figure III-7, predicting a negligible contribution towards QQ activity from the QQ bacteria located deep within the medium. However, the QQ activity of a QQ-HC (Thin) had a similar value to that of a QQ-HC (Med). Considering that pore distribution on surface of the media is similar (Figure III- 10), it seems that, at this point, the contribution towards QQ activity from the amount of entrapped QQ bacteria became significant, exceeding that from surface area. In other words, there is a balancing point between the amount of entrapped QQ bacteria and surface area that maximizes the QQ effect of a QQ medium.



	Cylinder	HC (Thick)	HC (Med)	HC (Thin)
Cross-section	3.3 mm	1.1 mm, 3.3 mm	0.8 mm, 3.3 mm	0.6 mm, 3.3 mm
Number of media	10	10	10	10
Media volume (mL)	1.63	1.53	1.21	1.07
Entrapped QQ bacteria (mg)	~ 11.4	~ 10.7	~ 8.5	~ 7.5
Total surface area (cm^2)	~ 17	~ 28	~ 31	~ 35

Figure III- 9. Comparison of QQ activity between four different QQ cylinders that have the same outer diameter but different inner diameters. Error bar: standard deviation (n = 3).

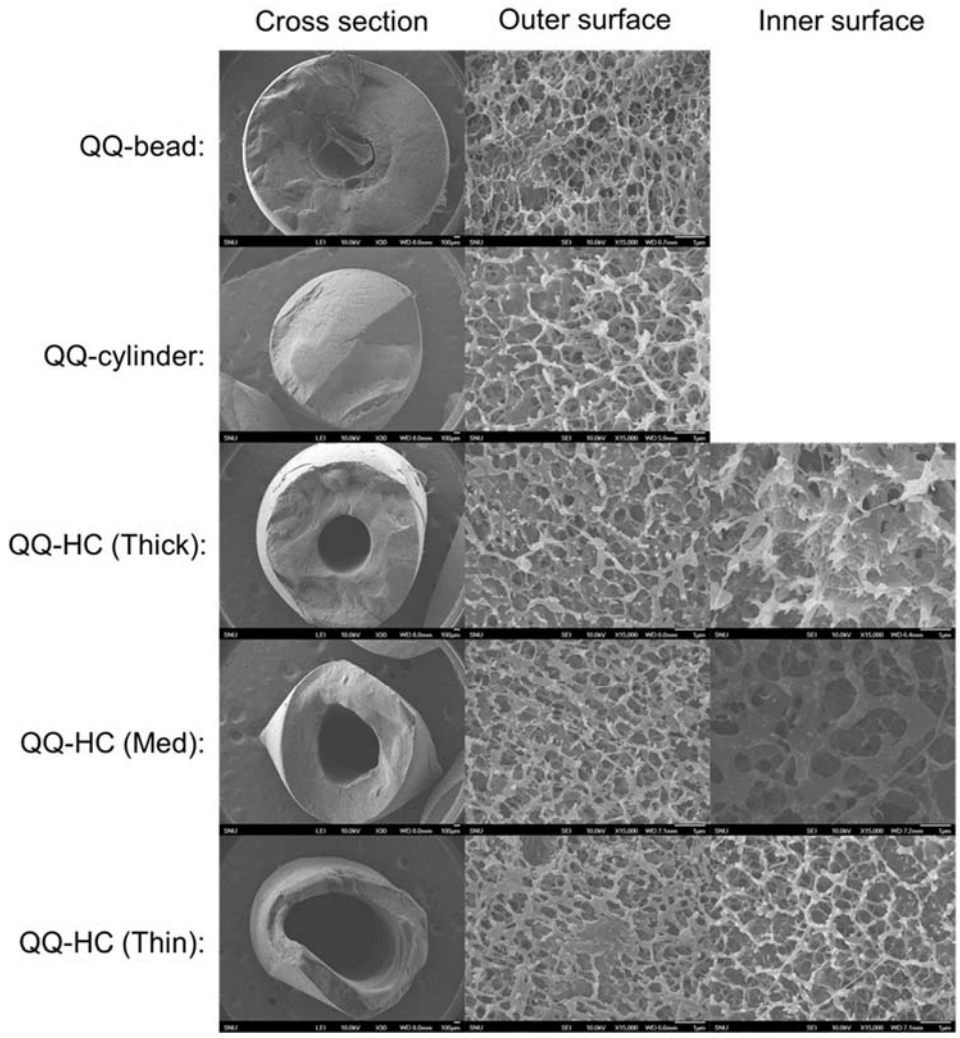


Figure III- 10. SEM images of various QQ media after freeze-drying. Left column: cross-section of media (magnification: X 30); center column: close up (X 15,000) of outer surface; right column: close-up (X 15,000) of inner surface.

III.3.3. Distribution of AHL QS Signal Molecules in MBR

In this section, hypothetical view on AHL concentration in an MBR and AHL mass transfer is discussed, combining previously published studies and results from this study.

Sources of AHL in an MBR can be divided into two: 1) microorganisms in mixed liquor and 2) microorganisms in biofilm. Figure III- 11 shows AHL level in biofilm and mixed liquor of an MBR. AHL extract samples from biofilm and mixed liquor were 500 times concentrated for analysis The AHL level is expressed in term of relative luminescence unit from *A. tumefaciens* A136 bioassy. Given that analyzed biomass was fixed to 1.6 g, AHL level was significantly higher in biofilm than in mixed liquor. AHL level in the extracted sample from mixed liquor was undetectable which coincides with the result from a previous study. However, in-situ detection of AHL with JB525 reporter strain resulted in positive response as shown in Figure III-12 which is also evidenced in a study by Song et al. (2014). This indicates that small amount of AHLs does exist around microbial flocs in the mixed liquor. Furthermore, previous study by Jahangir et al. (2012) demonstrated that when a microbial vessel is inserted in a bioreactor, increase in recirculation rate between the bioreactor and membrane tank enhanced fouling mitigation by the microbial vessel. Also, fouling is further reduced when the microbial vessel is inserted in the membrane tank, i.e. closer to the membrane.

Above data suggests that AHL concentration is the highest on the membrane surface and lowest in the mixed liquor generating a concentration gradient of AHL shown in Figure III- 13.

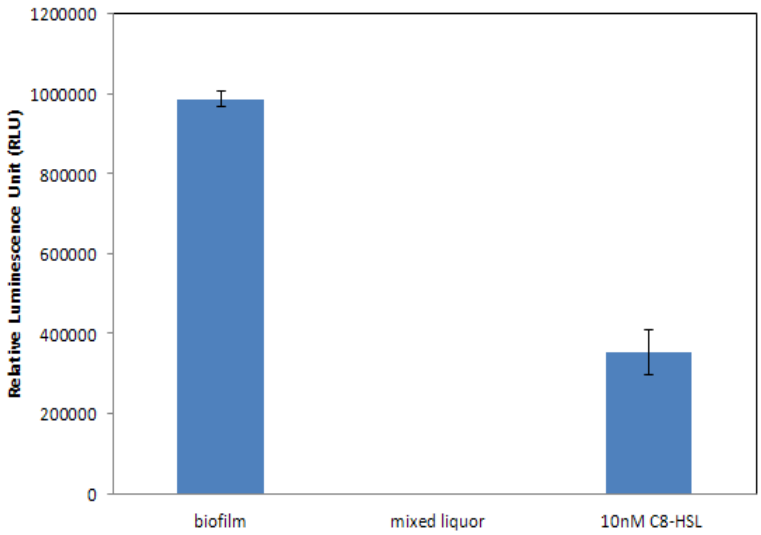


Figure III- 11. Comparison of AHL level in biofilm and mixed liquor. A. tumefaciens A136 bioassay was used to estimate the AHL concentration in terms of relative luminescence unit. 1.6 g of biomass samples were used for extraction of AHL. Error bar: standard deviation, n = 3.

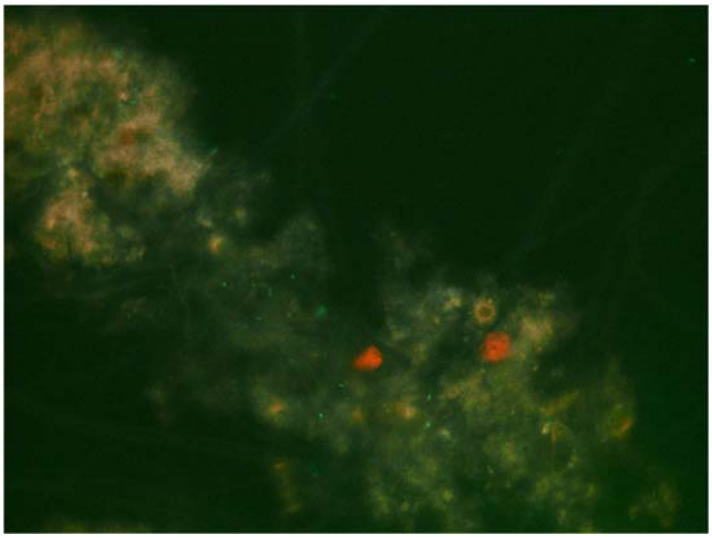


Figure III- 12. Detection of AHL in a floc of mixed liquor using JB525. A sample of mixed liquor was mixed with JB525 and observed with a fluorescence microscope after 2 hours of incubation. The green spots represent expression of GFP or presence of AHLs.

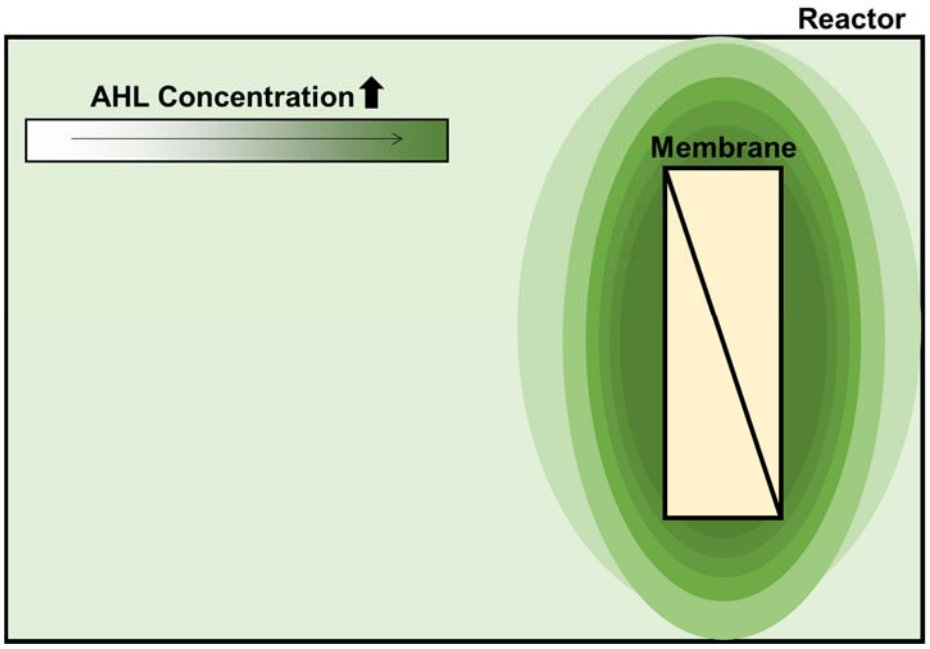


Figure III- 13. A hypothetical description of AHL concentration gradient in an MBR.

