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Characterization of Activated Sludge and Biofilm in MBR with Bacterial Quorum Quenching

정족수 감지 억제 적용에 따른 수처리용 분리막 생물반응기의 활성 슬러지와 생물막의 특성 변화

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김 선 의

Abstract

Characterization of Activated Sludge and Biofilm in MBR with Bacterial Quorum Quenching

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Recently, quorum sensing (QS) has been found to play a key role in biofilm formation on the membrane surface in membrane bioreactors (MBRs). Thus quorum quenching (QQ) which interrupts QS systems has received great attention as a fundamental solution to control biofouling in MBRs.

Previous studies have proved that the bacterial intra-species QQ with lactonase producing bacteria, *Rhodococcus* sp. BH4, could efficiently alleviate the biofouling in MBRs. In addition to delay in transmembrane pressure (TMP) which is a fouling index, it has been also reported that QQ led to change in the production of extracellular polymeric substances (EPS) in biofilm. However, the analyses of previous studies were only performed considering the EPS in biofilm. In this study, we aimed to further characterize the effect of QQ considering both EPS in biofilm and mixed liquor while applying *Rhodococcus* sp. BH4 in two different types of MBRs.

The first set of experiment was conducted with an anoxic/oxic combined MBR. In this set, soluble microbial product (SMP) was investigated in priority. In addition to examination of protein and polysaccharide, size exclusion chromatography (SEC) equipped with fluorescence detector was used to qualitatively analyze protein-like substances (Ex/Em wavelengths: 280/350 nm). Significant decrease in aromatic protein-like substances with molecular weight range of 100-1000 kDa was observed. Also, the filterability of sludge supernatant which depends on fouling tendency of SMP was evaluated by dead-end filtration with 150 kDa membrane. It was observed that QQ resulted in better filterability, shown by 2-3 times lower cake layer resistance.

Another set of experiment with aerobic MBR was designed to study the QQ effect of *Rhodococcus* sp. BH4, considering not only SMP but also bound EPS of floc and biocake. Aromatic protein-like and humic acid-like substances (Ex/Em wavelengths: 280/350 nm and 345/443 nm, respectively) were analyzed with SEC. In case of EPS bound to floc, neither aromatic protein-like nor humic acid-like substances showed apparent difference. However, both components have decreased in biocake EPS, where humic acid-like substances were mostly removed by QQ.

Keywords: Biofouling control, Extracellular polymeric substances (EPS), Membrane bioreactor (MBR), Quorum sensing (QS), Quorum quenching (QQ)

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Chapter 1. Introduction

1.1. Background

Membrane bioreactor (MBR) process is the combination of the conventional activated sludge (CAS) process and membrane technology. MBR process has emerged as an innovative technology for effective water treatment and reuse and developed over decades. Despite its recognizable advantages over CAS process such as smaller size of plant and higher quality of the treated water, there are still some obstacles yet to be solved, one of which is biofouling on filtration membrane.

Biofouling is regarded as the biggest issue in MBR process since it causes a severe decrease in flux and lifetime of filtration membrane and eventually results in the cost increase. Statistically, it was reported that around 60% of the cost is directly related to biofouling (Judd and Judd 2006). Various attempts to alleviate biofouling such as engineering (Yeon, Park et al. 2005), modification of membrane (Yu, Xu et al. 2007) and addition of chemicals (Lee, Kim et al. 2001) have been made, however, they were not able to fundamentally prevent biofouling.

Biofilm formation has been reported as one of group behaviors induced by quorum sensing (QS) (Davies, Parsek et al. 1998), a cell-to-cell communication between microorganisms with various types of signaling molecules controlled by their population density (Miller and Bassler 2001). Therefore, interruption of QS (quorum quenching, QQ) has been paid attention as a fundamental solution for biofilm formation and investigated in diverse fields (Choudhary and Schmidt-Dannert 2010).

Recently, the concept of QS and QQ was introduced to MBR fields and it was revealed that membrane biofouling was closely associated with N-acyl homoserine lactone (AHL) type autoinducer-1 (AI-1) (Yeon, Cheong et al. 2009). QQ in MBR has been studied in various ways such as enzymatic QQ (Yeon, Lee et al. 2009, Kim, Choi et al. 2011), bacterial QQ (Oh, Yeon et al. 2012) and design of bacterial carriers (Kim, Oh et al. 2013).

Previous studies have proved that the intra-species QQ by *Rhodococcus* sp. BH4, which are known as QQ-lactonase producing bacteria, could efficiently alleviate the biofouling in MBRs. In addition to delay in transmembrane pressure (TMP) which is a fouling index, it has been also reported that QQ led to change in the production of extracellular polymeric substances (EPS) (Kim, Oh et al. 2013). However, the analyses were only performed considering the EPS in biofilm. In this study, therefore, we aimed to further investigate the activated sludge and biofilm with bacterial QQ of *Rhodococcus* sp. BH4 using size exclusion chromatography (SEC) and dead-end filtration.

1.2. Objectives

The objective of this study was to characterize the activated sludge and biofilm with the application of QQ enzyme producing bacteria, *Rhodococcus* sp. BH4. Two independent sets of experiments were designed in order to achieve our goal.

(1) Anoxic/Oxic MBR

Characterization of soluble microbial products (SMP) in mixed liquor was performed using size exclusion chromatography (SEC) and dead-end filtration.

(2) Aerobic MBR

Characterization of SMP, bound EPS in mixed liquor and biocake EPS was performed using SEC.

Chapter 2. Literature Review

2.1. Membrane Bioreactor (MBR)

2.1.1. Concept and Process

Membrane bioreactor (MBR) is the combination of membrane process and conventional activated sludge systems (CAS) process. In addition to CAS process in which organic matters in wastewater are degraded by microorganisms in the bioreactor as substrates for their maintenance and growth, membrane was introduced to separate treated water from activated sludge rather than secondary clarifiers (Hardt, Clesceri et al. 1970). As a result, the MBR system has many advantages over CAS process.

Among numerous advantages of MBR over CAS process, the most recognized ones are that (Judd and Judd 2006):

(1) High quality, clarified and disinfected effluent are produced. The filtration membrane has an effective pore size which is significantly smaller than the pathogenic bacteria and viruses in the sludge.

(2) Solids and hydraulic retention time (SRT and HRT, respectively) can be controlled independently. The particles in MBR only need to be larger than the membrane pore size while retention times are controlled to have sufficient particle size for sedimentation in CAS process.

(3) The MBR can be operated at higher mixed liquor suspended solids (MLSS) concentration, which reduce the required reactor size and promote the development of specific nitrifying bacteria, thereby enhancing ammonia removal.

(4) Less excess sludge is produced due to longer SRT. It is the smaller size of the plant and the superior quality of the treated product water that are generally most important in practical wastewater treatment applications.

The MBR process also has constraint compared to CAS process, of which the most important ones are that:

(1) Higher energy consumption is occurred in MBR process. It is reported that 10 ~ 20 times the energy is required in MBR than in CAS process to treat same amount of wastewater (Yamamoto, Hiasa et al. 1989). However, the total cost is approximately equal in both cases due to smaller footprint.

(2) The membrane module of MBR requires higher capital equipment and operating costs. However, these costs are gradually decreasing with the mass production of filtration membrane.

(3) The removal efficiency of nitrogen and phosphorus which are closely related to eutrophication are relatively low while the organic matters are appropriately treated.

In addition to these problems, the greatest problem of MBR process is membrane contamination caused by biofilm formation on the filtration membrane. Biofouling is regarded as the biggest problem in MBR process since it causes a severe decrease in flux and the lifetime of filtration membrane and eventually results in the cost increase.

2.1.2. Development of MBR

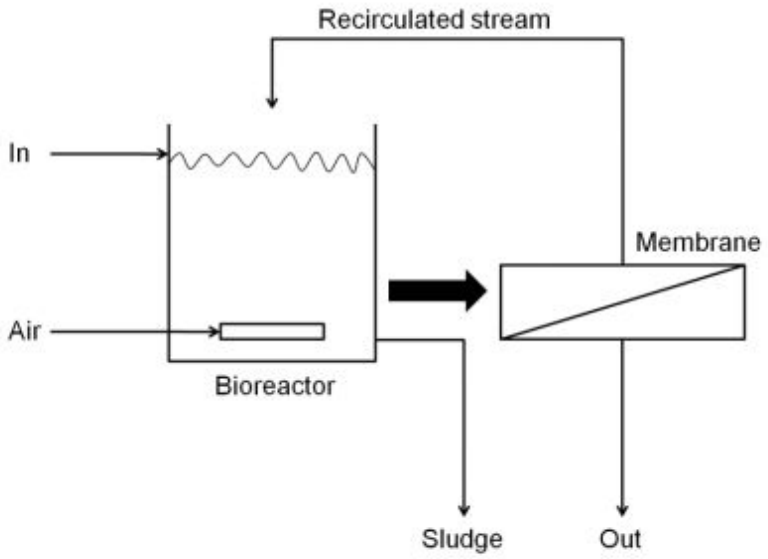
MBR process was first invented in the late 1960s by Dorr-Oliver with the idea of combining CAS process and ultrafiltration (UF) membrane process. The Dorr-Oliver system successfully established the principle of MBR to concentrate the biomass and produce the effluent of high quality, simultaneously. Around the same time, another lab-scale membrane separation system linked with a CAS process was reported (Hardt, Clesceri et al. 1970). These systems in the early stage were all based on external filtration (side-stream) MBR (Figure 1a).

From 1980s to early 1990s, in Japan, the government instigated water recycling project based on the work by Yamamoto, Hiasa, Mahmood & Matsuo. They developed a hollow fiber (HF)-ultra filtration (UF) submerged MBR process, as well as a flat sheet (FS)-microfiltration (MF) submerged MBR (Yamamoto, Hiasa et al. 1989) (Figure 1b). 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 side stream 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.

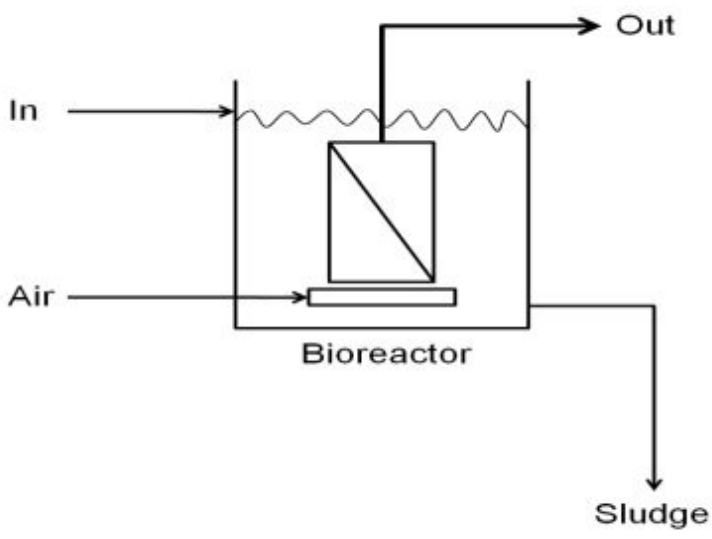
In 1997, the first Kubota municipal wastewater treatment works installed outside Japan was at Porlock in the United Kingdom. And the first Zenon membrane-based plant of similar size installed outside of the USA was the Veolia *Biosep*® plant at

pethes 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.

The first half of the 1990s saw the launch of only three major immersed MBR membrane products, originating from just two countries (USA and Japan). The first five years of the following decade saw the launch of at least 10 products originating from seven countries. For 12 major suppliers as at 2010, there were either existing or planned MBR installations of more than 10 MLD capacities (Table 1).



(a)



(b)

Figure 1. Configuration of (a) side stream MBR and (b) submerged MBR

Table 1. MBR Membrane module products, bulk municipal market

Installation	Supplier	Date	PDF	ADF
Brightwater, WA, USA	GE	2011	170	117
Qinghe, China	OW/MRC	2011	150	150
North Las Vegas, NV, USA	GE	2011	133	95
Yellow River, GA, USA	GE	2011	111	69
Shiyan Shendinghe, China	OW/MRC	2009	110	110
Aquaviva, Cannes, France	GE	2012	106	59
Bus an City, Korea	GE	2012	100	100
Guangzhou, China	Memstar	2010	100	–
Wenyuhe, Beijing, China	OW/Asahi Kasei	2007	100	100
Johns Creek, GA, USA	GE	2009	94	42
Awaza, Turkmenistan	GE	2011	87	69
Jordan Basin WRF, UT, USA	GE	2012	79	53
Beixiaohe, China	Siemens	2008	78	–
Al Ansab, Muscat, Oman	Kubota	2010	77	55
Cleveland Bay, Australia	GE	2009	75	29
Broad Run WRF, VA, USA	GE	2008	71	38
Christies Beach, Australia	GE	2011	68	27
Incheon, Korea	Econity	2012	65	–
Lusail, Qatar	GE	2011	61	61
Ecosama, Sao Paulo, Brazil	Koch	2012	61	57

(Judd, S., the MBR site, http://www.thembrsite.com/features.php)

(MRC–Mitsubishi Rayon Corporation, OW–Origin Water, PDF–peak daily flow, ADF–average daily flow)

2.2. Fouling Control in MBR Process

As described previously, the most important factor which hampers the MBR process is a contamination of the filtration membrane. Thus, various studies about the mechanism and control technology have been performed over 20 years through physical, chemical or material approaches.

2.2.1. Physical Approach

Physical cleaning methods are normally approached either by back flushing (reversing the flow) or relaxation (ceasing permeation) while continuing to scour the membrane with air bubbles. Intermittent aeration and permeation was studied to find a better aeration conditions for lower the fouling (Pollet, Guigui et al. 2009). They found out that discontinuous aeration helped filtration cake more likely to be removed. Filtration module design to improve the mobility of membrane was also studied to remove fouling layer on the membrane using physical force induced by hydrodynamics in the bioreactor (Yeon, Park et al. 2005, Hai, Yamamoto et al. 2008).

2.2.2. Chemical Approach

Physical cleaning mentioned in previous section should be supplemented with chemical cleaning to completely remove the residual and irreversible fouling on the membrane. It was reported that colloid and biopolymers, known as major foulants in bioreactors, can be removed from the MBR by chemicals such as mineral,

organic acids, caustic soda or, sodium hypochlorite. Such type of cleaning should be done on a fortnightly to monthly basis, designed to remove residual fouling and intensive chemical cleaning (once or twice a year) to remove the irreversible fouling.

Another type of chemical approach is the chemical additives. This approach is to remove the major foulants (small colloid or biopolymers) through the addition of chemicals. Since the pore size of MF membrane used in conventional MBR process is around 0.04 to 0.4 ㎛, colloids particles can block the membrane pore which result in filtration resistance.

