공학박사 학위논문

Encapsulation of Quorum Quenching Bacteria in Ceramic Microbial Vessel and its Application for Biofouling Control in MBR

세라믹 용기에 정족수 감지억제 미생물의 고정화 및 분리막-생물반응기에서 생물막 오염제어를 위한 활용

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Abstract

Encapsulation of Quorum Quenching Bacteria in Ceramic Microbial Vessel and its Application for Biofouling Control in MBR

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Commercial use of membrane bioreactors (MBRs) has increased dramatically over the last two decades due to the high quality of its effluent and compactness. However, biofouling, the filterability loss caused by natural biocake formation on the membrane surface, still remains unsolved and significantly reduces efficiency of MBR.

Quorum quenching (QQ) with a microbial vessel has recently been reported as an economically feasible biofouling control platform in MBR for wastewater treatment. In this study, a quorum quenching MBR with a ceramic microbial vessel (CMV) was designed to introduce ‘inner flow feeding mode’ which is expected to overcome the extremely low food-to-microorganism ratio (F/M ratio) inside a microbial vessel. The inner flow feeding, under

which fresh feed was supplied to the MBR only through the center lumen, facilitated nutrient transport to QQ bacteria in the CMV and thus enabled relatively long-term maintenance of cell viability.

The CMV was prepared with a monolithic ceramic micro-porous membrane and indigenous QQ bacteria producing AHL-acylase [1], *Pseudomonas* sp. 1A1. The CMV encapsulated *Pseudomonas* sp. 1A1 proved substantial inhibition of the membrane biofouling. The quorum quenching effect of CMV on controlling membrane biofouling in the MBR was more pronounced with the inner flow feeding mode, which was identified by the slower increase in the trans-membrane pressure (TMP) as well as by the visual observation of less biocake formation on the used membrane surface. In the QQ MBR with the CMV, the concentrations of extracellular polymeric substances (EPS) were substantially decreased in the biocake on the membrane surface compared with those in the conventional MBR. The CMV also showed its potential with effective biofouling control over long-term operation of the QQ MBR.

Successful mitigation of biofouling in a lab-scale MBR suggests that the biofouling control through the CMV with inner flow feeding mode could be expanded to the plant scale of MBR and various environmental engineering

[1] A kind of quorum quenching enzyme

systems with economic feasibility.

Keywords

Membrane bioreactor (MBR), Biofouling control, Quorum sensing, Quorum quenching bacteria, Ceramic microbial vessel, Inner flow feeding mode

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Chapter 1.

Introduction

1.1. Backgrounds

The market growth of MBR technology has been accelerated by rapid increase in implementation of water reuse technologies which are necessitated by the combination of elevated water scarcity and increasingly stringent legislation (Santos et al. 2011). However, membrane biofouling which causes severe flux decline, short membrane life-span, and increase of energy consumption still remains one of major research issues in this area, restricting widespread application of MBR technology (Drews 2010, Chang et al. 1998).

Recently, the concept of quorum sensing has been introduced as a novel biofouling control strategy since Yeon et al. (2009a) revealed that quorum sensing is closely related to biofouling in MBRs for wastewater treatment. Quorum sensing (QS) is the density-dependent regulation of versatile microbial functions such as biofilm formation using signaling molecules called autoinducers (Fuqua et al. 1994, Hammer and Bassler 2003, Parsek and Greenberg 2005, Ponnusamy et al. 2010). Based on QS mechanisms, decomposition of signal molecules, i.e., quorum quenching (QQ) has been reported as an effective membrane biofouling control technique (Kim et al. 2009, Yeon et al. 2009b, Xiong and Liu 2010, Oh et al. 2012, Kim et al. 2013a). For example, acylase which can hydrolyze *N*-acyl homoserine lactone (AHL) type autoinducers from Gram-negative bacteria was successfully applied to mitigate biofouling in MBRs as well as in nanofiltration with

various enzyme immobilization techniques (Yeon et al. 2009b, Kim et al. 2011, Jiang et al. 2012). Energy saving potential of QQ technique was also investigated. Jahangir et al. (2012) reported energy saving equivalent to fifty to sixty percent reduction in aeration rate through the quorum quenching technique in MBR.

To achieve economic feasibility of quorum quenching for anti-biofouling in MBRs, 'enzymatic quorum quenching' has been replaced by 'bacterial QQ' using 'microbial vessel' or 'cell entrapping beads', respectively. Oh et al. (2012) isolated QQ bacteria, *Rhodococcus* sp. BH4 from a real MBR plant and prepared a 'microbial vessel encapsulating QQ bacteria (BH4) to insert the 'microbial vessel' into an MBR. Kim et al. (2013), meanwhile, prepared 'free moving beads' by entrapping the same QQ bacteria. Both 'microbial vessel' and 'moving beads' were successful in substantially mitigating biofouling in MBRs. For practical application, the microbial vessel could be used with both hollow fiber and flat sheet membranes, whereas the moving beads often made trouble with hollow fiber membrane modules because the beads were easily trapped between fibers. Hence, with hollow fiber membrane modules, the microbial vessel is more desirable than the moving beads.

However, in QQ MBR with a polymeric microbial vessel (Oh et al. 2012, Jahangir et al. 2012), the food-to-microorganism (F/M) ratio inside the microbial vessel was extremely small compared to that in a mixed liquor due

to the high population density (M) of the QQ bacteria (> 100,000 mg/L) but the relatively lower substrate available (F) in the vessel than in the broth (< 50 mg COD/L). Such an abnormally small F/M ratio could cause the growth inhibition of QQ bacteria, and eventually lead to the loss of their QQ activity.

The ‘moving beads’ also faces the similar problems when applied to MBR for biofouling mitigation. Therefore more effective and universal quorum quenching methods are required to the successfully control biofouling in real MBR.

1.2. Objectives

The objective of this study was to devise a feasible solution for biofouling control in MBR, especially upgrade previous QQ microbial vessel using a monolithic ceramic micro-porous membrane with a novel indigenous QQ bacterium. To achieve this goal, the overall experiments were carried out in three phases: The specific objectives of this study are as follows:

(1) Design of quorum quenching microbial vessel to enhance cell viability in MBR

A novel QQ microbial vessel, ceramic microbial vessel (CMV) was presented for biofouling control in MBR. The CMV enabled the mechanical nutrient feeding, so called 'inner flow feeding mode' to the QQ bacteria in the microbial vessel. The 'inner flow feeding mode' of CMV was expected to facilitate the mass transfer of air and nutrients and thus augment the local F/M ratio, eventually enhancing the QQ bacterial viability in the CMV.

(2) Isolation and identification of indigenous bacteria producing quorum quenching enzyme

An indigenous QQ bacterium, *Pseudomonas* sp. 1A1, was isolated from a real MBR plant for application of the CMV. Various

characteristics of strain 1A1 were investigated, especially compared with the previous QQ bacterium, *Rhodococcus* sp. BH4.

(3) Application of CMV encapsulated *Pseudomonas* sp. 1A1 for biofouling control in MBR

The CMV containing *Pseudomonas* sp. 1A1 was applied to control biofouling in lab-scale MBR. Its biofouling inhibition effect was confirmed by profile of TMP rise up and observation of confocal laser scanning microscopy (CLSM). Furthermore, the effect of the CMV on SMP and EPS production in MBRs were investigated.

Chapter 2.

Literature Review

2.1. Biological wastewater treatment

2.1.1. Historical overview

Biological wastewater treatment uses live microorganisms to remove contaminants from sewage so that water quality and aquatic lives would not be harmed. Even though the fertilizer value of human excreta was already recognized in early days, sewage from human activity has for a long time been considered a potential health risk and nuisance in urban society. And then the ancient Greeks and the Romans used public latrines which drained into sewers conveying the sewage and storm water to a collection basin outside the city.

During mid-19th century, several epidemics of waterborne diseases such as cholera and typhoid fever ravaged throughout Europe. The emerging knowledge of the role of microorganisms and sanitary systems for the spreading of disease resulted in the construction of sewer systems in several large cities. In the late-19th century, the vast population increase in urbanized areas led to severe pollution of rivers and lakes, creating a demand for wastewater treatment. The first wastewater treatment was the natural treatment on irrigation fields. However, soon artificial treatment plants were used in Europe, which were simple and consisting mainly of primary treatment, i.e. screens, grits, strainers and settling tanks (Seeger 1999). In UK, the leading nation on wastewater treatment of this time, a full-scale biological treatment plant employing biofilm technique (trickling filter) was operated as

early as the 1880s (Lazarova et al. 2000). The activated sludge process was discovered in succession (Figure 2.1). Believing that the sludge has been activated, in a manner similar to activated carbon, the process was named "activated sludge" (Ardern et al. 1914). Widespread large scale biological wastewater treatment was established in Europe during the first half of the 20th century.

In the second half of the 20th century a new problem, eutrophication, in surface water emerged. Eutrophication stands for the explosive growth of algae and other water plants due to the fertilizing effect of nitrogen (N) and phosphorus (P) discharged to surface water. Soon, it was realized that the nitrate produced by nitrification could be used by some heterotrophic bacteria instead of oxygen and converted into nitrogen gas. This insight led to the nitrification-denitrification activated sludge system, in which parts of the reactor were not aerated to induce denitrification.

To inhibit eutrophication, solely nitrogen removal is not sufficient. Phosphorus, mainly in the form of ortho-phosphate from detergents and human waste, also needed to be removed because in many ecosystems phosphorus proved to be the main limiting element for eutrophication. Phosphorus removal by chemical precipitation followed by tertiary filtration appeared during the 1970s. The ambition to achieve a biological treatment set up resulted in the introduction of the enhanced biological phosphorus removal

process (EBPR) in the 1980s.

The increased amounts of wastewater, stricter discharge regulations, energy crisis and lack of space in urbanized areas accelerated the development of alternative methods for biological wastewater treatment. This resulted in a boost of research on biofilm systems leading to the development of innovative and flexible processes including various designs of both fixed and moving bed biofilm reactors (Lazarova et al. 2000). In the Netherlands, processes were developed such as the Single reactor system for High activity Ammonium Removal Over Nitrite (SHARON®), ANaerobic AMMonia Oxidation (ANAMMOX) and Biological Augmentation Batch Enhanced (BABE®) processes for improved nitrogen removal and mineral crystallization processes for phosphorus precipitation for phosphorus recovery and reuse.

Nowadays, improved analytical tools have led to the discovery of a new type of micro-pollutants (Dove 2006, Ternes 2007), which have potential endocrine disrupting effects and might accumulate in the water cycle or effect natural ecosystems. Water shortage comes from global weather accident also leads to further development and implementation of technologies for water reclamation and reuse.

Today we have a problem of the wastewater treatment as never seen before. Therefore, enormous research and development on the efficient wastewater treatment is carried out continuously.

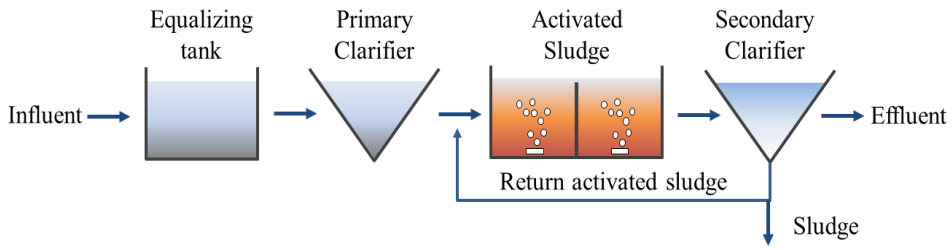


Figure 2.1. Conventional activated sludge process

2.1.2. Biological wastewater process

Due to the obvious economic advantage, both in terms of capital investment and operating costs, of biological treatment over other treatment processes like chemical oxidation, biological treatment using aerobic activated sludge process has been in practice for well over a century.

The purpose of wastewater treatment is to remove pollutants that can harm the aquatic environment if they are discharged into it. The major biological processes used for wastewater treatment are identified. These are five major groups: aerobic processes, anoxic processes, anaerobic processes, combined aerobic, anoxic, and anaerobic processes, and pond processes. The individual processes are further subdivided, depending on whether treatment is accomplished in suspended-growth systems or in attached-growth systems. The brief classification of biological wastewater treatment methods is described in Figure 2.2.

Municipal wastewater is composed of organic material, i.e. proteins, carbohydrates, fats and oils; nutrients, mainly nitrogen and phosphorus; as well as trace amounts of recalcitrant organic compounds and metals (Bitton 2005). Biodegradable organic material is biochemically oxidized by heterotrophic bacteria under aerobic conditions resulting in production of carbon dioxide, water, ammonia and new biomass (Madigan 2005).

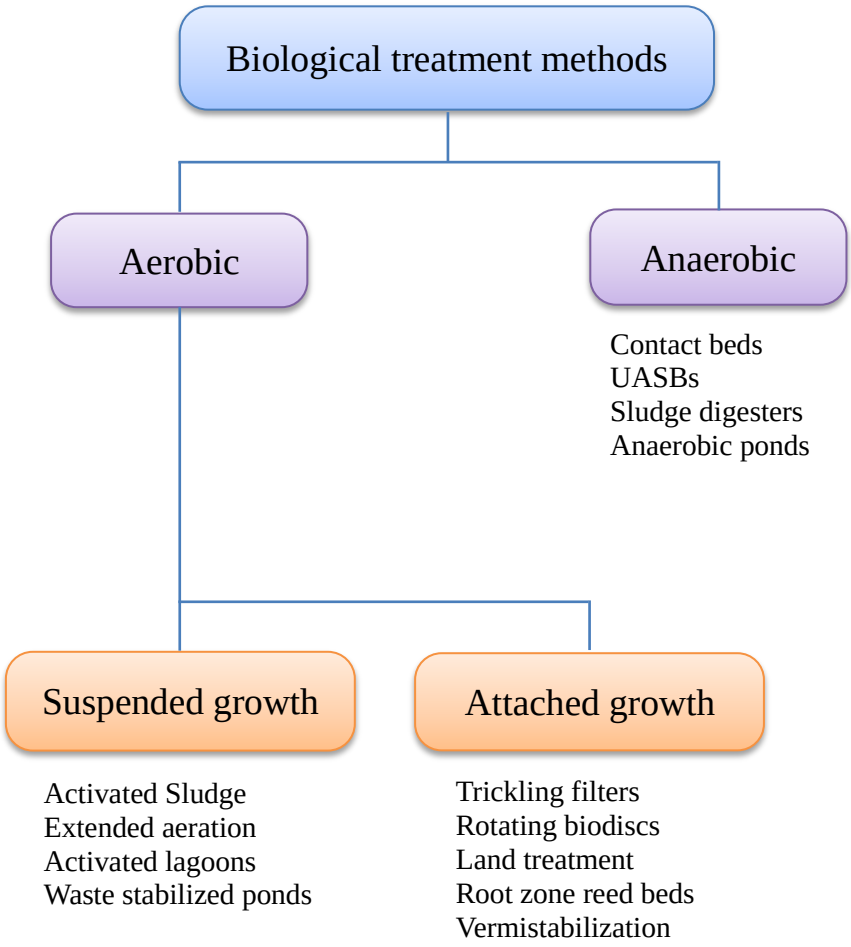


Figure 2.2. Classification of biological wastewater treatment process (Yung-Tse et al. 2012)

2.1.3. Operation parameters

(1) Air or Oxygen supply

The importance of dissolved oxygen (DO) in aerobic wastewater treatment is well-known as inadequate oxygen supply may be a primary cause of process failure. A minimum dissolved oxygen level of 2 mg/L is commonly accepted as a requirement for proper performance of aerobic systems. Low dissolved oxygen levels at high loadings promote sulfide production within a thick biofilm, which enhances the growth of sulfide-oxidizing organisms such as *Beggiatoa*. Sulfide present in influent wastewater can also stimulate *Beggiatoa* growth. *Beggiatoa*, a filamentous organism, structurally reinforces biofilm, thus complicating biomass control (Vesilind 2003).

(2) Food-to-Microorganisms ratio

Organic loading rate is an important design and controlling parameter in biological wastewater treatment process. It is measured by the amount of food provided to a unit amount of biomass (or reactor volume) for a unit period of time. Food-to-microorganisms (F/M) ratio is based on the amount of biomass while Food-to-volume (F/V) ratio is based on reactor volume. The F/M and the F/V are calculated using the following equations.

$$F/M = \frac{QS_0}{XV} \qquad \text{……..} \quad (1)$$

$$F/V = \frac{QS_0}{V} \qquad \text{……..} \quad (2)$$

Where, F/M = food-to-microorganism ratio (g BOD/g MLSS/day)

F/V = food-to-volume ratio (g BOD/L Tank/day)

Q = influent flow rate (m^3/day)

S_0 = influent BOD (mg/L)

X = MLSS in aeration tank (mg/L)

V = tank volume (m^3)

Four different F/M ratio exist depending on the unit used for food and microorganisms. Those four are inter-convertible using the ratio of COD/BOD and MLVSS/MLSS as shown in Table 2.1, where COD/BOD and MLVSS/MLSS were assumed at 2.0 and 0.8, respectively, for municipal wastewater.

Typically MBR runs at lower F/M ratio than conventional activated sludge (CAS) process in order to mitigate membrane fouling and maintain high oxygen transfer efficiency. The preferred F/M ratio range in MBR is approximately a third to a half of that in CAS, *i.e*. 0.05-0.15 g BOD/g MVLSS/day or 0.1-0.3 g COD/g MLVSS/day in municipal MBR. It is noticeable that nitrogen is not taken into consideration when F/M and F/V are calculated, but it contributes to the oxygen demand significantly (Grady Jr. et al. 2011).

Table 2.1. Comparison of preferred range of operating parameter in MBR and CAS (conventional activated sludge)

Design Parameter	Unit	MBR	CAS
F/M	g BOD/g MLSS/day	0.04-0.12	0.16-0.24
	g COD/g MLSS/day	0.08-0.24	0.32-0.48
	g BOD/g MLVSS/day	0.05-0.15[1), 2)]	0.2-0.3
	g COD/g MLVSS/day	0.1-0.30[3)]	0.4-0.6
F/V	g BOD/L/day	0.5-1.5	0.6-0.9
	g COD/L/day	1.0-3.0	1.2-1.8
MLSS	g/L	8-12[1)]	2-4
MLVSS	g/L	6-10	1.7-3.4
SRT	days	10-30	5-10
OUR	mg O_2/L/hr	15-50	20-40
DO	mg/L	1-2	1-2

1) Judd, 2006

2) Brepols, 2010

3) Converted from the BOD based F/M assuming COD/BOD=2.0 for municipal wastewater

(3) Temperature

Variations in temperature affect all biological processes. Just as with pH, each species is characterized by a minimum, optimum, and maximum temperature that will support growth. There are three temperature regimes: the mesophilic over a temperature range of 4 to 39^{o}C, the thermophilic which peaks at a temperature of 55 ^{o}C, and the psychrophilic which operates at temperature below 4 ^{o}C. For ecomomic and geographical reasons, most aerobic biological treatment processes operate in the mesophilic range. In the mesophilic range, the rate of the biological reaction will increase with temperature to a maximum value at 31 ^{o}C for most aerobic waste system. A temperature above 30 ^{o}C will result in a decreased rate for mesophilic organisms. At temperatures above 35.5 ^{o}C there is deterioration in the biological floc. Protozoa have been observed to disappear at 40 ^{o}C and a dispersed floc with filaments to dominate at 43.3 ^{o}C (Eckenfelder 1989).

(4) Potential of hydrogen ion: pH

As a chemical component of the wastewater, pH has direct influence on wastewater treatability. The optimal range for the activated sludge process is a pH in the range of 7 to 7.5. Outside this range the biological activity diminishes more or less in the same fashion as with the temperature changes. pH monitoring and control is important in the operation of those municipal

wastewater treatment plants that use biological methods of secondary treatment and those using chemical treatment processes such as chlorination. In addition, pH control of sewage in collection systems and in certain sludge treatment processes is essential.

(5) Toxicity

Toxic chemicals in the wastewater can enter the bacteria and inhibit one or more enzymes of the pathways involved in either anabolism or catabolism. If the catabolic reactions of respiration are affected, the rate of respiration and energy production is reduced and the rate of growth is reduced, and this accompanied by a fall in the rate of respiration, as the requirement for energy is reduced. Conventionally, toxicity is expressed as the EC_{50}, EC_{20}, or EC_{10} i.e. the concentration causing an inhibition of 50%, 20% or 10% of the respiration rate.

2.2. Membrane Bioreactor (MBR)

2.2.1. MBR History

The MBR is a technology which combines an activated sludge reactor with a membrane filtration unit for the advanced wastewater treatment and reuse. The application of membrane technology in the water pollution control field was implemented by Dorr-Oliver, Inc. (Milford, Connecticut, U.S.) in the early 1960s for the separation of fine particulate from waste. In 1974, Dorr-Oliver engineers entered into a licensing agreement with Sanki Engineering Company (Tokyo, Japan). The Japanese government had mandated that wastewater is reclaimed and reused to reduce the need for potable water as well as the amount of wastewater being discharged to wastewater treatment works. Sanki installed approximately 20 membrane wastewater reclamation systems similar to the one used in the Pikes Peak visitors' center. These facilities incorporated modules that held cartridges of flat-plate membranes installed in a horizontal plane and referred to as the *membrane wastewater treatment system* (Bemberis 1971). Other bench-scale membrane separation systems linked with a CAS were reported at around the same time (Bailey et al. 1971). These systems were all based on what have come to known as 'sidestream' configuration (Figure 2.3(b2)).

The major breakthrough, which allowed the MBR to grow much faster, was the development in Japan. From 1980s to early 1990s, the Japanese

government instigated water recycling program which prompted pioneering work by Yamamoto, Hiasa, Mahmood & Matsuo (1989) to develop an immersed hollow fiber (HF)-ultra filtration (UF) MBR process, as well as the development of an flat sheet (FS)-microfiltration (MF) submerged membrane MBR by the agricultural machinery company, Kubota (Yamamoto et al. 1989).

By the end of 1996, there were already 60 Kubota plants installed in Japan for domestic wastewater treatment. At the same time as Kubota were developing their products, in the USA Thetford Systems were developing their *Cycle-Let*® process, another sidestream process. Zenon Environmental, a company formed in 1980 and who subsequently acquired Thetford System were developing an MBR system. By the early 1990s, the *ZenoGem*® immersed HF-UF MBR process had been patented and introduced to the market in 1993.

