Bacterial Adaptation for the Inhibition of Quorum Sensing of Gram-Negative Bacteria

（グラム陰性細菌群菌のクォーラムセンシングの阻害に対する細菌学的適応機能）

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ABSTRACT

Quorum sensing (QS) is a strategic system to control the production of virulent factors and enzymes. A general way to inhibit the bacterial QS is known as quorum quenching (QQ) which can be triggered by some inhibitors of QS (e.g. compounds and enzymes). However, a recent study demonstrates that resistance toward the QQ occurs through a sequence of bacterial adaptation. Hence, the purpose of my Ph. D. study is to investigate the adaptation of bacteria under a treatment of QQ in the systems of monoculture and microbial consortia. In the monoculture system using *Pseudomonas aeruginosa* PA14, two main approaches were investigated to see the bacterial adaptation for 1) C-30 furanone as a QQ compound and 2) AiiM, a lactonase enzyme as a QQ enzyme. In the system of a microbial consortium, C-30 and AiiM were added to waste sewage sludge (WSS) to evaluate methane fermentation, which can be handled by the following hydrolysis, acidogenesis, and methanogenesis steps. In particular, the interaction between Gram-negative and Gram-positive bacteria could be important at the stages of hydrolysis and acidogenesis.

As a result, in the first approach of monoculture system, *P. aeruginosa* PA14 was able to degrade C-30 to C-56 according to the accumulation of bromine ions whereas a PA14 mutant showed less ability to degrade C-30. In addition, *P. aeruginosa* PA14 was able to utilize AiiM enzyme as a nutrient for the growth and some of the clinical isolates of *P. aeruginosa* showed a resistant ability towards AiiM by increasing the protease activity. In the microbial consortium using WSS, the addition of AiiM to WSS triggered a low methane production and lowered pH through the production of organic acids. In addition, the percentage of Gram-positive bacteria which may be functional as a repressor of methane fermentation increased in the presence of AiiM by changing the production of antimicrobial compounds in WSS with or without AiiM. The same result was obtained in the presence of C-30 at which a low percentage of Gram-negative bacteria was detected. Moreover, methane production increased in a condition that lysozyme was added to WSS.

Taken together, all the outcomes of this study should contribute to better understanding the adaptation of bacteria towards QQ along with the competitive relationship between Gram-negative and Gram-positive bacteria in the bacterial consortia under the regulation of QS system.

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LIST OF ABBREVIATIONS

Quorum sensing	:	QS
Quorum quenching	:	QQ
Quorum sensing inhibitor	:	QSI
Waste sewage sludge	:	WSS
Acyl-homoserine lactones	:	AHL
Autoinducer-2	:	AI-2
N-(3-oxododecanoyl)-L-homoserine lactone	:	3-oxo-C_{12}-HSL or OdDHL
N-butyryl homoserine lactone	:	C_4-HSL or BHL
2-heptyl-3-hydroxy-4-quinolone	:	PQS
2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde:		IQS
Luria-Bertani	:	LB
Terrific Broth	:	TB
Phenylmethane sulfonyl fluoride:	PMSF	
High-performance liquid chromatography	:	HPLC
Quantitative real time PCR	:	qRT-PCR
Gas chromatography–mass spectrometry	:	GC-MS
Pseudomonas aeruginosa PA14	:	PA14
Bromophenol blue	:	BPB
Isopropyl β-D-1-thiogalactopyranoside	:	IPTG
Pyoverdine	:	PVD
Pyocyanin	:	PCN
Phenylmethane sulfonyl fluoride	:	PM