Aluminum sulphate and ferric chloride have been used to coagulate small biological colloids in activated sludge (Lee, Kim et al. 2001). It was also reported that coagulants like ferric sulfate and aluminum chloride had removed the contaminants forming gel layer as well as separating the foulants from the membrane surface (Wu, Chen et al. 2006).

Extracellular polymeric substance (EPS) and soluble microbial product (SMP) began to be regarded as one of important factors for membrane fouling after the role of physiological status of microorganisms was revealed. Those biopolymers consist of protein, polysaccharide, lipid, nucleic acid and humic acid. Those components are anionic polymers so that they can be alleviated with positively charged components. For example, Nalco Company has developed the cationic polymers with the trade mark of ‘MPE (Membrane Performance Enhancer)’. MPE was reported to reduce the level of polysaccharide (Yoon, Collins et al. 2005, Yoon

and Collins 2006, Guo, Vigneswaran et al. 2008) and was successfully applied to pilot- and full scale MBRs (Collins, Yoon et al. 2006).

Addition of adsorbents into biological treatment systems decreases the level of organic compounds. Dosing with powdered activated carbon (PAC) produces biologically activated carbon (BAC) which adsorbs and degrades soluble organics and has been shown to be effective in reducing SMP and EPS levels (Kim and Lee 2003).

2.2.3. Material Approach

The fundamental factor for the fouling in MBR is an interfacial phenomenon between filtration membrane surface and mixed liquor containing organic matters and microorganisms. Therefore, fouling can be alleviated with the increased resistance of membrane surface against biofilm formation.

It was reported that the TMP increase rate of hydrophobic membranes was higher than that of hydrophilic ones (Futamura, Katoh et al. 1994). This suggests that fouling resistance of the membrane can be enhanced by regulating the hydrophilicity of the surface. Membrane surface modification by ozone treatment followed by graft polymerization also showed the enhanced filtration performance (Sainbayar, Kim et al. 2001, Yu, Xu et al. 2007).

Polypropylene membrane surface was modified by photoinduced graft polymerization using acrylamide which showed the improved permeability compared to wild type membrane (Yu, He et al. 2007).

Antibacterial treatment on the surface which can repress the microbial growth can also be another strategy. An antimicrobial property to microporous polyurethane membrane surface was imparted by grafting N-halamine precursor and subsequent chlorine bleaching, which showed reasonable antibacterial effect but its fouling control efficiency in the MBR system was not tested (Tan and Obendorf 2007).

2.2.4. Biological Approach

In the early stage, biofouling control was only studied in physic-chemical way since the fouling layer was considered as an abiotic layer. Afterward, the concept of biofilm was suggested and defined as a layer of microorganisms with high density presenting at the interface. Thus some biological control strategies against biofouling have been reported, including (1) inhibition of quorum sensing, (2) nitric oxide-induced biofilm dispersal, and (3) enzymatic disruption of extracellular polysaccharides, proteins, and DNA. Biological-based antifouling strategies are a promising constituent of an effective integrated control approach, since they target the essence of biofouling problems. However, biological-based strategies are still in their developmental phase, and several questions need to be addressed to set a roadmap for translating existing and new information into sustainable and effective control techniques.

2.3. Quorum Sensing (QS) System

2.3.1. Definition and Mechanism

Quorum sensing (QS) system refers to a series of phenomenon where bacteria accumulate small signaling molecules called autoinducer to maintain or increase their own cell population density. The activation of an autoinducer, regulatory proteins and sensor kinase is carried during QS. The independent cells of the same species of microbes recognize and react with each other, which leads to the expression of certain genes (Fuqua, Winans et al. 1994, Withers, Swift et al. 2001, Waters and Bassler 2005). Thus the concentration of autoinducers represents the population of microorganisms and its threshold concentration means quorum. Quorum sensing system results in group behaviors such as virulence, biofilm formation, conjugation and sporulation.

QS was discovered and described over 35 years ago in two luminous marine bacterial species, *Vibrio fischeri* and *Vibrio harveyi* (Nealson and Hastings 1979). In both species the enzymes responsible for light production are encoded by the luciferase structural operon *luxCDABE* (Engebrecht and Silverman 1984), and light emission was determined to occur only at high cell-population density in response to the accumulation of secreted autoinducer signaling molecules (Nealson and Hastings 1979).

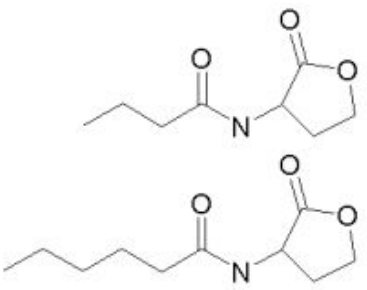
The QS systems are categorized into three based on the type and form of autoinducers (Figure 2):

(1) Gram-negative bacteria: LuxI/LuxR type QS

(2) Gram-positive bacteria: modified oligopeptides mediated QS

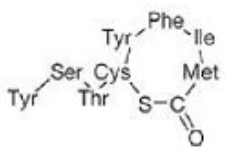
(3) Autoinducer-2 for the interspecies communication.

N−acyl homoserine lactone (AHL) for gram negative bacteria



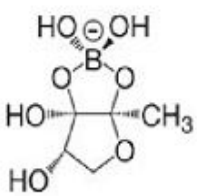
Pseudomonas aeruginosa

Oligopeptide for gram positive bacteria

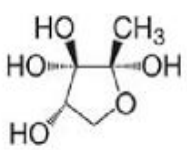


Staphylococcus aureus

Autoinducer−2 (AI−2) for interspecies communication



Vivrio harveyi



Salmonella enterica serovar Typhimurium

Figure 2. Representative signal molecules of each QS system

2.3.2. Gram-Negative Bacteria: LuxI/LuxR Type AI-1 QS

General mechanism of LuxI/LuxR type QS of gram-negative bacteria is shown in Figure 3 (Fuqua and Greenberg 2002). This type of QS employs N-acyl homoserine lactone (AHL) as an autoinducer. AHL molecules consist of homoserine lactone ring and fatty acid and vary according to the number of carbons in fatty acid (Figure 4). The Lux-I like proteins synthesize a specific AHL by producing amide linkage between an acyl moiety from an acyl-acyl carrier protein (acyl-ACP) and S-adenosylmethionine (SAM) and its subsequent lactonization (Figure 5) (Miller and Bassler 2001).

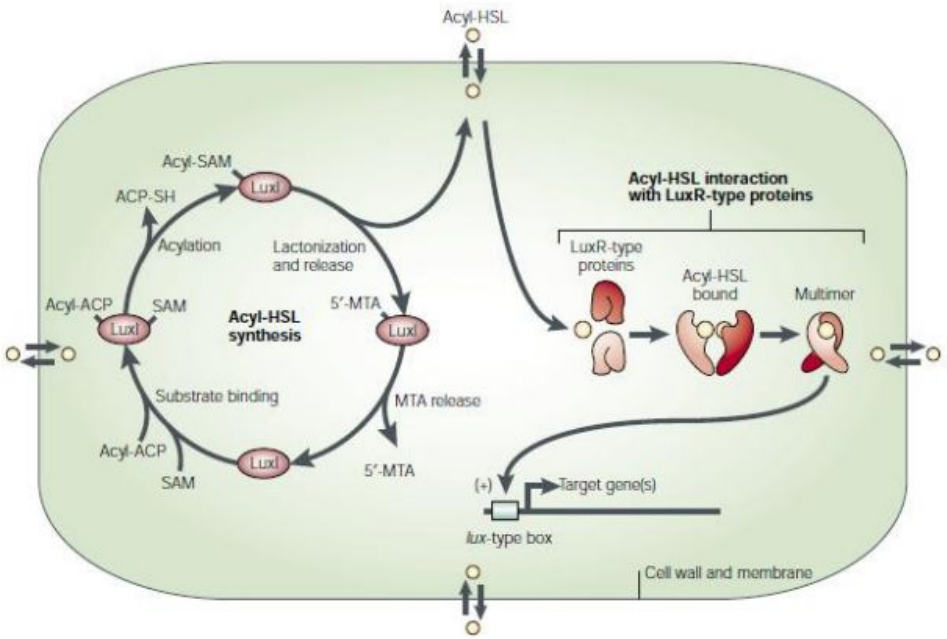


Figure 3. Model of AHL-QS in a single generalized bacterial cell (Fuqua and Greenberg 2002)

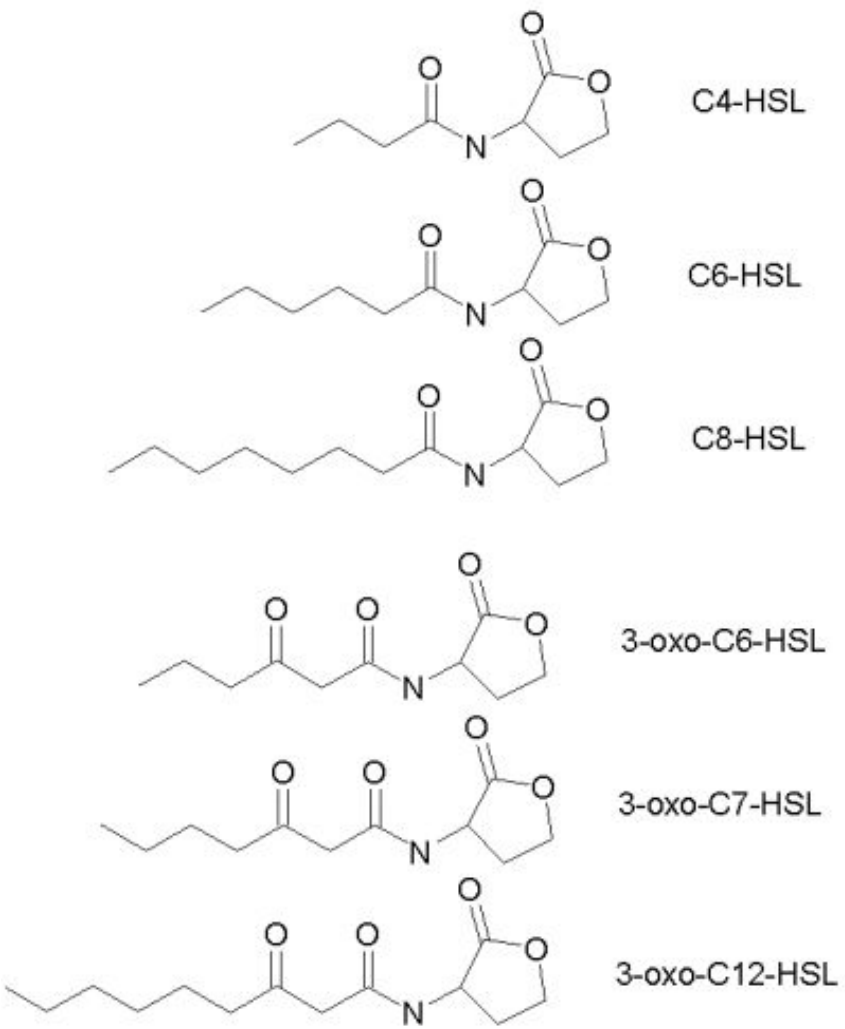


Figure 4. Molecular structure of each AHL autoinducer

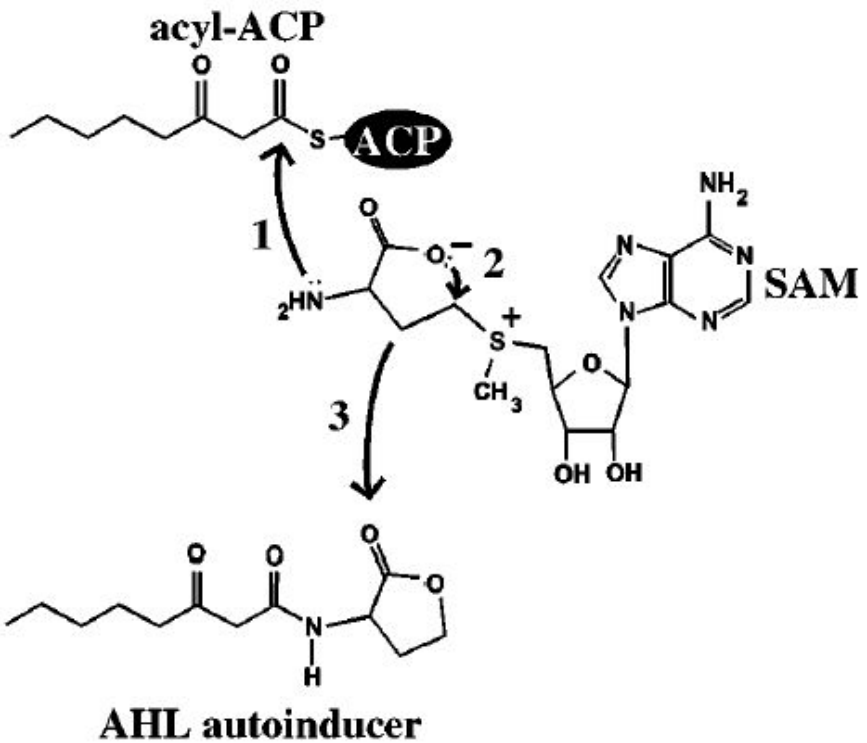


Figure 5. Lux-I directed biosynthesis of acyl-homoserine lactone autoinducers (Miller and Bassler 2001)

2.3.3. Gram-Positive Bacteria: Modified Oligopeptide Mediated AI-1 QS

In the same manner as gram-negative bacteria, gram-positive bacteria also regulate a variety of processes in response to increasing cell-population density. However, in contrast to gram-negative bacteria which employ AHL molecules as autoinducer, gram-positive bacteria use peptide for their quorum sensing. In general, the autoinducing peptides (AIPs) are secreted via a dedicated AP-binding cassette (ABC) transporter as an autoinducer for QS.

Figure 6 shows the general mechanism of gram-positive bacteria (Miller and Bassler 2001). A peptide signal precursor locus 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 residue (D). The phosphorylated response regulator activates the transcription of target gene(s).

In contrast to gram-negative bacteria, gram-positive bacteria utilize two response proteins systems to detect autoinducers. These two sensor kinase are used to recognize secrete peptide signaling molecules. The interaction between peptide

ligand induces phosphorylation of cognate response regulator protein, which results in the expression of protein used in QS system.