Considering above hypothesis on AHL concentration gradient in an MBR, one can seek to enhance QQ effect in an MBR by a QQ medium in two ways: 1) by facilitating mixing in the reactor thereby dispersing AHLs in the biofilm and increasing AHL concentration in the bulk or mixed liquor so that diffusion into QQ media is increased, and 2) by enhancing AHL degradation performance of a QQ medium. Since the former involves increasing the aeration rate, i.e. additional input of energy, this strategy was not considered. Enhancing AHL degradation performance of a QQ medium can be achieved by either increasing surface area or reducing mass transfer resistance within the medium. Although, reducing mass transfer resistance within the medium could be achieved by increasing pore size of the medium but such task requires meticulous tuning of pore size because too large pore size may result in leakage of QQ bacteria. Furthermore, given the current preparation method, it was confirmed that AHL degradation rate was faster than the diffusion rate as shown in Figure III- 7, thus, QQ bacteria entrapped in the core region of a QQ-bead were stripped off of the opportunity to degrade AHL signal molecules.

Therefore, enhancing surface area of a QQ medium through making an open structure was proposed as a more viable option in our study. As a result, a hollow cylindrical medium was developed. As shown in Figure III- 8, it was able to degrade AHL signal molecules not only at its outer surface but also at its inner surface and as a consequence it showed greater QQ activity than QQ-bead did.

It was interesting to note that the enhancement in QQ activity resulting from the hollow cylindrical shape of QQ-HC resembles the application of raschig ring in fluidized beds, distillation columns in the field of chemical engineering (Barker, 1965, Costa et al., 1986). It was reported that hollow cylindrical particles possess very high surface area to volume ratio, hence they can facilitate higher mass transfer rate (Jena et al., 2009).

III.3.4. Comparison of QQ Activity between QQ-HCs and QQ-beads

The QQ activity of a QQ-HC was compared with that of a QQ-bead because the latter has been often reported in studies on QQ MBRs(Lee et al., 2016c). The volume of the media was fixed at 1 mL, which corresponds to approximately 7 mg of the entrapped BH4. Then the degradation rate (moles/min) of QS signal molecules (C8-HSLs) by the media was measured over a 60-minute period. The result indicates higher QQ activity for HCs than for beads (Figure III- 14) ($p < 0.05$, one-tailed t-test with unequal variance). Because the media volume was the same for both media, the HC resulted in a higher total surface area due to its greater "surface area to volume ratio" at the given dimension. Because higher surface area can facilitate higher mass transfer of signal molecules through the media, the higher surface area of HC is thought to enhance its QQ activity over that of a bead.

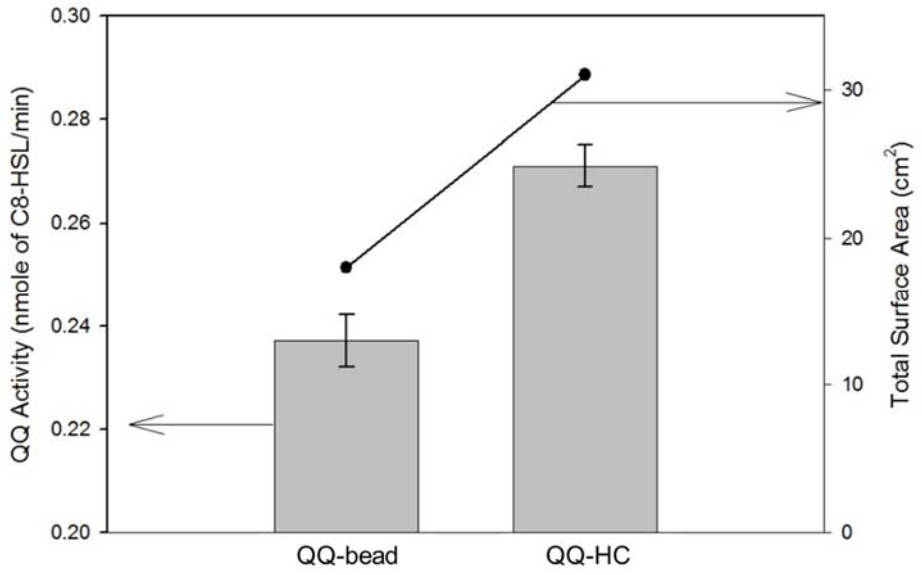


Figure III- 14. Comparison of QQ activity (columns) and surface area (line) between QQ-beads (diameter ~ 3.3 mm) and QQ-HCs (inner diameter ~ 2.1 mm, outer diameter ~ 3.3 mm, and length ~ 20 mm). Error bar: standard deviation (n= 3).

III.3.5. Comparison of Physical Washing Effect between Vacant-HC and Vacant-Bead

As QQ media circulate all the time together with activated sludge under the aeration environment in an MBR for wastewater treatment, they may reduce biofouling, not only through biological QQ, but also through collisions with and removal of attached biocakes on a membrane surface (i.e., physical washing) (Kim et al., 2013b, Kim et al., 2015, Maqbool et al., 2015, Lee et al., 2016c). Figure III-15 shows the comparison of physical washing effect between vacant-beads (diameter ~ 3.3 mm) and vacant-HCs (inner diameter ~ 2.1 mm, outer diameter ~ 3.3 mm, and length ~ 20 mm) at the same loading volume of 1% of bioreactor volume. Three batch reactors were operated in parallel with ten-fold concentrated synthetic wastewater and 5 mL activated sludge inoculum. The same medium loading volume of 1% (v/v) resulted in 780 pieces of vacant-beads and 280 pieces of vacant-HCs. The reactors were run with aeration for 24 hours, after which the PC module was taken out of each reactor and stained with crystal violet.

The y-axis represents the amount of biofilm formed, derived from measuring the concentration of crystal violet, which is proportional to the amount of biofilm attached to a PC plate. The statistical analysis showed that the physical washing effects of the three cases were different at medium loading of 1% (v/v) ($p < 0.05$, one-way ANOVA test). In detail, the conventional reactor, having no physical washing due to a lack of mobile media, had the highest amount of biofilm on the PC plate, whereas the reactor with vacant-beads had the second highest amount of biofilm and the reactor with vacant-HCs had the lowest amount of biofilm despite the fact that the number of HCs was approximately more than three times fewer than

the number of beads. This result is indicative of the greater cleaning efficiency of HCs, possibly due to HCs' larger contact area with the membrane surface because of their cylindrical geometry. In addition, the mass per unit medium of an HC (0.109 g/HC) was greater than that of a bead (0.035 g/bead). Hence, HCs may have had higher impact momentum than beads, which facilitated the detachment of biofilms that were more strongly adhered to the PC coupon. Recollecting that the inner part of QQ media had trivial contribution towards QQ activity, we became interested in testing whether the inner part of a medium would influence its physical washing efficiency.

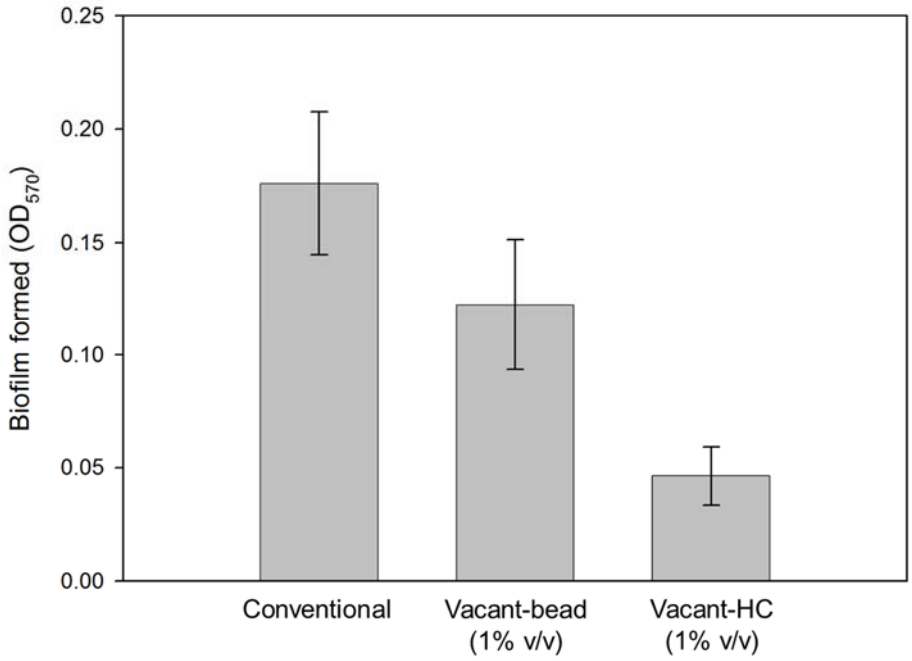


Figure III- 15. Comparison of physical washing effect between vacant-beads (diameter ~ 3.3 mm) and vacant-HCs (inner diameter ~ 2.1 mm, outer diameter ~ 3.3 mm, length ~ 20 mm) at the loading volume of 1% of the bioreactor. Error bar: standard deviation (n = 4).

III.3.6. Assessment of Physical Washing Effect of Inner Part of QQ Media

To examine closely whether the inner part of a medium influence its physical washing effect, vacant-cylinders (D_{in} = 0), vacant-HCs (Thick) (D_{in} = 2.1 mm), and vacant-HCs (Thin) (D_{in} = 1.1 mm) were prepared and their physical washing effects were assessed (Figure III- 16). For this comparison, instead of loading every medium at 1% (v/v), the same number of pieces (178), which translates into a net volume of 30 mL (1% (v/v)) of vacant-cylinder, 26 mL (0.9% (v/v)) of vacant-HC (Thick) and ~ 17 mL (0.6% (v/v)) of vacant-HC (Thin), for each medium loaded. The result shows there was no significant difference in physical washing effect among the cylinders regardless of their inner diameter size ($p > 0.05$, one-way ANOVA test). It is interesting to note that the inner material of a mobile medium does not seem to contribute significantly towards physical washing of a membrane. The hydrogel used to prepare QQ media and the MLSS have wet densities that are very close to that of water (approximately 1.0 g/mL) and the cylindrical media have almost the same outer dimension (D_{out} ~ 3.3 mm and length ~ 20 mm) as the HC media. Consequently, the filling the lumen of HC with MLSS may make the effective mass of the HC similar to the mass of a cylinder, thereby rendering the impact momenta of an HC and a cylinder indifferent. In addition, their similar outer diameter (~ 3.3 mm) and length (~ 20 mm) may result in similar membrane contact areas. For such reasons, the physical washing effects of HCs and a cylinder may not have seemed different. However, further analysis would be required to confirm the exact mechanism and key parameters in physical scouring of a membrane surface by mobile media in a submerged MBR.

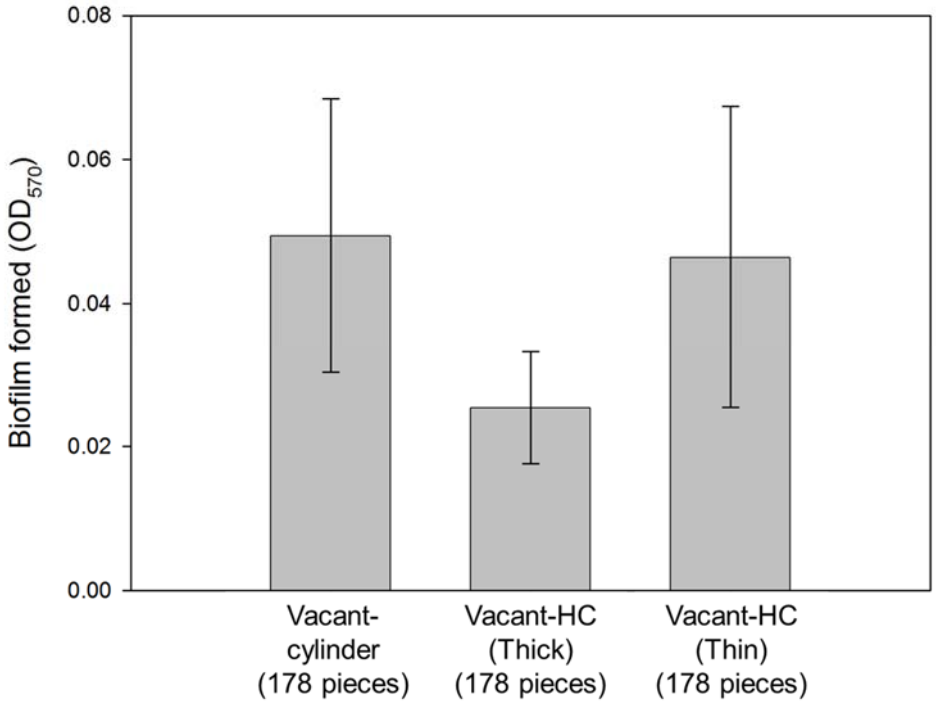


Figure III- 16. Physical washing effect comparison between vacant cylinders with the same outer diameter (~ 3.3 mm) but different inner diameter (vacant-cylinder: 0 mm, vacant-HC (Thick): 1.1 mm, and vacant-HC (Thin): 2.1 mm). Medium loading was 178 pieces each. Error bar: standard deviation (n = 4).