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1. Introduction

Quorum sensing (QS) has a main role to activate bacterial functions through small signaling molecules called autoinducers. It is a key system for controlling the gene expression of bacteria in which signal molecules from Gram-positive bacteria, such as autoinducer oligopeptides (Sturme et al., 2002) or from Gram-negative bacteria in which acyl-homoserine lactones (AHL), autoinducer-2 (AI-2), cholera autoinducer (CAI-1), and indole are used as a signal molecule (Galloway et al., 2011; Lee et al., 2015; Whitehead et al., 2001) were used. These autoinducers regulate numerous functions in bacterial communities including the productions of enzymes and virulence factors, biofilm formation, or plasmid conjugal transfer (Huang et al., 2016) which should be related to inactivate the functions of other bacteria, fungi, protozoa, and nematodes (Burgess et al., 1999; Dubuis et al., 2007) (Fig. 1.1). Since QS system is a important role in the expression of those functions, the prevention for these signaling processes known as the inhibition of QS, has been proposed as an alternative approach to regulate the virulence factors from pathogens and the biofilm formation (Dong et al., 2007). On the other hand, QS inhibition is a method of interrupting cell signaling without any direct selective pressure, so it is generally considered that QS inhibition is difficult to acquire tolerance. For that reason, the establishment of QS inhibition method has a great potential and is expected as one of the next generation pathogenic treatment methods to replace antibiotic (Bjarnsholt et al., 2010). For Gram-negative bacteria, quorum sensing inhibitors (QSI) or quorum quenching (QQ) is the general method to inhibit bacterial QS. QQ prevents QS for the Gram-negative bacteria through the following three ways; 1) to degrade autoinducers by some enzymes such as AHL lactonase and AHL acylase, 2) to block the synthesis of autoinducers, and 3) to interrupt the interaction with receptor

proteins and autoinducers (e.g. brominated furanone, cyclodextrin) (Dong et al., 2000; Guendouze et al., 2017; Mayer et al., 2015). Among QSI, C-30 brominated furanone interacts to the transcriptional regulator protein, LuxR; therefore, the C-30 compound interrupts QS (Maeda et al., 2012). Besides, among the QQ enzymes, AHL lactonases have the function to inhibit QS through the catalytic cleavage of the lactrone ring of the AHL signal molecules. For example, AiiM AHL lactonase (derived from *Microbacterium testaceum* StLB037) inactivates the QS of the plant pathogen *Pectobacterium carotovorum* subsp. *carotovorum* (Wang et al., 2010a). Also, AiiM inhibited the production of virulence factors mediated by AHLs and alleviate cytotoxicity in human lung epithelial cells when *aiiM* gene was expressed in *Pseudomonas aeruginosa* PAO1 (Migiyama et al., 2013). To date, QSI compounds and AHL lactonases are mainly investigated to understand the influence of QS inhibition in the monoculture and some of bacterial systems (Cheong et al., 2013; Dong et al., 2000; Guendouze et al., 2017; Kim et al., 2011; Tan et al., 2015).

However, the resistance of bacteria to QSI has been described in several recent reports. Defoirdt et al (2010) mentioned that the genes regulating QS were expressed in different ways within strains of the same species and found that if this variation enhances fitness changes in the presence of QQ systems, it becomes a risk of resistance (Defoirdt et al., 2010). The first demonstration that bacterial can evolve resistance to QSI (C-30 furanone) was a publication from Maeda et al (Maeda et al., 2012). According to the authors, there are two mutants can enhance the efflux of the C-30 furanone out of the cells, hence increasing QQ resistance ability in *P. aeruginosa* PA14 (Maeda et al., 2012). Afterward, García-Contreras et al (2013) found that the clinical isolates can resist to C-30 furanone and one of the resistant strains was unlikely to active efflux. Hence, there could exist other ways for bacteria to evolve resistance to QQ compound rather than the reported efflux pump. Otherwise, many studies usually focus on the effect of QSI in dual-culture bacterial systems rather than the interaction of

bacterial consortia under the treatment of QSI (Tan et al., 2015; Cheong et al., 2013; Oh et al., 2013). Few studies mentioned to the competitive relationship among species in bacterial community via the control of the QS system (Hsiao et al., 2014; Rutherford et al., 2012). Therefore, our efforts are to investigate on the single strain bacteria adaptation towards QSI and QQ enzyme, and to understand the bacterial interaction in bacterial consortia under the treatment of QSI and QQ enzyme.

In the first part of this study, *P. aeruginosa* PA14 was treated with C-30 furanone. According to Maeda et al. (2012), *P. aeruginosa* PA14 mutant *mexR* appeared and became resistant to C-30 in the QS minimal medium. The resistance for furanone compounds might arise in *P. aeruginosa* PA14 (Maeda et al., 2012). Therefore, under the treatment of C-30 furanone in M9 minimal medium, we investigated the ability of PA14 in degrading C-30, identified the C-30 metabolites, and also screening the possible mutants which have low activity in degrading the C-30.