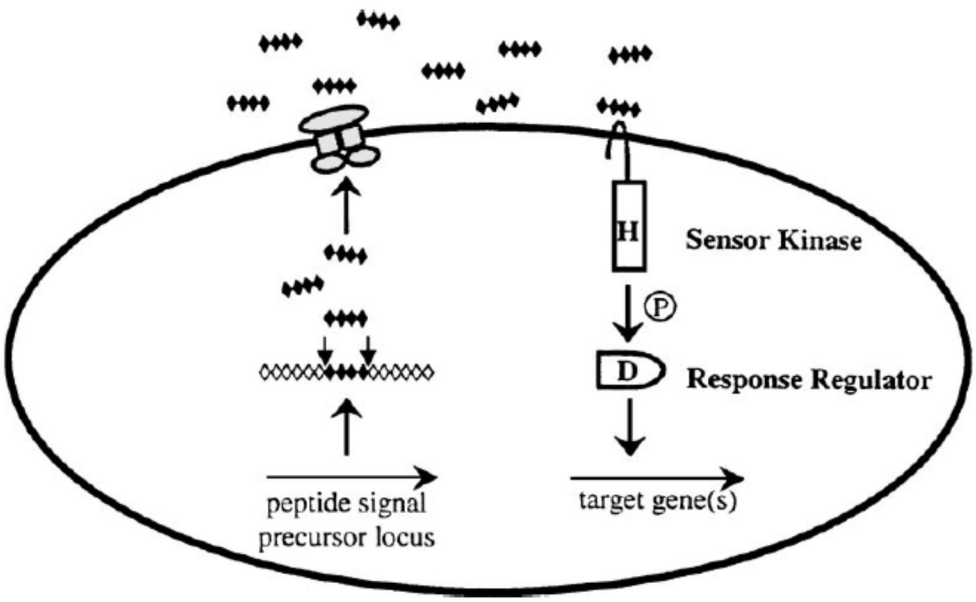


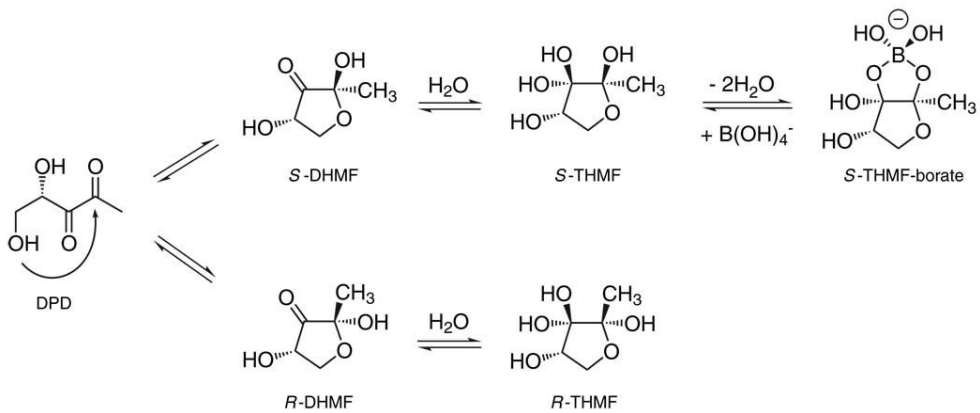
Figure 6. General model of QS in Gram-positive bacteria (Miller and Bassler 2001)

2.3.4. Interspecies Communication: AI-2 QS

AHLs and peptides represent the two major classes of known bacterial QS molecules, used by Gram-negative and Gram-positive bacteria, respectively, for intraspecies communication. Recently, a family of molecules generically termed autoinducer-2(AI-2) has been found (Chen, Schauder et al. 2002) (Figure 7). It has been proposed that AI-2 is an interspecies signal molecule among Gram-negative and Gram-positive bacteria.

Bassler and co-workers first identified AI-2 based QS mechanism of Vibrio harveyi in the early 1990s (Bassler, Wright et al. 1993, Bassler, Wright et al. 1994). It was observed that an AHL-deficient strain of the bacterium remained capable of producing bioluminescence even in the absence of the AHL autoinducer. This suggested that a second QS pathway, employing a different signaling molecule, was operating. This novel AI, whose structure at the time was unknown, was termed AI-2. It was subsequently shown that cell-free culture fluids from a number of bacterial species were capable of stimulating activity in a *V. harveyi* AI-2 reporter strain (Bassler, Greenberg et al. 1997). This suggested that the AI-2 signal may be produced by numerous bacterial species. Later work demonstrated that the same gene was responsible for AI-2 biosynthesis in *V. harveyi*, *E. coli*, and S. *typhimurium* (Surette, Miller et al. 1999). This gene, designated *luxS*, has been found in over 70 bacterial species (Lowery, Dickerson et al. 2008). These observations have led to the proposal that AI-2 is a universal signaling molecule for interspecies communication. It should be noted that the product of the *luxS* gene, the enzyme LuxS, is thought to have a metabolic role in cells, in addition to being

responsible for AI-2 biosynthesis (Vendeville, Winzer et al. 2005, Lowery, Dickerson et al. 2008). This may provide and alternative explanation for the widespread conservation of luxS. In spite of this controversy, there is a growing body of evidence that AI-2 does indeed represent a universal language for interspecies communication.



DPD: 4,5-Dihydroxy-2,3-pentanedione

DHMF : 2,4-dihydroxy-2-methyldihydrofuran

THMF: 2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran

Figure 7. Chemical structures of representative AI-2 molecules (Camilli and Bassler 2006)

2.3.5. Role of QS in Biofilm Formation

Generally, microorganisms in biofilm have higher cell density than free-floating, planktonic cell population. As a consequence, bigger amount of metabolites and other secreted or excreted microbial factors are produced in biofilm. According to previous studies, mobility, homogeneity and production of extracellular polymeric substances (EPS) of microorganisms influence the structure of biofilm (Hentzer, Teitzel et al. 2001).

AHL-based QS has been shown to influence biofilm maturation for the gram-negative bacterium *Serratia liquefaciens* (Labbate, Queck et al. 2004). Quorum sensing regulates swarming motility in S. liquefaciens (Eberl, Winson et al. 1996). AI-2 based *LuxS* type QS has also proved its important role in biofilm formation, in the mutant of StreptococcusA *luxS* mutant biofilm grown on hydroxyapatite disks was loose and rough in appearance compared with smooth and confluent biofilm of the wild-type strain (Wen and Burne 2004). In addition, AI-2 has been found to influence biofilm formation in mixed=species biofilm between *Streptococcusgordonii* and *Porphyromonas gingivalis* (McNab, Ford et al. 2003). Addition of AI-2 to *Escherichia coli* increased their biofilm mass 30 times (González Barrios, Zuo et al. 2006). These reports confirm that AI-2 based QS plays an important role in biofilm formation.

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, *Pseudomonas aeruginosa* (Parsek and Greenberg 2000), *Burkholderia cepacia*, and *Aeromonas hydrophila*

require functional AHL-mediated QS system for formation of biofilm (Davies, Parsek et al. 1998, Huber, Riedel et al. 2001, Lynch, Swift et al. 2002). The biofilm formation control by inhibiting QS signal molecules will be described in more detail in the next section.

2.3.6. Control Strategies of LuxI/LuxR Type AI-1 QS

Bacteria use QS to coordinate their group behaviors which are biofilm formation, swarming, motility, production of extracellular polysaccharides, etc. (Li, Attila et al. 2007, Lowery, Dickerson et al. 2008, Ng and Bassler 2009). Also, QS can occur within single species bacteria community as well as interspecies bacteria community. The QS mechanism is achieved by producing, releasing, and detecting small signal molecules known as AHLs. These signal molecules are synthesized by generator protein which is called LuxI homologue.

Therefore, QS systems generally offer three points of attack: the signal generator, the signal molecule and the signal receptor (Rasmussen and Givskov 2006, Roy, Adams et al. 2011). Therefore, QS inhibition strategy can be divided into blockage of signal molecule synthesis, interference with signal receptor and inactivation of signal molecule. Figure 8 describes three strategies to control the AHL type QS system.

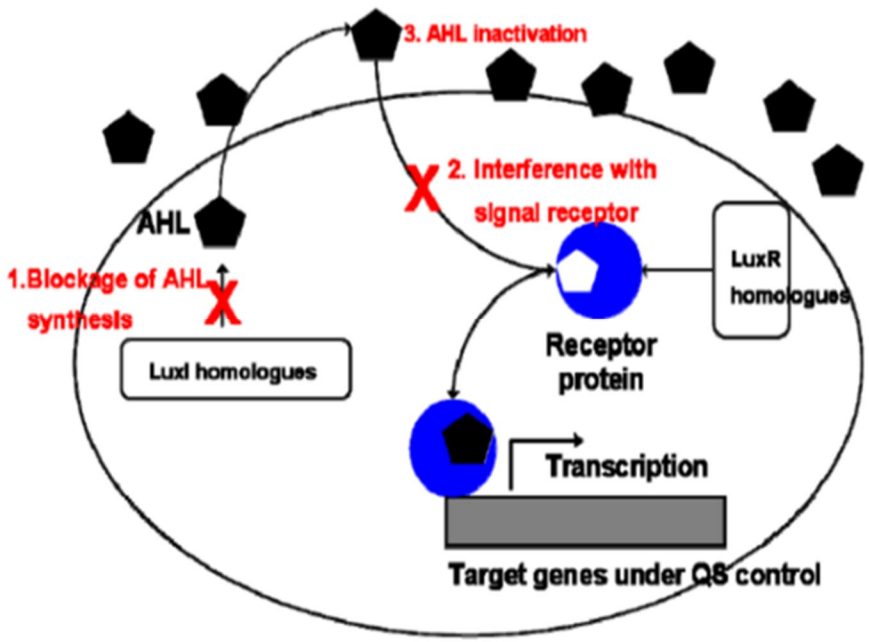


Figure 8. Three strategies to control of LuxI/R type

Ⅰ. Blockage of AHL Synthesis

It has been found that analogues of these AHL building blocks such as holo-ACP, L/D-*S*-adenosylhonocysteine, sinefungin and butyryl-*S*adenosylmethionine (butylryl-SAM) were able to block AHL production in vitro (Parsek, Val et al. 1999). 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. In addition, it was reported that curcumin inhibits PAO1 virulence factors such as biofilm formation, pyocyanin biosynthesis, elastase/protease activity, and AHL production. However, the exact inhibition mechanism of curcumin was not revealed (Rudrappa and Bais 2008).

Ⅱ. Interference with Signal Receptor

AHL QSI can be obtained from natural sources and chemical synthesis

Inhibitors from natural sources

In a recent screening of 50 *Penicillium* species grown on different growth media, a remarkably high fraction, 66% were found to produce secondary metabolites with QS inhibition activity. Two of the compounds were identified as penicilic acid and patulin produced by *Penicillium Radicicola* and *Penicillium coprobium*, respectively.

Diketopiperazines (DKPs) are a family of cyclic dipeptides isolated from the supernatant of numerous cultures of bacterial species, such as *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Cotrobactor freundii* and Enterobacter agglomerans. DKPs can regulate several strains of quorum-dependent phenotypes in several different species of bacteria by acting as AHL antagonists in LuxR based QS system (Holden, Ram Chhabra et al. 1999).

Inhibitors from chemical synthesis

One widely explored method is to block the receptor with an analogue of the AHL autoinducer molecule. AHL analogs can be substituted in either the side chain or the ring moiety. Analogs of the 3-oxo-C6 HSL molecule with different substituents in the side chain are able to displace the native signal from the LuxR receptor. However, most of these compounds also exhibit agonist effects, which limit their use as QS inhibitor. Another strategy is to substitute the entire ring structure. For example, 2-amino-3-oxo-C12- inhibits QS system by regulating LasR-dependent expression of LasI AHL synthesis enzyme.

Ⅲ. Inactivation of AHL Signal Molecules

Chemical degradation: Lactonolysis

A simple way to achieve inactivation of the AHL signal molecules is to increase pH to above 7.0 (Yates, Philipp et al. 2002), which causes ring opening of the AHL

(lactonolysis). A number of higher organisms employ this strategy in defense against invading QS bacteria. The infection of some plants *Erwinia carotovora* results in tissue-macerating plant pathogen which increases pH by inactivation of QS signal molecules and blocking expression of QS controlled genes (Byers, Lucas et al. 2002).

Enzymatic degradation: Quorum quenching

Another approach to inactivate AHL molecules is to degrade cyclic ester or amide linkage of AHLs by enzymatic hydrolysis. Only two enzyme families in the microorganism have the capability of cutting AHL structures; the AiiA-like AHL-lactonases and the AiiD-like AHL acylases have been demonstrated to be involved in the real cleavage of the QS signal molecules, although a large diversity of QQ microbes have been identified. Figure 9 shows each quorum quenching pathway by lactonase or acylase. In addition to these two major quorum quenching enzymes, oxidoreductase also can inactivate AHLs by structural modification.

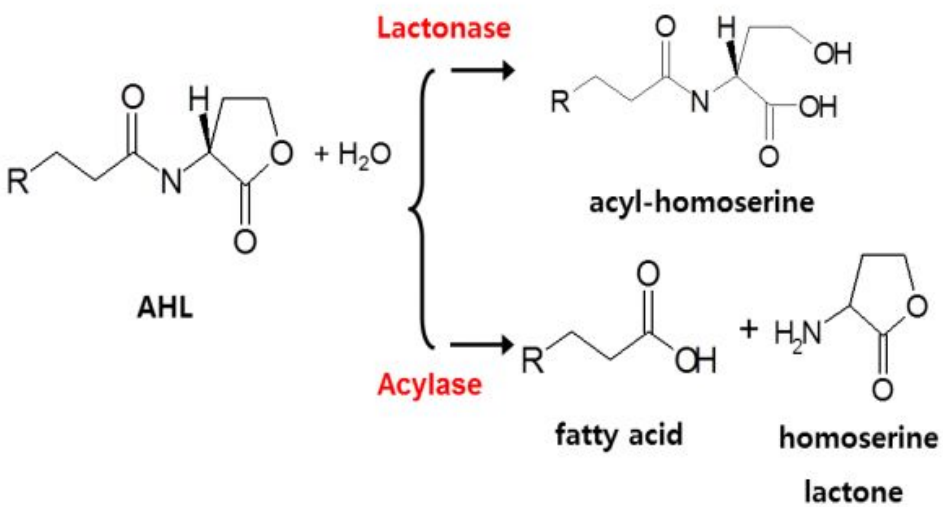


Figure 9. Enzymatic inactivation of AHL autoinducers by QQ enzymes

2.4. Quorum Quenching (QQ) Application in MBR

Recently, the concept of quorum quenching has been proposed as a novel biofouling inhibition strategy in MBR. As most of aquatic bacteria are gram-negative, controlling AHL molecules was regarded as a key factor for inhibition of biofilm formation in MBRs. Indeed, it was reported that biofouling and AHL concentration increased with same tendency (Yeon, Cheong et al. 2009).