In 1997, the first Kubota municipal wastewater treatment works installed external Japan was at Porlock in the United Kingdom. And the first Zenon membrane-based plant of similar size installed outside of the USA was the Veolia *Biosep*® plant at Perthes en Gatinais in France in 1999. Both these plants have a peak flow capacity just below 2 mega liter per day (MLD), and represent landmark plants in the development and implementation of immersed MBR technology.

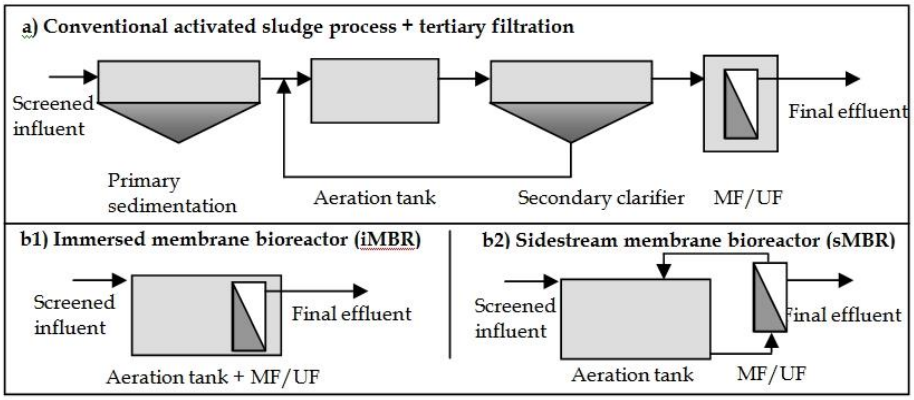


Figure 2.3. Conventional activated sludge process (a) and MBR in both configurations: immersed (b1) and sidestream (b2) (Delgado et al. 2011)

2.2.2. Market and Research

(1) MBR market

The global MBR market was worth $838.2 million in 2011 and is expected to witness positive growth and revenue sales through 2018 (Frost & Sullivan 2013). As shown in Figure 2.4, it is rising at 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 2010). In addition, more than 4,400 MBR plants have been installed worldwide by the end of 2009 (Judd 2010).

(2) Research : Membrane Fouling

The research trend in MBR technology was reviewed by the data on the number of articles published since year 1995. As shown in Figure 2.5, number of published papers increased dramatically since year 2005 in similar way with global market value, which suggests growing acceptance of MBR technology and escalating technical demand to overcome the perceived drawbacks of MBR such as its complex and small scale nature, high costs and operator skill requirements.

The performance of MBR process is mainly deteriorated by the membrane fouling which is the occlusion or blocking of membrane pores at the surface of the membrane. This membrane fouling causes severe operation

problems of flux decline, short membrane life-span, and increases of energy consumption and therefore, has been regarded as a main obstacle restricting the development of MBR technology. As clearly shown in Figure 2.5(a), about 50% of MBR researches were dedicated to the topic of fouling including investigation of the mechanism and development of the control techniques.

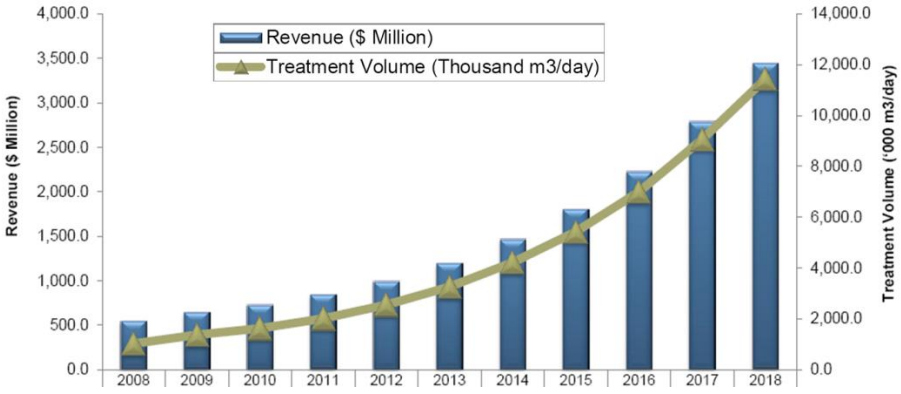
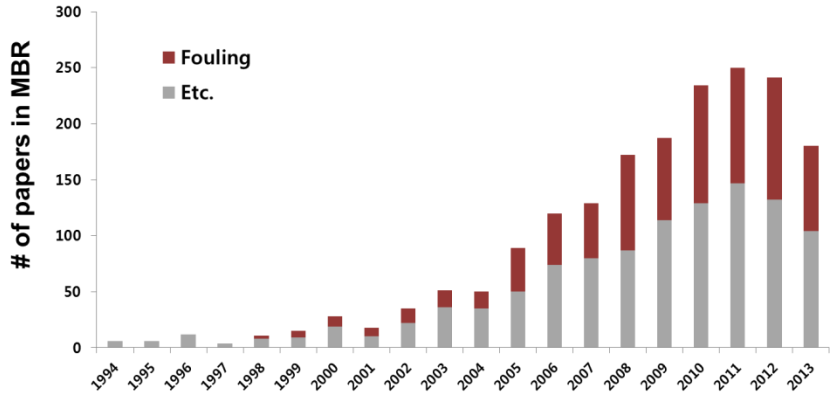
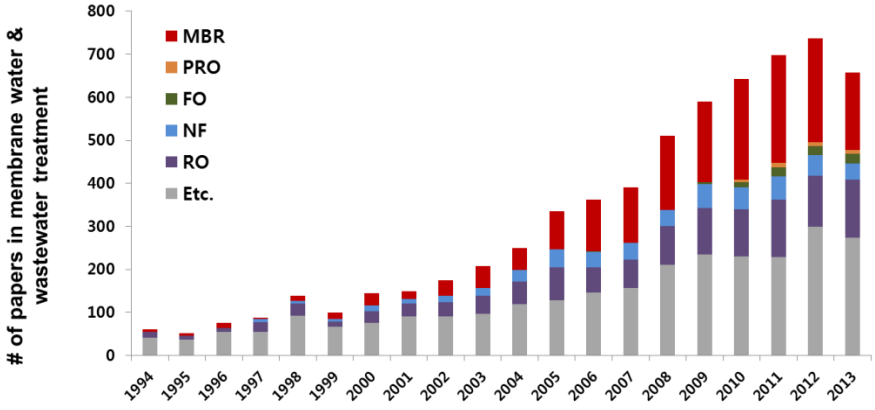


Figure 2.4. Global MBR market value MBR and treatment volume (Frost & Sullivan 2013, Sze Chai Kwok 2010)



(a)



(b)

Figure 2.5. Number of research articles published since 1994 regarding (a) MBR, and (b) wastewater treatment (Source ISI)

2.2.3. Advantage and Disadvantage of MBR

The advantages offered by MBR over conventional activated sludge processes (CAS) are widely recognized and of these the ones most often cited are (Judd 2008):

(1) Production of high quality, clarified and largely disinfected permeate product in a single stage; the membrane has an effective pore size <0.1 mm – significantly smaller than the pathogenic bacteria and viruses in the sludge.

(2) Independent control of solids and hydraulic retention time (SRT and HRT, respectively). In a conventional ASP separation of solids is achieved by sedimentation, which then relies on growth of the mixed liquor solid particles (of flocs) to a sufficient size (>50 mm) to allow their removal by settlement. This then demands an appropriately long HRT for growth. In an MBR the particles need only be larger than the membrane pore size.

(3) Operation at higher mixed liquor suspended solids (MLSS) concentrations, which reduces the required reactor size and promotes the development of specific nitrifying bacteria, thereby enhanced ammonia removal.

(4) Reduced sludge production, which results from operation at long SRTs

because the longer the solids are retained in the bioreactor the lower the waste solids (sludge) production.

Of these advantages, it is the intensity of the process (i.e. the smaller size of the plant compared to conventional treatment) and the superior quality of the treated product water that are generally most important in practical wastewater treatment applications. An MBR displaces three or four individual process, demanding only that the initial screening stage be upgraded to limit the impact of large gross solids (>1–3 mm in size) on clogging of the membrane flow channels.

Having said that, compared with conventional bio-treatment processes MBRs are to some extent constrained, primarily by:

(1) Greater process complexity; membrane separation demands additional operational protocols relating to the maintenance of membrane cleanliness.

(2) Higher capital equipment and operating costs; the membrane component of the MBR incurs a significant capital cost over and above that of an CAS and maintaining membrane cleanliness demands further capital equipment and operating costs. This is only partly offset by the small size of the plant.

2.3. Quorum Sensing (QS)

2.3.1. Definition and Mechanism

Bacteria perceives its own cell population density and communicate with each other using small signaling molecules called autoinducers and, as a result, coordinate expression of specific genes to fluctuations in cell-population density. This “bacterial signaling” is generally described as “quorum sensing (QS)” in reference to the frequent observation that the signals only accumulate in environments that support a sufficiently dense population (a quorum) of signal producing bacteria.

QS system can be divided into four general classes based on the type of autoinducer signal and the apparatus used for its detection (Fuqua and Greenberg 2002):

① Gram-negative bacteria typically have LuxI/LuxR type QS

② Gram-positive bacteria which use modified oligopeptides autoinducers

③ Autoinducer-2 (AI-2) for the interspecies communication

④ AI-3 and *Pseudomonas* quinolone signal (PQS)

(1) Gram-Negative Bacteria: LuxI/LuxR type QS

General mechanism of LuxI/LuxR type quorum sensing system of gram negative bacteria was depicted in Figure 2.6. In this type of QS system, *N*-acylated homoserine lactones (AHLs) which consist of a homoserine lactone

ring joined to a fatty acid side that can vary in the number of carbons and modifications on the third carbon is used as an autoinducer (Figure 2.7).

In these gram-negative bacterial QS circuits contain, at a minimum, homologues of two *Vibrio fischeri* regulatory proteins called LuxI and LuxR. The LuxI-like proteins synthesize a specific AHL signaling molecule ligating a specific acyl moiety from an acyl–acyl carrier protein (acyl-ACP) to the homocysteine moiety of *S*-adenosylmethionine (SAM). In detail, the LuxI-type protein directs the formation of an amide linkage between SAM and the acyl moiety of the acyl-ACP. Subsequent lactonization of the ligated intermediate with the concomitant release of methylthioadenosine occurs. This step is results in the formation of the AHL autoinducers (Figure 2.8).

The autoinducer concentration increases with increasing cell-population density. The LuxR-like proteins bind cognate AHL autoinducers that have achieved a critical threshold concentration, and the LuxR-autoinducer complexes also activate target gene transcription

The LuxI/R systems have been identified in over 25 species of gram-negative bacteria and these bacteria were summarized at Table 2.2. Among the 25 species of bacteria that mediate QS by means of a LuxI/LuxR type circuit, the *Vibrio fischeri*, *Pseudomonas aeruginosa*, *Agrobacterium tumefaciens*, and *Erwinia carotovora* systems are the best understood, and descriptions of each of these systems are provided below.

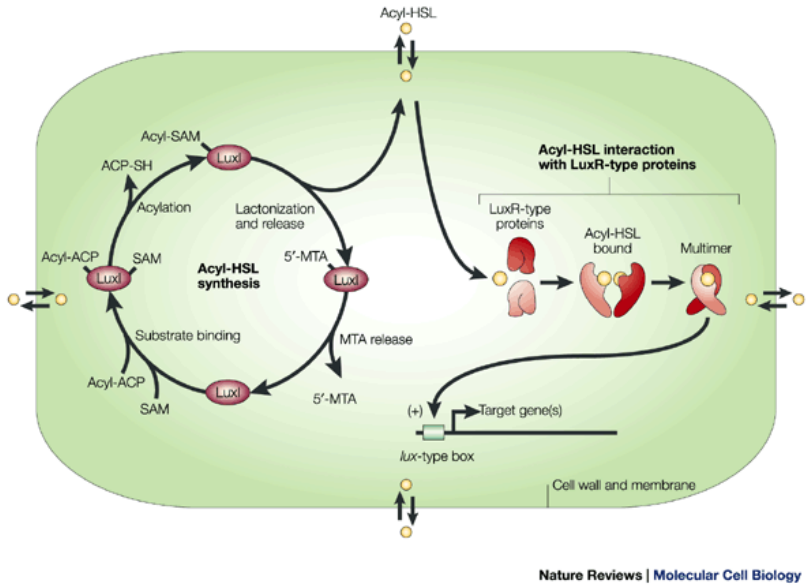


Figure 2.6. Model of AHL QS in a single generalized bacterium (Fuqua and Greenberg 2002).

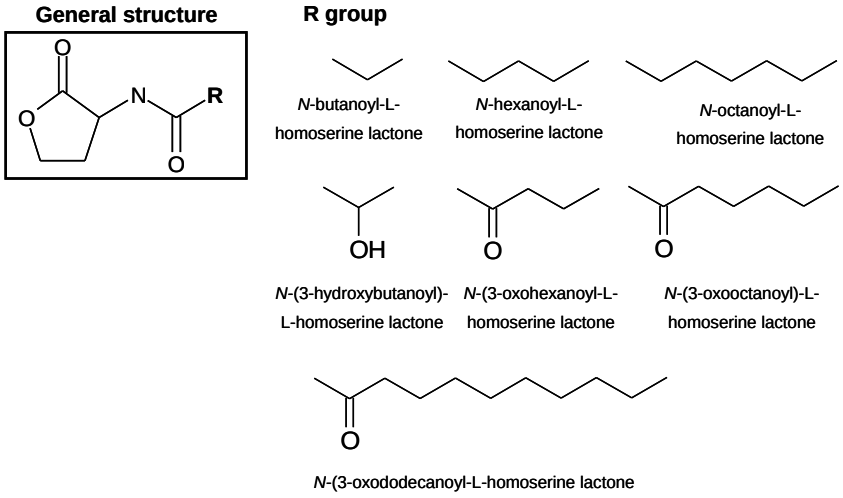


Figure 2.7. Molecular structure of each AHL autoinducer

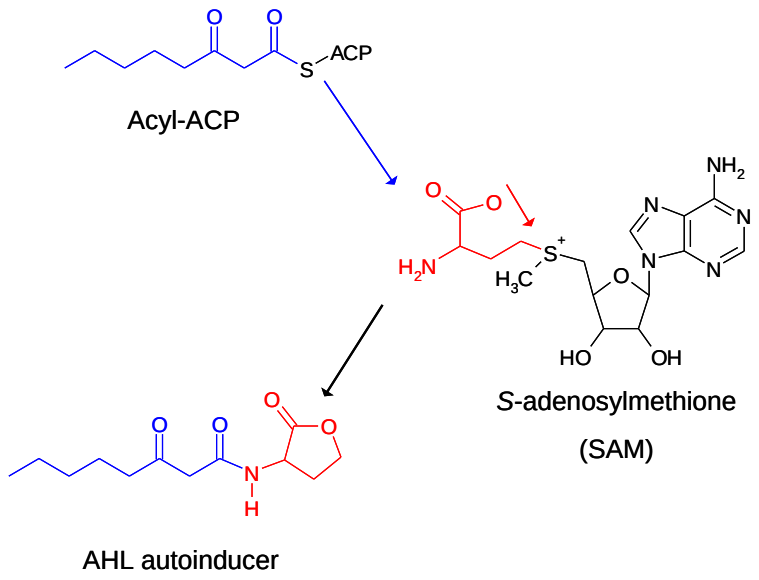


Figure 2.8. LuxI-directed biosynthesis of AHL autoinducers. Shown in the figure is the *N*-(3-oxooctanoyl)-L-homoserine lactone(3-oxo-C8-HSL), which is synthesized by TraI of *Agrobacterium tumefaciens*.

Table 2.2. Organisms possessing LuxI/LuxR homologues : the regulatory proteins, the AHL autoinducers, and the regulated functions(Miller and Bassler 2001)

Organism	LuxI/LuxR Homologue	Autoinducer Identity	Target genes and functions
Vibrio fischeri	LuxI/LuxR	3-oxo-C6-HSL	*luxICDABE* (bioluminescence)*(Eberhard et al. 1981)*
Aeromonas hydrophila	AhyI/AhyR	C4-HSL	Serine protease and metalloprotease production*(Swift et al. 1997)*
Aeromonas salmonicida	AsaI/AsaR	C4-HSL	*aspA* (exoprotease)*(Swift et al. 1999)*
Agrobacterium tumefaciens	TraI/TraR	3-oxo-C8-HSL	*tra, trb* (Ti plasmid conjugal transfer)*(Piper et al. 1993)*
Burkholderia cepacia	CepI/CepR	C8-HSL	Protease and siderophore production*(Lewenza et al. 1999)*
Chromobacterium violaceum	CviI/CviR	C6-HSL	Violacein pigment, hydrogen cyanide, antibiotics, exoproteases and chitinolytic enzymes*(McClean et al. 1997)*
Enterobacter aglomerans	EagI/EagR	3-oxo-C6-HSL	Unknown*(Swift et al. 1993)*
Erwinia Carotovora	(a) ExpI/ExpR (b) CarI/CarR	3-oxo-C6-HSL	(a) Exoenzyme synthesis*(Pirhonen et al. 1993)* (b) Carbapenem antibiotic synthesis*(Bainton et al. 1992)*
Erwinia hrysanthemi	ExpI/ExpR	3-oxo-C6-HSL	*pecS* (regulator of ectinase synthesis)*(Reverchon et al. 1998)*
Erwinia stewartii	EsaI/EsaR	3-oxo-C6-HSL	Capsular polysaccharide Biosynthesis, virulence*(Vonbodman and Farrand 1995)*

Table 2.2. (Continued)

Organism	LuxI/LuxR Homologue	Autoinducer Identity	Target genes and functions
Pseudomonas aereofaciens	PhzI/PhzR	C6-HSL	*phz* (phenazine antibiotic biosynthesis) *(Wood et al. 1997)*
Pseudomonas aeruginosa	(a) LasI/LasR (b) RhlI/RhlR	(a)3-oxo-C12-HSL (b) C4-HSL	(a) *lasA*, *lasB*, *aprA*, *toxA* (exoprotease virulence factors), biofilm formation *(Pearson et al. 1994)* (b) *lasB*, *rhlAB* (rhamnolipid), *rpoS* (stationary phase)*(Pearson et al. 1995)*
Ralstonia solanacearum	SolI/SolR	C6-HSL C8-HSL	Unknown *(Flavier et al. 1997)*
Rhizobium etli	RaiI/RaiR	Multiple, unconfirmed	Restriction of nodule number *(Rosemeyer et al. 1998)*
Rhizobium leguminosarum	(a) RhiI/RhiR (b) CinI/CinR	(a) C6-HSL (b) 3-OH-7-cis-C14-HSL	(a) *rhiABC* (rhizosphere genes) and stationary phase *(Rodelas et al. 1999)* (b) QS regulatory cascade *(Lithgow et al. 2000)*
Rhodobacter sphaeroides	CerI/CerR	7,8,-cis-C14-HSL	Prevents bacterial aggregation *(Puskas et al. 1997)*
Serratia liquefaciens	SwrI/SwrR	C4-HSL	Swarmer cell differentiation, exoprotease*(Givskov et al. 1997)*
Vibrio anguillarum	VanI/VanR	3-oxo-C10-HSL	Unknown *(Milton et al. 1997)*
Yersinia enterocolitica	YenI/YenR	C6-HSL, 3-oxo-C6-HSL	Unknown *(Throup et al. 1995)*
Yersinia pseudotuberculosis	(a)YpsI/YpsR (b)YtbI/YtbR	(a) 3-oxo-C6-HSL (b) C8-HSL	Hierarchical QS cascade regulating bacterial aggregation *(Atkinson et al. 1999)*

(2) Gram-Positive Bacteria with modified autoinducers

Gram-positive bacteria also regulate a variety of processes in response to increasing cell-population density. However, in contrast to Gram-negative bacteria, which use HSL autoinducers, Gram-positive bacteria employ secreted peptides as autoinducers for quorum sensing. In general, the peptide is secreted via a dedicated ATP-binding cassette (ABC) transporter as an autoinducer for QS.

A general model for quorum sensing in Gram-positive bacteria is shown in Figure 2.9 (Miller et al. 2001). In Gram-positive bacteria, a peptide signal precursor locus is translated into a precursor protein (black and white diamonds) that is cleaved (arrows) to produce the processed peptide autoinducer signal (black diamond). Generally, the peptide signal is transported out of the cell via an ABC transporter (gray protein complex). When the extracellular concentration of the peptide signal accumulates to the minimal stimulatory level, a histidine sensor kinase protein of a two-component signaling system detects it. The sensor kinase autophosphorylates on a conserved histidine residue (H), and subsequently, the phosphoryl group is transferred to a cognate response regulator protein. The response regulator is phosphorylated on a conserved aspartate residue (D). The phosphorylated response regulator activates the transcription of target gene(s). The oval represents a bacterial cell. The “P” in the circle represents the phosphorylation

cascade. Note that the lengths of the precursor and processed peptides are not meant to signify any specific number of amino acid residues.

In contrast to Gram-negative bacteria, Gram-positive bacteria use two-component adaptive response proteins for detection of the autoinducers. Two-component sensor kinases are the detectors for the secreted peptide signals. Interaction with the peptide ligand initiates a series of phosphoryl events that culminate in the phosphorylation of a cognate response regulator protein. Phosphorylation of the response regulator activates it, allowing it to bind DNA and alter the transcription of the quorum sensing–controlled target genes.