III.3.7. Comparison of Biofouling Mitigation among Various Media in Continuous MBRs

TMP rise-up was monitored in MBRs with different media (Figure III- 4), and the profiles of such are shown in Figure III- 17. In addition, for quantitative comparison, T_{TMP40} was calculated and its value for each MBR was compared (Table III- 1). Fouled membrane modules were cleaned and reused in the next cycle. The possible change in intrinsic membrane resistance resulting from the different number of chemical washing in Reactors A and B was assumed to be negligible.

In Phase 1, Reactors A and B were operated in conventional mode meaning no medium was inserted. Almost identical TMP profiles yielded a ratio of T_{TMP40}s between the two reactors of 1.0, which indicates that the two reactors have similar microbial broths and thus similar fouling tendencies. In Phase 2, QQ-HCs were inserted into Reactor A (QQ-HC-MBR), which had been operated as a conventional-MBR. Though there were four TMP-Jumps in the conventional MBR (T_{TMP40} = 4.8 days), only one TMP-Jump occurred in the QQ-HC-MBR (T_{TMP40} = 21.6 days) over 22 days of operation. This translates into a ratio of T_{TMP40} values of 4.5, meaning the occurrence of TMP-Jump was approximately 4.5 times more frequent in the conventional MBR than the QQ-HC-MBR. In Phase 3, vacant-HCs were added to Reactor B (vacant-HC-MBR), which had been operated as the conventional MBR. The T_{TMP40}s for the QQ-HC-MBR and vacant-HC-MBR were 25.9 and 12.8 days, respectively, and the ratio of their T_{TMP40} values was 2.0. Because the number of hollow cylinders inserted in both reactors was the same and hence, it could be assumed that the physical washing effect in the two reactors was the same, the difference in the T_{TMP40} values (i.e., greater membrane biofouling retardation in QQ-

HC-MBR) is likely attributable to the greater QQ activity of QQ-HCs than of vacant-HCs. In Phase 4, vacant-HCs were removed and QQ-beads were inserted, in Reactor B. Additionally, the QQ-HCs in Reactor A were replaced by freshly prepared QQ-HCs, so that the comparison between the two reactors was done with equally aged QQ bacteria. The ratio of T_{TMP40} values of the QQ-HC-MBR to the QQ-bead-MBR was calculated as 2.3, which indicates that the QQ-HC was more than twice as effective as the QQ-bead was in mitigating biofouling in an MBR under the operating conditions in this study. As demonstrated in batch studies, the enhancement of mitigation of membrane fouling in a QQ-HC-MBR may be attributed to the higher QQ activity and greater physical washing effect of QQ-HCs than those of QQ-beads.

It is worth noting that the operating parameters such as MLSS and COD removal were maintained within an acceptable range and particle size did not show a notable difference in the two MBRs throughout the operation (Figure III- 18). This coincides with the previous results in which QQ was found to have negligible impact on effluent quality of an MBR.

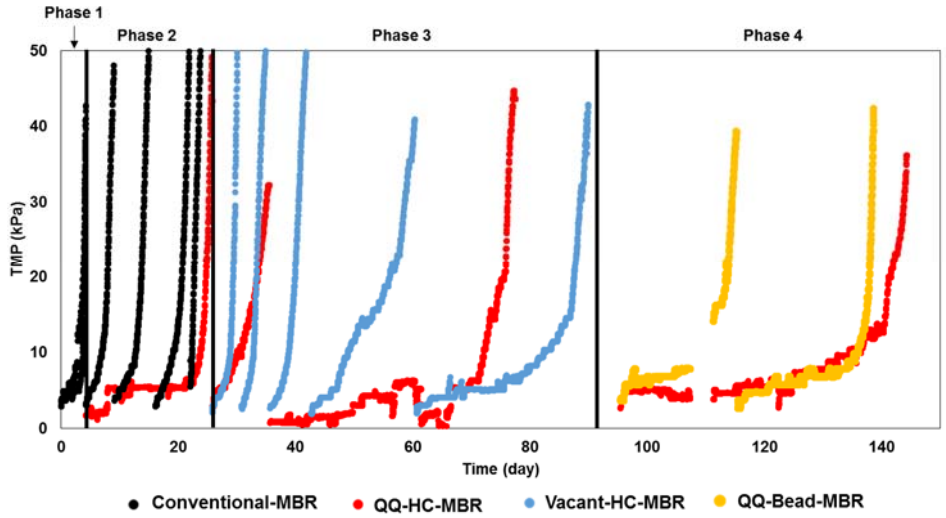


Figure III- 17. TMP profiles of MBRs with different media.

Table III- 1. Average number of days for one TMP-Jump at 40 kPa, $\mathbf{T_{TMP40}}$, to occur in Reactors A & B in each of the four phases.

Phase	Reactor A	Reactor B	Ratio (A/B)
1	4.2 days (conventional-MBR)	4.1 days (conventional-MBR)	1.0
2	21.6 days (QQ-HC-MBR)	4.8 days (conventional MBR)	4.5
3	25.9 days (QQ-HC-MBR)	12.8 days BR (Vacant-HC-MBR)	2.0
4	48.8 days (QQ-HC-MBR)	21.6 days (QQ-bead-MBR)	2.3

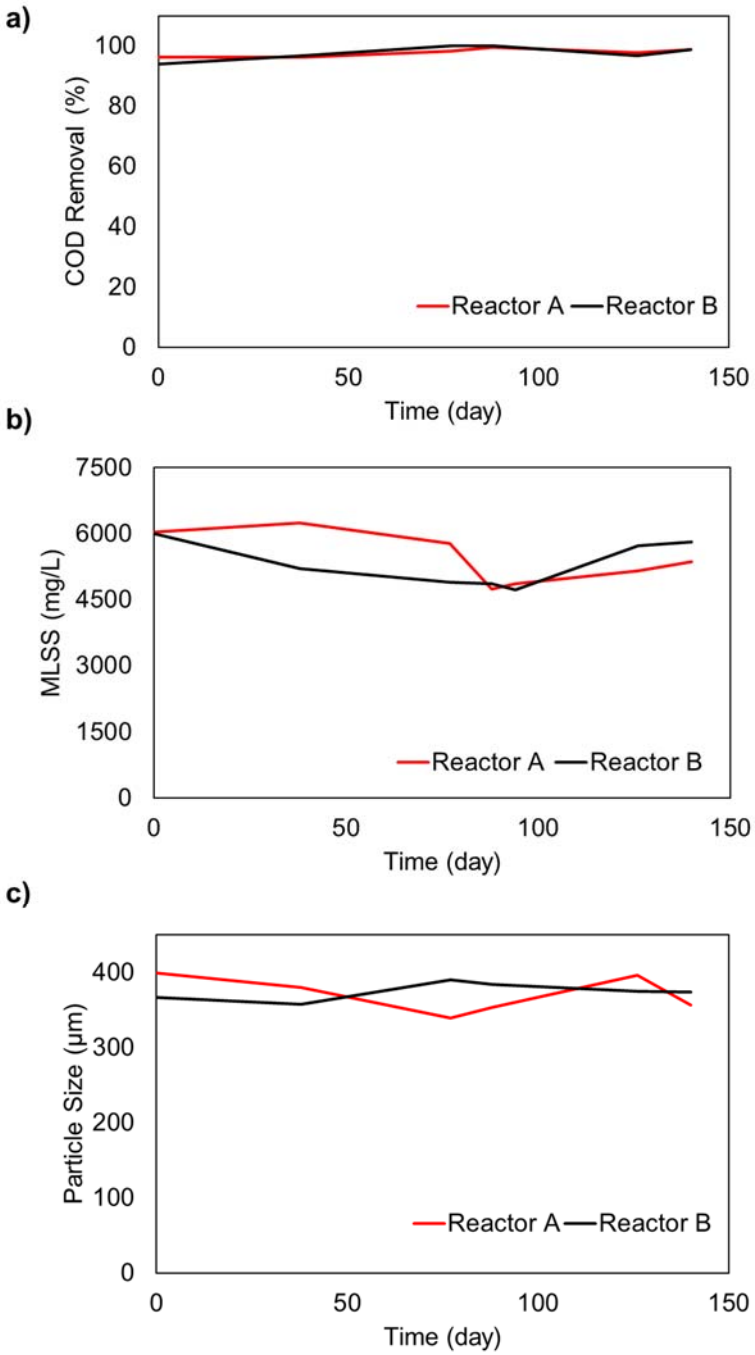


Figure III- 18. COD removal (b), MLSS (a), and floc size (c) recorded during MBR operation.

III.4. Conclusions

The interaction between AHL QS signal molecules and QQ media was studied. Based on the information from the analysis a more efficient QQ-HC was developed. Following conclusions were made from this chapter:

- QQ bacteria entrapped at the core, away from the surface, were found to exert negligible QQ activity towards the overall AHL degradation activity of a QQ-bead. It was speculated that most of AHL signal molecules were firstly degraded by the QQ bacteria in the region near the surface thereby they are deprived of the opportunity to encounter and degrade QS signal molecules.

- QQ-HCs, having no core, was shown to exhibit higher QQ activity (by approximately 13%) and physical washing effect (by approximately 140%) than QQ-beads due to increased surface area and more efficient use of media material. The inner parts of a hydrogel media did not seem to contribute to the physical washing effect of a medium.

- Such properties of QQ-HCs enabled enhanced fouling control than QQ-beads in a lab-scale MBR. Also, application of QQ media had no significant effect on effluent quality.

Chapter IV

Enhancing Physical Properties and Lifespan of Bacterial Quorum Quenching Media through Combination of Ionic Cross-linking and Dehydration

IV.1. Introduction

Immobilization of cells in a hydrogel network allows a selective culture of a microorganism of interest while facilitating sufficient transport and diffusion of essential nutrients, dissolved oxygen, extracellular metabolic products across the hydrogel matrix (Jen et al., 1996). As such, hydrogel media in which cells are immobilized have been extensively used in the fields of environmental, chemical, and biomedical processes for removal of pollutants (de-Bashan and Bashan, 2010, Hashimoto and Furukawa, 1987, Magri et al., 2012) (organics, nutrients, and etc.) as well as for production of commercial compounds(Cruz-Ortiz et al., 2011, Dave and Madamwar, 2006, Idris et al., 2008, Nunes et al., 2010), and biofuels(Hanaki et al., 1994), and pharmaceuticals(Kamoun et al., 2015, Swamy et al., 2012).