In the second part of this study, AiiM was cloned and used as a quorum quenching enzyme in the treatment with *P. aeruginosa* PA14. AiiM is a protein enzyme, so it can be easily degraded by protease enzyme. Besides, PA14 have ability in producing alkaline protease to degrade protein (García-Contreras et al., 2013b; Ołdak et al., 2005). Therefore, the interaction of PA14 with AiiM was investigated in both rich medium and QS minimal medium. According to García-Contreras et al. (2013), QSI (including C-30 and 5-fluorouracil) can be resisted by some clinical isolates and their alkaline protease activity was also increased. Therefore, these clinical isolates were also used to check the resistance towards AiiM.

In the third part of this study, AiiM was added into waste sewage sludge (WSS) that was known as a source of bacterial consortia. AHL molecules present in the microbial consortia may be important for regulating bacterial functions (Tan et al., 2015), and the AHL

signal molecules may control the activity of Gram-negative bacteria (Huang et al., 2016). In some of the recent reports, the interaction between QS bacteria and QQ bacteria has been described as well as the impact of QQ enzymes in the processes of wastewater treatment, which are handled by microbial community; for example, QS system may changed in the presence of QQ bacteria (Cheong et al., 2013; Oh et al., 2013). Besides, QS systems regulate the production of antimicrobial compounds in both the Gram-negative and Gram-positive bacteria (Dubuis et al., 2007; Duerkop et al., 2009). In this study, the impact of AHL lactonase, AiiM, by which QS system via AHLs in the Gram-negative bacteria can be inactivated, was investigated during the methane fermentation. In addition, the dynamics of antimicrobial activities in the presence of AiiM lactonase were evaluated.

The last part of this study is linked with the purpose of the third part. Gram-negative and Gram-positive bacteria always present in WSS microbial community which may have a important function during the anaerobic digestion of WSS. Since WSS is a bacterial community, QS systems from Gram-negative and Gram-positive bacteria can be utilized to regulate bacterial functions. Hence, the purpose of this study was to understand the effect of QS system and the power balance between the Gram-negative and the Gram-positive bacteria in the process of methane fermentation using WSS. In this part, two approaches were investigated. The first approach is to the effect of brominated furanone QSI, C-30, by which QS system via AHLs can be inactivated during the methane fermentation. Another approach is lysozyme, a Gram-positive bacteria killing compound that was utilized to evaluate its function in methane fermentation of WSS.

Overall of the studies, QS system of Gram-negative bacteria in monoculture (*P. aeruginosa* PA14) and microbial consortia (WSS) were inactivated by QSI (C-30) and QQ enzyme (AiiM) through the evaluation on the adaptation mechanism of single bacteria as well as the interaction of bacterial consortia under the inhibition of QS system.