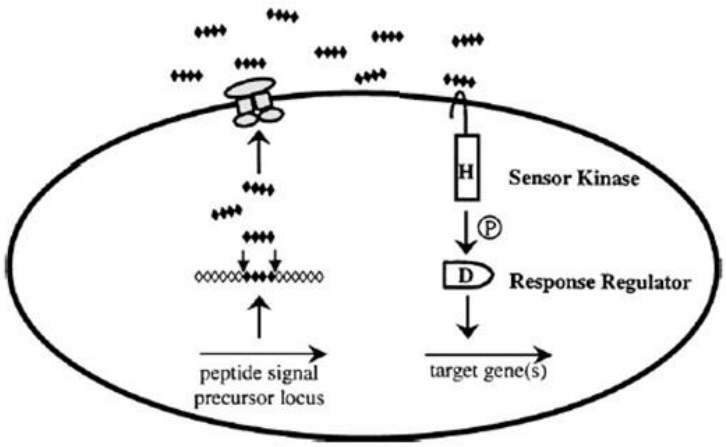


Figure 2.9. A general model for quorum sensing in Gram-positive bacteria (Miller and Bassler 2001)

(3) Autoinducers-2 (AI-2) for the Interspecies Communication

The *Vibrio harveyi* quorum sensing circuit possesses features reminiscent of both Gram-negative and Gram-positive quorum sensing systems. Similar to other Gram-negative bacteria, *V. harveyi* produces and responds to an acylated homoserine lactone. In contrast to Gram-negative bacteria, but analogous to Gram-positive bacteria, quorum sensing signal transduction in *V. harveyi* occurs via a two-component circuit (Bassler 1999). Additionally, *V. harveyi* possesses a novel signaling molecule, denoted AI-2 (Bassler et al. 1993, Bassler et al. 1994).

This molecule and the gene required for its production have recently been shown to be present in a variety of Gram-negative and Gram-positive bacteria (Surette et al. 1999, Surette and Bassler 1998). AI-2 could be the link that ties together the evolution of the two major classes of quorum sensing circuits. The structure of *V. harveyi* AI-2 signaling molecule was recently determined to be a novel furanosyl borate diester with no similarity to other autoinducers.

Response to AI-2 was graphically depicted in Figure II-15 (Vendeville et al. 2005). The clearest evidence that demonstrates that AI-2 can control gene expression through quorum sensing comes from studies of *Vibrio* spp., in particular the organism in which it was first identified, *V. harveyi*. AI-2 is one of three quorum sensing signal molecules produced by *V. harveyi* that control

bioluminescence. AI-2 can gain access to the periplasm of *V. harveyi*, where it interacts with the periplasmic binding protein LuxP, which in turn interacts with a membrane-bound histidine protein kinase, LuxQ (Chen et al. 2002). In the absence of AI-2, LuxQ autophosphorylates (Freeman and Bassler 1999a, b, Freeman et al. 2000) and transfers a phosphate to LuxU, which subsequently passes it to the response regulator, LuxO. Phospho-LuxO, with σ54, activates expression of small regulatory RNA (sRNA), which, with the help of the chaperone Hfq, destabilizes the mRNA that encodes the activator protein LuxRVh. As LuxRVh is required for transcription of the luciferase-encoding genes, no light is generated. At high AI-2 concentrations (indicating high cell density), LuxQ functions as a phosphatase, reversing the flow of phosphates and turning on light production (Figure 2.10).

The sensors of the other two autoinducers (AI-1 and CAI-1 (cholerae AI-1)) feed into the same pathway at the level of LuxU. The components of the AI-2 response network are shared by other *Vibrio* species (including *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Vibrio vulnificus* and *Vibrio fischeri*, as well as the renamed *Listonella anguillaru* (Hardt et al. 1970, Lenz et al. 2004). This mechanism integrates the sensory inputs from all the autoinducers; however, *V. harveyi* can distinguish between conditions in which all autoinducers are present (the 'coincidence state', characterized by complete dephosphorylation of LuxO) and all other variations (Henke and Bassler 2004,

Mok et al. 2003). So in *V. harveyi*, the individual presence of an autoinducer initiates a phosphatase activity that exceeds the remaining kinase activity, but is not sufficient to deplete phospho-LuxO completely.

Therefore, the simultaneous presence of all three autoinducers will result in a more dramatic effect on gene regulation. The sensitivity of this system might even enable *V. harveyi* to respond to changes in the ratio between AI-1 and AI-2 (Mok et al. 2003). The involvement of sRNAs in this system might impart the ability to behave as a sensitive, definitive on/off switch leading to an all-or-nothing response (Lenz et al. 2004). Moreover, it is not dedicated to the control of bioluminescence, but can modulate other phenotypes in *V. harveyi* (Lilley and Bassler 2000).

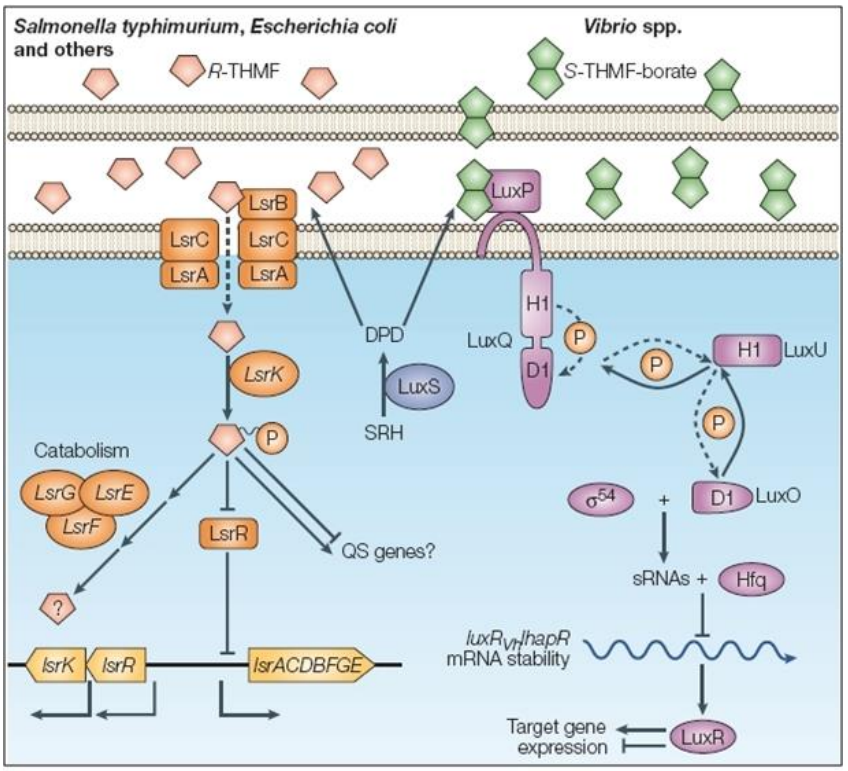


Figure 2.10. Current scheme of autoinduce-2 (AI-2) response (Vendeville et al. 2005)

(4) Other Quorum Sensing Systems

At least, two additional QS systems have been identified in gram-negative bacteria. These include autoinducer 3 (AI-3), which is associated with virulence regulation in EHEC O157:H7 and the *Pseudomonas* quinolone signal (PQS), which is associated with *Pseudomonas aeruginosa* (Mashburn and Whiteley 2005).

AI-3 is associated with luxS homologs in EHEC O157:H7, but this signal itself is hydrophobic and thus chemically distinct from the polar AI-2 signals. AI-3 is also biologically distinct from AI-2. During EHEC pathogenesis, both AI-3 and host epinephrine, but not AI-2 stimulates expression of the 'locus of enterocyte effacement (LEE)' genes and thus provide evidence of bacteria and host cross-talk during this infection (Walters and Sperandio 2006).

A novel, additional autoinducer has recently been demonstrated to be involved in quorum sensing in *Pseudomonas aeruginosa*. This signal is noteworthy because it is not of the homoserine lactone class. Rather, it is 20-heptyl-3-hydroxy-4-quinolone (denoted PQS for *Pseudomonas* quinolone signal) (Pesci et al. 1999). PQS partially controls the expression of the elastase gene *lasB* in conjunction with the Las and Rhl QS systems. The expression of PQS requires LasR, and PQS in turn induces transcription of *rhlI*. These data indicate that PQS is an additional link between the Las and

Rhl circuits. The notion is that PQS initiates the Rhl cascade by allowing the production of the Rhl-directed autoinducer only after establishment of the LasI/LasR signaling cascade. PQS molecules are quite hydrophobic and have been shown to be transported between cells by outer membrane vesicles. There is also strong evidence that the PQS actually induces the formation of these vesicles through interference with Mg^{2+} and Ca^{2+} ions in the outer membrane. In a recent review, it was suggested that membrane vesicles may represent a mechanism for inter kingdom signaling in the plant rhizosphere.

2.3.2. Role of QS in Biofilm

In general, biofilm cells encounter much higher local cell densities than free-floating, planktonic cell populations. An obvious consequence of this is the elevated levels of metabolic by-products, secondary metabolites and other secreted or excreted microbial factors that biofilm cells encounter. Of particular interest is intercellular signaling or QS molecules. Because biofilms generally consist of aggregates of cells, one could argue that they present an environmentally relevant context for QS. This idea that the biofilms are optimum sites for expression of phenotypes regulated by QS has led to numerous studies of QS mechanism in the bacterial biofilms including its various phenotypes.

The maturation of a biofilm community occurs downstream of adherence. Several factors have been shown to influence biofilm architecture, including motility, homogeneity of microorganisms, extracellular polymeric substance matrix production and rhamnolipid production (Klausen et al. 2003, Hentzer et al. 2001, Davey et al. 2003).

AHL-based QS has been shown to influence biofilm maturation for the gram-negative bacterium *Serratia liquefaciens* (Labbate et al. 2004). QS Regulates swarming motility in *S. liquefaciens* (Eberl et al. 1996). Wild-type *S. liquefaciens* biofilms are heterogeneous, consisting of cell aggregates and long filaments of cells. A mutation in the AHL synthesis gene, *swr*I, resulted

in thin biofilms that lacked aggregates and filaments. Two regulated genes, *bsm*A and *bsm*B, were implicated in biofilm development. The CepI/R QS system of *Burkholderia cepacia* H111 has also been shown to control biofilm maturation. Huber et al. (Huber et al. 2001) showed that strains with mutations in either *cep*I or *cep*R formed biofilms that were arrested at the microcolony stage of growth, whereas the wild-type strain formed more robust biofilms that covered the attachment surface. The AhyI/R AHL QS system of *Aeromonas hydrophila* has also been shown to be required for biofilm formation (Lynch et al. 2002). A strain harboring an *ahy*I mutation formed biofilms that were structurally less differentiated than the wild-type strain. For all three of the systems mentioned, the functional consequence of this altered architecture is unclear.

The LuxS type QS system in *Streptococcus* mutants is also involved in biofilm development. A mutation in *lux*S resulted in mature biofilms with less overall biomass (Wen and Burne 2004). The architecture of the mature biofilm was different for the mutant strain. A *lux*S mutant biofilm grown on hydroxyapatite disks was loose and rough in appearance compared with that of the wild-type strain, which formed biofilms that were smooth and confluent. There are growing evidences that QS constitutes a global regulatory system in many different parts. Many studies have shown that QS affects the biofilm development for several bacterial species. For example, In *Pseudomonas*

aeruginosa (Parsek and Greenberg 2000) (Figure 2.11), *Burkholderia cepacia*, and *Aeromonas hydrophila*, are known to be require a functional AHL-mediated QS system for formation of biofilms (Huber et al. 2001, Lynch et al. 2002, Davies et al. 1998). A common theme in the studies in these studies is that mutants are constructed in key QS regulators and then biofilm phenotypes are evaluated. In detail, biofilms formed by QS mutant or biofilm treated with quorum sensing inhibitors were much more susceptible of the antibiotics or biocides. From these researches, QS can be a highly attractive target for chemotherapy against biofilm chronic formations.

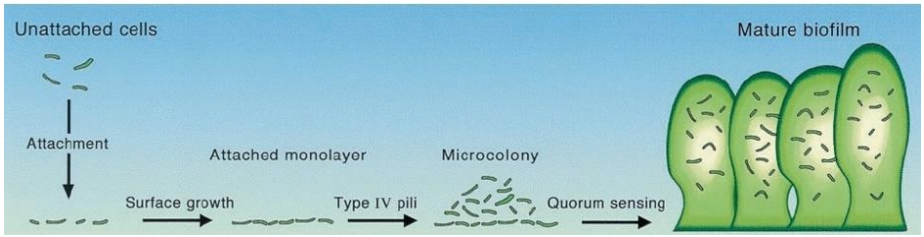


Figure 2.11. Diagram of the *P. aeruginosa* biofilm-maturation pathway. Unattached cells that approach a surface may attach. Attachment involves specific functions. Attached cells will proliferate on a surface and use specific functions to actively move into micro-colonies. The high-density micro-colonies differentiate into mature biofilms by a 3-oxo-C12-HSL-dependent mechanism

2.3.3. Quorum sensing control Strategy

There are three strategies to control the AHL type QS system of gram-negative bacteria (Figure 2.12). Focal points for controlling the QS system are listed below: The signal generator (LuxI homologue), the signal receptor (LuxR homologue), and the signal molecule (AHL)

Therefore, AHL QS inhibition strategy can be divided into three ways: First, Blockage of AHL synthesis, second, Interference with signal receptor, and third, Inactivation of AHL molecules

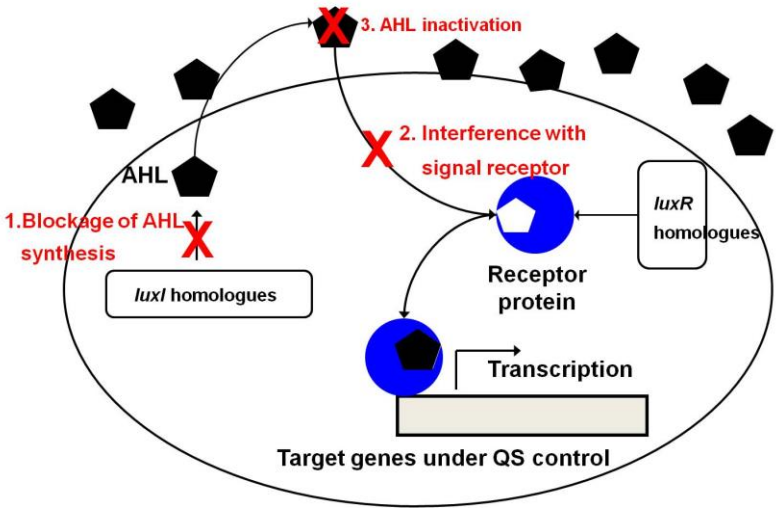


Figure 2.12. Three strategies to control AHL-type QS system of gram-negative bacteria

(1) Blockage of AHL synthesis

To date, the least investigated strategy to interfere with quorum sensing is blockage of AHL production. The LuxI family proteins use *S*-adenosyl-L-methionine (SAM) and specific acyl-acyl carrier proteins (acyl-ACP) as substrates for AHL autoinducer biosynthesis. The LuxI type proteins direct the formation of an amide linkage between SAM and the acyl moiety of the acyl-ACP. Subsequent lactonization of the ligated intermediate with the concomitant release of methlythioadenosine occurs. This step results in the formation of the AHL.

Parsek et al. (Parsek and Greenberg 2000) have found that analogues of these AHL building blocks such as L/D-*S*-adenosylhomocysteine, sinefungin and butylryl-*S*-adenosylmethionine (butylryl-SAM) were able to block AHL production in vitro. However, none of them have been tested on bacteria in vivo and how these analogues of AHL building block, SAM and acyl-ACP, which are also used in central amino acid and fatty acid catabolism, would affect other cellular functions in presently unknown. And also, curcumin inhibits PAO1 virulence factors and biofilm formation with AHL production. But, exact inhibition mechanism of curcumin was not revealed (Rudrappa and Bais 2008).

(2) Interference with signal receptor

In recent screening of 50 *penicillium* species grown on different growth media, 66% were found to produce secondary metabolites with QS inhibition activity. Two of compounds were identified as penicillic acid and patulin produced by *Penicillium radicicola* and *Penicillium coprobium*, respectively. DNA microarray-base transcriptomics showed that patulin targets 45% of the QS genes in *Pseudomonas aerugonisa*.

Diketopiperazines (DKPs) are a family of cyclin dipeptides that have been isolated in the supernatant of numerous cultures of bacterial species, including *Psedomonas aeruginoas, Proteus mirabilis, Cotrobacter freundii*, and *Enterobacter agglomerans* (Holden et al. 1999), as well as from protein hydrolysates and fermentation broths from yeast, lichen, and fungi (Prasad 1995). DKPs can modulate quorum-dependent phenotypes in several different species of bacteria by acting as AHL antagonists in some LuxR-based QS systems and as agonist in others (Holden et al. 1999). Differential recognitions of various DKPs by different LuxR homologue suggest that these AHL mimics may play a role in both intra- and interspecies QS regulation.

Blocking the QS signal receptor, by applying analogues of the AHL autoinducer molecules, is one of the most researched ways. Analogues of the AHL autoinducers can be substituted in either the side chain or the ring moiety. Analogs of the 3-oxo-C6 HSL molecules with different substituent in

the side chain (Figure 2.13) are able to displace the native signal from the LuxR receptor. However, most of these compounds also exhibit agonists effects, which limit their use as QS inhibitor (Schaefer et al. 1996). If the C-2 atom in the side chain is replaced by a sulfur atom (Figure 2.13), it will produce a potent inhibitor of both the *lux* and *las* systems (Persson et al. 2005).

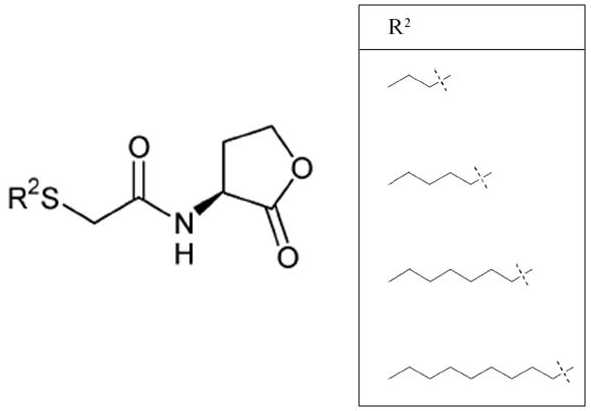


Figure 2.13. QS Inhibitor. C-2 atom in the side chain is replaced by a sulfur atom

Instead of substitutions at acyl side chain, the entire ring can be exchanged with another cyclic structure. For example, 3-oxo-C12-(2-aminocyclohexanone, Figure 2.14a) is an inhibitor of the LasR-based QS systems. It is able to down-regulate LasR dependent expression of LasI AHL synthase. When applied in a concentration of 100 μM (which is relatively high for a QS inhibitor), the compound significantly reduced the production of exopreoteins. An even more potent inhibitor of LasR is 3-oxo-C12-(2-aminophenol, Figure 2.14b), which is able to abolish production of pyocyanin and elastase; in addition, it can disrupt normal biofilm formation by *Pseudomonas aeruginosa.* Interestingly, the very similar molecule, 3-oxo-C12-(2-aminocyclohexanol) with the phenol replaced by a hexane ring, is a potent agonist of the *las* system. Likewise, if the HSL ring of the 3-oxo-C6 HSL from the lux system is replaced by a hexane ring, the ability to activate LuxR is retained.

On the other hand, if the HSL ring is replaced by a benzyl group, an inhibitor of LuxR is generated (Reverchon et al. 2002). Ishida et al. (2007) have synthesized a series of structural analogs of *N*-octanoyl cyclopentylamide with 4-12 carbon. (Figure 2.14c) They also have reported that *N*-octanoyl cyclopentylamide inhibited production of virulence factors, including elastase, pyocyanin, rhamnolipid, and biofilm formation without affecting growth of *Pseudomonas aeruginosa* PAO1.

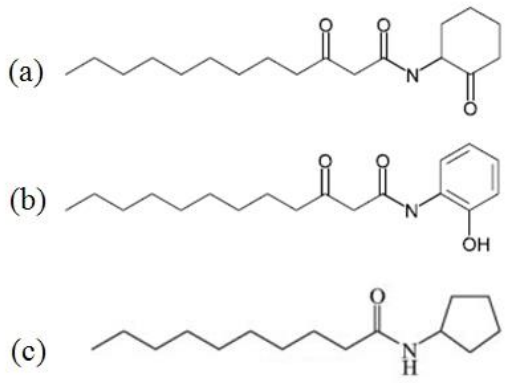


Figure 2.14. *N*-acyl homoserine lactone (AHL) analogs with exchange ring part. (a) 2-amino-3-oxo-C12-(2-aminocyclohexanone), (b) 2-amino-3-oxo-C12-(2-aminocyclohexanol) (c) *N*-decanoyl cyclopentylamide

(3) Inactivation of AHL signal molecules

Chemical Degradation: Lactonolysis

A simple way to achieve inactivation of the AHL signal molecules is by increasing the pH to above 7 (Yates et al. 2002). This causes ring opening of the AHL (lactonolysis). A number of higher organisms employ this strategy in defense against invading quorum sensing bacteria. When some plants are infected with *Erwinia carotovora*, causing the tissue-macerating plant pathogen, the plants will increase pH as a first response for attacking the virulence microorganisms by inactivation of QS signal molecules and blocking expression of QS controlled genes.

Enzymatic Degradation: quorum quenching

Some bacteria produce 'Lactonase' and 'acylase', which have enzymatic roles to hydrolyze the cyclic ester or amide linkage of the AHL type autoinducers and disrupt the cell-to-cell communication. Figure 2.15 show the quorum quenching pathway by lactonase or acylase, respectively. And Figure 2.16 show the possible ways of enzymatic degradation of AHLs.