2.4.1. Enzymatic QQ in MBR

The MBR with QQ acylase freely added or immobilized in magnetic enzyme carrier (MEC) enhanced the membrane permeability to a large extent compared with a conventional MBR with no enzyme (Yeon, Cheong et al. 2009, Yeon, Lee et al. 2009). Subsequent study investigated the changes in population dynamics and gene expression in the MBR with MEC using pyrosequencing and proteomics (Kim, Oh et al. 2013). It was reported that QQ influenced biofouling propensity and sludge characteristics such as settleability, particle size, extracellular polymeric substances (EPS), viscosity, zeta potential and the component of filtration resistances while not influencing pollutant degradation and biomass concentration (Jiang, Xia et al. 2013).

2.4.2. Bacterial QQ in MBR

To avoid practical issues regarding cost and stability of enzymes, more feasible and sustainable approach has been proposed. Recombinant *Escherichia coli* and

Rhodococcus sp. BH4 producing N-acyl homoserine lactonase encapsulated in hollow fiber membrane were applied in submerged MBR systems and successfully controlled biofouling (Oh, Yeon et al. 2012). In addition, it was reported that the QQ effect was largely dependent on the location of QQ bacterial carrier and recirculation rate of the mixed liquor between the bioreactor and the membrane reactor in external submerged type MBR (Jahangir, Oh et al. 2012). Subsequent study introduced another unit of bacterial carriers named cell entrapping beads (CEBs) which have both physical washing and biological effects of biofouling inhibition (Kim, Oh et al. 2013).

2.5. Extracellular Polymeric Substances (EPS)

2.5.1. Definition and Characteristics

Extracellular polymeric substances (EPS) are a complex mixture of polysaccharides, proteins, humic acids, nucleic acids and possibly other compounds. EPS forms a highly hydrated gel matrix, while soluble EPS (SMP, soluble microbial product) may form a gel on the membrane surface (Reid, Liu et al. 2008). These compounds not only impact the physical chemistry of the mixed liquor, but also provide nutrients and a habitat for microbes on the membrane surface. EPS and SMP compositions are known to be highly variable and dependent upon, amongst other things, microbial diversity and physiology. SMP and EPS provide adhesion of aggregates to maintain the floc structure (Mikkelsen and Keiding 2002), but also impact upon sludge physicochemistry. The latter includes viscosity, filterability, dewaterability, hydrophobicity and surface charge, all of which may then influence membrane fouling (Laspidou and Rittmann 2002).

Biofilms are microbial communities encased in a layer of extracellular polymeric substances (EPS). The EPS matrix provides several functional purposes for the biofilm, such as protecting bacteria from environmental stresses, and providing mechanical stability. It is known that EPS consist of polysaccharides, proteins and humic-like substances as major constituents (Wingender, Neu et al. 1999), whereas nucleic acids and phospholipids are minor EPS constituents (Frølund and Keiding 1994). Molecular weight of proteins of EPS from various activated sludge varies from small (10 kDa) to large (600 kDa) sizes (Garnier, Gorner et al. 2005).

Furthermore, the wastewater type and operating conditions of the treatment plant have an influence on the composition of the EPS (Sponza 2002).

2.5.2. EPS Analysis by Size Exclusion Chromatography (SEC)

The EPS characterization is essential from different points of view (composition, size distribution, ability to link metal ions, electric charge) in order to understand their role in (i) bacterial aggregates and consequently in the wastewater treatment process, or (ii) the fate of metals in the environment (Guibaud, Tixier et al. 2003, Sheng, Yu et al. 2010). However, current methods like colorimetric methods give only quantitative information (Wingender, Neu et al. 1999) while qualitative analysis are also important to understand the characteristics of EPS.

Size exclusion chromatography (SEC) provides access to valuable information about the fingerprints and/or the distribution of apparent molecular weight (aMW) of EPS present in a sludge or biofilm. UV spectrometric detection at 210 or 280 nm (Frølund and Keiding 1994, Görner, de Donato et al. 2003, Comte, Guibaud et al. 2007) is usually used after separation by SEC or asymmetrical flow field-flow fractionation (Alasonati and Slaveykova 2011). It is assumed that a wavelength of 280 nm mainly corresponds to the protein fraction of the EPS (Görner, de Donato et al. 2003). However, it was specified that other conjugated macromolecules present in the EPS such as humic-like substances or nucleic acids, can also be detected at 280 nm and that UV absorbance at 210 nm corresponds to aliphatic-like

compounds where the whole organic and mineral compounds of EPS are detected (Simon, Pairo et al. 2009).

Fluorescence is an easy and non-degradative method for monitoring protein and humic-like substances based on the association of functional groups to excitation-emission region distribution. In various natural organic matters, five regions corresponding to protein and humic-like substances were differentiated: I (Aromatic proteins I, or tyrosine- like proteins), II (Aromatic proteins II or tryptophan-like proteins), III (Fulvic acids-like), IV (soluble microbial by-products-like) and V (humic-like substances) regions (Chen, Westerhoff et al. 2003). Several recent works have been performed with 3-D fluorescence spectroscopy to characterize EPS or the foulant materials of membrane bioreactors directly or on the collected fraction after SEC separation (Sheng and Yu 2006, Adav and Lee 2011, Liu, Chen et al. 2011).

2.5.3. EPS Analysis in MBR with QQ

Jiang et al. have studied the characteristics of EPS production in enzymatic QQ and control MBR (Jiang, Xia et al. 2013). They analyzed EPS not only qualitatively but qualitatively in many aspects. They found that relative hydrophobicity (RH) values of the polysaccharides and proteins in the QQ MBR were much lower than those in the control MBR, not only in the mixed liquor, but also on the membrane surface. Additionally, the RH values of all components in the biocakes were notably higher than in the mixed liquor for both MBRs, which is consistent with other studies (Tielen, Rosenau et al. 2010). Subsequently, they

analyzed composition of SMP and EPS of sludge and biocake with excitation-emission eatrix (EEM) fluorescence and molecular weight (MW) distribution. It was found out that, in tightly bound (TB) EPS and EPS extracted from biofilm on the membrane, a peak known to be related to humic acid-like substances (Chen, Westerhoff et al. 2003) decreased significantly in QQ MBR compared to control MBR. As quorum quenching promoted deflocculation of sludge flocs into small pieces, more humic acid-like substances were desorbed and appeared in the bulk liquid. Because humic-acid compounds have a high membrane fouling potential (Chuang, Chang et al. 2009, Wu and Lee 2011), the control MBR with a higher concentration of humic acid-like substances suffered from more severe fouling.

Chapter 3. Materials and Methods

3.1. Preparation of QQ Agents

3.1.1. Strains and Growth Conditions

AHL-Lactonase producing bacteria *Rhodococcus* sp. BH4 (Called BH4) were isolated from real wastewater treatment plant (Okchoen, Korea) by an enrichment culture method (Oh, Yeon et al. 2012). The single colony of BH4 was inoculated in Luria-Bertani (LB) (Miller, USA) broth then incubated at 30°C with orbital shaking (200 rpm) for 20 hours.

3.1.2. Preparation of Microbial Carriers

Prior to immobilization, culture of BH4 was centrifuged (7000 rpm, 15 min) and resuspended in DI water.

Ⅰ. Vessel for A/O MBR

Two sets of vessel were fabricated using microporous poly ethylene (PE) membrane (GE, USA) with pore size of 0.4 μm (Oh, Yeon et al. 2012). The surface area of each set of vessel was 0.013 m^2. The dry mass of BH4 was measured then 600mg were immobilized in one set of vessel using syringe (BH4 vessel). The other vessel without BH4 cells (vacant vessel) was used for the control experiment. Vessels were applied in anoxic reactor of anoxic/oxic (A/O) MBR.

Ⅱ. W-beads for aerobic MBR

W-beads were prepared with and without BH4 (BH4 w-beads and vacant w-beads, respectively) and applied in aerobic MBR. Microbial suspensions were mixed with synthetic polymer solution. The mixture was dropped into gelation solution using a syringe needle to form spherical beads and stirred for 12 hours.

3.2. MBR Set-up

3.2.1. Anoxic/Oxic (A/O) MBR

A lab-scale anoxic/oxic combined MBR (A/O MBR) which is composed of two separate reactors, an anoxic reactor with the volume of 5.4 L and an aerobic reactor with the volume of 12.6 L, was used to elaborate the QQ effect (Figure 10). Wastewater and activated sludge were provided from a municipal wastewater treatment plant located in Labege, France.

A filtration module containing a flat sheet microfiltration membrane (Lp_0=1110 L/ (m^2 h bar), Kubota, Japan) with an average pore size of 0.2 μm and an effective filtration area of 0.1 m^2 was submerged in the aerobic reactor. The activated sludge was recirculated with the ratio of 4. The MBR was operated at constant flux of 10 L/ (m^2 h), with an SRT of 30 days and an MLSS in aerobic reactor of 5000-6000 mg/L.

Two cycles of operation were performed sequentially during 13 days each, firstly without vessel (A/O MBR: w/o vessel) and then with vacant, BH4 vessel in an order. In the cycle with vessel, vacant vessel was applied for first 7 days (A/O MBR: Vacant vessel cycle) then replaced with BH4 vessel on the 8th day (A/O MBR: BH4 vessel cycle). Vessels were located in anoxic reactor in both cases.

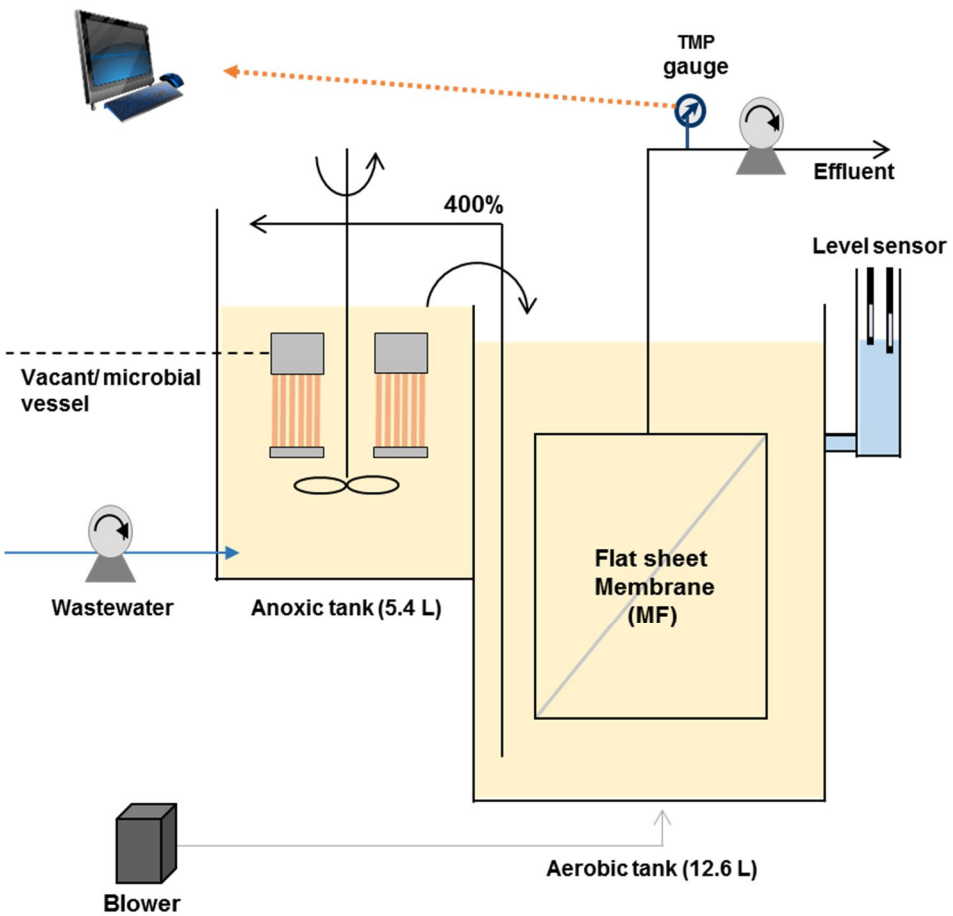


Figure 10. Schematic diagram of the A/O MBR

3.2.2. Aerobic MBR

Two lab-scale submerged aerobic MBRs with 2.5 L of working volume were used to investigate the QQ effect (Figure 11). Activated sludge was provided from a real wastewater treatment plant located in Sihwa, Korea. Wastewater was provided from local restaurant.

Two MBRs with 0.5 % (v/v) of BH4 w-beads or vacant w-beads were operated in parallel: MBR w/BH4 w-beads and MBR w/vacant w-beads. MBRs were operated with same conditions except the type of bead. The effective area and pore size of the hollow fiber filtration membrane module (Lp_0=1300 L/ (m^2 h bar), ZeeWeed 500, GE-Zenon, U.S.) was 0.0155 m^2 per MBR and 0.04 μm, respectively. MLSS in each reactor was maintained within the range of 3000 mg/L. The MBR was operated at constant flux of 15 L/ (m^2 h), with an SRT of 30 days and an HRT of 8 h.