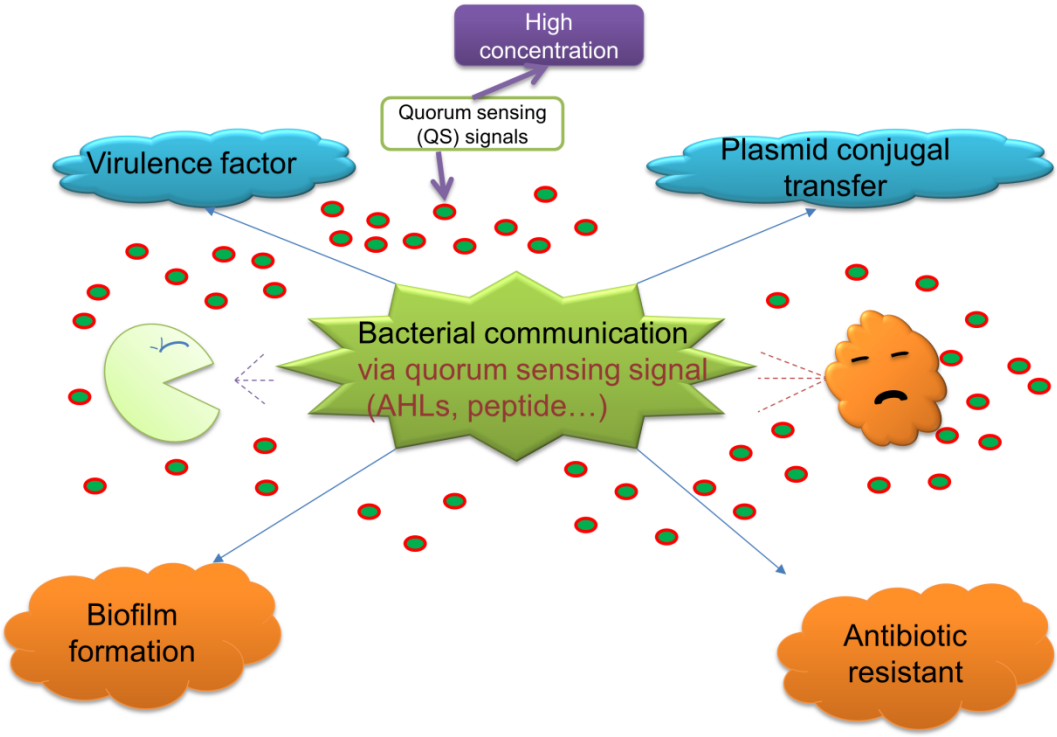

Figure 1.1. Bacterial communication via QS signal which regulate function genes

1.2. Quorum sensing system in *P. aeruginosa* PA14

Pseudomonas aeruginosa is a Gram-negative bacterium and one of the opportunistic human pathogens that involve many infection types and causes serious diseases. They include not only cystic fibrosis and other lung diseases but also corneal trauma, burn injury, Gustilo open fractures, long-term intubated patients, and the immunocompromised (Driscoll et al., 2007; Lee et al., 2015; Stover et al., 2000). Between *P. aeruginosa* species, the clinical isolate *P. aeruginosa* PA14 is significantly more virulent than PAO1 for a wide range of hosts (Lee et al., 2006).

P. aeruginosa uses a communication system which is called QS, to control the expression of certain genes related to virulence factors and biofilm formation. There are four QS signaling systems which are regulated by *las*, *iqs*, *pqs*, and *rhl* in *P. aeruginosa* (Lee et al.,

2015). The *las* system can produce and can be regulated by N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C_{12}-HSL or OdDHL), and *rhl* system can produce and can be regulated by N-butyryl homoserine lactone (C_4-HSL or BHL) (Ueda et al., 2009). Besides, both Las- and Rhl-QS can be regulated by 2-heptyl-3-hydroxy-4-quinolone (PQS) (Diggle et al., 2006). The fourth QS molecule, 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde (IQS), was identified as a new class of QS signal which can integrate environmental stress signals with the QS network (Lee et al., 2015). Several phenotypes such as biofilm formation, virulence production, or antibiotic resistance were regulated by these signals (Fig. 1.2).

In *P. aeruginosa*, the autoinducer OdDHL can be produced by LasI and responded by LasR. The complex of LasR and 3-oxo-C_{12}-HSL can regulate the transcription of several genes including *rhlR*, which encodes a second QS receptor. C_4-HSL binds to RhlR to create RhlR-C_4-HSL complex which also regulated a large of genes. This phenomen allows LasI/R to regulate the first expression of QS-related genes and RhlI/R to control the second ones (O'Loughlin et al., 2013). The *las* and *rhl* systems, therefore, control the production of virulence factors such as rhamnolipids, pyoverdine, pyocyanin, elastase, alkaline protease, lectines, and superoxide dismutase (Hirakawa et al., 2013).

P. aeruginosa also produces different QS molecule named "the *Pseudomonas* quinolone signal (PQS)" (Pesci et al., 1999). The PQS is created by PqsABCD and PqsH from anthranilate that is related to the tryptophan biosynthetic pathway. Some of studies suggested that PQS and AHL-QS (*las* and *rhl*) have the relationship. PQS could play a key role in producing virulence factors in the organism because *las* system control the production of PQS and PQS affects the expression of C_4-HSL-regulated genes in *rhl*-dependent and independent behaviors (Hirakawa et al., 2013).