One of the successful applications of cell immobilizing hydrogel medium is the entrapment of quorum quenching (QQ) bacteria in a polymeric composite hydrogel (QQ medium). Such QQ bacteria entrapping hydrogel media have been prepared and reported to inhibit quorum sensing (QS) induced biofilm formation and thus mitigate membrane biofouling in a membrane bioreactor (MBR) for wastewater treatment(Lee et al., 2016a, Lee et al., 2016b, Lee et al., 2016c, Lee et al., 2016d). The technology for quenching QS signal molecules in an MBR, known as QQ MBR, has evolved since 2009 from fundamental lab-scale studies(Kim et al., 2013a, Yeon et al., 2009a, Yeon et al., 2009b) to a practical one which assessed energy savings in a pilot-scale MBR when QQ media are applied(Lee et al., 2016c). In their study, the authors confirmed the anti-fouling property of QQ beads in a pilot plant MBR fed with real wastewater and analyzed energy savings based on the reduced aeration requirement in the membrane tank by QQ beads. They reported that the reduction in

the biofouling related energy reached approximately 60% by the addition of QQ media. More recent studies have focused on the preparation of various types of mobile QQ media and explored the effect of their geometry on the fouling mitigation performance in an MBR(Lee et al., 2016d, Lee et al., 2016b).

Although the results from above series of recent studies illuminate that researches on QQ-MBR have already crossed the borders between laboratory and fields, economic aspects of QQ media have been neglected in their studies. In order to bring current QQ technology closer to a practice, however, it would be required not only to enhance physical strength and entrapped cell viability of QQ media, but also to improve methods of storage and transport of QQ media.

In this study, enforcement of physical strength as well as an extension of the lifetime of a QQ bacteria entrapping hollow cylinder (QQ-HC) was sought by adding a dehydration procedure following the cross-linking of the polymeric hydrogel by inorganic compounds like Ca^{2+} and boric acid. Such prepared medium was characterized in terms of physical strength, QQ activity, shelf-life, and a cell viability of QQ bacteria. Lastly, practical implications of our findings regarding storage, transportation, and application of QQ media were also discussed.

IV.2. Material and Methods

IV.2.1. Preparation of QQ-Beads and QQ-Cylinders

QQ-HCs were prepared as described in the previous study (Lee et al., 2016d). *Rhodococcus* sp. BH4 was used as the QQ bacterium because its biofouling control performance has been numerously confirmed in previous studies(Oh et al., 2012, Oh et al., 2013, Kim et al., 2013b, Kim et al., 2015, Kose-Mutlu et al., 2016, Maqbool et al., 2015, Lee et al., 2016c, Lee et al., 2016d, Lee et al., 2016b). Hollow cylindrical media with QQ bacteria (i.e., QQ-HC) and without QQ bacteria (i.e., vacant-HC) were prepared from a mixture of polyvinyl alcohol and sodium alginate. For a QQ-HC, an aliquot amount of BH4 was added to the polymeric mixture so that the final concentration of BH4 is 7 mg BH4 (by dry weight) per g of QQ-HC (by dry weight). Whereas, for a vacant-HC, an equivalent volume of deionized (DI) water was added to the polymeric mixture instead of BH4. Cross-linking of the polymer mixture was done by extruding it through a nozzle into a $CaCl_2$ and boric acid solution followed by the second cross-linking in sodium sulfate solution. Media are cut into 2 cm pieces for QQ activity analysis and MBR operation.

Prepared media were either dried in an anhydrous magnesium sulfate containing airlock container in 30 °C oven or kept hydrated in 0.9% NaCl solution. Both dehydrated and hydrated media were stored in a 4 °C refrigerator until further analysis. Prior to analysis, the stored dehydrated media were immersed in either 0.9% NaCl solution or Luria-Bertani (LB) broth and then placed in a 30 °C incubator for 7 hours. Dehydrated media were addressed as D-HC. QQ-HCs kept hydrated in 0.9% NaCl were also placed in a 30 °C incubator for 7 hours prior to analysis and denoted

as S-HC.

IV.2.2. Measurement of QQ Activity of QQ Media and Biological Stability

QQ activity of prepared media was analyzed by measuring the degradation rate of commercially available QS signal molecule, N-Octanoyl-DL-homoserine lactone or C8-HSL. C8-HSL was used as the model QS signal molecule in this experiment because it was previously reported to be one of the major QS signal molecules in an MBR (Yeon et al., 2009a). Total of 10 pieces of each type of media (~ 1.1 g), which corresponds to approximately 8 mg of BH4, was added to a conical tube containing 20 mL of 1 μM C8-HSL (Sigma-Aldrich, US) dissolved in 20 mM phosphate buffer (PB) at pH 7. The mixture was placed on an orbital shaker at 70 RPM and 25 °C and let reacted for 60 min. 0.5 mL of the solution was sampled followed by filtration using a 0.45 μm syringe filter and immersion in a 100 °C water bath for 15 minutes. The quantification of C8-HSL was performed by using a reporter strain *A. tumefaciens* A136 as described in the previous study (Lee et al., 2016c, Lee et al., 2016d). In detail, the reporter strain *A. tumefaciens* A136 and samples were mixed and loaded into a 96-well plate and the plate was incubated at 30 °C for 90 minutes. Subsequently, A Beta-Glo® Assay System (Promega, US) was added to the wells and the plate was stored at 25 °C for 30 minutes. The luminescence intensity in the wells was measured by a luminometer (Synergy 2, Bio-Tek, US) and C8-HSL concentration was determined by plotting the calibration curve of standard C8-HSL. The QQ activity was calculated by computing the number of moles of C8-HSL degraded per min, over a period of 60 minutes.

Biological stability of QQ-HCs was assessed by periodically measuring QQ activities of used QQ-HCs during the MBR operation. The measurement of QQ activity was performed as described above.

IV.2.3. Tensile Test and Physical Stability Assessment

Texture analyzer CT3 4500 (Brookfield, US) with a TA-DGA probe was used to estimate physical strength of prepared vacant-HCs. The physical strength in this study was represented as the elongation work which is defined as the work (mJ) required for 250% elongation of vacant-HC at an elongation speed of 10 mm/s. The initial length of a vacant HC was 4 cm and it was elongated to 14 cm which resulted in 350% elongation. 6 independent samples of each type of media are used for the measurement and no rupture occurred during the elongation process. The outer diameters of HCs were 3.4 (± 0.1), 3.313 (± 0.110), 2.7 (± 0.1), 2.5 (± 0.1), 2.6 (± 0.1), 2.6 (± 0.1), and 2.5 (± 0.1) mm and inner diameters were 1.8 (± 0.1), 1.5 (± 0.2), 1.3 (± 0.1), 1.4 (± 0.1), 1.5 (± 0.1), 1.4 (± 0.1), and 1.4 (± 0.1) mm for dehydration times of 0, 0.25, 0.5, 1, 3, 5, and 10 days, respectively.

Physical stability of HC media was assessed by measuring the average wet weight of a medium during the MBR operation and comparing the values with the initial one. Wet weight of each of 10 QQ-HCs was obtained after removing excess water on the surface of a medium and their average value, termed as the unit mass, was considered for analyzing physical stability.

IV.2.4. Compression Test on Beads

In addition to tensile test on HCs, compression test on beads was

performed to confirm the increase in physical strength of the hydrogels by dehydration and re-swelling. Texture analyzer CT3 4500 (Brookfield, US) with a TA39 probe was used. Beads after dehydration for a time, t, was re-swelled and subjected for the compression. Dehydration times considered were 0, 0.5, 1, 2, and 4 days with 0 being a bead not underwent dehydration. The compression test was conducted as follows. A bead was compressed by a cylindrical probe (diameter = 2 mm) at a speed of 10 mm/sec. The work required to compress a bead to yield 50% deformation was recorded. Total of 6 beads per dehydration time was considered and their average values were plotted. The diameters of beads were 3.66 (± 0.09), 3.66 (± 0.09), 2.67 (± 0.10), 2.67 (± 0.14), 2.70 (± 0.07), 2.61 (± 0.13) mm for dehydration times of 0, 0.5, 1, 2, and 4 days, respectively.

IV.2.5. Determination of Gel Fraction

Gel fraction is an indirect measure of the degree of cross-linking in a hydrogel(Chen et al., 2008, Khan and Ranjha, 2014). Gel fraction before and after dehydration procedure was determined as follows. Wet weight (W_w) of a vacant medium is measured after removing excess water on the surface by patting the medium with lintless dry fabric. The medium was dried in an oven at 60 °C until there was no further change in the weight to obtain its dry weight (W_d). The gel fraction was calculated by the following Eq. (1).

$$\text{Gel fraction (GF\%)} = \frac{W_d}{W_w} \times 100 \qquad \text{Eq. (1)}$$

IV.2.6. MBR Operation

Two 3-liter volume lab-scale MBRs were operated in parallel as shown in Figure IV- 1. Activated sludge sampled from a real wastewater treatment plant (Tancheon, Korea) was inoculated to the reactors and it is acclimatized until operating parameters became stable. Synthetic wastewater was continuously fed into the reactors and the composition of the synthetic wastewater was as follows (all in mg/L)(Oh et al., 2012): glucose, 200; yeast extract, 7; bactopeptone, 57.5; $(NH_4)_2SO_4$, 52.4; KH_2PO_4, 10.88; $MgSO_4$, 7.82; $FeCl_3$, 0.038; $CaCl_2$, 1.23; $MnSO_4$, 0.9; and $NaHCO_3$, 127.8. A hollow fiber membrane (ZeeWeed 500, GE-Zenon, U.S.) with an effective filtration area of 186 cm^2 was used for filtration at a constant flux of 20 liter per m^2 per hour (LMH). Total of 261 pieces of QQ-media were applied to each reactor. The aeration intensity was kept constant at 2.0 L/min. Fouled membranes were taken out of the reactor and subjected to a chemical washing (1000 ppm NaOCl, 3 hours). The trans-membrane pressure (TMP) in each reactor was monitored to estimate the degree of membrane fouling in each MBR. In addition, for comparison of the anti-fouling performance of QQ-media, the number of days for one TMP-jump to occur at 30 kPa (T_{TMP30}) was considered.

Mixed liquor suspended solids (MLSS) and chemical oxygen demand (COD) were measured according to standard methods (Keith, 1998). The average floc size was measured using a particle size analyzer (Microtrac S3500, US).

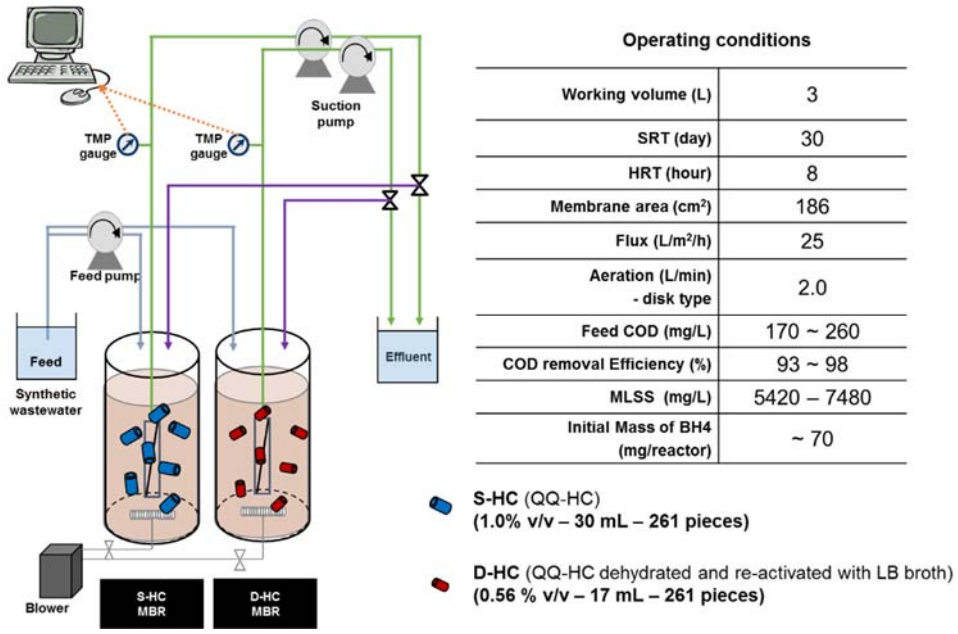


Figure IV- 1. Schematic, operating conditions of the two MBRs with S-HC and D-HC, respectively.