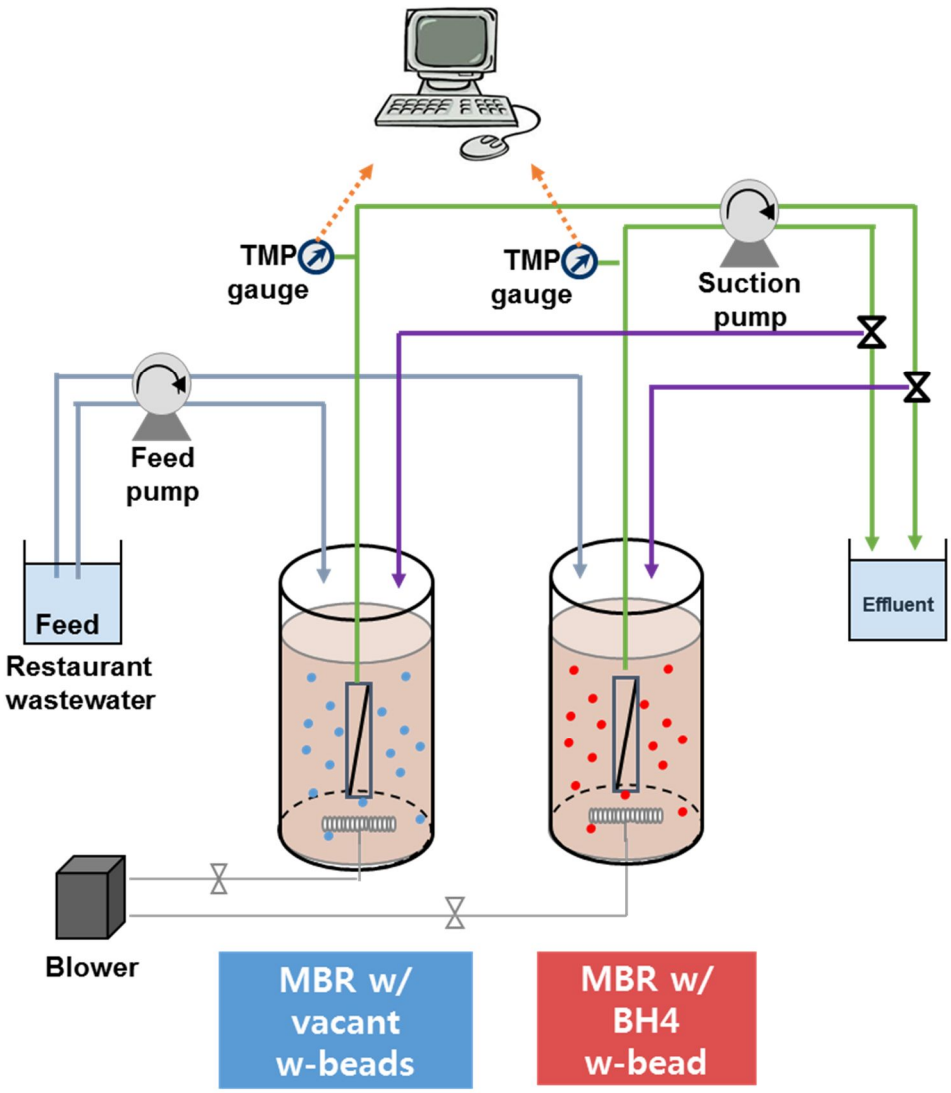


Figure 11. Schematic diagram of the aerobic MBR

3.3. Measurement of QQ Activity

3.3.1. Substrate and Reporter Strain

QQ activity of BH4 immobilizing agents was examined by degradation level of standard C8-HSL (N-N-octanoyl-DL-homoserine lactone) (Sigma-Aldrich), which is known as one of the most abundant signal molecules in the MBR for wastewater treatment (Yeon, Cheong et al. 2009). Degradation level of C8-HSL was evaluated based on a bioassay method with the reporter strain *Agrobacterium tumefaciens* A136 (Ti-)(pCF218)(pCF372) which is genetically modified not to produce AHL autoinducers. The single colony of the reporter strain was inoculated in Luria-Bertani (LB) (Miller, USA) broth supplemented with 0.1 % (v/v) of tetracycline and 0.5 % (v/v) of spectinomycin, then incubated at 30°C with orbital shaking (200 rpm) for 20 hours.

3.3.2. QQ Activity of BH4 Vessels for A/O MBR

The C8-HSL was added to 30 mL of sterile DI water to a final concentration of 200nM. A unit of BH4 vessel (containing 50 mg cells) and corresponding unit of vacant vessel were inserted in prepared C8-HSL solutions and the mixture was incubated at 30°C with orbital shaking (200 rpm) for different lengths of time. The remaining concentrations of C8-HSL in the solution were measured using bioassay.

The bioassay was carried on an indicating agar plate made by mixing LB agar and overnight culture of reporter strain A136 in the ratio of 9:1. Tetracycline (1 % (v/v) as A136), spectinomycin (5 % (v/v) as A136) and X-gal (4 % (v/v) as A136)

were also supplemented in the mixture. The indicating agar plates with AHL samples loaded in holes were incubated at 37°C for 20 hours.

As the reporter strain is known for blue color development in the presence of exogenous AHLs and X-gal, the concentration of AHL molecules can be determined according to the level of blue color development.

3.3.3. QQ Activity of W-beads for Aerobic MBR

The C8-HSL was added to 30 mL of sterile DI water to a final concentration of 200nM. 80 units of BH4 (containing 50mg cell) and corresponding units of vacant w-beads were inserted in prepared C8-HSL solution, separately. The remaining concentrations of C8-HSL in the solution were measured using bioassay.

Bioassay was performed using luminescence method. The reporter strain and each AHL sample were loaded in each well of microwell plate and mixed. The microwell plate was incubated at 30°C for 1.5 hours, and the Beta-Glo® Assay System (Promega, U.S.A) was added to each well for the luminescent reaction with β-galactosidase produced by the reporter strain. After 30 minutes, the luminescence was measured by a luminometer (Synergy2, Biotek®, U.S.A.). The amounts of AHLs were calculated using relationship equations based on the calibration curve derived from standard samples of AHLs.

3.4. Analytical Methods

3.4.1. A/O MBR

Figure 12 shows the TMP profile of A/O MBR during two cycles. Analyses for basic performances, SMP and filterability of sludge supernatant were performed on samples taken on the 19th day (vacant vessel cycle) and 24th day (BH4 vessel cycle) (marked on Figure 12).

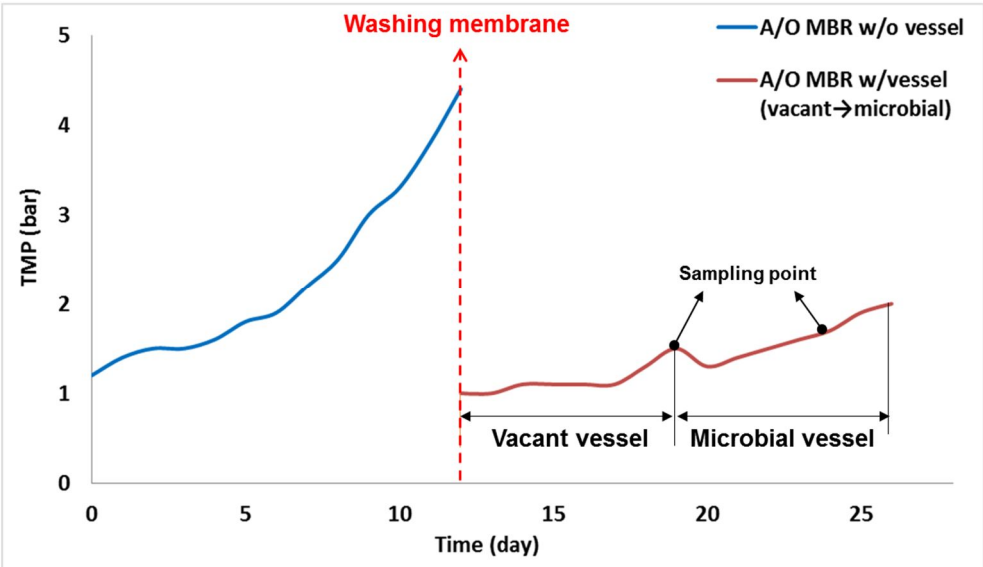


Figure 12. TMP profile of A/O MBR (sampling points)

Ⅰ. Basic analysis

The concentration of MLSS was measured by the standard method AFNOR NFR 90-105. Dissolved organic carbon (DOC) in liquid samples was analyzed with a Total Organic Carbon analyzer (Shimadzu, France). Chemical oxygen demand (COD), total nitrogen (TN) and ammonium contents were measured by spectrometry with reagent kits (Merck, U.S.A.). The analyses with supernatant (organic matter rejection and pollutant removal efficiency) and activated sludge (MLSS) were performed with samples from aerobic reactor. Samples were filtered through 0.45 μm membrane prior to DOC, COD, TN and ammonium analysis. Average floc size was measured by Malvern Mastersizer 2000.

Ⅱ. EPS analysis

Mixed liquor from the MBR was dewatered through centrifugation at 4000g for 15 min. The filtrate of sludge supernatant from this step was regarded as SMP. Protein and polysaccharide contents in SMP were quantitatively analyzed using the acid bicinchoninic (Smith, Krohn et al. 1985) and the Anthrone methods (Dreywood 1946), respectively.

More specific analysis of SMP was also conducted by size exclusion chromatography (SEC). Separation of SMP was carried out with Akta Purifer (GE Healthcare, USA) equipped with a column Protein Kw804 (Shodex, Japan), a silica-based column with an exclusion limit of 1000 kDa, and a fluorescence detector (Varian, USA). The eluent solution of the mobile phase consisted of 25

mM Na_2SO_4 and a phosphate buffer (2.4 mM NaH_2PO_4 and 1.6 mM Na_2HPO_4 at pH 6.8). Excitation/emission wavelengths were set at 280/350 nm, which are confirmed as the required values by EEM flourescence results, to detect aromatic protein-like substances (Chen, Westerhoff et al. 2003). The column was calibrated using proteins with known molecular size: thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), ribonuclease A (13.7 kDa) and aprotinin (6.5 kDa) purchased from GE Health care. Each test sample was filtered through a 0.45 μm filter before HPLC-SEC analysis.

Ⅲ. Filterability Test

Beside the experiments with continuous MBRs, the effect of QQ on filterability of supernatant was evaluated by dead-end filtration cell. All filtration tests were performed with an Amicon cell (Millipore, U.S.A.). The filtration module had an effective surface area of 13.4 m^2. Ultrafiltration membranes (PES, Lp_0=400-600 L/(m^2 h bar), Microdyn-Nadir GmbH, Germany) with a molecular weight cutoff (MWCO) of 150 kDa was used as a filtration membrane to filter out small components in the supernatant. A new membrane was used for each filtration. Membranes were soaked in deionized water overnight before using to open and maintain pores wet, and to remove the extra substances on the membrane. For each filtration test, influent (supernatant) was filtered through the membrane until the collected permeate volume reached 50 L/m^2. All data were normalized to 20°C.

Membrane fouling resistance was determined by the Equation (1)

$$J = \frac{\Delta P}{\mu(R_m + R_f)} \quad (1)$$

Where J is the permeate flux (L/(m^2 h)) at 20 °C ; ΔP is the transmembrane pressure (bar) ; μ is the dynamic viscosity at 20 °C (Pa s) ; R_m is the membrane resistance (m^{-1}) and R_f is the fouling resistance (m^{-1}).

Fouling layer properties were then determined in term of specific cake resistance multiplied by retained dissolved organic carbon according to the Equation (2).

$$\frac{t}{V} = \frac{\mu \alpha C}{2S^2 \Delta P} V + \frac{\mu R_m}{S \Delta P} \quad (2)$$

Where t is the filtration time (s); V is the permeate volume (m^3); μ is the dynamic viscosity at 20 °C (Pa s) ; α is the cake specific resistance (m kg^{-1}) ; C is the retained dissolved organic carbon (kg m^{-3}) ; S is the membrane surface (m^2) ; ΔP is the transmembrane pressure (bar) and R_m is the membrane resistance (m^{-1}).

3.4.2. Aerobic MBR

Ⅰ. Basic analysis

MLSS and COD were determined according to standard methods (Eaton, Clesceri et al. 1995). The mean particle size was measured using particle size analyzer (S3200, Microtrac, U.S.A.).

Ⅱ. EPS analysis

A two-step thermal extraction method was adopted to extract loosely bound EPS (LB-EPS) and tightly bound EPS (TB-EPS) from the sludge sample (Li and Yang 2007). Mixed liquor from the MBR (50 ml) was dewatered through centrifugation (Mega 17R, Hanil Science Industrial, Ltd., Korea) at 4000g for 5 min. The filtrate of sludge supernatant from this step was regarded as SMP. The sludge pellet was resuspended in 15 ml of 0.1 M potassium phosphate buffer (PB) and 35 ml of DI water, then immediately sheared by a vortex mixer (G-560, Scientific Industries, Inc., NY, USA) for 1 min. The sludge suspension was then centrifuged at 4000g for 10 min, and the organic matter in the supernatant was regarded as LB-EPS of the sludge mass. For TB-EPS extraction, the sludge pellet was resuspended in 0.1 M PB to its original volume of 50 mL. The sludge suspension was heated at 80 ℃ in a water bath for 30 min and then it was centrifuged at 4000g for 15 min. The organic material in the supernatant was regarded as the TB-EPS.

Biocake EPS was prepared by sonication for 2 hours, followed by thermal extraction method as TB-EPS in mixed liquor (80 ℃, 30 min). The LB-, TB-EPS and biocake EPS extracts were further analyzed for their protein and polysaccharide contents, measured using the modified Lowry and phenol/sulfuric acid methods, respectively.

Qualitative analysis on EPS was also conducted by size exclusion chromatography (SEC) equipped with a fluorescence detector (Varian, USA). Separation of EPS was carried out with Ultimate 3000 (Dionex, USA) equipped with a GPC column Ultrahydrogel Linear (Waters, U.S.A.). The eluent solution of the mobile phase consisted of 50 mM sodium phosphate buffer (pH 7.0). Excitation/emission wavelengths were set at 280/350 nm and 345/443 nm, which are confirmed as the required values by EEM flourescence results, to detect aromatic protein-like substances and humic acid, respectively (Chen, Westerhoff et al. 2003).

Chapter 4. Results and Discussion

4.1. A/O MBR

4.1.1. QQ Activity of BH4 Vessel

The QQ activity of BH4 immobilizing vessel was evaluated by the degradation level of standard C8-HSL. The activity can be interpreted from the variation in the blue area, which is the index for the concentration of C8-HSL as described in section 3.3.2. Standard samples made of standard C8-HSL and samples from vacant vessel were also analyzed for the calibration and control test, respectively (Figure 13c and 13a). The initial QQ activities of vessels were measured 1 day after the fabrication. The concentration of C8-HSL with vacant vessel kept same area, which means there was no degradation (Figure 13). In case of BH4 vessel, meanwhile, we observed an abrupt decrease in blue area, which reached around 10% of initial area after 45 minutes and almost 0% after 90 minutes (Figure 13b). Additionally, the QQ activity of BH4 vessel used in MBR for two weeks was also measured to evaluate its stability. They showed the same tendency as the initial activity, which shows the stability of BH4 vessel (Figure 13d).