The gene cluster *amb*BCDE which synthesize the non-ribosomal peptide synthase is involved in the synthesis of 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde (IQS). The disruption of the IQS synthesis triggers to reduce the production of PQS and BHL signals as well as the virulence factors such as elastase, pyocyanin and rhamnolipids. Furthermore, IQS has been revealed to contribute to the virulent production of *P. aeruginosa* in the infection to four different animal host models such as mouse, zebrafish, fruitfly, and nematode, emphasizing the important roles of IQS system in controlling bacterial pathogenesis. Significantly, the functions of the central *las* system (Lee et al., 2013) under the lack of phosphate can be take over by IQS, providing important evidences to understand about the reason that the clinical isolates of *P. aeruginosa* regularly harbour mutated *lasI* or *lasR* genes (Lee et al., 2015).

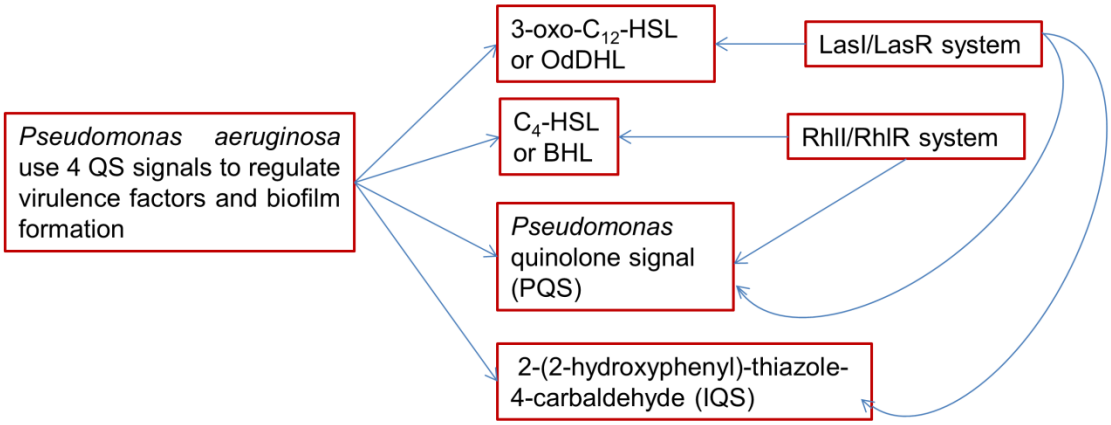

Figure 1.2. Schematic representation of the four QS signaling networks in *P. aeruginosa* and their regulations

1.3. Quorum sensing system in bacterial consortia

Bacteria use three classes of autoinducer for QS; modified oligopeptides or autoinducing peptides (AIP) produced by Gram-positive bacteria, AHLs produced by Gram-negative bacteria (Fig. 1.3), and autoinducer-2 (AI-2) which is used by both Gram-negative

and Gram-positive bacteria (Huang et al., 2016). Among them, AHL is commonly utilized as one of the autoinducers in the Gram-negative bacteria and widely studied (Hirakawa et al., 2013). The structure of AHL contains N-acylated homoserine-lactone ring along with a veriety of a 4 to18 carbon acyl chain (Galloway et al., 2011).