IV.3. Results and Discussion

IV.3.1. Comparison of Tensile Strength between S-HC and D-HC

The elongation work, defined by the work required for 250% elongation of vacant media is measured with respect to the dehydration time (0, 0.25, 0.5, 1, 3, 5, and 10 days) to estimate physical strength of a medium. The media of dehydration time at zero was S-HC. Media subjected to dehydration are re-swelled prior to the measurement of elongation work. Total of 6 independent samples were measured for tensile test at each dehydration time given above and their averages and standard deviations are plotted in Figure IV- 2. The elongation work was trending upward over dehydration time. There was a rapid rise in the elongation work over the first day and it gradually leveled off as the dehydration time was continued till the 10th day. Since the slope of the plot is abruptly lowered after day 1, it was speculated that dehydration time of 1 day would be the most efficient. Hence, D-HCs dried for 1 day was used in following experiments.

The increase in physical strength of the hydrogel after dehydration might be attributed to the enhanced physical cross-linking through hydrogen bonding between polymer chains and/or increase of crystalline region. Gel Fraction (GF) is reported to be related to the degree of cross-linking in a hydrogel (Chen et al., 2008, Khan and Ranjha, 2014). GF of HC that was not subjected to dehydration (S-HC) was 6.7 (± 0.2) %, whereas that of HC subjected to dehydration and subsequent re-swelling (D-HC) was 16.6 (± 0.7). Consequently, an increase of the degree of cross-linking in D-HC may have elevated its physical strength. Furthermore, as shown in the SEM images of cross-sections of S-HC (Figure IV- 3a) and D-HC (Figure IV-

3b), the hydrogel shrinks significantly in its volume upon dehydration and re-swelling. When the inner structure of S-HC (Figure IV- 3a') and D-HC (Figure IV-3b') is observed more closely, the denser inner structure is evidenced in the cross-section of D-HC than that of S-HC. The densification of the inner structure may also support the increased degree of cross-linking in the hydrogel through the dehydration process.

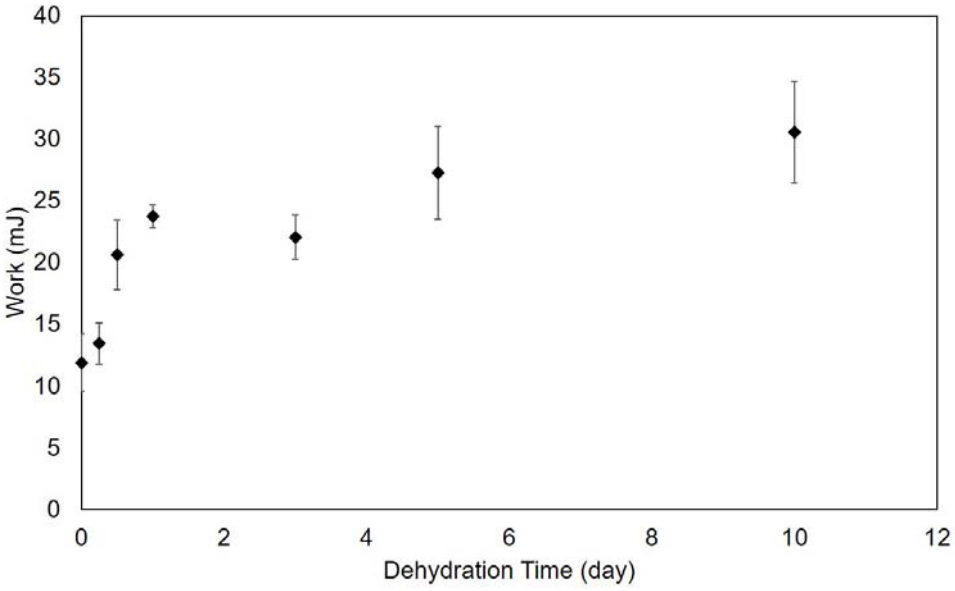


Figure IV- 2. Measurement of elongation work, represented as the work required 250% elongation of a vacant medium with respect to dehydration time. Initial length of the medium was 4 cm and the elongation speed was 10 mm/s. Error bar: standard deviation (n = 6)

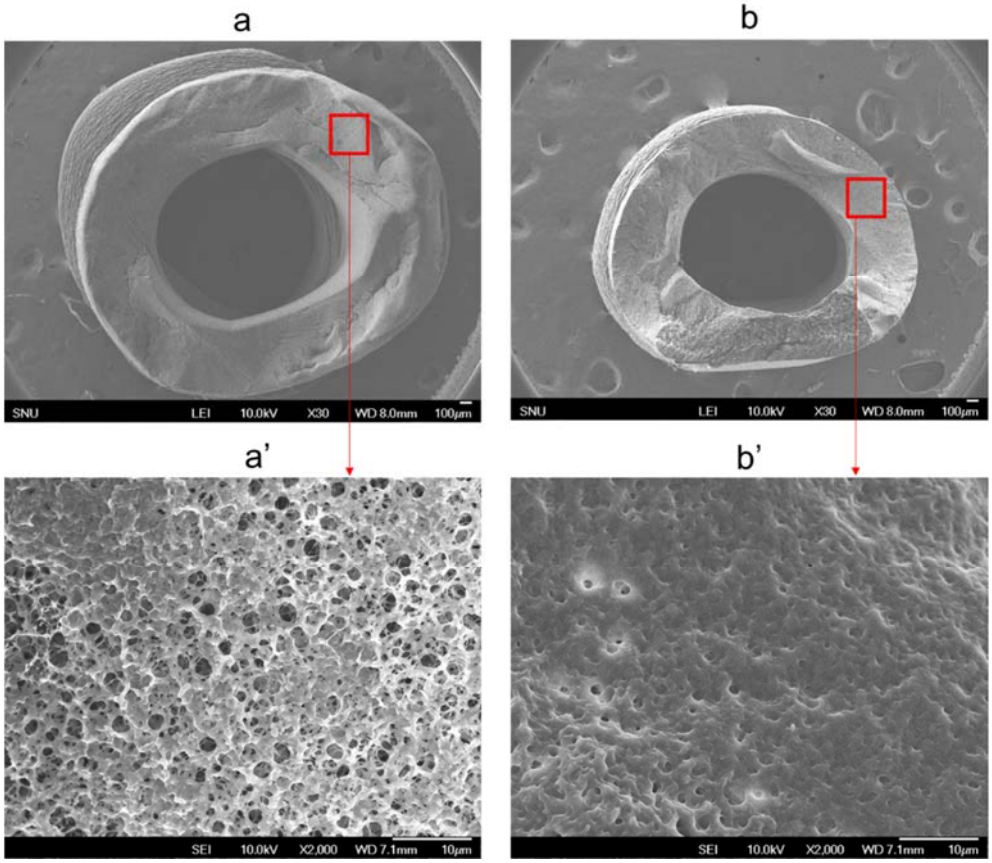


Figure IV- 3. SEM images of cross-sections of a vacant HC before dehydrating (S-HC) at magnification of X 30 (a) and X 2000 (a') and of a vacant HC dried and then re-swelled with 0.9% NaCl solution (D-HC) at magnification of X 30 (b) and X 2000 (b').

IV.3.2. Effect of Dehydration on Compressive Work of Beads

The result on the compression test on beads is shown in Figure IV- 4. It shows similar trend as experienced in the tensile test on HC: longer the dehydration time, greater the work required in compressing beads. However, a steeper rise in work was observed until the dehydration time of 2 days, instead of 1 day in tensile test on HC, and the rise of the work seemed to reach a plateau. This result confirms the empowerment of physical strength of hydrogel through addition of dehydration procedure to previously known method of gel preparation. The diameters of beads were 3.66 (± 0.09), 3.66 (± 0.09), 2.67 (± 0.10), 2.67 (± 0.14), 2.70 (± 0.07), 2.61 (± 0.13) mm for dehydration times of 0, 0.5, 1, 2, and 4 days, respectively.

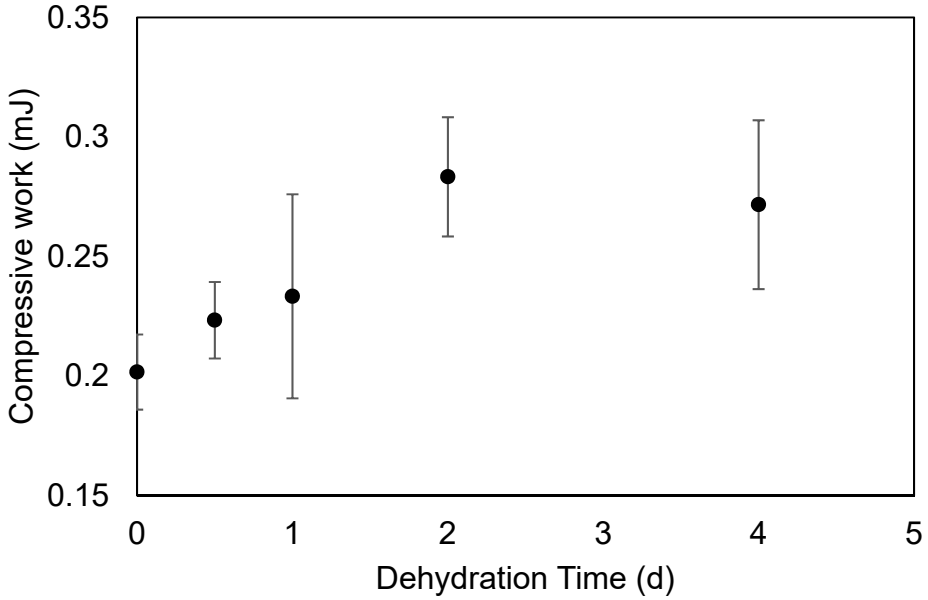


Figure IV- 4. Measurement of compressive work, represented as the work required 50% deformation of a bead with respect to dehydration time. The compression speed was 10 mm/s. Error bar: standard deviation (n = 6)

IV.3.3. Effect of Storage Method on QQ Activity of QQ Media

Exposure to desiccation could impose an adverse effect on a bacterium's viability. It is reported that Gram-positive bacteria, including the QQ bacterium *Rhodococcus* sp. BH4 in this study, tend to be the most desiccation tolerant (Potts et al., 2005). Nevertheless, loss of water may augment intracellular solute concentration and osmotic pressure within the cell which can cause cell death (Lederberg, 2000) and thus, significant loss of QQ activity of QQ media might occur. Therefore, it was crucial to investigate the dependence of the QQ activity of QQ media on their storage methods, whether they are kept dehydrated or hydrated.

Figure IV- 5 shows the QQ activities of QQ-HCs depending on their storage methods, in saline water (S-HC) or in dried state (D-HC). There was a notable loss of the QQ activity (approximately 23%) in D-HC but upon re-activation with LB broth (re-activated D-HC), the activity was recovered close to the QQ activity of S-HC. The loss of QQ activity from dehydration could be attributed to either the partial loss of cell viability during the dehydration process or the reduced mass transfer resulting from reduced porosity of hydrogel network or the combination of the two cases. Thus, the re-activation of QQ-HCs with LB broth may have increased the number of viable cells and/or have recovered the porosity of media, which enabled the recovery of lost QQ activity to a greater degree than the initial value.