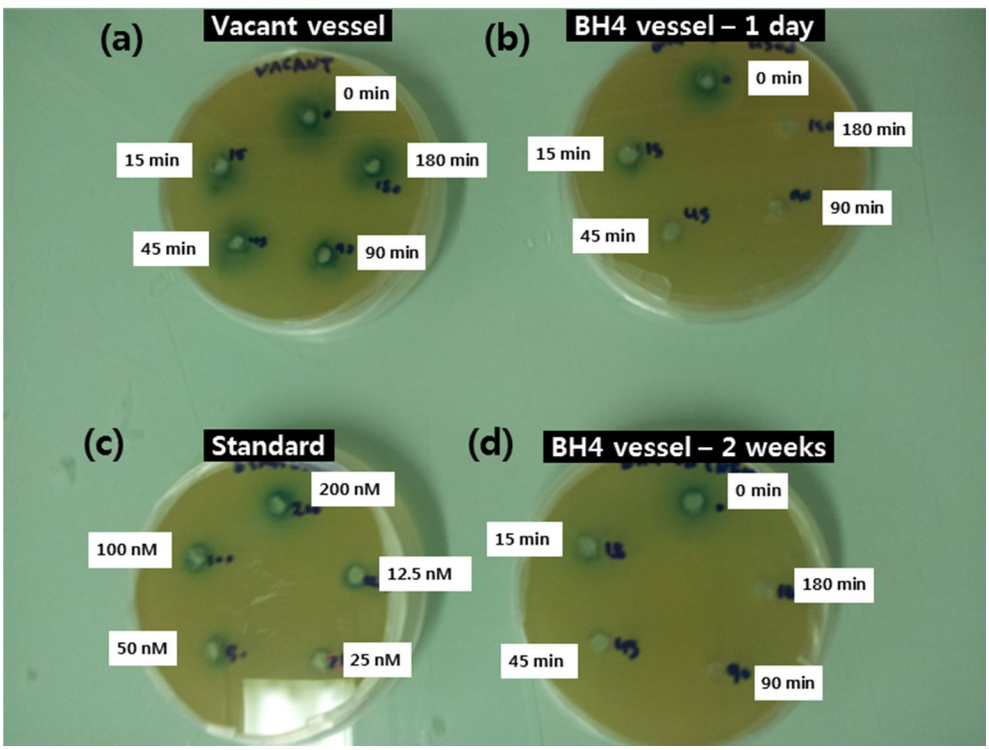


Figure 13. Quorum quenching activity of (a) vacant vessel, (b) BH4 vessel - 1 day, (c) standard samples with C8-HSL and (d) BH4 vessel - 2 weeks

4.1.2. General MBR Performances

Table 2 shows the basic performances of A/O MBR. It is shown that organic matter rejection, pollutant removal efficiency and MLSS were not affected by QQ, which corresponds to the previous study (Jiang, Xia et al. 2013). Additionally, particle sizes of floc from both aerobic and anoxic reactors were observed to be slightly decreased around 5-10 %.

Table 2. Performances of A/O MBR

		Vacant vessel cycle	BH4 vessel cycle
Organic matter rejection (%)			20.5
COD removal efficiency in Permeate (supernatant) (%)		98.6 (92.3)	98.2 (95.0)
Total nitrogen removal efficiency in Permeate (%)		90.3	88.6
Ammonium removal efficiency in Permeate (%)		94.9	93.9
MLSS (mg/L)		5-6000	5-6000
Particle size (μm)	Anoxic reactor	180.6	162.6
	Oxic reactor	174.7	164.6

4.1.3. Characterization of SMP

Ⅰ. Quantitative analysis: protein and polysaccharide

Concentration of protein and polysaccharide in SMP from each reactor was measured to verify the effect of QQ (Figure 14). The concentration of protein and polysaccharide in SMP from both reactors decreased around 10 % and 20 %, respectively. The result of protein analysis largely corresponds to the result of SEC where we observed decrease in aromatic protein-like substances.

Ⅱ. Size exclusion chromatography: Aromatic protein-like substances

The effect of QQ on characteristics of SMP was examined by HPLC-SEC equipped with a fluorescence detector with excitation/emission wavelengths of 280/350 nm which are reported to target aromatic protein-like substances (Chen, Westerhoff et al. 2003). The separation of SMP from A/O MBR was performed using GPC column Protein Kw804 (Shodex, Japan).

Figure 15 shows the profile of molecular weight distribution of aromatic protein-like substances in SMP from A/O MBR of vacant or BH4 cycle. Generally, bimodal distribution which represent two groups of protein-like substances, one with molecular size of 100-1000 kDa (around 7ml of elution volume) and the other with 10-100 kDa (around 12ml of elution volume) were detected.

Significant decreases were detected for both peak groups, of which the peak for molecules in 100-1000 kDa range showed greater difference than that in 10-100

kDa. This implies that protein-like substances with bigger molecular size were more related to QS.

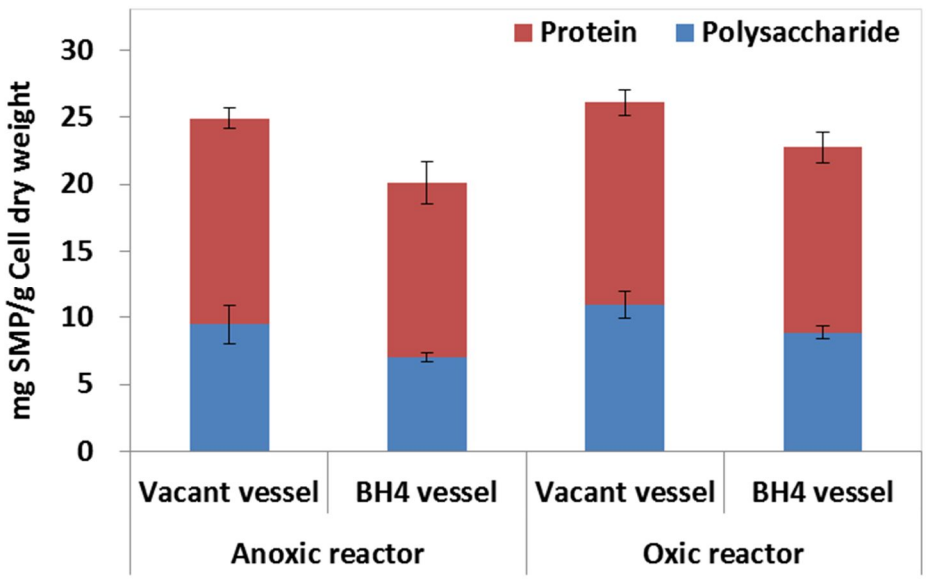
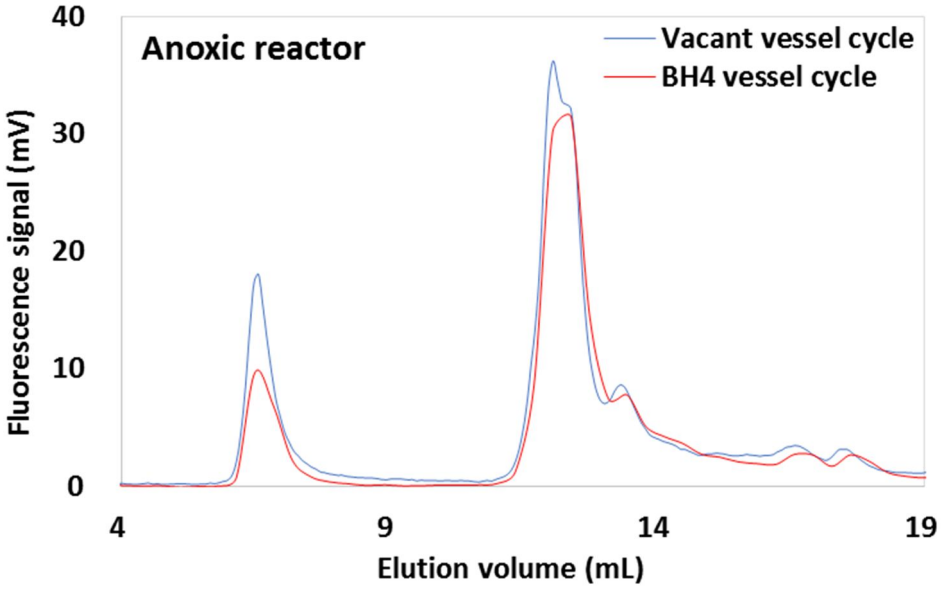
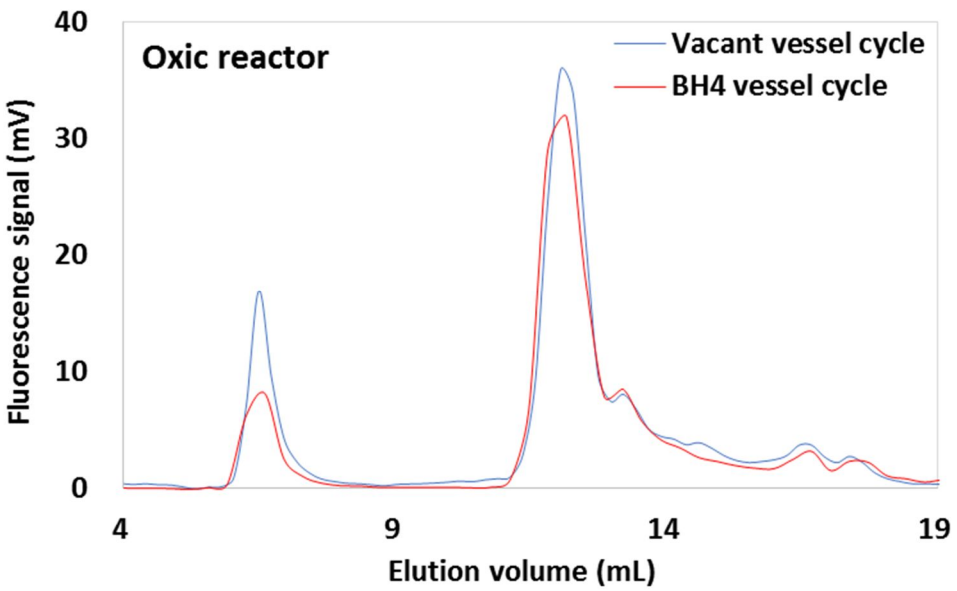


Figure 14. Concentration of polysaccharide and protein in SMP from aerobic and anoxic reactors of the A/O MBR



(a)



(b)

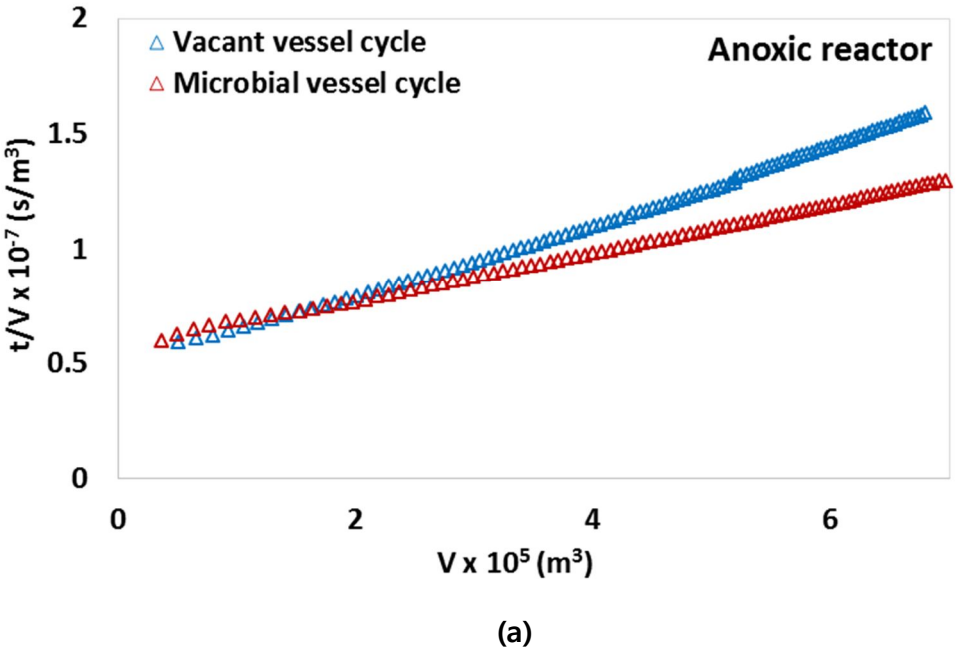
Figure 15. Chromatograms of aromatic protein-like substances in SMP from (a) anoxic and (b) oxic reactor of the A/O MBR (Ex/Em wavelength:

4.1.4. Filterability of Sludge Supernatant

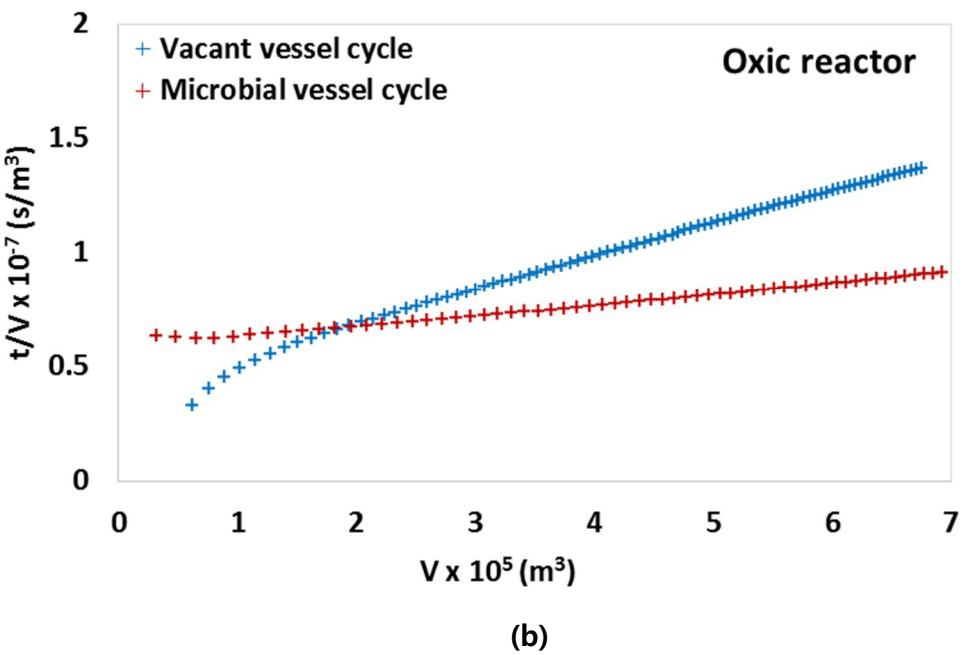
Membrane used in the A/O MBR was in microfiltration range with pore size of 0.2 μm with which most of soluble organic matters may not be retained. In order to evaluate the cake forming tendency and filterability of soluble organic compounds in supernatant, therefore, we used a membrane with smaller pore size. Dead-end filtration module equipped with PES membrane with pore size of 150 kDa was used.

Figure 16 shows fouling tendency of supernatant from each reactor of the A/O MBR at 0.7 bar. The Slopes represent the cake specific resistance (α) which is regarded as fouling index. The application of QQ significantly decreased the fouling level of supernatant from both reactors. This result can be related with SMP analysis. Since the fouling phenomenon mostly depends on dissolved organic matters in case of dead-end filtration, higher concentration of EPS contents in supernatant may have caused more fouling.