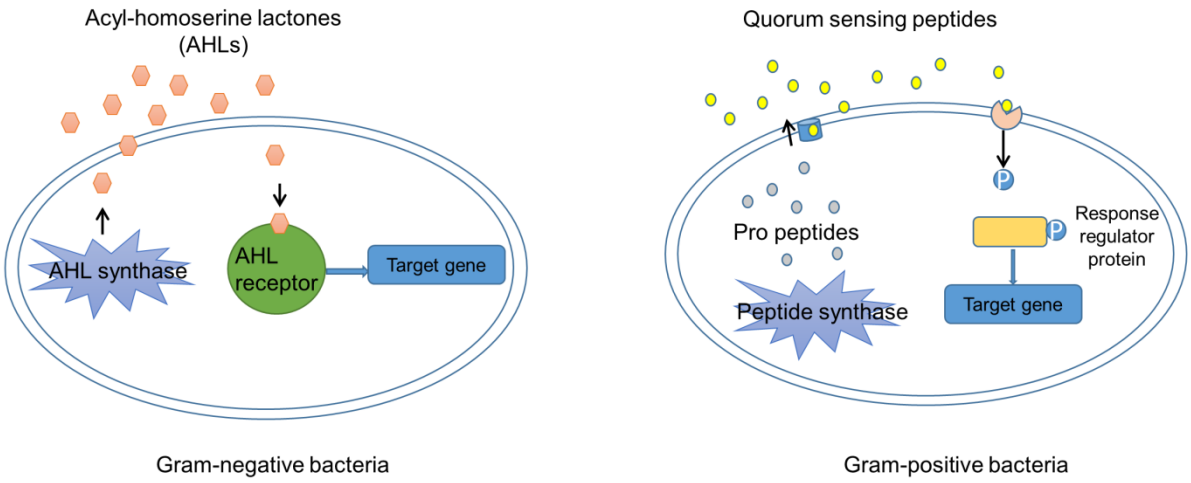

Figure 1.3. Quorum sensing system in Gram-negative and Gram-positive bacteria

The first AHL molecule to be described was 3-oxohexanoylhomoserine lactone (3-oxo-C-HSL) from *Vibrio fischeri* (Hirakawa et al., 2013). This bacteria and other Gram-negative bacteria use a common genetic system which is called the LuxI-LuxR QS system (Galloway et al., 2011). AHL molecules are released and received in this QS system. When the concentration of the AHL signals reach a high level, the AHLs uptake and bind to the *lux*R system in bacterial cell and regulating for the transcription of the associated genes.

While the signaling molecules in the LuxI-LuxR system are specific, the autoinducer molecules utilized in AI-2 QS system are all matching. Therefore, the AI-2 QS system is a universal system, which can be utitilized in both inter- and intra-species communication (Papenfort et al., 2016). The luxS gene moderated the AI-2 system that encodes for S-ribosyl homocysteine lyase, a Fe^{2+} dependent enzyme which cleaved bonds in the S-ribosylhomocysteine (SRH) to create the precursors to AI-2 signals. The function of the AI-2 signal varies and belongs to the associated genes (Montgomery et al., 2013).

Gram-positive bacteria use peptide-based signaling systems. Some peptide pheromones work through cell surface receptors, which are often membrane-bound histidine protein kinases. An oligopeptide permease transports other peptide pheromones into the cell and these peptide cooperate with intracellular receptors to control gene expression. The production of these molecules has been dependent on cell density and this indicates as a form of QS (Montgomery et al., 2013).

Some species of bacteria such as *P. aeruginosa* can produce and respond to various QS signals (J.-H. Lee et al., 2015). Others are even can use more than one QS system. For example, *V. harveyi* uses three different QS systems to control their haviour including bioluminescence and biofilm formation (Montgomery et al., 2013).

Besides several studies concentrated to the ability of microbes in sending QS signals, other studies found that some bacteria could not send signals, but still can receive signals and respond to them. For example, *Salmonella* sp. does not possess any LuxI-LuxR homologue ; however, it has a LuxR-like receptor called SdiA, by which the cell can recognize AHL signals produced by other microorganisms (Montgomery et al., 2013).

In dual-culture between species or in bacterial consortia, QS can be considered as a community event. A study of Pacheco et al. in 2009 had been contributed for these definitions, in which, they demonstrated that QS can control the heterogeneity in gene expression in inter-kingdom via intracellular signals (Pacheco et al., 2009).

QS can regulate biofilm formation, the production of virulence factors and antibiotic compounds (Rutherford et al., 2012). Otherwise, the functions of QS can be affected by the mixed communities and the different growth conditions. For example, the development of dental plaque and multispecies biofilms formation can be controlled by AI-2 signals in cavity of the mouth, which has the similar conditions as above. In other biofilm communities, QS encourage the competition between species which are not related to each others. For instance,

V. cholera can produce type VI under the control of QS to lysis of neighboring non-relationship cells, therefore, collecting the DNA from lysed cells (Rutherford et al., 2012).

Recently, AI-2 was described to encourage the higher developement of *Firmicutes* than *Bacteroidetes* after the treatment of antibiotic, which recommends that QS at least partially arranges the composition of the microbiota in the gut (Thompson et al., 2015). Moreover, AI-2 produced by *Blautia obeum* - the gut commensal bacterium - limits *V. cholera* virulence, which is involved in the recovery of cholera (Hsiao et al., 2014).