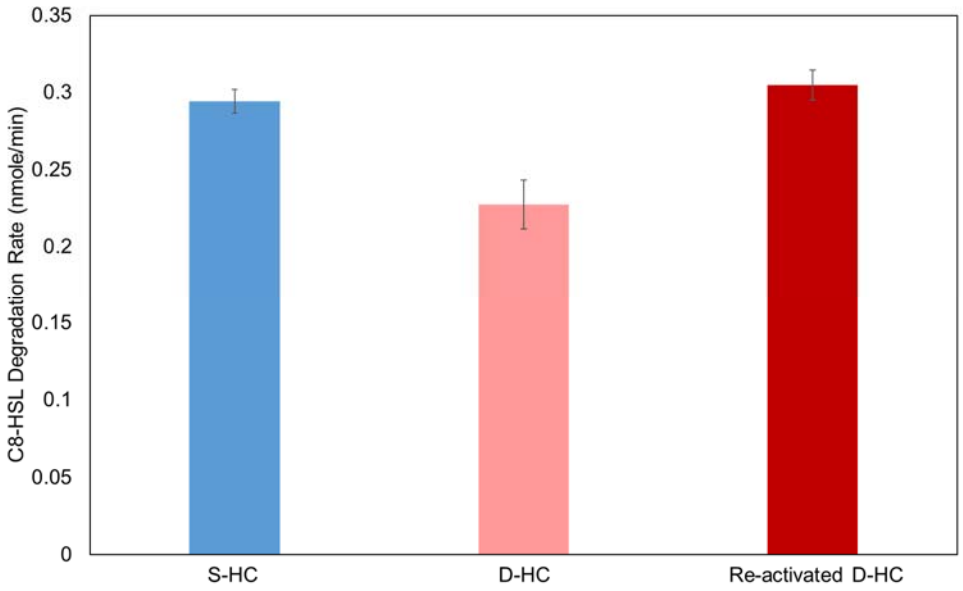


Figure IV- 5. Change in QQ activity depending on storage methods. S-HC: QQ-HCs that were stored in 0.9% NaCl solution for one day; D-HC: QQ-HCs that were stored in dried state for one day and then re-swelled with 0.9% NaCl solution for 7 hours prior to activity test; re-activated D-HC: QQ-HCs that were in dried state for one day and then re-activated with LB broth for 7 hours prior to activity test. Error bar: standard deviation (n = 3 as the technical replicate).

IV.3.4. Long-term Monitoring of QQ Activity of QQ Media

Dehydration of QQ media will certainly offer a more efficient way of storing cell entrapping hydrogel because the shape and activity of hydrogel media are preserved even after dehydration and re-swelling. In addition, dehydrating cells makes them less susceptible to microbial contamination (Lederberg, 2000). Furthermore, reduced weight of dried media makes handling and transportation highly convenient and economical. Thus, it is important to check the shelf life of D-HCs, i.e., whether QQ activity of a QQ medium is well preserved during long-term storage under dehydrated condition.

To assess the shelf life of QQ media, total of 6 batches of QQ bacteria entrapping S-HCs and 12 batches of QQ bacteria entrapping D-HCs were prepared. Each batch contained 10 pieces of QQ media and they were subjected to QQ activity measurement at a time point during 200 days of storage. Prior to the activity test for each batch, the stored D-HCs for a given time were immersed in either 0.9% NaCl solution (D-HC) or LB broth (re-activated LB) and placed in a 30 °C incubator for 7 hours. QQ-HCs kept hydrated in 0.9% NaCl (S-HC) were also placed in a 30 °C incubator for 7 hours prior to analysis. Resulting QQ activity (i.e. the number of moles of C8-HSL degraded per min over a period of 60 minutes) for each batch is plotted in Figure IV- 6.

Initially, S-HCs showed greater QQ activity than D-HCs which coincides with the result from the previous section. However, the QQ activity of S-HCs was monotonically decreased with respect to the storage time while that of D-HCs was maintained at a relatively constant value during 200 days of storage. It was interesting to note that sometime between storage days 90 and 200, the QQ activity

of S-HCs fell below that of D-HCs. This suggests that if the storage time is expected to last over 90 days, it could be more beneficial to keep QQ media dehydrated. In addition, upon re-activating D-HCs with LB broth, the QQ activities were recovered to their initial values. Although the lost QQ activity of D-HCs was demonstrated to be restored by re-activation with nutrients, a continual reduction in QQ activity of S-HCs may imply a steady decline in viability of QQ bacteria which could lead to a permanent loss of QQ activity if the storage time is prolonged. In order to correlate the QQ activity result with cell viability, BH4's cell viability in the QQ media was analyzed.

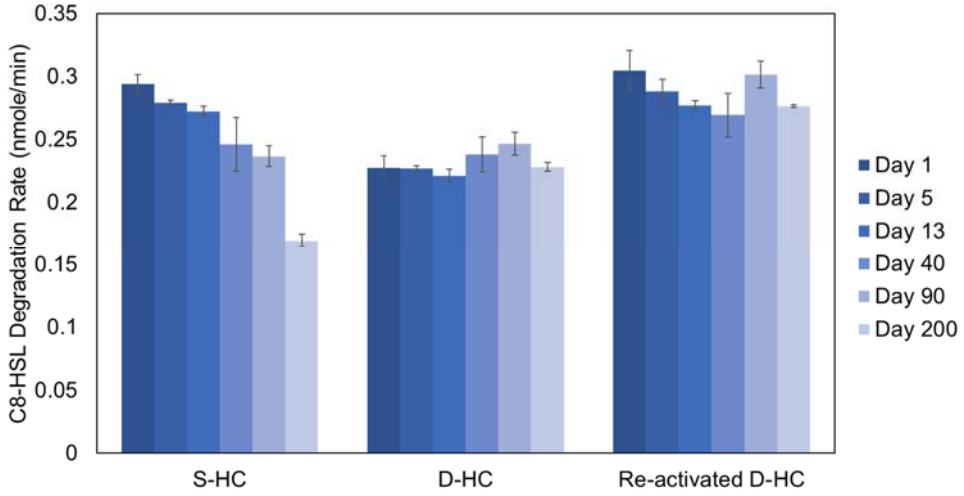


Figure IV- 6. Long-term monitoring of QQ activity depending on storage methods. S-HC: QQ-HCs that were stored in 0.9% NaCl solution, D-HC: QQ-HCs that were stored in dried state and then re-swelled with 0.9% NaCl solution prior to activity test, re-activated D-HC: QQ-HCs that were in dried state and then re-activated with LB broth prior to activity test. Color gradient of columns indicates different storage time. Error bar: standard deviation (n= 3 as the technical replicate).

IV.3.5. Correlation of Cylinder Size with the Physical Washing Effect

To analyze how storing QQ media under dehydrated condition would affect cell viability, QQ media after 200 days of storage were stained with a LIVE/DEAD BacLight Bacterial Viability Kit and their cross-section was observed through a CLSM. Total of 6 independent samples per type of QQ media were subjected to observation and the cell viability was calculated by taking the ratio of green (SYTO 9) and red (propidium iodide) fluorescence intensity through Leica LAS AF Lite software.

Figure IV- 7 shows the average values of cell viability in QQ-HCs. It is notable that cell viability was higher in D-HCs (5.3 ± 0.4) than in S-HCs (2.6 ± 0.4). It was reported that cells under low water condition drastically reduces their metabolic activity and tend to survive for a longer period than cells under water-rich condition (Lederberg, 2000). Our result also suggests that QQ bacterium, *Rhodococcus* sp. BH4, in its dried state, was more capable of surviving than when it was in a hydrated state. As such, the higher QQ activity of D-HCs than that of S-HCs is likely to be attributed to the greater cell viability of D-HCs.

In summary, the addition of dehydration step after the ionic cross-liking procedure in the preparation of QQ bacteria entrapping hydrogel not only enhanced physical but also increased QQ media's shelf-life by improving cell viability.

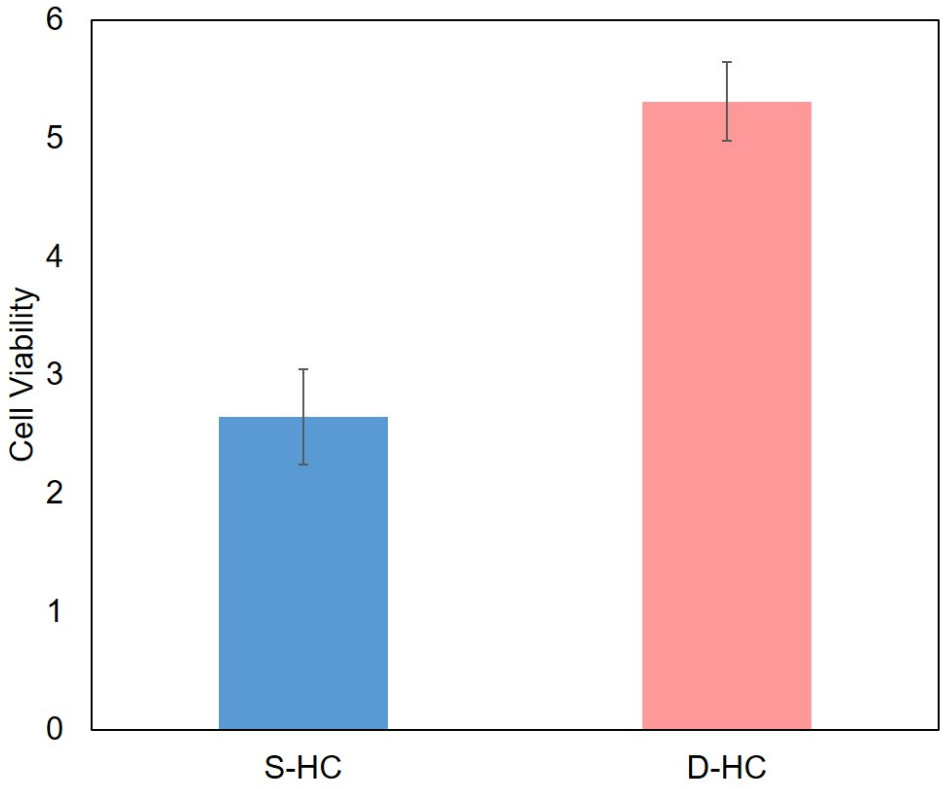


Figure IV- 7. Cell viability in QQ-HC before dehydrating (S-HC) and dried QQ-HCs re-swelled with 0.9% NaCl solution (D-HC). The cell viability was derived from taking the ratio of average intensities of green fluorescence (SYTO® 9) and red fluorescence (propidium iodide) from 6 independent samples which were kept in storage for 200 days. Error bar: standard deviation (n= 6).

IV.3.6. Comparison of Biofouling Mitigation between S-HC and D-HC in MBR

To test how the addition of dehydration step in the preparation of QQ media would improve their performance in mitigating biofouling in an MBR, hydrated QQ-HCs (S-HCs) and dehydrated QQ-HC (D-HCs) were inserted in each of two lab-scale MBRs and the extent of membrane fouling (i.e. transmembrane pressure) in each MBR was monitored. D-HCs were re-activated with LB broth for 7 hours at 30 °C prior to application to set the initial QQ activities of the two types of QQ-HC at a similar level. Total of 216 pieces of each of S-HCs and D-HCs, which correspond to approximately 1.0 and 0.56% (v/v – volume of media / volume of reactor) respectively, were used.