Table 3 shows the ratio of final flux to initial flux and αC values calculated from the filterability test of supernatant at 3 pressures. Generally, lower flux decrease and αC values were observed with supernatant of BH4 vessel cycle at each TMP. Besides, increase in αC value was more significant when TMP increased from 0.5 bar to 0.7 bar than with TMP increase from 0.3 bar to 0.5 bar, for every case of supernatant. It may indicate that fouling layer was more compressible at higher TMP.



(a)



(b)

Figure 16. Fouling tendency of supernatant from (a) anoxic and (b) oxic reactor of the A/O MBR at 0.7 bar

Table 3. Filterability test of supernatant from A/O MBR

	ΔP (bar)	Anoxic reactor		Oxic reactor	
		J/J0 (%) at 0.05 m^3/m^2	αC x 10^{-13} (m^{-2})	J/J0 (%) at 0.05 m^3/m^2	αC x 10^{-13} (m^{-2})
Vacant vessel cycle	0.3	30	2.5	29	2.7
	0.5	27	3.2	28	2.8
	0.7	22	4.1	27	3.8
BH4 vessel cycle	0.3	41	1.9	60	0.7
	0.5	36	2.0	54	0.7
	0.7	30	2.7	47	1.2

4.2. Aerobic MBR

4.2.1. QQ Activity of BH4 W-bead

The QQ activity of BH4 encapsulating w-beads was evaluated by the degradation level of standard C8-HSL. The activity was interpreted from luminescence intensity which represents to the concentration of C8-HSL as explained in section 3.3.3.

As shown in Figure 17, the degradation efficiency of C8-HSL with the BH4 w-beads was measured to be 65 % in the reaction time of 120 minutes. As we can assume that the adsorption of C8-HSL on the w-beads was negligible with control experiment with vacant w-beads, the decrease in the concentration was attributed mainly to its degradation by QQ with BH4 in w-bead.

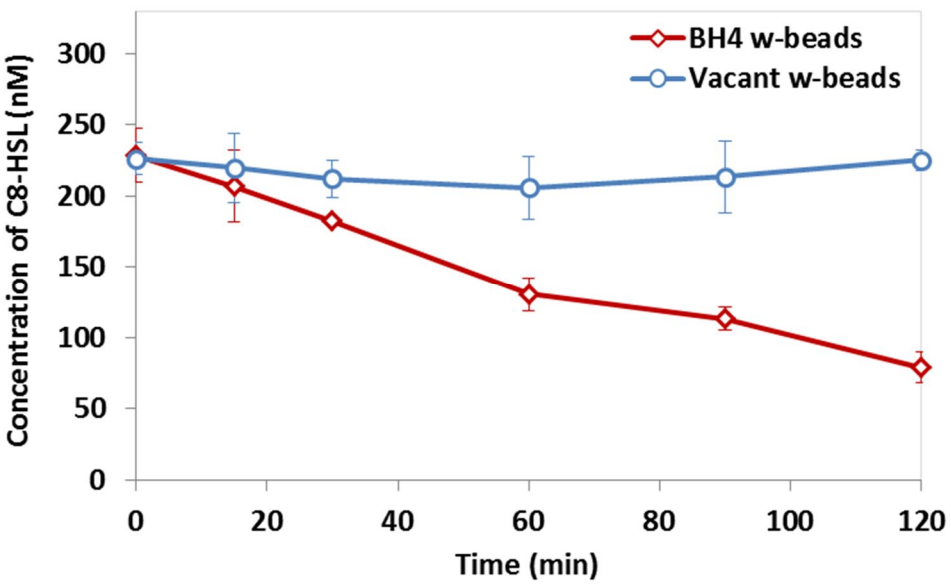


Figure 17. Quantitative quorum quenching activity of w-beads

4.2.2. General MBR Performances

Table 4 shows the basic performances of each aerobic MBR at the sampling points. It is shown that COD removal efficiency and MLSS were not affected by QQ.

Table 4. Performances of aerobic MBR

	MBR w/vacant w-beads	MBR w/BH4 w-beads
COD removal efficiency in broth (%)	93	92
MLSS (mg/L)	5840	5060
Particle size (μm)	222	242

4.2.3. Characterization of Mixed Liquor EPS

SMP was only analyzed quantitatively due to low concentration for fluorescence detector.

Ⅰ. Quantitative analysis of protein and polysaccharide

Figure 18 shows the concentration of protein and polysaccharide in SMP, LB- and TB-EPS in mixed liquor of aerobic MBR. Protein and polysaccharide contents in both LB- and TB- bound EPS did not change with QQ. In SMP, however, polysaccharide showed 30 % of decrease while protein did not have significant change.

Ⅱ. Size exclusion chromatography

For SEC analyses, two sets of excitation/emission wavelength were selected: 280/350 nm and 345/443 nm which are reported to selectively detect aromatic protein-like substances and humic acid-like substances, respectively. The separation of EPS was performed using GPC column Ultrahydrogel Linear (Waters, U.S.A.).

Aromatic protein-like substances

Figure 19 shows the profile of molecular weight distribution of aromatic protein-like substances in LB- and TB-EPS from the aerobic MBR. The first notable point compared to the chromatogram of SMP from A/O MBR (Figure 15) is the general

pattern of peaks. While bimodal distribution was observed in Figure 15, in chromatograms of LB- and TB-EPS from aerobic MBR, we could observe 5-6 peaks. This may have been resulted from different type of wastewater, according to the result of Sponza (Sponza 2002).

The differences in peak pattern caused by QQ are more significant for smaller molecules (after 15ml of elution volume) than in bigger molecules (before 15ml of elution volume). In detail, QQ resulted in less production of molecules separated at around 16 mL of elution volume and more production of those at 18-19 mL of elution volume in both LB- and TB-EPS, of which the gap was greater in LB-EPS.

Humic acid-like substances

Figure 20 shows the profile of molecular weight distribution of humic acid-like substances in- and TB-EPS from the aerobic MBR. In general, QQ did not have notable effect on the composition of humic acid-like substances, especially in TB-EPS. The only change resulted from QQ was slightly more production of molecules at 8-13 mL of elution volume in LB-EPS.

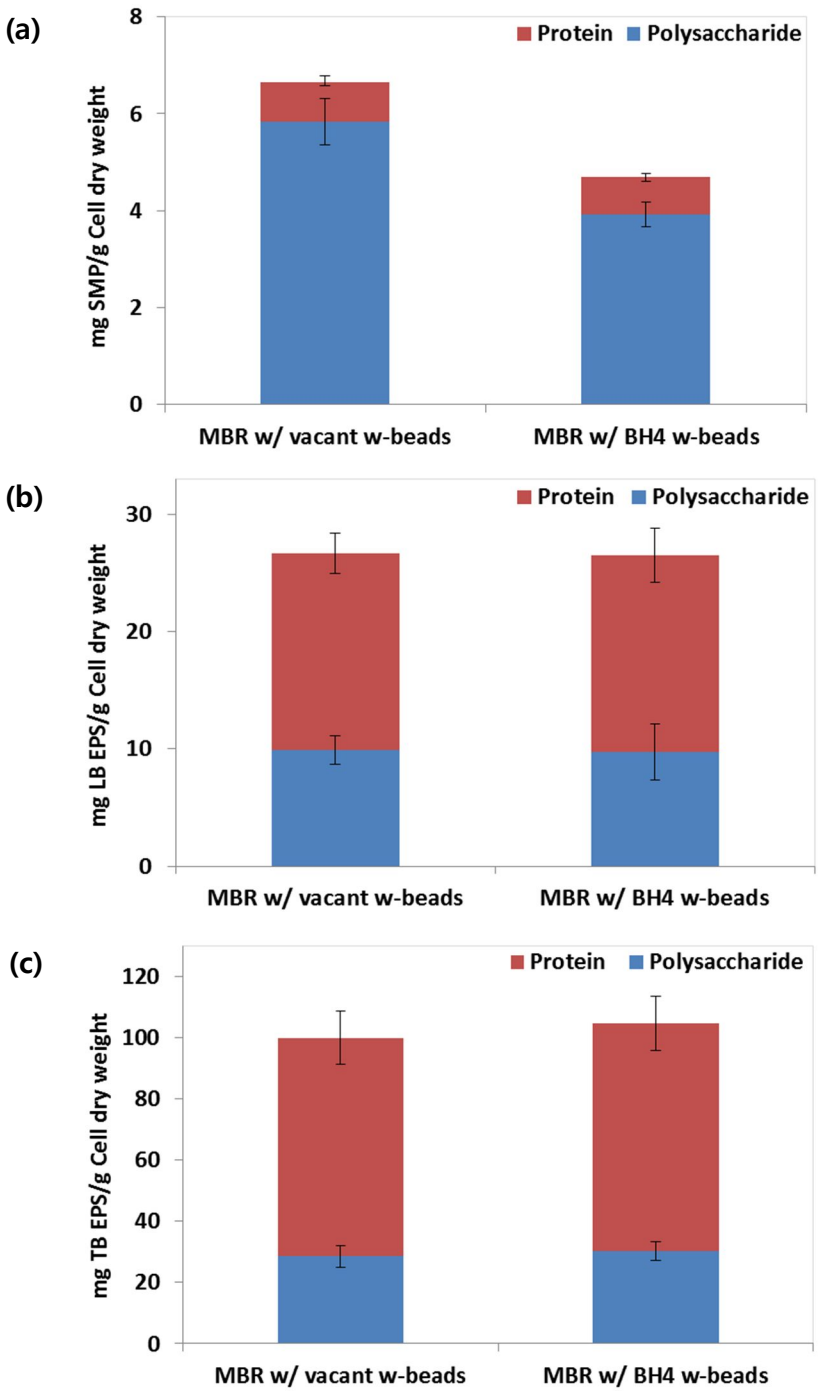
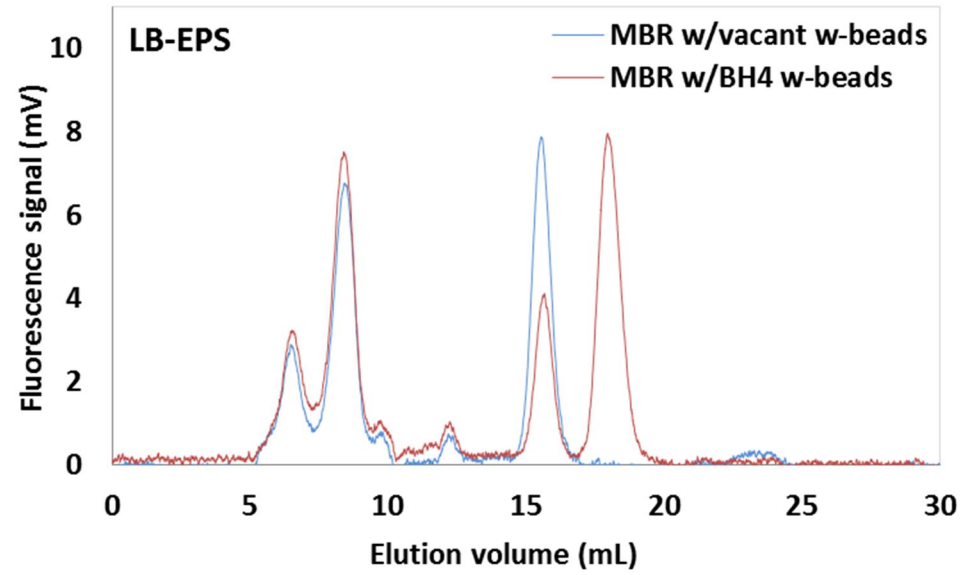
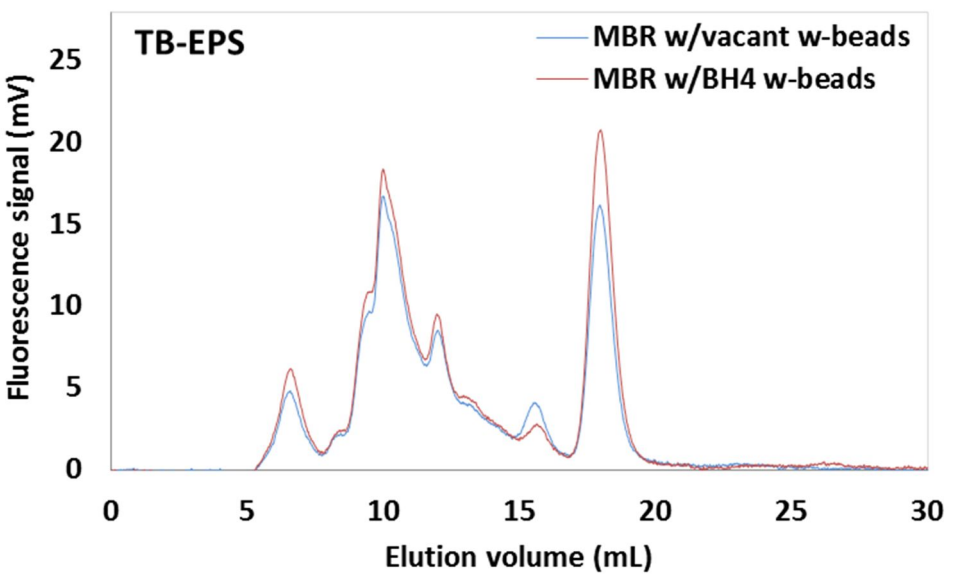


Figure 18. Concentration of polysaccharide and protein in (a) SMP, (b) loosely bound EPS and (c) tightly bound EPS from the aerobic MBR

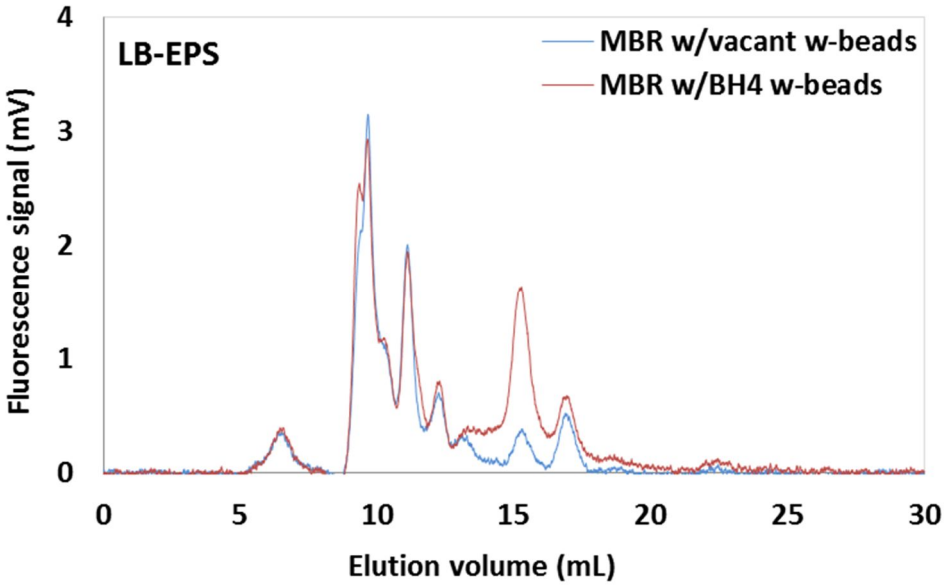


(a)