The communication among bacteria through QS is a important characteristics of bacterial life that allow bacteria to investigate the population and recognize who their neighbours are, whether they have the relationship or non-relationship, and/or cooperator or competitor. QS allows bacteria to organize cooperative behaviours (Papenfort et al., 2016).

1.4. QS inhibitor

Some methods have been known to inhibit QS by 1) competing the binding of signal molecules to the LuxR-family receptor, 2) inhibiting the production of signals, 3) degrading signals, 4) trapping signals, and 5) suppresing the activities, stabilities, or productions of synthases and receptors (Hirakawa et al., 2013). In this study, we focus on two methods – competition signal binding and degrading signals.

1.4.1. Competition signal binding to LuxR-family receptor

Several natural and synthetic compounds were used to test about the antagonistic activity. The algae *Delisea pulchra* which is endemic to the South-eastern coast of Australia produces a halogenated furanone compound capable of inhibiting the swarming activity of *Serratia liquefaciens* and inhibiting the biofilm formation by *P. aeruginosa* (Manefield et al., 1999; Rasmussen et al., 2005). These compounds compete the binding site – LuxR receptor

protein with the 3-oxo-C6-HSL signal, thus inhibiting the QS-regulated gene expression (Manefield et al., 1999). Among them, synthetic furanones C-30 and C-56 have been extensively studied since they can inhibit virulence factors which are controlled by QS, enhance the sensity to antimicrobial agents on biofilm cells, decrease bacteria in the lung of infected mice and prolong the survival time of the mice (Hentzer et al., 2002; Lönn‐Stensrud et al., 2007; Wu et al., 2004). Taha et al. (2006) used two bromated furanone compounds, C-30 and C-56 as a model compound for the *in silico* screening with pharmacophore modeling to identify three LasR antagonists. Meanwhile, a series of furanone antagonists were reported to possess the structure which are similar to AHL. They have a furanone in the structure instead of the homoserine lactone ring of AHL. These molecules bind to the LuxR receptor (Estephane et al., 2008). Furthermore, other natural AHL inhibitors such as patulin and penicillic acid have been found to be produced by fungi (Rasmussen et al., 2005). In addition, ajoene from garlic (Jakobsen et al., 2012b) or iberin from horseradish extracts (Jakobsen et al., 2012a) are also indentfied. These AHL inhibitors disrupt the QS of *P. aeruginosa* and affect to the *las* and/or *rhl* systems. Among the AHL inhibitors, the structure of patulin and penicillic acid is similar to the furanone compounds from *Delisea pulchra*; however, iberin and ajoene can inihibit *P. eruginosa* QS system depite they are not smilar to the furanone compounds (Hirakawa et al., 2013).

1.4.2. Degrading enzyme

Lactonase and acylase are two classes of enzymes, which were known to degrade AHL signals. Acylase catalyzes the hydrolysis of an amide bond between a fatty-acyl group and the homoserine lactone moiety while lactonase degrade AHL compound by cleaving the homoserine lactone ring.

AHL acylase was first isolated and purified from *Variovorax paradoxus*, a strain can utilize AHLs signals as source of energy and nitrogen for their growth. In the first reaction, AHL acylase catalyze to cleave AHLs to homoserine lactone moiety and a fatty acid, then the homoserine lactone is degraded into ammonium chloride and carbon dioxide, while the fatty acid becomes an energy material by beta-oxidation (Hirakawa et al., 2013). AHL acylases were then identified in *P. aeruginosa* (Huang et al., 2003) and in *Shewanella* sp. (Morohoshi et al., 2008). Most of AHL acylases prefer to degrade long-chain AHLs. QQ activity of AHL acylases has been proved through various systems, suggesting that these enzymes could be utilized to control AHL-regulated pathogen (Fetzner, 2015).