The TMP profiles from the two MBRs operated in parallel for over 75 days are shown in Figure IV- 8a. The number of days for one TMP-jump to occur (T_{TMP30}) were plotted in Figure IV- 8b. T_{TMP30} values were computed when the TMP reached 30 kPa except for the last point in MBR with D-HCs. As shown in Figure 7b, T_{TMP30} values from both reactors seem close to each other until about 50 days of operation, although fluctuations are observed. However, after 50 days, T_{TMP30} in the MBR with S-HC drops while that in the MBR with D-HCs remained relatively stable. The decrease in T_{TMP30} values implies exacerbation of membrane fouling in an MBR. It is speculated that the increase in the fouling tendency in MBR with S-HCs may have been caused by the diminished QQ effect by S-HCs due to the possible wearing of S-HCs. Whereas, the relatively stable fouling tendency in MBR with D-HCs may have been attributed to the enhanced physical and biological (QQ activity) stabilities obtained through the addition of dehydration step in the preparation of QQ media. It

is worth noting that the operating parameters such as MLSS and COD removal were maintained within an acceptable range and particle size did not show a notable difference in the two MBRs throughout the operation (Figure IV- 9).

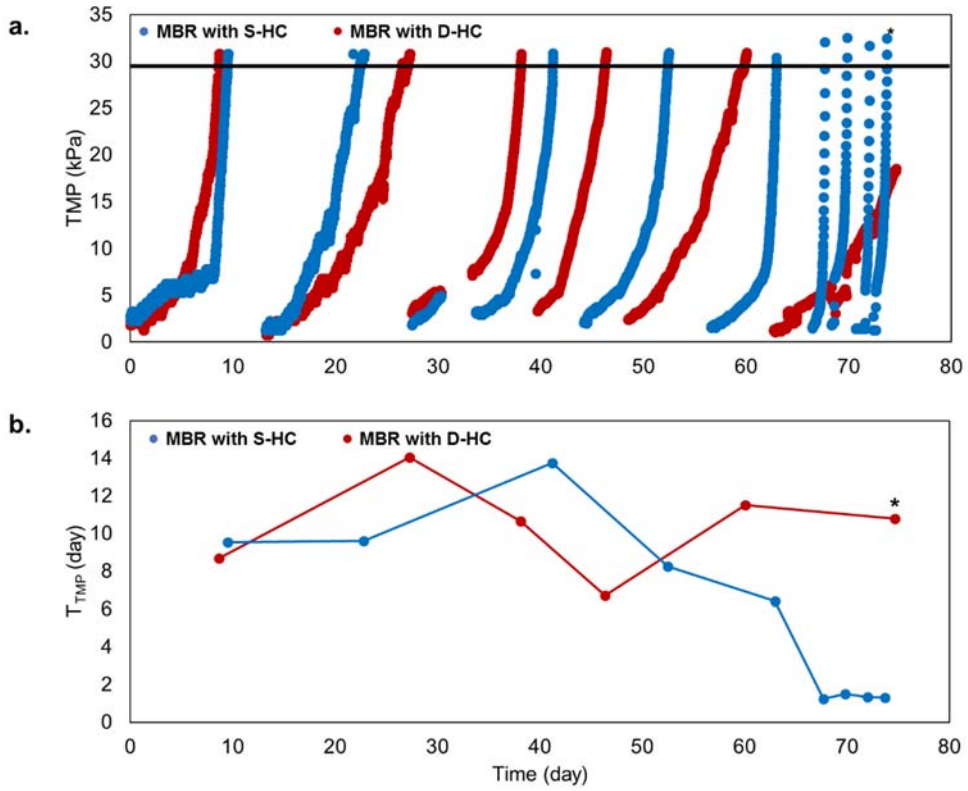


Figure IV- 8. TMP profiles (a) and $\mathbf{T_{TMP30}}$ values (b), measured at 30 kPa, from MBR operation with D-HC and S-HC. $\mathbf{T_{TMP30}}$: time till TMP to reach 30 kPa. *T_{TMP} value measured at 18 kPa.

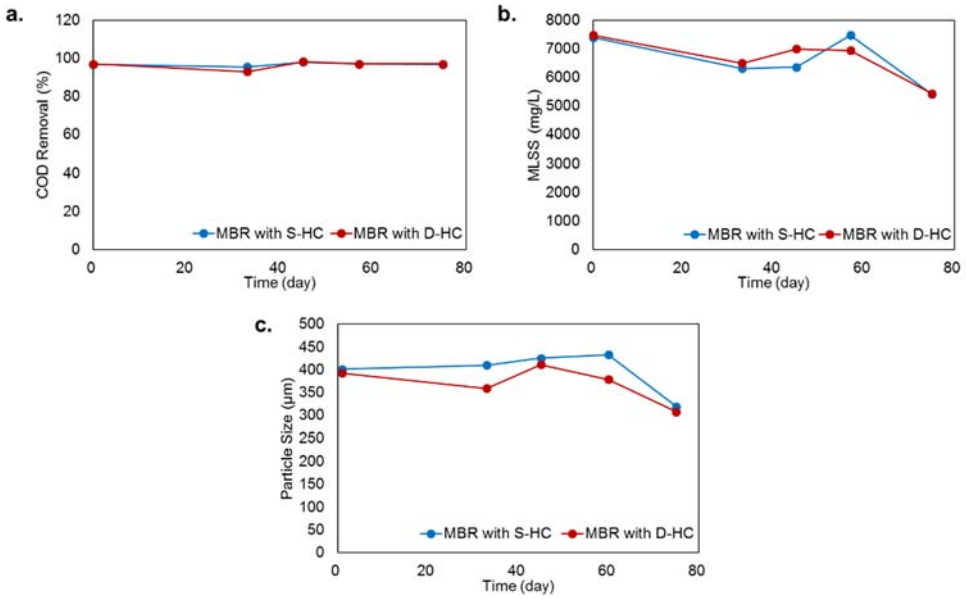


Figure IV- 9. COD removal (b), MLSS (a), and floc size (c) recorded during MBR operation.

IV.3.7. Comparison of Physical and Biological Stabilities between S-HC and D-HC

To investigate the reasons why the dehydrated QQ media (D-HCs) maintained longer their QQ activity than the hydrated QQ media (S-HCs) did, physical and biological stabilities of QQ-HCs were analyzed during the MBR operation. QQ-HCs applied to the MBR were 2 cm long, whereas the minimum length of a QQ-HC required for tensile strength measurement was 4 cm. Therefore, instead of measuring the tensile strength of QQ-HCs, unit mass (i.e., wet weight of one medium) was measured for assessment of physical stability. Biological stability was represented by the QQ activity. The unit mass and QQ activity of 10 pieces of each medium were measured at the operation days of 30 and 73 for both MBRs and the values relative to their initial values were plotted.

As shown in Figure IV- 10, the unit mass and the QQ activity of used S-HC and D-HC relative to their initial values show drastic discrepancies between the two media. In detail, unit mass (wet weight) of D-HCs was initially increased to approximately 120% of initial value while that of S-HCs experienced an abrupt fall. The increment in the mass of D-HCs is possibly due to the partial loosening of physical cross-linking between polymer chains which caused water to fill up the gap. The loss of media's mass could be related to the hydrolysis of polymer chains by microbes (Kawai and Hu, 2009, Kitamikado et al., 1992) and reversal and destabilization of ionic cross-linking in presence of non-gel-including ions such as Mg^{2+}, Na^{+}, PO_4^{2-}, citrate, etc. (Smidsrod and Skjakbraek, 1990). Picture of used media in Figure IV- 11 shows that physical integrity of S-HCs is highly impaired when compared to that of D-HCs. This may have downgraded the physical scouring

of the membrane by S-HCs to a greater extent resulting in a reduced biofouling mitigation performance of S-HCs.

Furthermore, the loss of media's mass may have exacerbated the loss of QQ activity of S-HCs during MBR operation through leakage of QQ bacteria as reported by a previous study (Kim et al., 2015). Especially, significant loss of QQ activity near the end of MBR operation (Day 73) due to decline of physical integrity of S-HCs may have led to the accelerated membrane fouling as shown in Figure IV- 9 in the MBR with S-HCs.

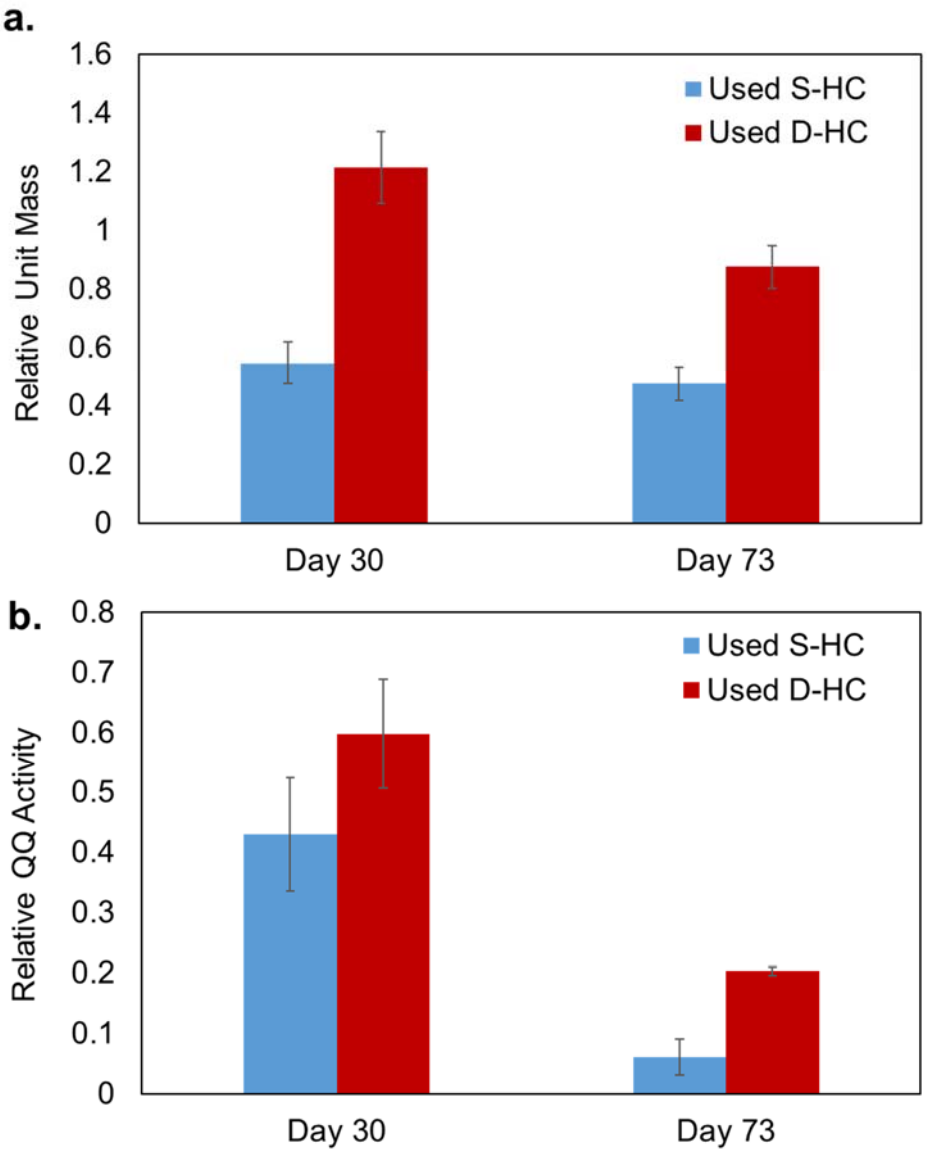


Figure IV- 10. Change of unit mass (wet weight of one QQ medium) (a) and QQ activity (b) of D-HC and S-HC relative to their initial values during MBR operation. Error bar: standard deviation (n= 10 for (a) and n=3 as the technical replicate for (b)).