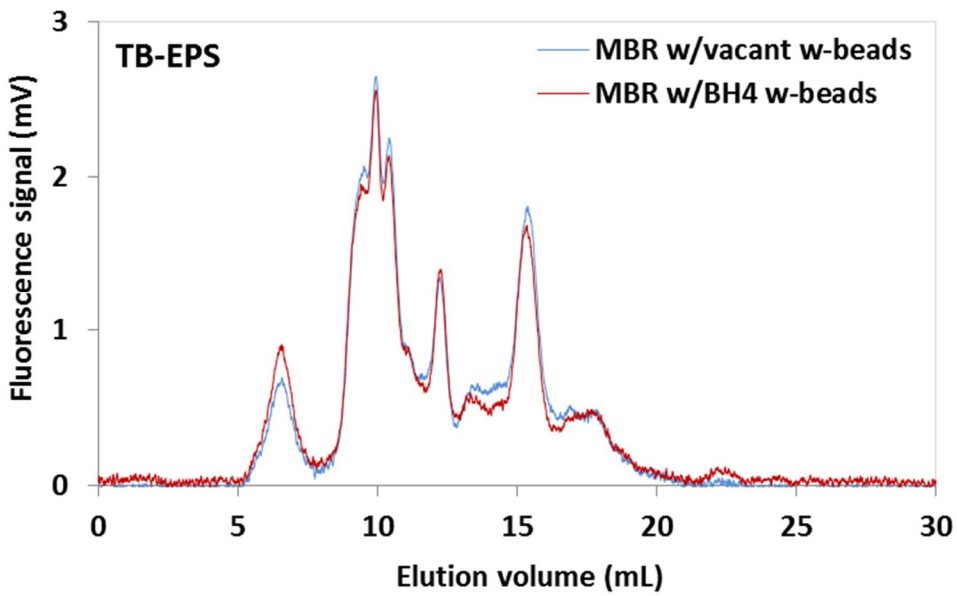


(b)

Figure 19. Chromatograms of aromatic protein-like substances in (a) loosely bound EPS and (b) tightly bound EPS from the aerobic MBR (Ex/Em wavelength: 280/350 nm)



(a)



(b)

Figure 20. Chromatograms of humic acid-like substances in (a) loosely bound EPS and (b) tightly bound EPS from the aerobic MBR (Ex/Em wavelength: 345/443 nm)

4.2.4. Characterization of Biocake EPS

Biocake EPS samples were prepared when TMP reached 40 kPa in each MBR.

Ⅰ. Quantitative analysis of protein and polysaccharide

Figure 21 shows the concentration of protein and polysaccharide in EPS extracted from biocake on the filtration membrane of aerobic MBR. Decrease in polysaccharide contents was over 35 % while protein contents only decreased 5 %.

Ⅱ. Size exclusion chromatography

Aromatic protein-like substances

Figure 22a shows the molecular weight distribution of aromatic protein-like substances in EPS extracted from biocake. The first notable point is that components with relatively small molecular weight (after 15ml of elution volume) were barely detected while floc EPS showed broad distribution (Figure 19). This may imply that aromatic protein-like substances composing biocake are mostly of bigger molecules. Generally, we could observe slight decrease in aromatic protein-like components.

Humic acid-like substances

Figure 22b shows the molecular weight distribution of humic acid-like substances in EPS extracted from biocake. Same as protein-like substances,

biocake EPS only contained big components of humic acid-like substances. We could observe that humic acid-like substances were mostly removed by QQ except two small peaks at 10 and 22 ml of elution volume. It may imply that humic acid compounds are significantly related to QS so that they are more likely to be affected by QQ. Indeed, humic acid compounds are known to have a high membrane fouling potential compared to other EPS components (Chuang, Chang et al. 2009).

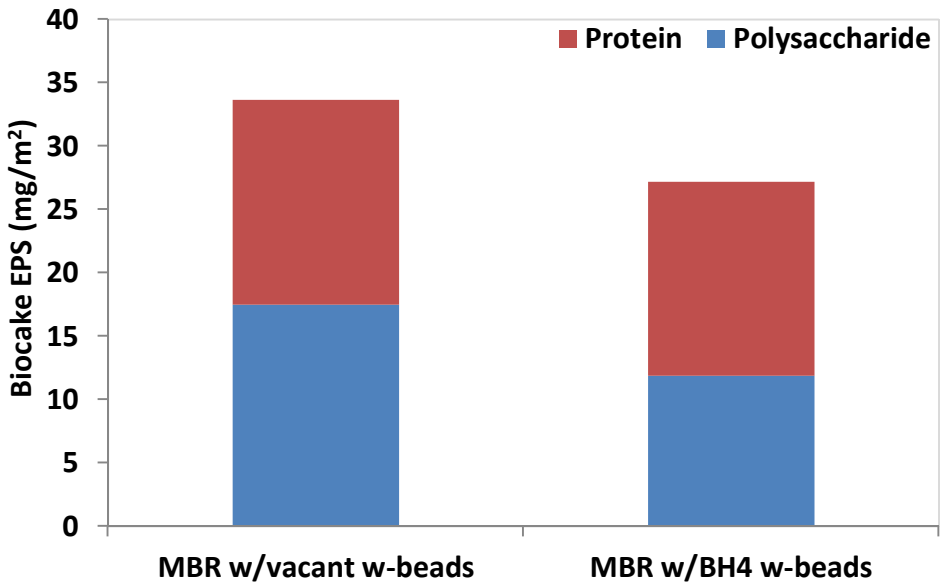


Figure 21. Concentration of polysaccharide and protein in biocake EPS

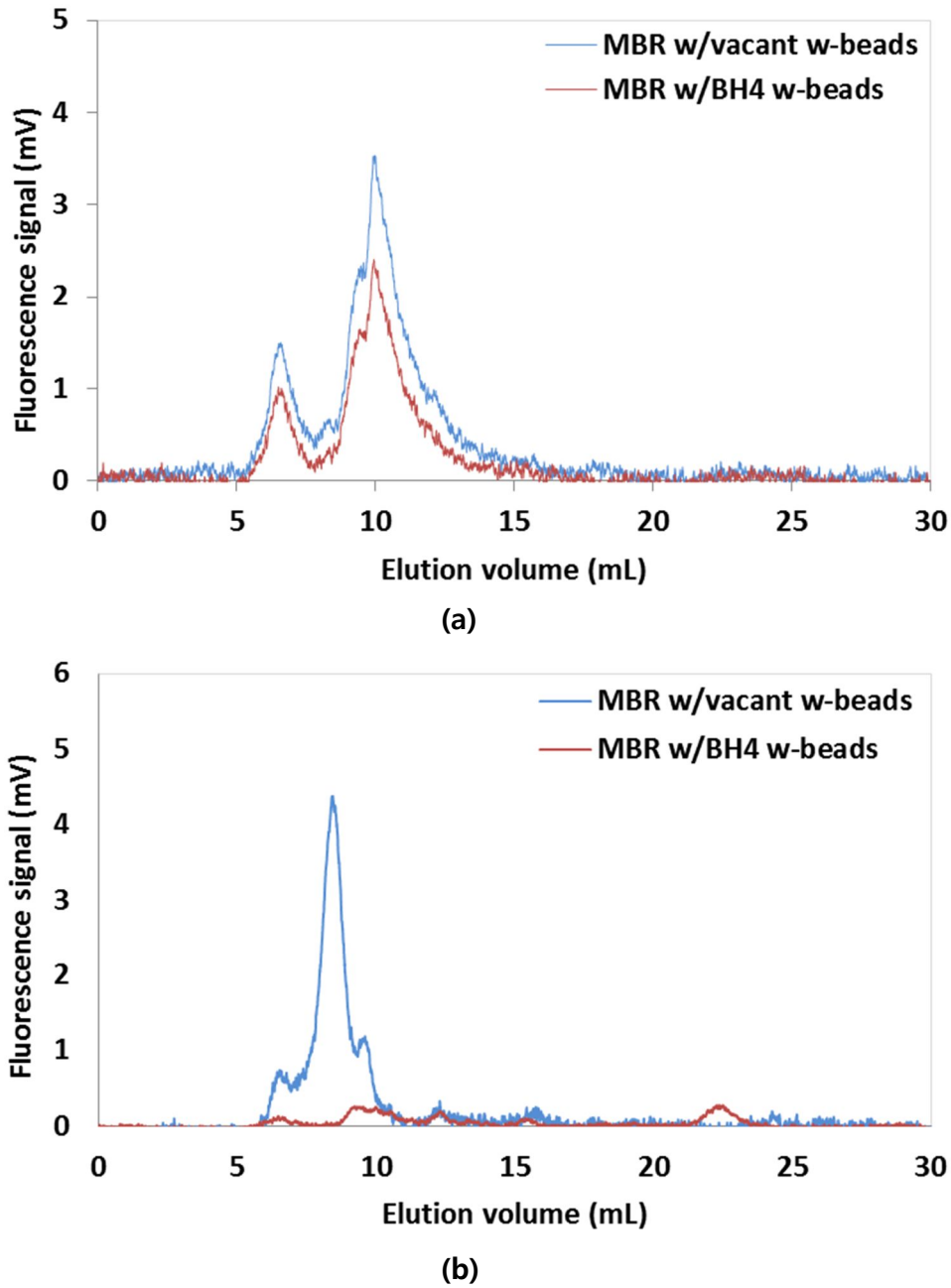


Figure 22. Chromatograms of (a) aromatic protein-like substances and (b) humic acid-like substances in biocake EPS from the aerobic MBR

4.2.5. Comparison Between Mixed liquor and Biocake EPS

To sum up the results of broth and biocake EPS analysis, there were no apparent difference in floc bound EPS while SMP and biocake EPS showed a meaningful decrease with the bacterial QQ. This may indicate that the EPS bound to the flocs are not the main target of quorum quenching, furthermore that there is no considerable level of QS by the microbial community in the broth condition. Indeed, it was reported that microbial community structures in membrane biofilms are different from those in the broth, regardless of quorum quenching. The microbial composition in biocake was much different from that in mixed liquor even in the same time (Kim, Oh et al. 2013). Subsequently, they proved that quorum quenching bring a big difference in microbial communities only in the mature biofilm, not in broth, which corresponds to our results.

Chapter5. Conclusion

The objective of this study was to characterize the extracellular polymeric substances (EPS) and soluble microbial product (SMP) with bacterial quorum quenching (QQ). QQ lactonase producing bacteria, *Rhodococcus* sp. BH4, were applied in two different sets of MBR. Main results of this study are as below.

(1) A/O MBR: SMP analysis

① Aromatic protein-like substances in soluble microbial products (SMP) decreased with QQ. Furthermore, relatively big components (100-1000 kDa) were more affected by QQ than small components (10-100 kDa).

② Filterability of supernatant was improved with QQ (lower cake layer resistance).

(2) Aerobic MBR: broth and biocake EPS amalysis

① In loosely and tightly bound EPS of floc, no apparent effect was observed in either aromatic protein-like or humic acid-like substances.

② Both aromatic protein-like and humic acid-like substances have decreased in biocake EPS, of which humic acid-like components were mostly removed.

초록

분리막 생물반응기 (membrane bioreactor, MBR) 공정은 고도의 하폐수 처리기술로 주목 받는 한편 필연적으로 분리막 표면에 형성되는 생물막에 의한 막오염 (membrane fouling)의 문제점을 지닌다. 최근 미생물 간의 신호전달 물질을 통한 정족수 감지 (quorum sensing) 기작이 MBR 의 생물막 형성에 중요한 역할을 한다는 사실이 밝혀졌으며, 정족수 감지 억제 (quorum quenching) 기술을 MBR 공정에 적용하여 막오염 저감 효과를 증명하였다.

정족수 감지 억제 효소인 Lactonase 를 생산하는 미생물인 *Rhodococcus* sp. BH4 의 분리막 오염 저감 효과를 증명하는 연구가 수차례 보고되었는데, 미생물이 분비하는 체외고분자 물질 (extracellular polymeric substances, EPS) 또한 정족수 감지 기작에 영향을 받아 감소한다는 사실이 밝혀졌다. 하지만 기존 연구의 EPS 분석은 단백질과 탄수화물의 총량 변화만 다루었다는 점에서 한계를 가진다. 따라서 본 연구에서는 BH4 를 고정한 담체를 두 종류의 MBR 에 적용하였을 때 미생물 플록 (floc), 상청액 (supernatant) 및 분리막 표면에 형성된 생물막에서 추출한 EPS 의 변화를 사이즈 배제 크로마토그래피를 이용하여 심층 분석하였다.

첫 번째로 무산소/산화조 (anoxic/oxic) MBR 에 BH4 고정 담체를 적용한 후 가용성 미생물 생성물 (soluble microbial products, SMP) 의

단백질을 분석해 본 결과 BH4 를 적용하지 않았을 경우에 비해 감소하는 것을 확인하였으며 이러한 감소 경향은 10-100 kDa 의 작은 분자량을 가지는 물질보다 100-1000 kDa 범위의 분자량을 가지는 큰 물질에서 더 크게 나타났다. 또한 기공 크기가 150 kDa 인 한외여과 (ultrafiltration, UF) 막을 이용한 상청액의 전량 여과 (dead-end filtration) 실험을 통해 BH4 의 적용이 상청액의 여과성을 향상시킨다는 것을 증명하였고, 이는 SMP 의 감소와 높은 상관관계를 가지는 것으로 보인다.

두 번째로는 호기성 (Aerobic) MBR 에 BH4 를 적용한 후 플록 및 생물막에서 추출한 EPS 내의 단백질과 부식산 (humic acid)을 분석하였다. 플록에 결합된 EPS 의 경우 두 가지 물질 모두 BH4 의 적용에 큰 영향을 받지 않은 반면 생물막의 EPS 에 부식산은 BH4 의 적용으로 인해 대부분 제거되는 것을 확인하였다.

주요어: 생물막오염 제어, 미생물 체외고분자 물질, 분리막 생물반응기, 정족수 감지, 정족수 감지 억제

학 번: 2012-20935

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