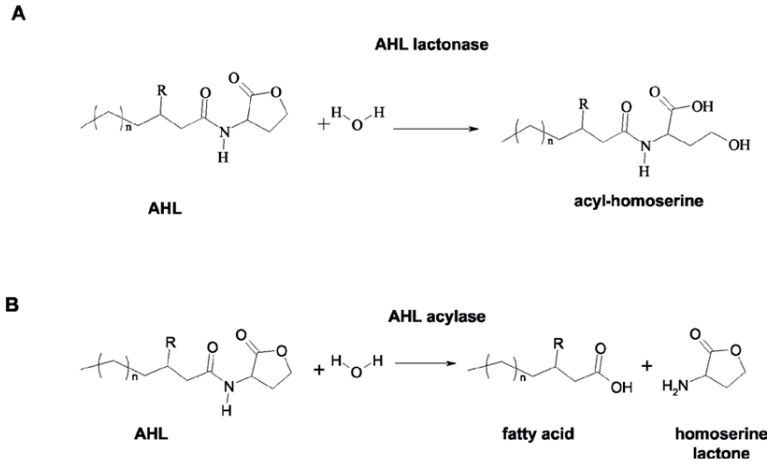


Figure 2.15. Enzymatic disruption of AHL autoinducers by quorum quenching enzymes. A. AHL- lactonase, B. AHL- acylase

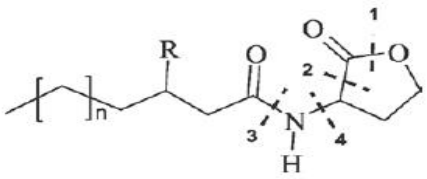


Figure 2.16. Possible ways of enzymatic degradation for removal signal molecules

2.4. Quorum Quenching (QQ)

2.4.1. Enzymatic Biological processes

The AHL inactivation scheme is the ‘quorum quenching’, which is defined as the enzymatic degradation of signal molecules. Some bacteria produce lactonase and acylase, which can disrupt cell-to-cell communication by hydrolyzing the cyclic ester or amide linkage of the AHL autoinducers respectively. Figure 2.15 shows quorum quenching pathways by lactonase and acylase respectively. Microorganisms which have quorum quenching activity were summarized in Table 2.3.

Kim et al. (2013) investigated the variation of microbial communities under the enzymatic quorum quenching. They reported microbial community in the control and quorum quenching MBRs were not significantly different, however, those in membrane biofilms and broth were very different. (Figure 2.17)

Table 2.3. Organisms identified to date exhibiting AHL-degrading activity

Strains	Activity	Notes
Bacillus sp. 240B1	Lactonase	*aiiA* gene, 3-oxo-C6-HSL, 3-oxo-C8-HSL, 3-oxo-C10-HSL (Dong et al. 2000)
Bacillus strain COT1	Lactonase	*aiiA* homologues, 3-oxo-C6-HSL with different efficiency (Dong et al. 2002)
Bacillus thuringiensis	Lactonase	*aiiA* homologues, 3-oxo-C6-HSL with different efficiency (Dong et al. 2002, Lee et al. 2002)
Bacillus cereus	Lactonase	*aiiA* homologues ,3-oxo-C6-HSL with different efficienty (Dong et al. 2002)
Bacillus mycoids	Lactonase	*aiiA* homologues, 3-oxo-C6-HSL with different efficiency (Dong et al. 2002)
Arthrobacter sp. IBN110	Lactonase	*ahlD* gene, C6-HSL, 3-oxo-C6-HSL, 3-oxo-C12-HSL, C4-HSL, C10-HSL (Park et al. 2003)
Klebsiella pneumoniae	Lactonase	*ahlK* gene (Park et al. 2003)
Agrobacterium tumefaciens C58	Lactonase	*attM*, *aiiB* gene, C6-HSL, 3-oxo-C8-HSL (Carlier et al. 2003)
Rhodococcus strain LS31	Lactonase	Rapidly degraded a wide range of AHLs (Park et al. 2006)
Rhodococcus strain PI33	Lactonase	Less activity towards 2-oxo substituents (Park et al. 2006)
Comamonas strain D1	Acylase	AHLs with acyl side chains ranging from 4~16 carbons (Uroz et al. 2007)
Ralstonia strain XJ12B	Acylase	*aiiD* gene, C4-HSL, 3-oxo-C6-HSL, 3-oxo-C8-HSL, 3-oxo-C12-HSL (Lin et al. 2003a)
Pseudomonas aeruginosa PAO1	Acylase	PA2385 gene encode an acylase (Sio et al. 2006)
Streptomyces sp. strain M664	Acylase	has a broad substrate specificity (Park et al. 2005)
Streptomyces albus strain A66	Not identified	Attenuated the biofilms and inhibited QS of *Vibrio* strains (You et al. 2007)
Bacillus megaterium	CYP101A1	Oxidation at the ω-1, ω-2, ω-3 carbons of the acyl chain (Chowdhary et al. 2007)

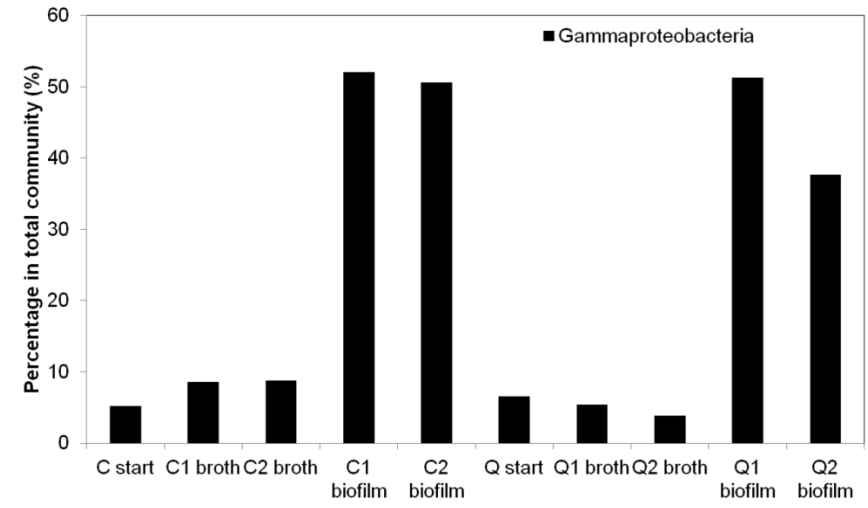


Figure 2.17. The distribution of Gammaproteobacteria at a class level in broth and biofilm samples of control and quorum quenching MBRs. (C: Control MBR; Q: Quorum quenching MBR)

(1) Quorum Quenching by Lactonase

Lactonolysis of AHLs can also be accomplished by enzymatic activity. Members of the genus *Bacillus* including *Bacillus cereus* (Dong et al. 2000), *Bacillus mycoides* (Dong et al. 2002), and *Bacillus thuringiensis* (Lee et al. 2002) produce an enzyme, AiiA, specific for degradation of AHLs. The activity of these enzymes lowers the amount of bioactive AHL signal molecules by catalyzing the ring-opening reaction. Within 2 h, up to 20 mM 3-oxo-C6 HSL can be completely inactivated by a suspension culture producing the enzyme. When *Erwinia Carotovora* is transformed with a plasmid carrying the *aiiA* gene, its virulence against potatoes and eggplants is attenuated.

In addition, when the plant-colonizing bacterium *Pseudomonas fluorescens* was transformed with the aiiA gene it was able to prevent soft rot in potatoes caused by *Erwinia Carotovora* and crown gall disease in tomatoes caused by *Agrobacterium tumefaciens*.

Furthermore, expression of *aiiA* in transgenic tobacco plants made them much less vulnerable to infection by *Erwinia Carotovora* compared to their wild type counterparts

This indeed indicates that enzymatic degradation of AHLs would be useful as a means of biocontrol. Production of AHL lactonases is not limited to *Bacillus* species. Several bacteria including *Pseudomonas aeruginosa* PAI-

A, *Arthrobacter* sp., *Klebsiella pneumoniae*, *Agrobacterium tumefacien*s and *Rhodococcus* sp. have been found to produce AiiA homologues.

A drawback of this lactonolysis reaction is that it is reversible at acidic pH. That is to say, a ring-opened AHL molecule spontaneously undergoes ring formation if the environment is not alkaline, regardless of the method by which it was opened (chemical or enzymatic). One way to prevent this could be by chemically modifying the ring-opened AHL (e.g. by mild nucleophilic substitution or reduction of the carboxylic acid), thus preventing reconversion to the ring form. Blocking QS in the environment may have the unintentional effect of interfering with beneficial bacteria.

Pseudomonas chlororaphis controls production of an antibiotic with QS. Under normal circumstances, this bacterium and its antibiotic can be used to control tomato vascular wilt caused by Fusarium oxysporum. In an experiment where the bacterium was co-cultured with an AiiA-producing bacterium, the biocontrol activity was lost, rendering the plants susceptible to infection.

(2) Quorum Quenching by Acylase

AHL autoinducers can also be degraded by the acyl-amide cleavage which is catalyzed by acylase. Various microorganisms including *Ralstonia* (Lin et al. 2003a) and *Variovorax* species (Leadbetter and Greenberg 2000) have been reported to produce quorum quenching acylase.

Especially, Park et al. (Park et al. 2005) found the Streptomyces species strain M664 which secrete an AHL degrading enzyme. They also have reported that the addition of AhlM protein to the growth medium reduced the accumulation of AHLs and decreased the production of virulence factors including elastase, total protease, and LasA in *Pseudomonas aeruginosa*.

Xu et al. (2003) have shown that porcine kidney acylase I was able to degrade *N*-acyl homoserine lactones and can reduce the biofilm growth in aquarium water sample.

(3) Other Quorum Quenching Method

A different, enzyme-based method to inactivate the signal molecules is simply to metabolize the AHLs. Both *Variovorax paradoxus* (Leadbetter and Greenberg 2000) and *Pseudomonas aeruginosa* PAO1(Huang et al. 2003) are able to proliferate with AHLs as sole source of energy, carbon and nitrogen. The bacteria produce an amino acylase which cleaves the peptide bond of the signal molecule. The side chain is used as carbon source, the nitrogen from the amide bond is made available as ammonium via the action of lactonase and the ring part is used as energy donor.

Chowdhary et al. (2007) have reported that CYP102A1, a widely studied cytochrome P450 from *Bacillus megaterium*, is capable of very efficient oxidation of AHLs and their lactonolysis products acyl homoserines.

2.4.2. Application of QQ to the Control of Biofouling

Recently, the concept of bacterial quorum sensing was also introduced to an MBR as a new biofouling control paradigm. In detail, it was experimentally observed that quorum sensing is closely associated with the formation of a biofouling layer on the immersed membrane surface in a submerged MBR for wastewater treatment. Furthermore, AHL-acylase which degrades the *N*-acyl homoserine lactone (AHL) type quorum sensing signal molecules has proven its potential to inhibit biofouling when the AHL-acylase immobilized magnetic carriers were put into the submerged MBR (Yeon et al. 2009a) (Figure 2.18).

Kim et al. (2011) attempted to immobilize the AHL-acylase directly onto the NF membrane surface, aiming at the inhibition of quorum sensing between microorganisms in the biocake on the membrane and thus the reduction of biofouling (Figure 2.19).

Oh et al. (2012) isolated QQ bacteria, *Rhodococcus* sp. BH4 from a real MBR plant, prepared a ‘microbial vessel encapsulating QQ bacteria (BH4) and inserted the ‘microbial vessel’ into an MBR (Figure 2.20).

Kim et al. (2013) prepared ‘free moving beads’ by entrapping the QQ bacteria (BH4). CEB could successfully mitigate biofouling in MBR using QQ effect and physical cleaning (Figure 2.21)

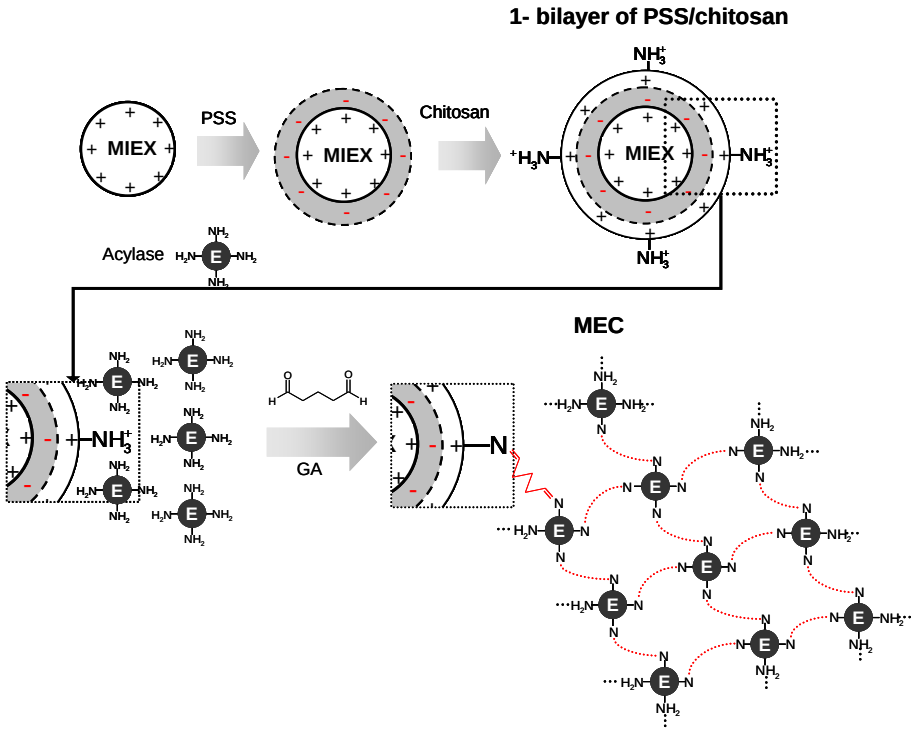


Figure 2.18. Schematic diagram showing the preparation of the MEC through LBL deposition of PSS/chitosan on MIEX resin and enzyme immobilization via glutaraldehyde treatment (Yeon et al. 2009a)

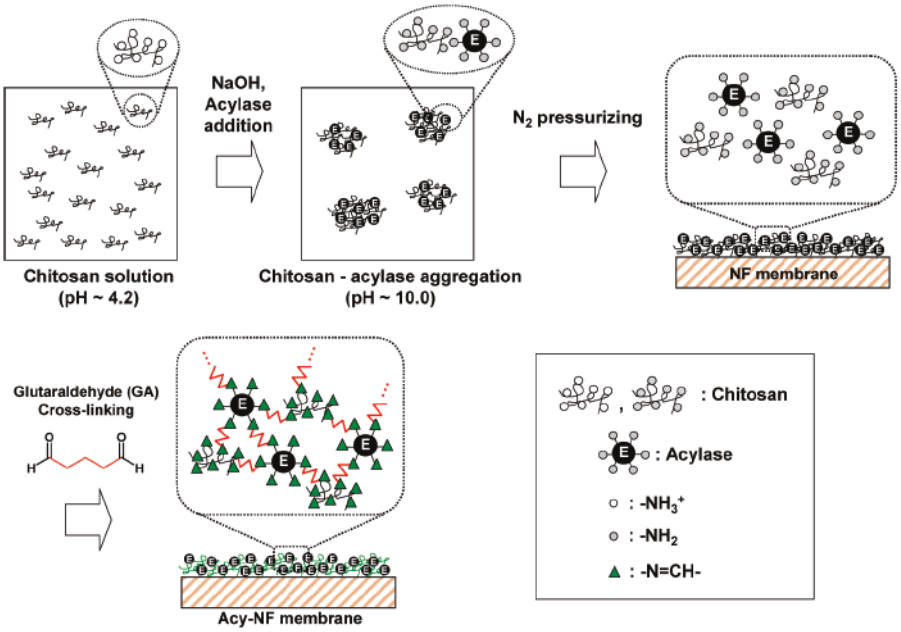


Figure 2.19. Schematic diagram of acylase immobilization onto the nanofiltration membrane surface by forming a chitosan-acylase matrix (Kim et al. 2011)

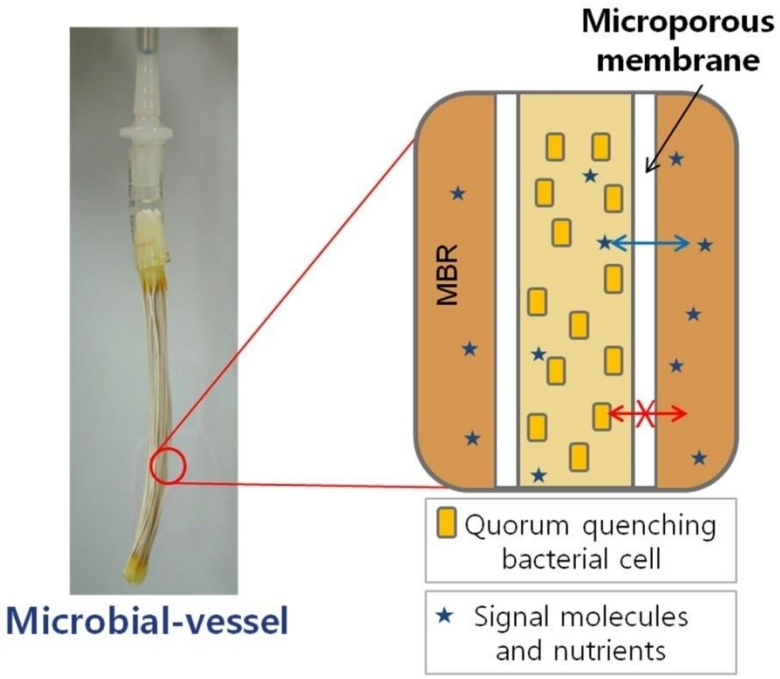


Figure 2.19. Photograph and enlarged diagram of a Microbial-vessel (Oh et al. 2013)

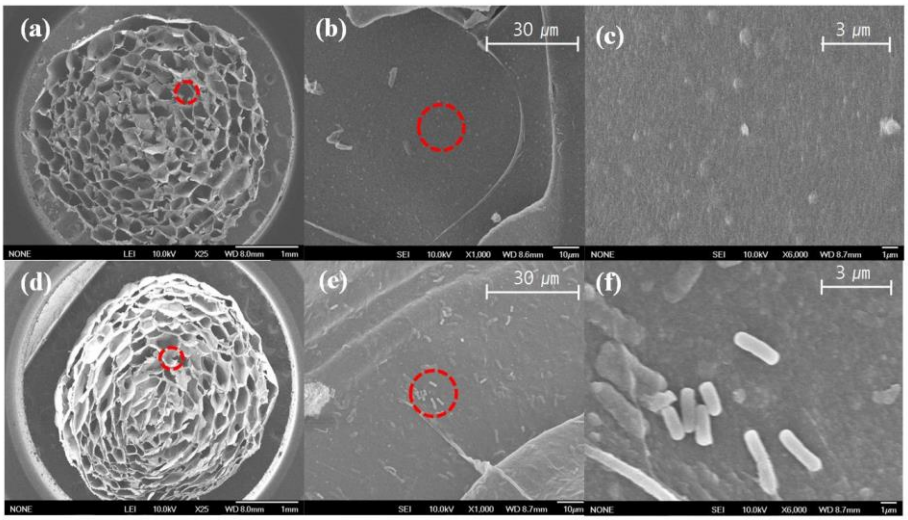


Figure 2.20. SEM microphotographs of the CEB: Cross-section of a vacant bead (a) ×25; (b) ×1000; (c) ×6000. Cross-section of a CEB (d) ×25; (e) ×1000; (f) ×6000.

Chapter 3.

Design of Quorum Quenching Microbial Vessel to Enhance Cell Viability in MBR

3.1. Introduction

As was shown in the previous section, interspecies quorum quenching (QQ) by bacterial cell has been reported to control biofouling in MBR. From an applicative point of view, the microbial vessel containing QQ bacteria has been demonstrated a successful mitigation of biofouling in lab-scale MBR and expected to be an economical method (Oh et al. 2012, Jahangir et al. 2012).

However, in the previous quorum quenching microbial vessels, the food-to-microorganism (F/M) ratio inside the microbial vessel was extremely small compared to that in a mixed liquor due to the high population density (M) of the QQ bacteria (> 100,000 mg/L) but relatively lower concentration of substrate (F) is available in the vessel than in the broth (< 50 mg COD/L). Such an abnormally small F/M ratio could inhibit growth of QQ bacteria, and eventually lead to the loss of their QQ activity.

In this chapter, a novel microbial vessel was designed using a monolithic ceramic micro-porous membrane with seven lumens to augment the F/M ratio in the microbial vessel. Both air and fresh feed were directly supplied through one lumen located in the center of the ceramic microbial vessel (CMV), while the AHL degrading QQ bacteria were entrapped inside the remaining six lumens in the CMV. Such a so called ‘inner flow feeding mode’ was expected to facilitate the mass transfer of air and nutrients and thus augment the local

F/M ratio, eventually enhancing the QQ bacterial viability in the CMV.

To achieve this goal, the overall experiments were carried out in three phases: (1) design of CMV, (2) evaluation of mass-transfer efficiency and (3) evaluation of cell viability in the CMV during the MBR operation.

3.2. Experimental Section

3.2.1. Design of Ceramic Microbial Vessel (CMV)

A monolithic ceramic micro-porous membrane with a nominal pore size of 0.45 μm (KERASEP^{TM}, Novasep, France) was chosen as a supporting component of the CMV. The monolithic module consists of seven lumens, one located in the center and the other six in a circle (Figure 3.1). The bottom sides of the lumens except for the center one were completely sealed with silicon glue and then the quorum quenching (QQ) bacteria culture was injected into the six lumens in a circle using a sterilized syringe. After cell encapsulation, the top sides of all six lumens were covered with a silicon cap which can be opened in case of cell replacement.

The surface of the CMV was investigated by a scanning electron microscope (JSM-6701F, JEOL, Japan). The SEM images of the CMV were obtained at an accelerating voltage of 10.0 kV and magnification of 100×, 200× and 800×. Before the analysis, the SEM sample was coated with platinum by sputter coater (108auto, Cressington Scientific Instruments, UK). The detailed specifications of the CMV are given in Table 3.1.