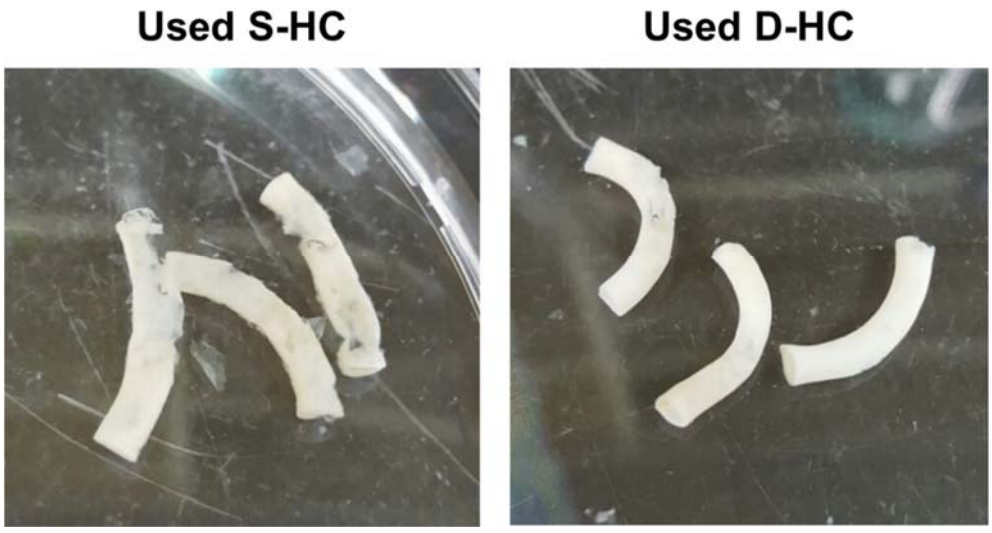


Figure IV- 11. Images of used media after 75 days of operation in MBR.

IV.3.8. Practical Re-activation of Dehydrated QQ Media

In practice, re-activation of D-HCs with LB broth may not be feasible due to the additional reagents, equipment, and labor needed to prepare a large volume of LB broth. Hence, it was desired to develop a more economical and convenient way of re-activating D-HCs.

Figure IV- 12 shows the QQ activity of S-HC, and D-HCs re-activated with saline solution (S-D-HC), feed wastewater from a municipal wastewater treatment plant (Daejeon, Korea) (W1-D-HC), and wastewater from Seoul National University cafeteria (Seoul, Korea) (W2-D-HC). Wastewaters were filtered through 0.45 μm syringe filter prior to the re-activation of D-HCs and the COD values for the municipal wastewater treatment plant feed and cafeteria wastewater were 76 ± 1 and 581 ± 4 mg/L respectively. Re-activation was performed in a shaking incubator at 30 °C for 24 hours.

The result shows that the wastewater with a low concentration of carbon source yielded a minuscule rise in QQ activity whereas the wastewater with a high carbon content restored QQ activity close to the initial value. This demonstrates that re-activation of D-HC with wastewater is feasible as long as there are sufficient carbon sources for QQ bacteria. In addition, use of on-site wastewater instead of LB broth or other commercial culture media could be a viable option for re-activating D-HCs in real MBR plants.

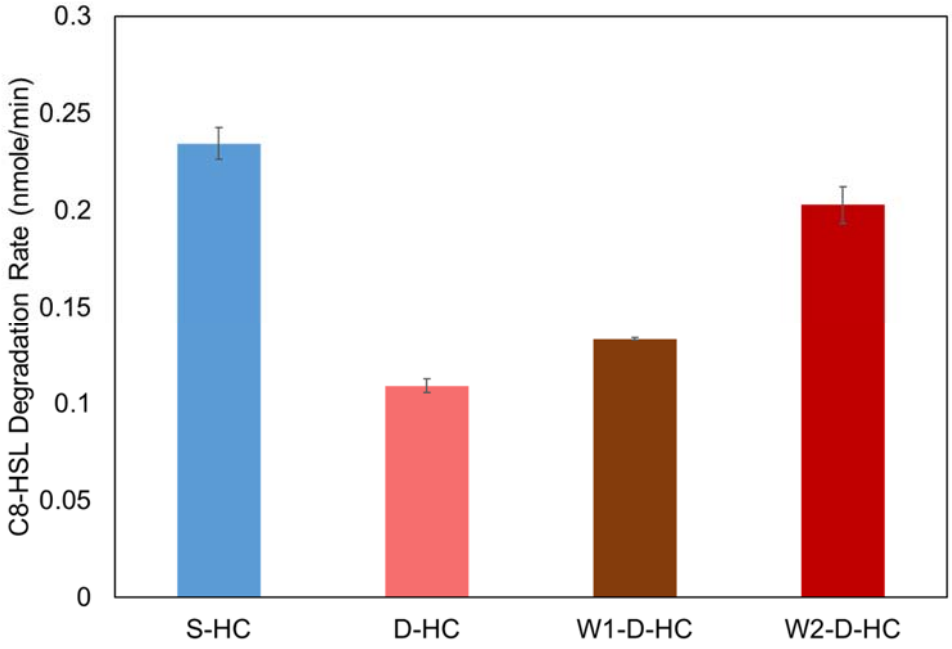


Figure IV- 12. QQ activity of dehydrated HCs re-activated saline solution (D-HC), wastewater from a municipal wastewater treatment plant (Daejeon, Korea) (W1-D-HC), and wastewater from Seoul National University cafeteria (Seoul, Korea) (W2-D-HC) in comparison with hydrated QQ-HC (S-HC). 10 pieces of each medium were used for measurement. Error bar: standard deviation (n= 3 as the technical replicate).

IV.4. Conclusions

The purpose of this chapter was to enhance physical and biological properties of QQ media. Specifically, enforcement of physical strength as well as an extension of the lifetime of a QQ bacteria entrapping hollow cylinder (QQ-HC) was sought by adding a dehydration procedure following the first ionic cross-linking. Based on the results of this study, the following conclusions were made:

- Physical strength of a QQ-HC was increased by addition of dehydration procedure. The elevation of physical strength was related to the dehydration time: it steeply increased until 1 day of dehydration and showed minimal increments afterwards.

- In terms of biological properties, the lost QQ activity from dehydration could be easily recovered through re-activation of QQ-HCs by either LB broth or real wastewater. Furthermore, storing QQ-HCs dehydrated offered greater cell viability when storage time lasted more than 90 days.

- The enhancement in physical and biological properties of QQ-HCs after dehydration resulted in a longer lifetime of QQ-HCs which led to prolonged biofouling mitigation performance in an MBR.

- The reduced weight from dehydration could make application of QQ media more economical and convenient in terms of transporting and handling.

Chapter V

Conclusions

V. Conclusions

A hollow cylinder QQ medium (QQ-HC), was developed based on the information obtained from studying the interaction between QS signal molecules and a QQ-bead. The physical (physical washing effect) and biological (QQ activity) aspect of fouling control of QQ-HC were improved. Addition of dehydration step in preparing QQ-HCs further enhanced physical (strength) and biological (cell viability) properties of QQ-HCs. Based on the experiments, following conclusions were made:

- Increased surface area and more efficient use of medium material allowed QQ-HCs to have higher QQ activity (approximately by 13%) than its predecessor, QQ-beads.
- Larger contact area of a QQ-HC than that of a QQ-bead resulted in enhanced physical washing effect (approximately by 140%) of QQ-HCs.
- Such combinatory improvement in QQ-HCs resulted in greater biofouling control in an MBR.
- Dehydration of QQ-HCs for 1 day enhanced physical strength approximately by 100% compared to the one prepared without the dehydration step.
- QQ activity was partially lost after dehydration but it was easily recovered by re-activating the media with either LB broth or real wastewater.
- Storing QQ-HCs dehydrated resulted in a greater cell viability when storage time exceeded 90 days.
- Such enhanced physical and biological properties resulted in extended lifetime of QQ-HCs in an MBR which led to prolonged biofouling mitigation.

- Reduced weight and improved cell viability from dehydration would allow more economical and convenient storage, transportation, and handing of QQ media in practice.

국문초록

분리막 생물반응기(Membrane bioreactor)는 고도 하·폐수처리 공정으로 각광받고 있지만, 분리막에 생물막오염으로 인한 투수도 감소, 분리막 수명 단축, 운전비 증가 등의 문제점들이 발생하게 된다. 최근에 정족수감지(Quorum sensing)가 생물막형성에 중요한 역할을 한다는 것이 밝혀졌고, 정족수감지 억제(Quorum quenching) 미생물을 담체에 고정해 적용하여 분리막 생물반응기의 생물막오염을 저감을 시킨 연구결과들이 보고되고 있다. 하지만 이전 연구결과들은 QQ 미생물 고정 담체(QQ담체)의 소재와 적용 방법을 달리해 효율을 증가시키고자 하였지만, 정작 중요한 QS 신호분자가 어떻게 QQ담체에 의해 분해되는지에 대해서는 아직 보고 되지 않았다. 또한, QQ담체의 경제성을 확보하기 위해서는 담체의 물리, 생물학적 안정성이 향상될 필요성이 있었다.

따라서 본 연구에서는 QS 신호분자와 QQ담체사이 물질 전달을 조사하였고 이 정보를 바탕으로 더 효율적인 QQ담체를 개발하였다. 추가로, 담체 제조과정에 건조과정을 추가해 담체의 물리, 생물학적 안전성을 향상 시켜 수명을 증가시켰다. 구체적인 연구내용은 다음과 같다.

첫째, 리포터 균주 (reporter strain, *Escherichia coli* JB525)을 이용해 QQ-bead 내부의 QS 신호분자를 시각화 하였다. 이를 통해 담체 내부에 고정된 QQ 미생물은 활성이 기여하지 못한다는 것을 밝혔다. 이 결과를 바탕으로 중공사 형태인 QQ hollow cylinder (QQ-HC)를 개

발하였다. QQ-HC의 내부 빈 공간을 조절함으로써, 고분자 재료의 사용을 줄이면서도, 담체의 비표면적을 증가 시킬 수 있었고, 그로 인해 기존의 QQ-bead 보다 QQ활성이 약 13% 증가됨을 확인하였다. 또한 QQ-HC의 물리세정 효과가 기존 QQ-bead 보다 약 140% 향상되었는데 이것은 분리막과 점으로 충돌하는 QQ-bead보다 선으로 충돌할 수 있는 QQ-HC가 분리막과의 충돌 면적이 크기 때문이라고 생각된다. 실험실 스케일의 MBR에 적용했을 시에도 QQ-HC가 기존의 QQ-bead보다 뛰어난 생물막오염 저감 성능을 보였다. 이는 기초실험에서 확인했듯 QQ-HC의 증가된 QQ활성과 물리세정효과에서 기인한 것으로 보인다.

둘째, 기존 QQ 담체 제법에 건조과정을 추가해 QQ-HC의 물리강도를 약 100% 증가시켰다. Gel fraction과 SEM 분석 결과, 담체를 이루는 고분자 사이 가교결합의 증가로 인해 담체의 물리강도가 증가한 것으로 추측된다. 또한 건조 과정 중 감소된 QQ활성은 LB 배지나 실폐수로 배양하여 초기 값으로 회복이 가능하였다. 건조 된 상태로 보관기간이 90일 넘어갈 시에는 건조 후 보관한 QQ-HC이 생리식염수에 보관 된 QQ-HC보다 QQ 미생물의 생존도 (cell viability) 유지에 더 유리한 것으로 확인되었다. 그 결과 건조과정을 거친 QQ-HC는 실험실 MBR에서 더 높은 수명을 유지하였고 생물막오염 저감 효과 또한 더 오래 유지된 것을 확인하였다. 더 나아가, QQ담체를 건조시키면 부피와 무게가 크게 감소하게 되는데 이는 QQ담체의 운반이나 취급을 경제적이고 용이하게 할 것으로 예상된다.

주요어: 분리막 생물반응기, 생물막오염 억제, 정족수감지, 정족수감지 억제, 담체, 가교

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