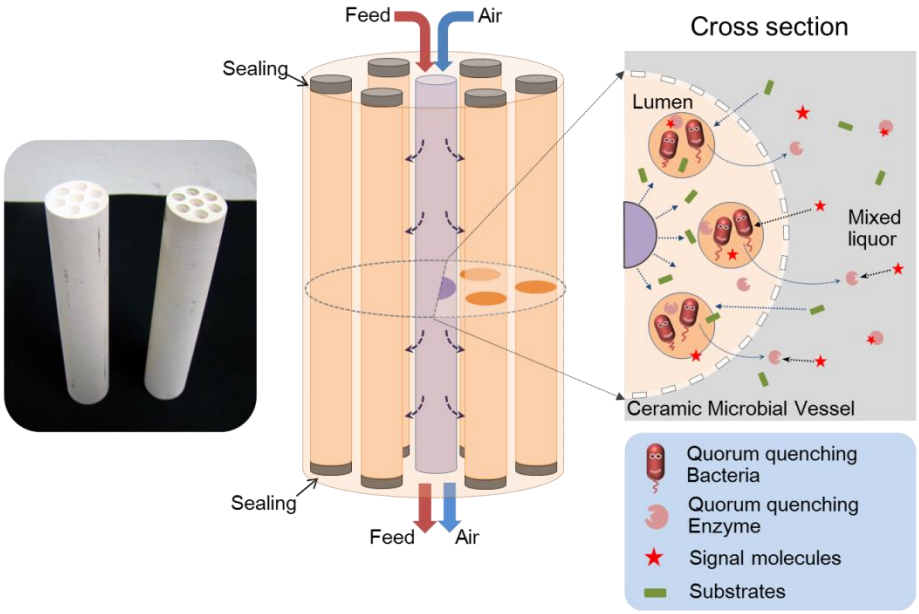


Figure 3.1. Schematic diagram of the ceramic microbial vessel under the inner flow feeding mode

Table 3.1. Specifications of the ceramic microbial vessel (CMV)

Membrane material	TiO_2-Al_2O_3
Nominal pore size	0.45 μm
Number of lumens	7
Internal diameter of lumen	4 mm
External diameter of module	20 mm
Total length	100 mm

3.2.2. Measurement of the nutrient transport rate

Because the purpose of the 'inner flow feeding mode' in the CMV was to promote the mass transfer of nutrients into the lumens entrapping the QQ bacteria, it was necessary to compare the mass transfer rate between the normal feeding and inner flow feeding modes in MBR.

Firstly, in order to compare the mass transport rate in ceramic vessel under the inner flow feeding mode, we examined ceramic vessel as following procedure using model chemicals. The six lumens in the circle of ceramic vessel were filled with powdered activated carbon (PAC, Calgon, USA) instead of QQ bacteria with the packing ratio of 79.6 mg PAC/cm^3-lumen. This PAC vessel was completely immersed into batch reactor with 25 mL of crystal violet solution (2 μM). As shown in Figure 3.2a, crystal violet solution was circulated with tree different operation modes. The decrease of absorbance at 590 nm (Safarik and Safarikova 2002) caused by adsorption of crystal violet on PAC was continuously monitored for the estimation of mass transport rate through pore of ceramic vessel.

Secondly, we tried to investigate directly the nutrient in the ceramic vessel during operation of MBR. The six lumens in the circle of both ceramic vessels were filled with deionized water instead of QQ bacteria and then both ceramic vessels were immersed into two MBRs, respectively (Figure 3.2b). The normal feeding mode was applied to Reactor-1 in which fresh synthetic

wastewater was fed into the reactor (that is into the mixed liquor), whereas the inner flow feeding mode was applied to Reactor-2 in which the same wastewater was fed directly to the lumen that was in the center of the ceramic vessel. After the operation of both MBRs for 24 hours, each ceramic vessel was taken out of the MBR to measure and compare the total nutrient concentrations inside the six lumens located in the circle of each vessel.

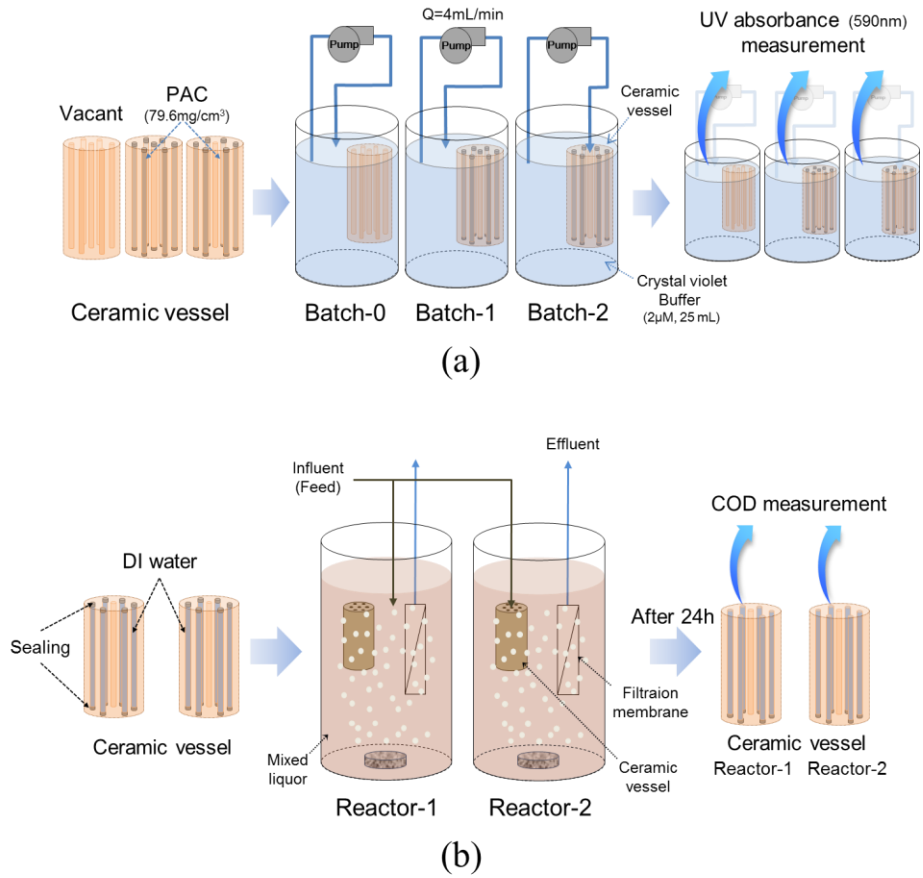


Figure 3.2. Experimental set-up for the measurement of (a) the model chemical and (b) the nutrient transfer rate under different feeding modes

Batch-0: *Vacant CMV under the normal feeding mode*

Batch-1: *CMV with PAC under the normal feeding mode*

Batch-2: *CMV with PAC under the inner flow feeding mode*

Reactor-1: *CMV with distilled water under the normal feeding mode*

Reactor-2: *CMV with distilled water under the inner flow feeding mode*

3.2.3. Analysis of cell viability in the CMV

In order to investigate the cell viability in the CMV under the different feeding modes, Two CMVs containing QQ bacteria (142.6 mg biomass/cm^3-lumen) were prepared. Initial viability of QQ bacteria were investigated by the microscope before the test. Both CMVs were immersed into two MBRs respectively and operated under the different feeding modes. After 72 hours of operation, the viability of QQ bacteria from the CMVs was monitored again (Figure 3.3). The LIVE/DEAD® BacLight™ Bacterial Viability Kit (Molecular Probe, USA) was used to determine the active state of the biomass in the CMV according to the manufacturer's instructions.

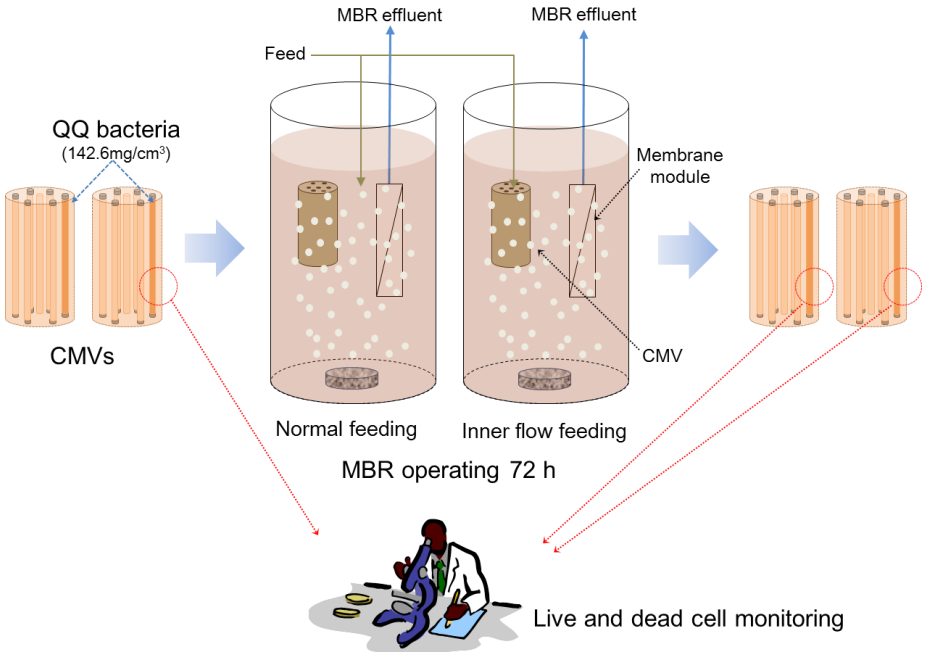


Figure 3.3. Experimental set-up for the measurement of cell viability in CMVs under the different feeding modes

3.3. Results and Discussion

3.3.1. Investigation of the morphology of CMVs

The SEM images of the CMV have shown in Figure 3.4. The active layer protecting QQ bacteria from others microorganism was located at the inside of lumens. However, outside of CMV was covered with a macro-porous layer, which means that CMVs are able to endure from a harsh environment e.g. scratch, grits or vigorous aeration.

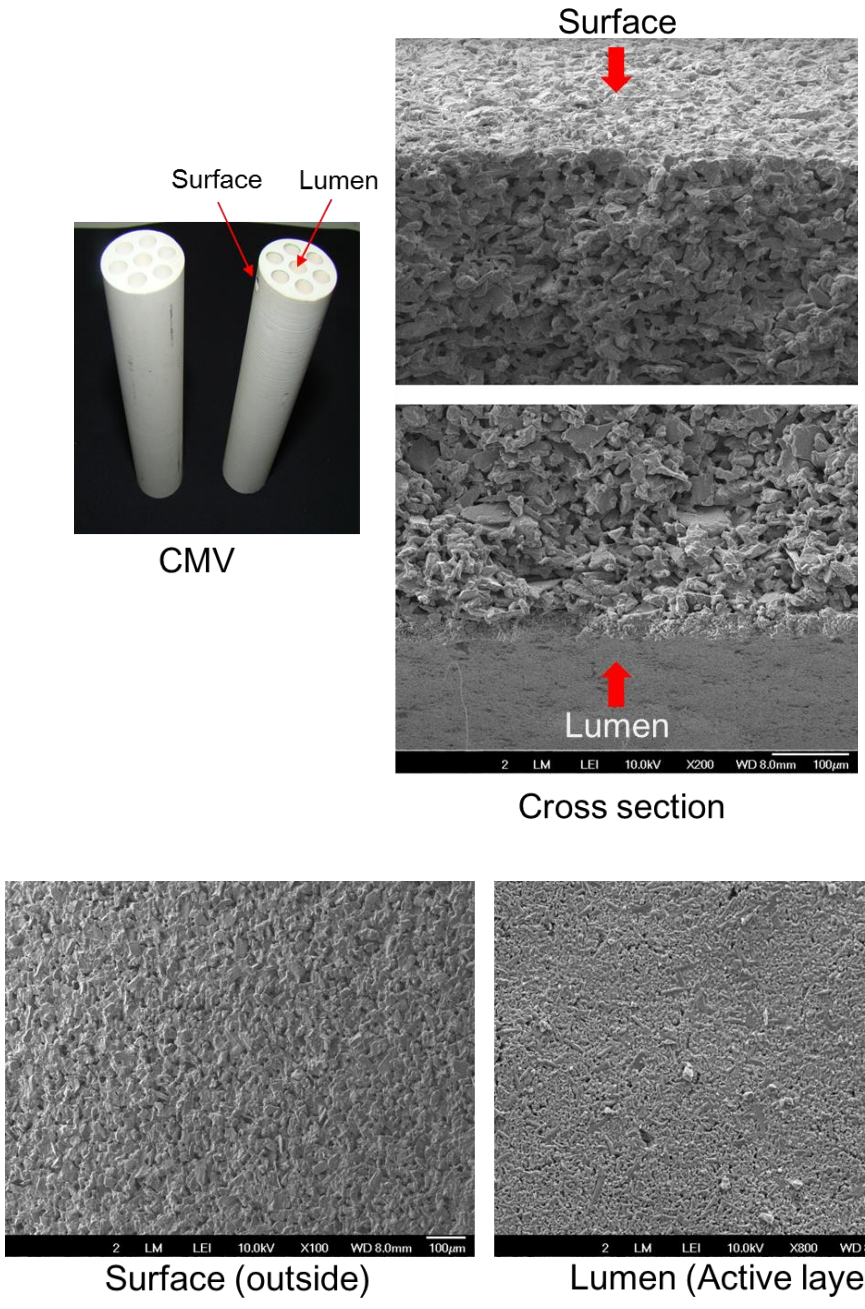


Figure 3.4. SEM image of ceramic microbial vessel

3.3.2. Effect of the inner flow feeding mode on the rate of nutrient transfer to the CMV

In order to test the mass transfer efficiency of CMV under the inner flow feeding mode, model crystal violet buffer was pumped into the empty center lumen with flow rate of 4 mL/min and its removal rate of crystal violet was compared with that of only diffusion condition (Figure 3.2a). The Batch-0 of the Figure 3.2a was the empty ceramic vessel considered to calibrate the adsorption of crystal violet on the ceramic membrane. Each crystal violet decrease rate of 'normal feeding mode' and 'inner flow feeding mode' set was determined to 0.013 and 0.031 μg crystal/min (Figure 3.5). Therefore, CMV with inner flow feeding mode showed higher mass transfer efficiency rather than normal feeding mode.

To further clarify the greater mass transfer in Reactor-2 under the inner flow feeding mode than that in Reactor-1 under the normal feeding mode, the mass transfer rate of nutrients into the six lumens of each ceramic vessel was estimated in terms of the COD level (Figure 3.2). After the operation of both MBRs for 24 hours as shown in Figure 3.2, each ceramic vessel was taken out of Reactor-1 & Reactor-2 and then the total nutrient concentration inside the six lumens located in the circle of each vessel were measured and compared (Figure 3.6). Under the normal feeding mode, the soluble COD in the lumen-1 (25.4 mg/L) became almost the same as that of the bulk phase (26.3 mg/L)

after the operation of the MBR for at least 24 hours, indicating the soluble COD in the feed (336 mg/L) was equally distributed in the ceramic vessel and in the mixed liquor of the activated sludge. However, the soluble COD in lumen-2 under the inner flow feeding mode (242 mg/L) was much higher than that in the mixed liquor. This implies that the inner flow feeding mode makes the COD level in the ceramic vessel approach close to that in the fresh feed. Consequently, it was anticipated that the inner flow feeding mode would help compensate the extremely low F/M ratio inside the CMV in the QQ MBR by forced feeding through the center lumen.

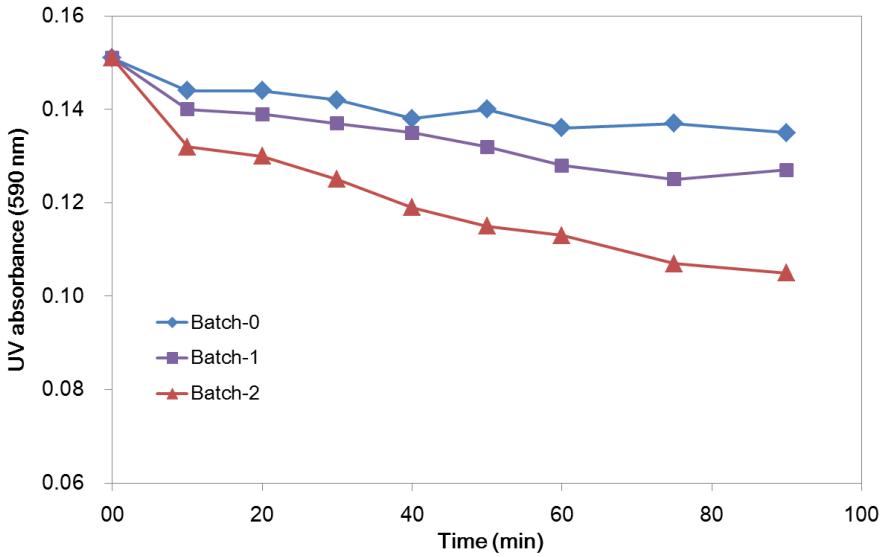


Figure 3.5. Comparison of mass transfer efficiency according to operation mode

Batch-0: *Vacant CMV under the normal feeding mode*

Batch-1: *CMV with PAC under the normal feeding mode*

Batch-2: *CMV with PAC under the inner flow feeding mode*

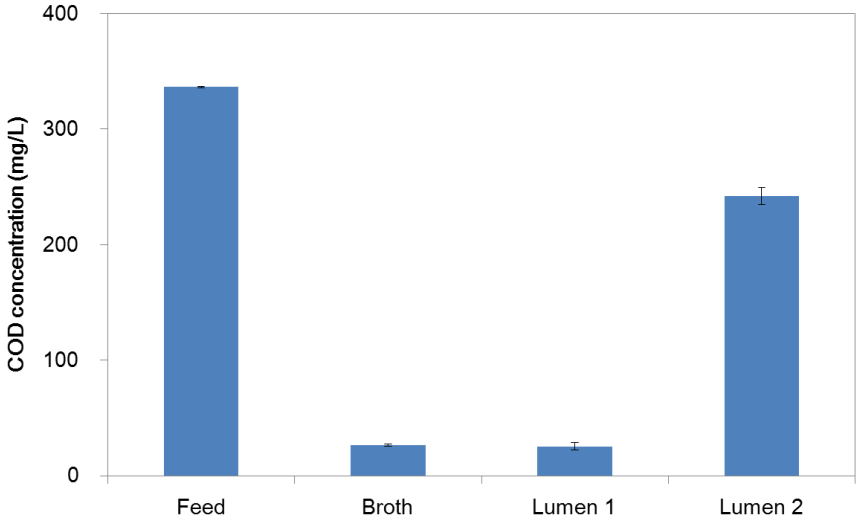


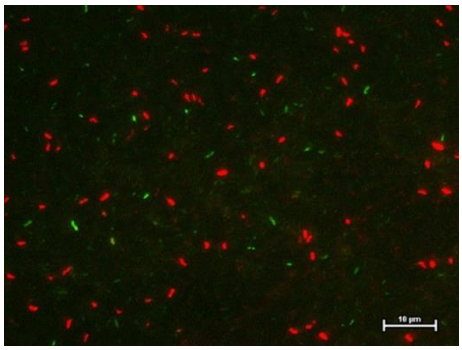
Figure 3.6. COD concentrations in the feed, broth and lumens under different feeding modes. Error bar: standard deviation (n=3).

Lumen 1: *COD in the lumen under the normal feeding mode*

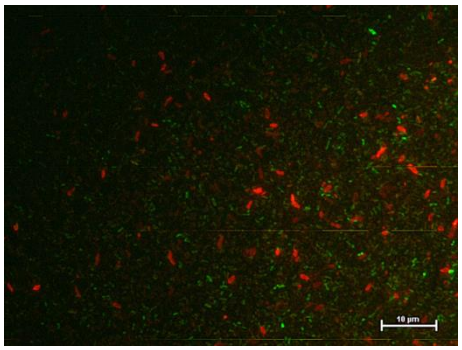
Lumen 2: *COD in the lumen under the inner flow feeding mode*

3.3.3. Comparison of cell viability of CMVs under the different feeding mode

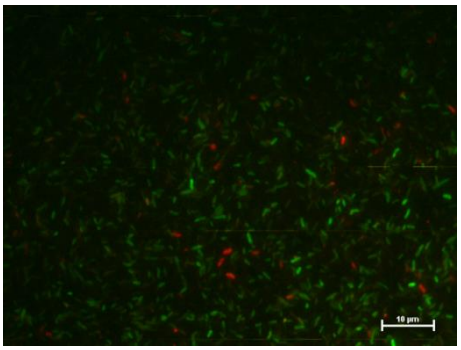
In order to compare the cell viability in the CMV under the different feeding modes, we investigated the condition of cell which was taken from the used CMV. The live/dead cell staining experiments was conducted to the biomass in each CMV to check its cell viability. Figure 3.7 clearly showed that degree of active cell population with inner flow feeding mode was much higher than normal feeding mode. The value of live/dead ratio of each condition was calculated to be 1.60 and 3.30. Thus, it was revealed that CMV with inner flow feeding mode can maintain the microbial activity of CMV through facilitate nutrients transfer.



(a)



(b)



(c)

Figure 3.7. Images of live/dead QQ bacteria from the lumens of the used CMVs (Green color: live cell; red color: dead cell)

(a) Initial QQ bacteria: 0 h

(b) QQ bacteria of CMV under the normal feeding mode: 72 h

(c) QQ bacteria of CMV under the inner flow feeding mode: 72 h

3.4. Conclusions

The purpose of this chapter was to devise a novel microbial vessel, ceramic microbial vessel for the biofouling control. Based on the results of this study, the following conclusions were drawn:

(1) A monolithic ceramic microbial vessel (CMV) which can efficiently increase the active state of quorum quenching bacteria was prepared for biofouling control in MBR.

(2) The key characteristic of CMV was its unique convective feeding of nutrients and oxygen to the bacterial cells encapsulated in the lumens of ceramic membrane (inner flow feeding mode).

(3) Inner flow feeding mode under which fresh feed is supplied to the MBR only through the center lumen in the CMV compensated for the extremely low F/M ratio inside the CMV, and thereby enabling the CMV to maintain greater bacterial QQ activity through the facilitated nutrient transfer.

Chapter 4.

Characterization of Indigenous Quorum Quenching Bacteria, *Pseudomonas* sp. 1A1

4.1. Introduction

The kind of quorum quenching bacteria was most important factor in the CMV for efficient performance. Oh et al. (2012) already isolated one of the QQ bacterium, *Rhodococcus* sp. BH4 from the real plant of MBR and showed that the stain BH4 had the *N*-acyl homoserine lactone hydrolase (AHL–lactonase) gene (Oh et al. 2013). Although there might be many other QQ bacteria in nature (Christiaen et al. 2011, Kraemer et al. 2012, Yin et al. 2012), we still do not know which strain is more appropriate for application of QQ to real wastewater MBR and especially application of CMV in MBR. Thus, continuous searching for novel QQ strains should be required for further development of the bacterial QQ to mitigate the biofouling in MBR.

In this study, *Pseudomonas* sp. 1A1 was chosen as a novel QQ bacterium, which was isolated by Chi-Ho Lee (2013) from the lab-scale MBRs in which sludge from real wastewater treatment plants were inoculated. Various characteristics of *Pseudomonas* sp. 1A1 were examined in terms of degradation rates of various AHLs, molecular weight of its enzyme, the specific growth rates, survival competition, etc., particularly comparing it with the previously reported QQ bacterium, *Rhodococcus* sp. BH4. It was because strain BH4 was only strain used in MBR as a QQ bacterium and no other strain having higher QQ activity than strain BH4 had not been yet discovered among the QQ isolates from the lab-scale MBRs (Lee et al. 2013).

4.2. Experimental Section

4.2.1. Indigenous QQ bacteria

We used the indigenous QQ bacterium, *Pseudomonas* sp. 1A1 which was isolated by Chi-Ho Lee (2013) from the lab-scale MBRs in which sludge from real wastewater treatment plants were inoculated. He confirmed its quorum sensing activity by cross-feeding assay for the detection of AHL production (Stickler et al. 1998).

4.2.2. Comparison of QQ enzyme localization between strain 1A1 and BH4

Pseudomonas sp. 1A1 was compared to another QQ bacterium, *Rhodococcus* sp. BH4 (Oh et al. 2012) with respect to the location of QQ enzyme. The indicating agar plate was prepared by mixing an overnight culture of *Agrobacterium tumefaciens* A136 (AHL biosensor)(Fuqua and Winans 1996), LB agar and X-gal. A sterilized 0.45 μm filter (Super 450, Pall Corporation, U.S.) was soaked in 100 mg/L of *N*-(decanoyl)-$_{DL}$-homoserine lactone (C10-HSL) and then put on the agar plate. On the filter, two strains were loaded separately. After overnight incubation, we checked whether or not a blue color develops around each colony in order to identify the location of QQ activity for each strain.

4.2.3. Characterization of QQ bacteria

The growth rate of *Pseudomonas* sp. 1A1 was compared with that of *Rhodococcus* sp. BH4. The two stains were inoculated separately in a sterilized volumetric flask with 100 mL of LB medium and incubated at 30^{o}C with orbital shaking (200 rpm). The cell concentrations were determined by measuring OD_{600} at intervals during incubation. The specific growth rates (μ) of *Pseudomonas* sp. 1A1 and *Rhodococcus* sp. BH4 were determined from the logarithmic scale of growth curve of each (Nair 2008).

We also investigated the survival competition between *Pseudomonas* sp. 1A1 and *Rhodococcus* sp. BH4. 500 L of each strain (OD_{600} 4.0) was mixed and the mixture was inoculated in a sterilized volumetric flask with 100 mL of LB medium and incubated at 30^{o}C with orbital shaking (200 rpm). The Gram staining procedures (Beveridge 2001) were applied at the beginning and 36 h of incubation to differentiate *Pseudomonas* sp. 1A1 (Gram-negative) from *Rhodococcus* sp. BH4 (Gram-positive).

The following eight AHL molecules (Sigma-Aldrich, U.S.) were chosen to compare their degradation rates by the *Pseudomonas* sp. 1A1: C6-HSL, C8-HSL, C10-HSL, *N*-(dodecanoyl)-$_{DL}$-homoserine lactone (C12-HSL), *N*-(3-oxo-hexanoyl)-$_{L}$-homoserine lactone (3-oxo-C6-HSL), *N*-(3-oxo-octanoyl)-$_{L}$-homoserine lactone (3-oxo-C8-HSL), *N*-(3-oxo-decanoyl)-$_{L}$-homoserine lactone (3-oxo-C10-HSL), and *N*-(3-oxo-dodecanoyl)-$_{L}$-homoserine lactone

(3-oxo-C12-HSL). Equal volumes of a culture medium (OD_{600} 1.0) and a solution containing 0.4 M AHLs were mixed and incubated (30°C, 200 rpm). The percentage of degraded AHL in 10 min was measured to determine the degradation rate of each AHL by strain 1A1.

4.2.4. Bioassay for detecting AHL molecules

The concentrations of AHL molecules were measured via luminescence method using the reporter strain of *A. tumefaciens* A136. First, the reporter strain and the AHL sample were mixed and loaded on the micro-well plate. The micro-well plate loaded with the mixed solution was placed on the incubator to keep the temperature at 30°C for 1.5 h, and then the Beta-Glo® Assay System (Promega, U.S.) was added to the solution for the luminescent reaction with β-galactosidase produced by the reporter strain. After 30 min, the luminescence was measured by a luminometer (Synergy 2, Biotek®, U.S.). The amounts of AHLs were calculated using relationship equations based on the calibration curve derived from standard samples of AHLs.

4.3. Results and Discussion

4.3.1 Localization of quorum quenching enzyme of the strain, *Pseudomonas* sp. 1A1

Prior to quorum quenching experiments in MBR, a bio agar assay was carried out to reconfirm the location of QQ enzymes of the strain *Pseudomonas* sp. 1A1 and *Rhodococcus* sp. BH4 although the location was already reported by Chi-Ho Lee (2013). A membrane filter (0.45 μm) with the signal molecule of C10-HSL was put on the indicating agar and inoculated with each culture of *Pseudomonas* sp. 1A1 and *Rhodococcus* sp. BH4, respectively. It was anticipated that a blue color would be developed all over the membrane except the region in which C10-HSL is degraded by the QQ enzyme of each strain. As shown in Figure 4.1, there was no blue color development inside the colonies of both strain 1A1 and BH4, whereas a white ring was observed only around the colony of strain 1A1. This implies that *Pseudomonas* sp. 1A1 produces QQ enzymes and excretes them out of the cell, whereas *Rhodococcus* sp. BH4 produces QQ enzymes and keeps them inside the cell.

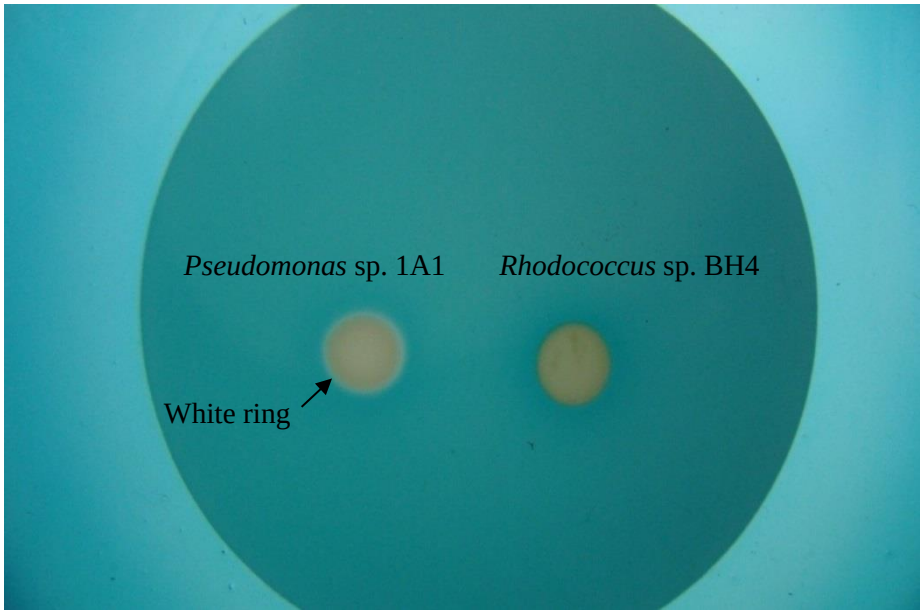


Figure 4.1. Bio agar assay for the locations of quorum quenching enzymes produced by *Pseudomonas* sp. 1A1 and *Rhodococcus* sp. BH4

4.3.2 Molecular Size of AHL-acylase of *Pseudomonas* sp. 1A1

The approximate molecular size of extracellular enzyme the strain 1A1 produces was estimated. The supernatant of strain 1A1 culture medium were separated into three fractions using MWCO centrifugal filters based on their molecular weight: < 3 kDa, < 50 kDa, and < 100 kDa. And then, the QQ activity of each fraction was measured. As shown in Figure 4.2, QQ activity was detected in the fraction under 100 kDa, whereas nearly no activity was observed in the fraction under 50 kDa. It means that the molecular weight of QQ enzyme produced by the strain 1A1 is within the range of 50 ~ 100 kDa. It was reported that the molecular weights of AHL-acylases are within the range of 60 ~ 90 kDa (Sio et al. 2006, Huang et al. 2006, Shepherd and Lindow 2009), whereas those of AHL-lactonases are between 28 kDa and 35 kDa (Bai et al. 2008, Wang et al. 2004, Liu et al. 2007). Therefore, the extracellular enzyme of strain 1A1 is highly likely to be an AHL-acylase.

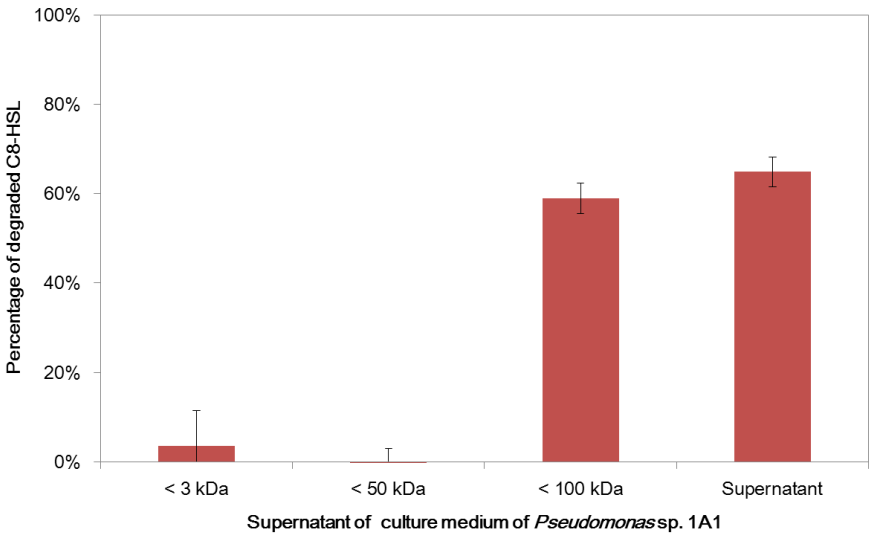
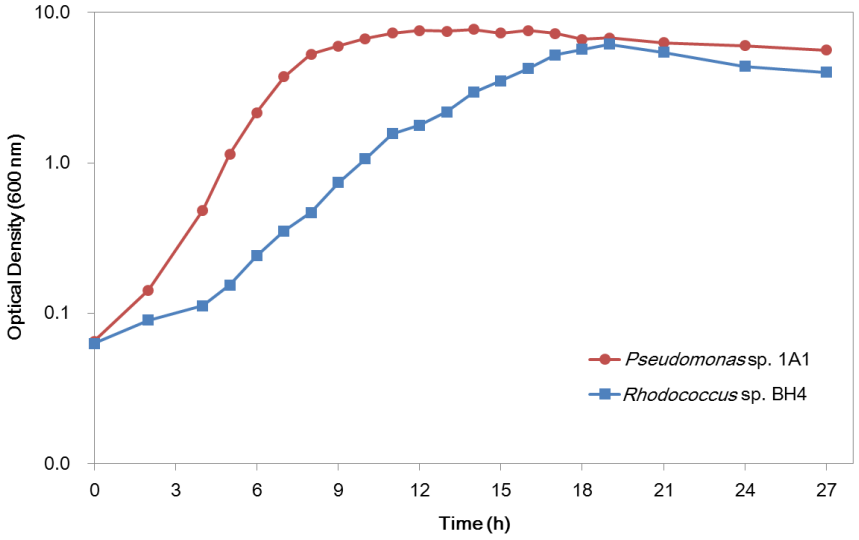


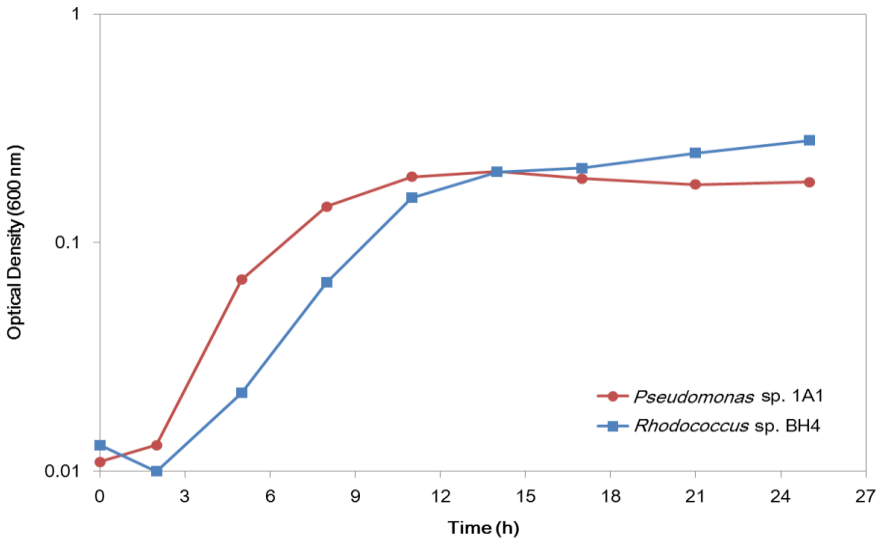
Figure 4.2. Quorum quenching activity of the supernatant of 1A1 culture. The supernatant of strain 1A1 culture medium was separated into three fractions using MWCO centrifugal filters: < 3 kDa, < 50 kDa, and < 100 kDa. Error bar: standard deviation (n=3).

4.3.3 Comparison of the Growth rate of *Pseudomonas* sp. 1A1

The microbial characteristics of *Pseudomonas* sp. 1A1 were investigated, if necessary, comparing with those of the previously reported *Rhodococcus* sp. BH4 in terms of growth rate and survival competition in population. As shown in Figure 4.3, 4.4, 4.5, the *Pseudomonas* sp. 1A1 grows faster than *Rhodococcus* sp. BH4 in the LB medium and synthetic wastewater. The specific growth rate of 1A1 during the exponential growth phase was 0.65 h^{-1} and 0.56 h^{-1}, whereas that of BH4 was 0.38 h^{-1} and 0.32 h^{-1}. Survival competition with each other was also put through the bi-culturing of two QQ bacteria. Gram-staining procedures were applied to visually separate *Pseudomonas* sp. 1A1 (Gram-negative) from *Rhodococcus* sp. BH4 (Gram-positive). As shown in Figure 4.6, although the population of each strain was almost same at the beginning of bi-culturing, the strain 1A1 became dominant after 36 h of incubation. This indicates that the strain 1A1 has grown more rapidly than the strain BH4 and survived better during bi-culturing. Thus, *Pseudomonas* sp. 1A1 is expected to be more advantageous for the long-term application in MBR than *Rhodococcus* sp. BH4. Furthermore, faster growth will result in higher productivity of biomass which provides an advantage from the economical point of view when commercializing the QQ bacteria for the biofouling control in MBR.


10.0
1.0
0.1
0.0
Optical Density (600 nm)
0
3
6
9
12
15
18
21
24
27
Time (h)
Pseudomonas sp. 1A1
Rhodococcus sp. BH4

(a)


1
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Optical Density (600 nm)
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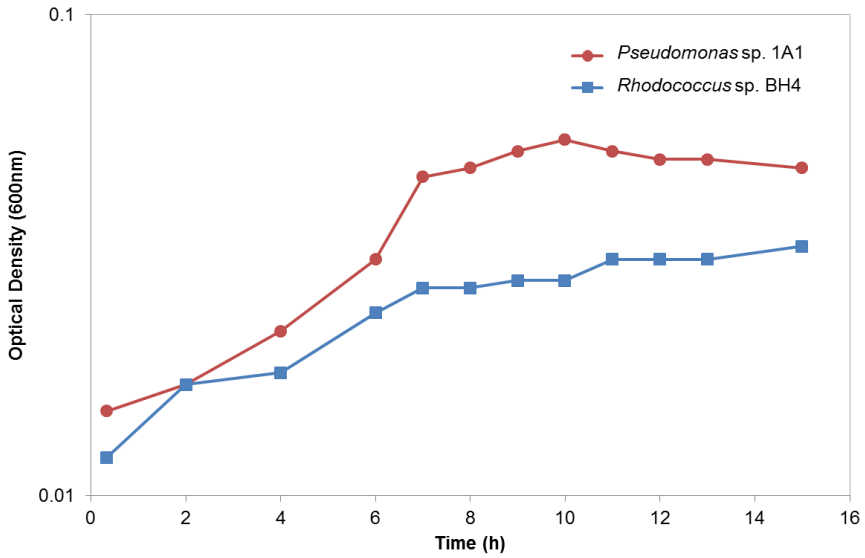
Time (h)
Pseudomonas sp. 1A1
Rhodococcus sp. BH4

(b)



(c)

Figure 4.3. Comparison of a growth rate in (a) LB medium (b) Synthetic wastewater (COD = 408mg/L) (c) domestic soluble sewage (COD = 240mg/L) between *Pseudomonas* sp. 1A1 and *Rhodococcus* sp. BH4

Table 4. 1. Comparison of a specific growth rate

	Strain BH4	**Strain 1A1**
LB medium	0.38	0.65
Synthetic W.W. (COD = 408 mg/L)	0.32	0.56
Domestic Soluble Sewage (COD = 240 mg/L)	0.07	0.15

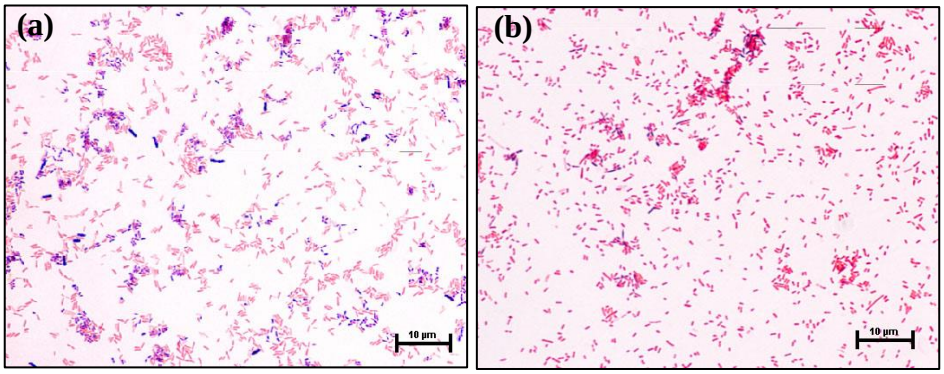


Figure 4.4. Gram staining of the mixture of *Pseudomonas* sp. 1A1 and *Rhodococcus* sp. BH4 (a) at the beginning, and (b) after 36 h of incubation (Blue: *Rhodococcus* sp. BH4, Red: *Pseudomonas* sp. 1A1)

4.3.4 QQ activity of *Pseudomonas* sp. 1A1 according to the Growth rate

We also investigated the QQ activity of strain 1A1 culture medium (whole cell and supernatant) according to its growth phase. During the incubation of strain 1A1 in LB medium, the culture medium was sampled at 5, 7, 9, 12, 15, and 21 h to compare its QQ activity. The strain 1A1 culture medium was constantly set to an OD_{600} value of 1.0 prior to the test of QQ activity. The QQ activity was measured by quantifying the degraded C8-HSL within 15 min. As shown in Figure 4.5, strain 1A1 culture medium degraded around 20% of C8-HSL at the early exponential phase (5 h). However, the degradation of C8-HSL, i.e., the QQ activity, increased to 44% in the late exponential phase (9 h). Furthermore, it continuously increased to around 80% even after the exponential phase (9 ~ 21 h). Considering that the OD_{600} value only represents an indirect cell concentration including both living and dead cell, it was interesting that QQ activity of strain 1A1 culture medium increased even after the exponential phase in which the concentration of dead cells would start to increase. It would be quite possible that the extracellular enzymes secreted from the strain 1A1 would accumulate in the culture medium, thereby increase the QQ activity of the culture medium.

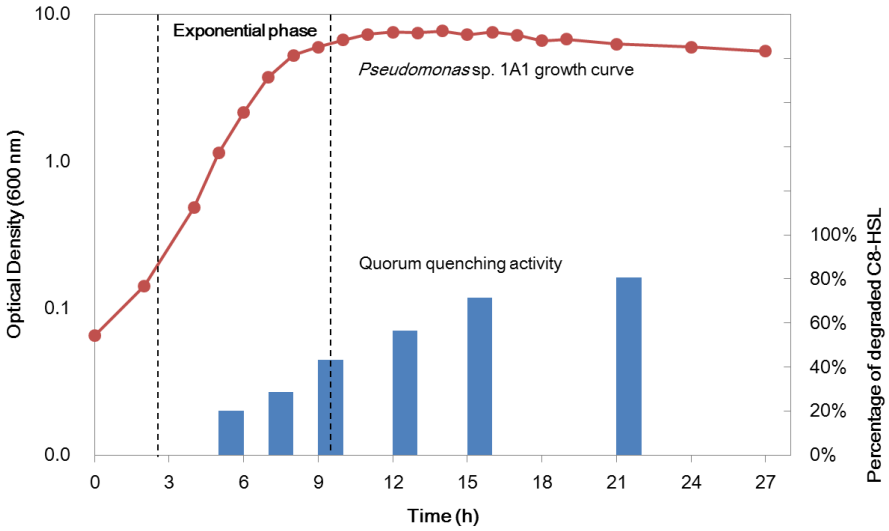


Figure 4.5. Degradation of C8-HSL by the strain 1A1 culture medium (whole cell and supernatant) according to its growth phase in LB medium. Equal volumes of a culture medium (OD_{600} 2.0) and a solution containing 0.4 µM C8-HSL were mixed and incubated for 15 min (30°C, 200 rpm).

4.3.5 Degradation of various AHLs by *Pseudomonas* sp. 1A1

The degradation rates of various AHLs by *Pseudomonas* sp. 1A1 were compared with one another. As shown in Figure 4.6, the degradation rate increased with the length of acyl chain in AHLs (C6 to C12), i.e., the AHL with longer acyl chain was more easily degraded. In case of the AHLs which contain additional oxo-functional group in the acyl chain, the strain 1A1 substantially degraded 3-oxo-C12-HSL with the longest acyl chain, but hardly did the rest of AHLs with oxo-functional group. Lin et al. also reported that AHL-acylase effectively degraded long-chain AHLs (Lin et al. 2003b). Bokhove et al. (2010) explained the reason as following: PvdQ, one of the AHL-acylase, has a distinct, unusually large, hydrophobic binding pocket, ideally suited to recognize C12 fatty acid-like chains of AHLs. Hence, it showed a strong degradation activity against AHLs with C12, whereas it did a weak degradation activity against AHLs with shorter acyl chains like C6, C8-HSL.

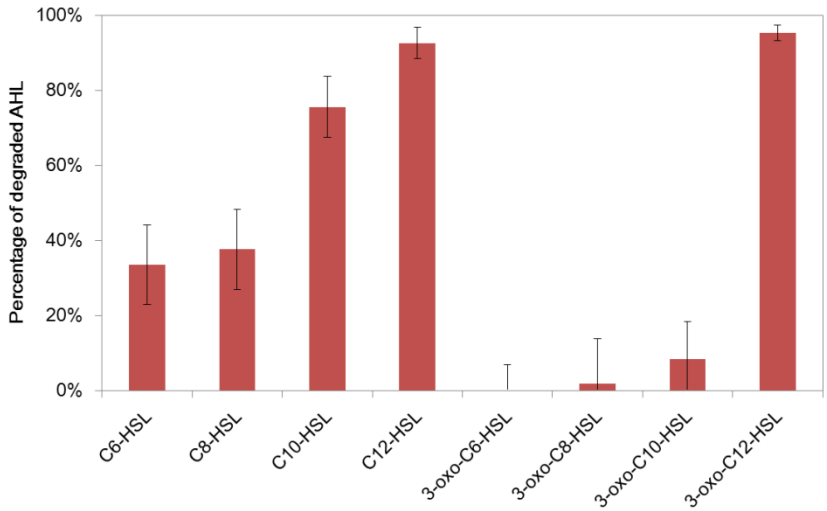


Figure 4.6. Degradation rates of various AHL molecules by *Pseudomonas* sp. 1A1. Equal volumes of culture medium (OD_{600} 1.0) and a solution containing 0.4 μM of each AHL were mixed and incubated (30°C, 200 rpm). Then, the percentages of degradation at 10 min were measured. Error bar: standard deviation (n=3)

4.4 Conclusions

The purpose of this chapter was to characterize an indigenous QQ bacterium, *Pseudomonas* sp. 1A1. Based on the results of this study, the following conclusions were drawn:

(1) The molecular weight of QQ enzyme produced by the strain 1A1 is within the range of 50 ~ 100 kDa.

(2) The specific growth rate of 1A1 during the exponential growth phase was 0.65 h^{-1} and 0.56 h^{-1}, whereas that of BH4 was 0.38 h^{-1} and 0.32 h^{-1}. The strain 1A1 has grown more rapidly than the strain BH4 and survived better during bi-culturing.

(3) The degradation rates by *Pseudomonas* sp. 1A1 increased with the length of acyl chain in AHLs (C6 to C12), i.e., the AHL with longer acyl chain was more easily degraded. In case of the AHLs which contain additional oxo-functional group in the acyl chain, the strain 1A1 substantially degraded 3-oxo-C12-HSL with the longest acyl chain, but hardly did the rest of AHLs with oxo-functional group.

Chapter 5.

Application of CMV encapsulated *Pseudomonas* sp. 1A1 for biofouling control in MBR

5.1 Introduction

In the previous chapter, we devised a ceramic microbial vessel with the inner flow feeding mode and an indigenous QQ bacterium, *Pseudomonas* sp. 1A1 which was applicable to CMVs. The CMV under the inner flow feeding mode was expected to facilitate the mass transfer of air and nutrients and thus augment the viability of QQ bacteria. Meanwhile, *Pseudomonas* sp. 1A1 had an extracellular QQ activity and degraded AHLs with longer acyl chain more effectively.

In this chapter, the application of the CMV encapsulated *Pseudomonas* sp. 1A1 to the lab-scale MBR was studied. The inhibition of biofouling in MBR using CMV could be elucidated by decreasing TMP rise-up. To further clarify the control of biofouling by CMV, the biofilm on the membrane surface during the MBR operation was investigated using confocal laser scanning microscopy (CLSM). Comparison of the inner flow feeding mode with normal feeding mode was another important point of view. Lastly, stability of CMV was monitored for 30 days operation.

5.2 Experimental Section

5.2.1 Preparation of the ceramic microbial vessel (CMV)

A ceramic membrane with a nominal pore size of 0.45 μm (KERASEP^{TM}, Novasep, France) was prepared. The monolithic module consists of seven lumens and the bottom sides of the lumens except for the center one were completely sealed with silicon glue and then the *Pseudomonas* sp. 1A1 culture was injected into the six lumens in a circle using a sterilized syringe (Figure III-1). After cell encapsulation, the top sides of all six lumens were covered with a silicon cap which can be opened in case of cell replacement. The bacterial population density in the CMV was set to 26.5 or 70.3 mg biomass/cm^3-lumen. *Pseudomonas* sp. 1A1 was inactivated with autoclave and injected into a ceramic vessel in the just same way as the CMV to prepare an 'inactive CMV' as a control group.

5.2.2 AHL bioluminescence assay

The concentration of the AHL molecules was measured with the luminescence method using the reporter strain *A. tumefaciens* A136 (Ti$^-$)(pCF218)(pCF372) (Oh et al. 2012, Kawaguchi et al. 2008, Yeon et al. 2009b). The reporter strain and the AHL sample were mixed and loaded onto a microwell plate. The microwell plate was placed in an incubator to maintain the temperature at 30°C for 1.5 hr, and then the Beta-Glo® Assay System (Promega, U.S.) was

added to the solution for the luminescent reaction with β-galactosidase produced by the reporter strain. After 40 min, the luminescence was measured by a luminometer (Synergy 2, Biotek®, U.S.). The amounts of AHL were calculated using relationship equations based on the calibration curve derived from standard samples of AHL.

5.2.3 Measurement of the QQ activity of the CMV

The QQ activity was determined by the degradation rate of *N*-octanoyl-$_L$-homoserine lactone (C8-HSL), which has been identified and reported as one of the major AHL molecules in a previous study (Yeon et al. 2009a). To measure the QQ activity of the CMV, C8-HSL was added to 20 mL of Tris-HCl buffer (pH 7, 50 mM) for a final concentration of 200 nM. Then, the CMV was inserted into the buffer and the mixture was incubated at 30℃ with orbital shaking (200 rpm) for 15, 45, 90, and 180 minutes. The remaining concentration of C8-HSL at each reaction time was measured using a bioluminescence assay.

5.2.4 MBR operation

Three laboratory-scale MBRs, each with a 3.0 L working volume were operated in parallel using synthetic wastewater (Figure 5.1). The composition of the synthetic wastewater was as follows (g/L)(Table 5.1): glucose, 0.3; peptone, 0.1; yeast extract, 0.014; $(NH_4)SO_4$, 0.1; KH_2PO_4, 0.02; $MgSO_4$, 0.03; $MnSO_4$, 0.003; $FeCl_3$, 0.0002; $CoCl_2$, 0.002; $CaCl_2$, 0.004 and $NaHCO_3$, 0.26.

MBR-A was operated under the inner flow feeding mode with the CMV filled with inactivated *Pseudomonas* sp. 1A1. MBR-B was operated under the inner flow feeding mode with the CMV filled with active *Pseudomonas* sp. 1A1. MBR-C was operated under the normal feeding mode with the CMV filled with active *Pseudomonas* sp. 1A1. The filtration membranes with an effective membrane area of 0.021 m^2 were prepared using hydrophilic polyvinylidenefluoride (PVDF) hollow fiber (ZeeWeed 500, GE-Zenon, US) with a nominal pore size of 0.04 μm. Other operating parameters are described in Table 5.2.

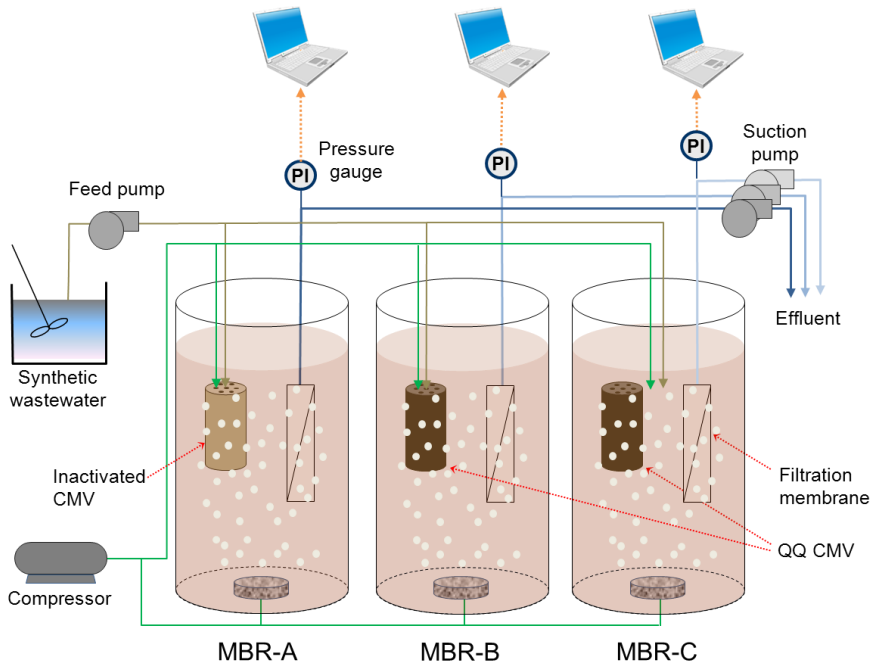


Figure 5.1. Schematic diagram of the three MBRs in parallel operation

MBR-A: *CMV with inactivated QQ bacteria under the inner flow feeding mode*

MBR-B: *CMV with QQ bacteria under the inner flow feeding mode*

MBR-C: *CMV with QQ bacteria under the normal feeding mode*

Table 5.1. Composition of the synthetic wastewater in lab-scale MBR operating conditions

Nutrient	Composition
Glucose	306.75 mg/L
Peptone	115 mg/L
Yeast extract	14 mg/L
$(NH_4)SO_4$	104.75 mg/L
KH_2PO_4	21.75 mg/L
$MgSO_4$ $7H_20$	32 mg/L
$MnSO_4$ $5H_20$	2.875 mg/L
$FeCl_3$ $6H_20$	0.125 mg/L
$CoCl_2$ $6H_20$	1.25 mg/L
$CaCl_2$ H_20	3.25 mg/L
$NaHCO_3$	255.5 mg/L

Table 5.2. Lab-scale MBR operating conditions

Working volume	3.0 L
SRT	60 d
HRT	6 h
Flux	25, 30, 35 L/m^2/h
MLSS	11,000 - 13,000 mg/L
Feed COD	400 - 570 mg/L
F/M ratio	0.12 - 0.21
pH	6.8 - 7.0
COD removal efficiency	95 - 99%

5.2.5 Analytical methods

Gram staining was carried out according to the procedure described by Holm and Jespersen (2003). The LIVE/DEAD® BacLight™ Bacterial Viability Kit (Molecular Probe, USA) was used to determine the active state of the biomass in the CMV according to the manufacturer's instructions.

The biocake that formed on the membrane surface during MBR operation was stained with SYTO 9 (Molecular Probe, USA), which has specificity for nucleic acids from bacterial cells (Kim et al. 2013a, Hwang et al. 2008). All of these fluorescently stained samples were immediately observed with a confocal laser scanning microscope (Nikon C1 plus, Japan). Images were recorded in the green channel (excitation 488 nm and emission 515/30 nm) and red channel (excitation 543 nm and emission 600/50 nm). A Z-section image stack for each green and red channel was constructed using the IMARIS software (Bitplane AG, Zurich, Switzerland).

Mixed liquor suspended solids (MLSS) and chemical oxygen demand (COD) were determined according to standard methods. Soluble microbial products (SMP) were obtained by centrifuging the mixed liquor (5000 g, 5 minutes) and filtering the supernatant through a glass microfiber filter (pore size of 1.2 μm). Extracellular polymeric substances (EPSs) were extracted from the biocake using the heat extraction method (Kim et al. 2013b). The proteins and polysaccharides in the SMP and EPS were quantitatively

analyzed using the modified Lowry method and phenol-sulfuric acid method (Judd 2010, Dubois et al. 1956), respectively.

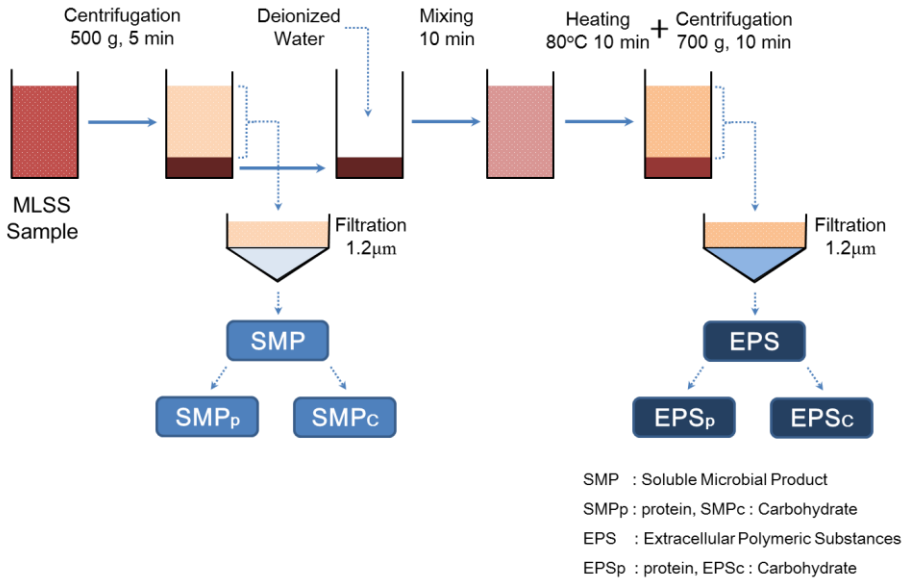


Figure 5.2. Method for EPS and SMP extractions and measurements (Judd 2010)

5.3 Results and Discussion

5.3.1 Quorum quenching activity of the ceramic microbial vessel

Two CMVs with different biomass packing densities (26.5 or 70.3 mg biomass/cm^3-lumen) were prepared and the AHL degradation activity of each was evaluated using C8-HSL as a signal molecule. One control CMV with inactivated *Pseudomonas* sp. 1A1 was also prepared to check the potential adsorption of C8-HSL by a ceramic vessel and the inactivated bacteria. As shown in Figure 5.3, the CMV with the inactivated QQ bacteria did not show a substantial decrease in the C8-HSL concentration, suggesting that its adsorption of C8-HSL was negligible. However, the AHL degradation rates of the two CMVs were 0.013 and 0.032 nmol C8-HSL/minutes for a 90-minute reaction time depending on the QQ bacterial weights in the CMVs, proving that the CMV has AHL quenching activity.

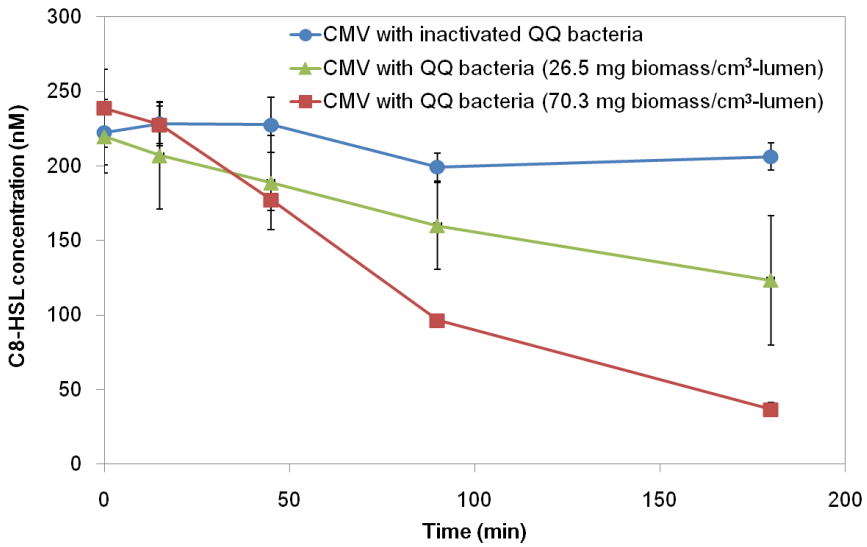


Figure 5.3. Quorum quenching activity of the ceramic microbial vessels with different microbial packing densities. Error bar: standard deviation (n=3).

5.3.2 Effect of the CMV on MBR biofouling

Two MBRs with different CMVs were run in parallel to investigate the effect of the CMV on membrane biofouling (MBR-A & -B in Figure 5.1): 1) MBR-A, CMV with inactivated QQ bacteria under the inner flow feeding mode and 2) MBR-B, CMV with QQ bacteria under the inner flow feeding mode. Membrane biofouling was monitored by TMP rise-up in the operation of the submerged MBRs. In the 1st cycle, the two MBRs were operated without the CMV at constant flux of 30 LMH to confirm there was little difference in the membrane fouling tendency among them (Figure 5.4). The two MBRs had similar rates of TMP rise-up, suggesting the population dynamics of the microorganisms in the mixed liquors were identical for the two MBRs.

In the 2nd cycle, two CMVs with a biomass packing density of 70.3 mg biomass/cm^3-lumen were installed into each reactor. MBR-A with inactivated QQ bacteria showed the same trend in TMP rise-up as in the 1st cycle, indicating QQ was not carried out because of the inactivation of the QQ bacteria. MBR-B with the QQ bacteria, however, showed a delay in the TMP rise-up compared to MBR-A. This proves the quorum quenching effect of the CMV with QQ bacteria on membrane biofouling under the inner flow feeding mode. When the flux was decreased to 25 LMH from 30 LMH at the 3rd cycle, the rate of TMP rise-up slowed down for both MBRs, but a large gap in TMP between MBR-A and -B was still maintained at the end of 10 days of

operation. To elucidate the discrepancy in TMP between the two MBRs, the used filtration membranes were taken out at points 1 and 2 in the 2nd cycle and the total attached biomass (TAB) accumulated on the surface of the filtration membrane was measured. The TAB in MBR-A was 119 (±5.0) mg, whereas that in MBR-B was only 44 (±1.0) mg. The difference in the TAB implies that the development of the biocake on the membrane surface was retarded in the MBR-B by the QQ bacteria in the CMV. It was also confirmed visually with CLSM photography of the used membranes at the same points (Figure 5.5). In detail, the reconstructed CLSM images of the used filtration membranes at points 1 and 2 clearly show that the biocake on the membrane surface was developed much less in MBR-B than in MBR-A.

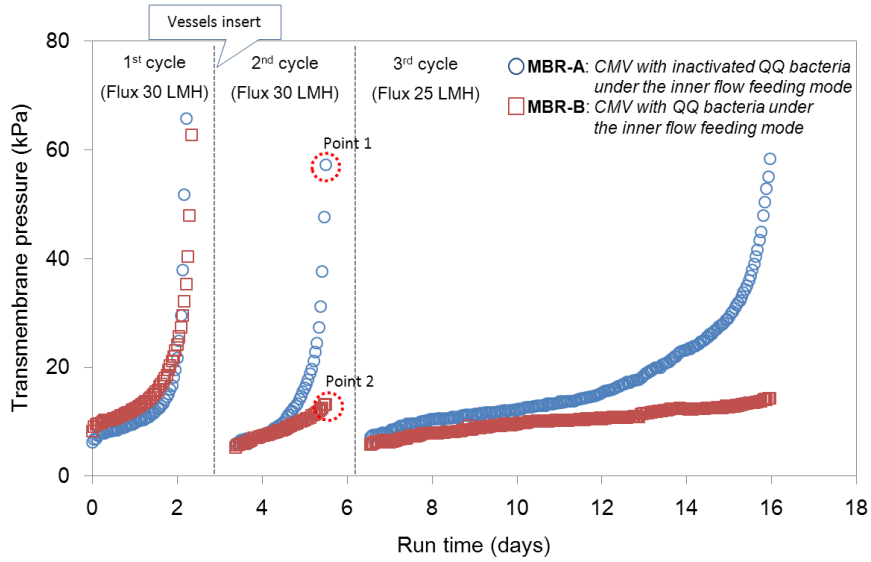


Figure 5.4. TMP profiles during 30 days of MBR runs (CMV with a packing density of 70.3 mg biomass/cm^3-lumen)

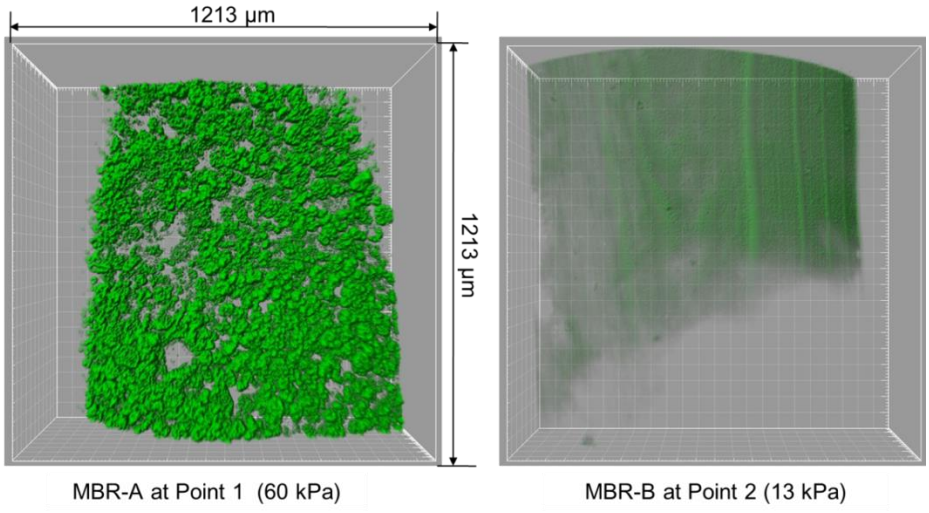


Figure 5.5. CLSM images of the biocakes on the surface of the used filtration membranes at points 1 & 2 in the 2nd cycle

5.3.3 Effect of the inner flow feeding mode on TMP rise-up in QQ MBR at constant flux

The main purpose of the 'inner flow feeding mode' through the CMV was to enhance the growth of the QQ bacteria by ensuring the nutrient supply, thereby mitigating membrane biofouling. In order to compare the biofouling mitigating effect of the inner flow feeding mode to that of the normal feeding mode, MBR-C, the MBR with the CMV under the normal feeding mode, was operated together with MBR-A and -B (Figure 5.1). After confirming that all three MBRs were in the same biological environment based on the TMP profile (the 1st cycle in Figure 5.6), the three CMVs with a biomass packing density of 26.5 mg biomass/cm^3-lumen were installed into each reactor before starting the 2nd cycle. The profiles of the TMP rise-up during three cycles for MBR-A and -B (Figure 5.6) were quite similar to those in the previous run (Figure V-4). In the 2nd cycle, MBR-C with the QQ bacteria in the CMV under the normal feeding mode showed retardation in the TMP rise-up compared to MBR-A (green color in Figure 5.6), but the extent of retardation was less than that in MBR-B under the inner flow feeding mode (red color in Figure 5.6). This result was attributed to the greater viability of the active QQ bacteria (*Pseudomonas* sp. 1A1) in the CMV under the inner flow feeding mode. The 3rd cycle of operation for the three MBRs further confirmed the reproducibility of the discrepancy in the rate of the TMP rise-up.

Of interest is that the MBR-C with the CMV under the normal feeding mode gradually lost most of its quorum quenching activity in the 3rd cycle. Because it was thought that the loss of QQ activity might be related to the deterioration of cell viability with the operation time due to the low F/M ratio in the CMV, the CMVs were taken out of MBR-B and MBR-C, respectively, right after the 3rd cycle of operation, and then the staining of live/dead cells inside the CMVs was conducted to check the cell viability in both CMVs. The portion of live cells (green color) in the CMV in MBR-B under the inner flow feeding mode (Figure 5.7a) was observed to be much higher than that in MBR-C under the normal feeding mode without the inner flow (Figure 5.7b). Quantitatively, the live/dead ratios in the CMVs of MBR-B and MBR-C were calculated as 4.8 (±0.5) and 1.3 (±0.3). It was concluded that the QQ MBR with the CMV could be more effective as a membrane biofouling control technique by adopting the inner flow feeding mode, which enabled the CMV to maintain a greater bacterial QQ activity through the facilitated nutrient transfer.

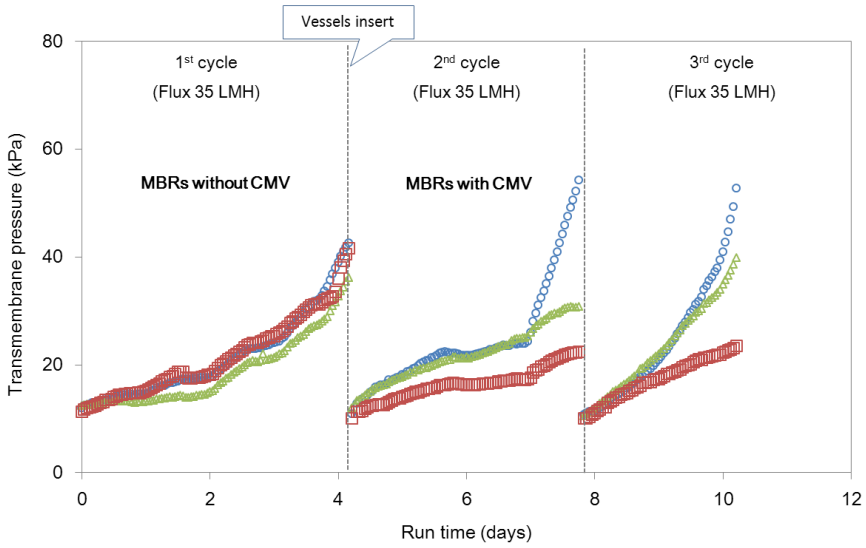
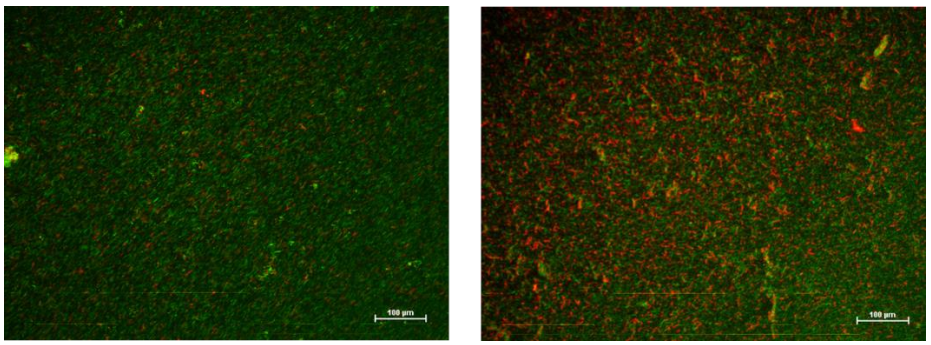


Figure 5.6. Effect of the CMV and its inner flow operation mode on MBR filterability. (CMV with a packing density of 26.5 mg biomass/cm^3-lumen)

- ○ **MBR-A:** *CMV with inactivated QQ bacteria under the inner flow feeding mode*
- □ **MBR-B:** *CMV with QQ bacteria under the inner flow feeding mode*
- △ **MBR-C:** *CMV with QQ bacteria under the normal feeding mode*



(a) **(b)**

Figure 5.7. 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.

5.3.4 Effect of the CMV on SMP and EPS production in the MBR

Polymeric substances, which are well known to be related to membrane biofouling, were analyzed in terms of soluble microbial products (SMP) in the mixed liquor and extracellular polymeric substances (EPS) in the membrane-biocake (Drews 2010, Jiang et al. 2012). It was reported that the QQ in MBR can reduce polysaccharides and proteins in both the mixed liquor and the biocake (Jiang et al., 2012). In order to investigate the effect of the CMV on SMP and EPS production in MBRs, polysaccharide and protein concentrations in the mixed liquor and biocakes were measured from the three MBRs at the end of the 2nd cycle in Figure 5.6. The CMV with QQ bacteria reduced substantially polysaccharides in the mixed liquor regardless of the feeding mode (Figure 5.8): from 23.6 (±0.6) to 8.9 (±1.1) for MBR-B or to 11.5 (±0.9) mg/L for MBR-C. However, the proteins in the mixed liquor from the three MBRs had a similar level and EPS in the mixed liquor were also a similar level (Figure 5.9).

In case of EPS in the biocake, the CMV with QQ bacteria reduced both polysaccharides and proteins (Figure 5.10): polysaccharide from 980 (±80) to 610 (±50) for MBR-B or to 650 (±40) mg/m^2-membrane for MBR-C; protein from 1,840 (±200) to 1,040 (±40) for MBR-B or to 1,290 (±40) mg/m^2-membrane for MBR-C. Taking into account that EPS and SMP are closely

associated with microbial physiology, the QQ bacteria in the CMV are presumed to have regulated the production of EPS and SMP by inhibiting quorum sensing between microorganisms in the MBRs.

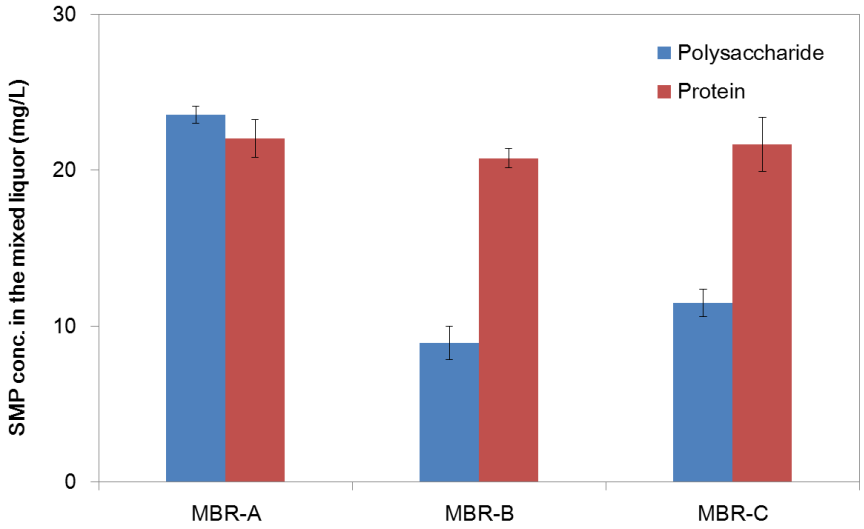


Figure 5.8. SMP in the mixed liquor. Error bar: standard deviation (n=3).

MBR-A: *CMV with inactivated QQ bacteria under the inner flow feeding mode*

MBR-B: *CMV with QQ bacteria under the inner flow feeding mode*

MBR-C: *CMV with QQ bacteria under the normal feeding mode*

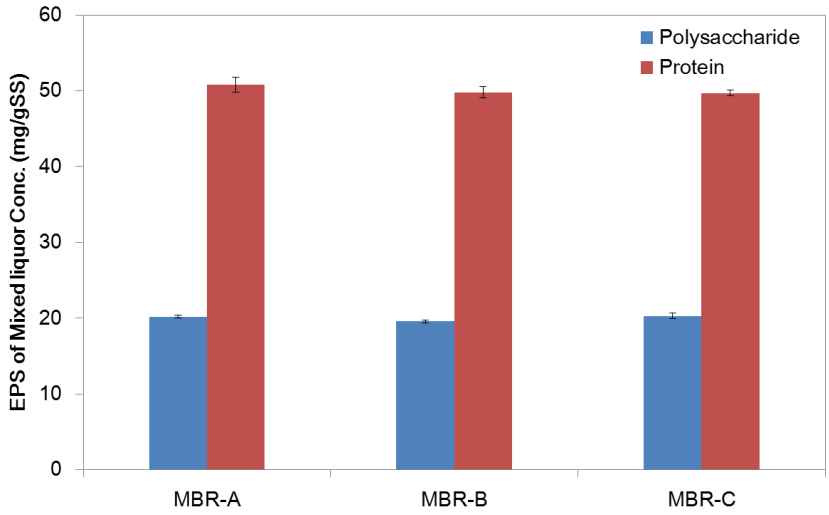


Figure 5.9. EPS in the mixed liquor. Error bar: standard deviation (n=3).

MBR-A: *CMV with inactivated QQ bacteria under the inner flow feeding mode*

MBR-B: *CMV with QQ bacteria under the inner flow feeding mode*

MBR-C: *CMV with QQ bacteria under the normal feeding mode*

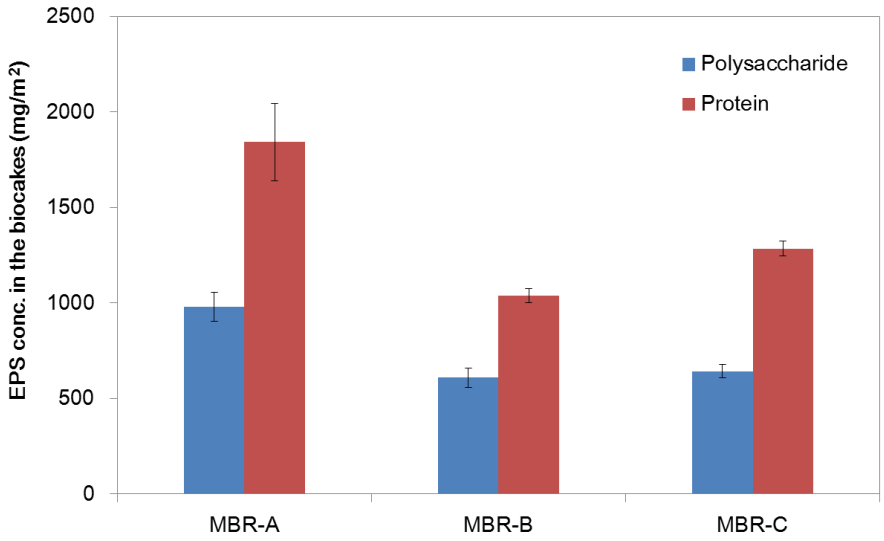


Figure 5.10. EPS in the biocakes on the membrane surface with the insertion of CMV with QQ bacteria. Error bar: standard deviation (n=3).

MBR-A: *CMV with inactivated QQ bacteria under the inner flow feeding mode*

MBR-B: *CMV with QQ bacteria under the inner flow feeding mode*

MBR-C: *CMV with QQ bacteria under the normal feeding mode*

5.3.5 Effect of the CMV on the biodegradation of organics in MBRs

Considering that quorum sensing (QS) regulates microbial physiology (Jiang et al., 2013), possible side effects of the CMV should be checked. As shown in Table 5.3, the biodegradation of organics in terms of COD removal was 98% - 99% for all MBRs, suggesting that the insertion of the CMV does not affect the microbial activity of the activated sludge for the biodegradation of organics.

Table 5.3. MBR conditions at the end of 2nd cycle

	MBR-A	MBR-B	MBR-C
COD removal rate	98 %	98 %	99 %
T-N removal rate	4 %	7 %	3 %
T-P removal rate	18 %	19 %	29 %
Floc size (MV)	259.5 μm	254.4 μm	253.0 μm
Floc size (MN)	76.8 μm	77.1 μm	77.5 μm
SVI	87.8 mL/g	82.6 mL/g	88.5 mL/g
Zeta potential	-16.05 mV	-7.06 mV	-9.82 mV

MBR-A: *CMV with inactivated QQ bacteria under the inner flow feeding mode*
MBR-B: *CMV with QQ bacteria under the inner flow feeding mode*
MBR-C: *CMV with QQ bacteria under the normal feeding mode*

5.3.6 Stability of the QQ activity in the CMV

Maintaining the QQ bacterial activity is one of the most important factors to be considered in QQ MBR with CMV. Consequently, the QQ activity of the CMV in QQ MBR under the inner flow feeding mode was periodically monitored during the operation of MBR-A and MBR-B for 30 days. For each time point at 15 and 30 days, the CMVs were taken out of the MBRs and then the QQ activity of the CMV was measured using C8-HSL as a standard signal molecule for the bioluminescence assay (Figure 5.11). The degradation rate of C8-HSL with a fresh CMV was 0.032 nmol C8-HSL/minute in a 90-minute reaction time, whereas that at 15 days was 0.047 nmol C8-HSL/minute, which was about 47% higher than the initial rate. This increase in activity was attributed to the good adaptation of the QQ bacteria to their new environment of the mixed liquor in the MBR as well as to their growth inside the CMV. Moreover, the QQ activity of the CMV at 30 days was 0.044 nmol C8-HSL/minute, indicating little loss of its activity. As a result, it was concluded that the CMV has the potential of facile and effective biofouling control in MBRs because it can maintain its quorum quenching activity at least during the test period without any further inputs.

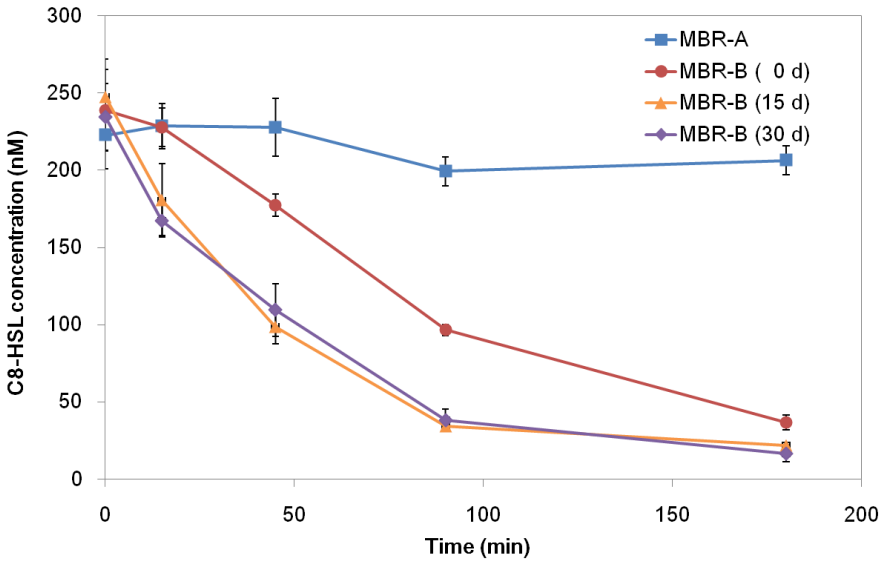


Figure 5.11. Quorum quenching activity of the CMV with C8-HSL as a standard during 30 days of MBR operation. Error bar: standard deviation (n=3).

MBR-A: *CMV with inactivated QQ bacteria under the inner flow feeding mode*

MBR-B: *CMV with QQ bacteria under the inner flow feeding mode*

5.4 Conclusions

In this chapter, a quorum quenching (QQ) MBR with a ceramic microbial vessel (CMV) was designed so as to augment the cell activity in the CMV which was prepared using a monolithic ceramic micro-porous membrane and AHL degrading QQ bacteria *Pseudomonas* sp. 1A1. The following conclusions were drawn:

(1) The quorum quenching effect of the CMV on the control of membrane biofouling was more pronounced when the feeding mode was changed from the normal mode to the inner flow mode.

(2) The QQ bacteria in the CMV are presumed to have inhibited the quorum sensing (QS) between the activated sludge in the MBR which regulates the production of EPS.

(3) The CMV showed little loss in its initial AHL degradation activity over 30 days of MBR operation, leading to the expectation for its potential in effective biofouling control in long-term MBR operations.

Recommendations for improving this research

The following recommendations are offered as possible ways to improve this study.

1. The QQ enzyme which was secreted from *Pseudomonas* sp. 1A1 has not clearly characterized in this study. Additional work on purification of the enzyme, its kinetics and specific productivity are required for further research.

2. The design factors of CMV are not defined yet. Optimizations of the length, internal diameters and external diameters, etc. are required for practical use.

3. The operational modelling of CMV is another task. Velocity or direction (up/down) of inner flow, aeration interval and amount, etc., should be investigated from the theoretical and practical point of view.

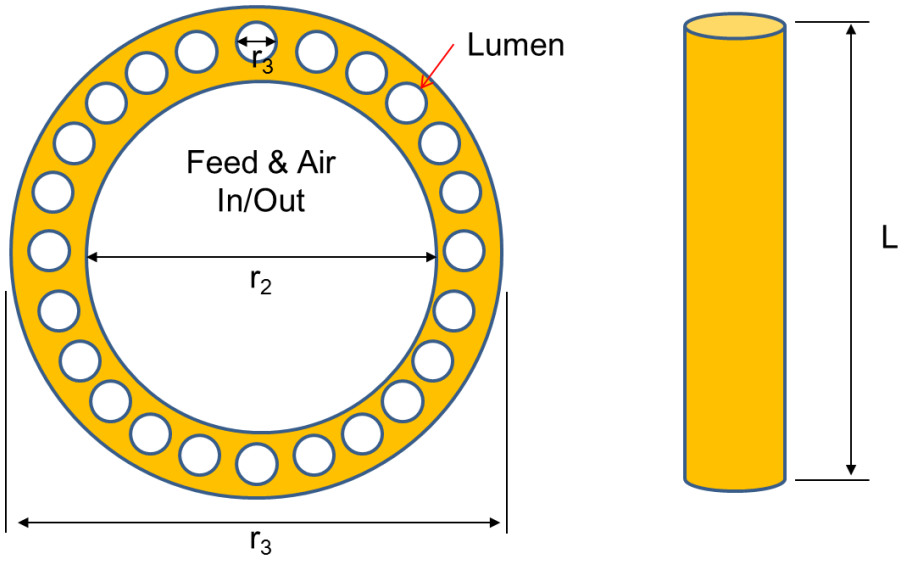


Figure 5. 12. Schematics of an ideal CMV and its design parameters

초 록

세라믹 용기에 정족수 감지억제 미생물의 고정화 및 분리막-생물반응기에서 생물막 오염제어를 위한 활용

최근 20년 분리막-생물반응기의 상업적인 활용은 탁월한 처리수질과 적은 소요부지 면적으로 인하여 급격히 증가하고 있다. 하지만 분리막 표면에 자연적으로 쌓여 여과성능을 떨어뜨리는 생물막 오염은 이 분야에서 여전히 풀지 못한 난제이며 분리막-생물반응기의 효율을 떨어뜨리는 주요 원인이다. 최근 폐수처리를 위한 분리막-생물반응기에서 미생물 용기를 이용한 정족수 감지억제 기술은 경제적으로 생물막 오염을 제어할 수 있는 방법으로 주목 받고 있다.

본 연구에서는 정족수 감지억제에 사용하는 미생물 용기 내부의 낮은 F/M 비를 개선하고자 세라믹 미생물 용기를 개발하였다. 세라믹 미생물 용기는 상용 세라믹 멤브레인과 정족수 감지억제 세균인 *Pseudomonas* sp. 1A1을 이용하여 구축하였다. 내부영양공급방식(inner flow feeding mode)은 세라믹 미생물 용기를 사용한 주목적으로서, 세라믹 미생물 용기 내부에 위치한 정족수 감지억제 미생물에게 영양물질을 용이하게 전달하여 보다 오랜 기간 동안 세포의 활성을 유지하도록 하였다.

Pseudomonas sp. 1A1을 담체한 세라믹 미생물 용기(CMV)는

내부영양공급방식으로 운전하였을 때 분리막 표면의 생물막 오염을 보다 효과적으로 제어하였으며, 또한 MBR 내에서 장기간 정족수감지억제 효과를 유지하였다. 이는 고분자 여과막의 차압상승과 공초점주사현미경에 의한 생물막 및 미생물의 직접 관찰로 확인하였다.

실험실 규모 MBR에서의 성공적인 생물막 오염제어는 내부영양공급방식을 이용한 세라믹 미생물 용기가 실규모 MBR에서도 효과적으로 생물막 오염을 제어할 수 있음을 보여 주었고 다양한 환경공학 분야에 응용가능성을 기대하게 한다.

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