The first lactonase "AiiA" was identified and purified from *Bacillus* strain, in which AiiA means autoinducer inactivation (Hirakawa et al., 2013). AiiA lactonase degrade the homoserine lactone ring in C4-HSLs to C12-HSLs, including HSLs signals which have the transposition at carbon three position such as 3-oxo-C6-HSL. Heterologous expression of the *aiiA* gene in *Erwinia carotovora* – a pathogen of plant- lead to the remarkable decreasing in the expression of QS-activating gene and reducing virulence to plants (Dong et al., 2001). AiiM is another kind of lactonase enzyme, this lactonase is produced by *Microbacterium testaceum*, a strain was isolated from a potato leaf. AiiM belongs to alpha/beta hydrolase fold family and degrades a wide range of HSLs, from C4 to C12-HSLs (Wang et al., 2010). The presence of AiiM inhibited 3-oxo-C6-HSL production in the plant pathogen *Pectobacterium carotovorum* subsp. *carotovorum*, lead to decrease virulence against the potato tissue (Wang et al., 2010). Moreover, the cloning of *aiiM* into *Pseudomonas aeruginosa* PAO1 reduced the production of AHL-regulated virulence factors and decreased cytotoxicity in human lung epithelial cells (Migiyama et al., 2013)

Besides the AHL degrading enzyme from microorganisms, mammalian enzymes also possess AHL lactonase activities (Hirakawa et al., 2013). There are three paraoxygenases

(PON1, PON2, and PON3) with a various substrate feature and expression type. These enzyme were repoted that they cleave lactone rings in a lot of AHLs (Hirakawa et al., 2013).

1.4.3. Quorum quenching in microbial consortia

To date, some of studies investigated the impact of QQ enzymes on the microbial communities as well as the relationship between QS-related bacterial and QQ-related bacterial in some system of microbial consortia (Kim et al., 2011; Cheong et al., 2013; Oh et al., 2013). Kim et al (2011) used an AHL acylase to inactivate the QS of microoragisms in the membrane biocake by connecting this enzyme on nanofiltration membranes. As a result, reduced biofouling in wastewater treatment is found (Kim et al., 2011). *Pseudomonas* sp. 1A1 isolated as a QQ bacterium has a cetain ability to regulate biofouling in a membrane bioreactor (MBR) during the wastewater treatment. This system was reported to reduce membrane biofouling via the AHL acylase enzyme which was produced by *Pseudomonas* sp. 1A1 (Cheong et al., 2013). A study from Oh and co-workers showed that strain *Rhodococcus* sp. BH4 can hydrolyze a broad range of AHL molecules and the QQ activity of *Rhodococcus* sp. BH4 in the batch test shows biofouling inhibition in a wastewater treatment MBR (Oh et al., 2013). In addition, when investigating the relationship between QS and QQ activities in floccular and granular sludge, Tan and co-workers found that a high QQ activity was found in the floccular sludge and the QQ activity is reduced during the transformation from the floccular biomass into the granular sludge (Tan et al., 2015). These studies demonstrate that the interaction between QS and QQ affects to the efficiency of each bacterial fermentation and explains the presence of AHL signals in environmental samples that contain QQ bacteria possessing AHL-degrading ability (Cheong et al., 2013; Mayer et al., 2015; Oh et al., 2013; Tan et al., 2015).

In addition, QQ could prevent AHL-mediated toxicity in microbial community (Grandclément et al., 2015). In bacteria, several genera such as *Bacillus, Halobacillus, Alteromonas*, and *Pseudomonas* can produce QQ molecules. For example, some strains belong to the *Bacillus* genus - Gram positive bacteria can produce AiiA lactonase (Dong et al., 2000). This AiiA lactonase had been demonstrated to degrade lactone ring of OC12-HSL and prevent it form to the tetramic acid derivative which becomes toxicity to Gram-positive bacteria (Kaufmann et al., 2005). Therefore, AiiA's ecological function in Gram-positive bacteria could be aligned toward both detoxification and quenching. Otherwise, *Bacillus* are sensitive to AHLs and AHLs derivatives demonstrating that AHLs could become antibiotics toward *Bacillus* and the genes encoding for AHL-lactonase could be antibiotic resistance genes (Grandclément et al., 2015). In fact, bacteria often live in diverse species environments where competition for nutrients is ferocious, and they need to be able to avoid harm from antagonistic bacteria as well as to produce antibiotic compounds to inhibit the growth of competitors or kill competitors. The synthesis of these antibiotic compounds is induced by QS mechanisms (Dubuis et al., 2007; Duerkop et al., 2009). QQ inhibit QS therefore could be affected to the survival of antibiotic producing bacterial.

1.5. Waste sewage sludge (WSS)

WSS is a by-product in the biological wastewater treatment process. WSS contains a huge population of microorganisms that exist in flocs, granules and biofilms (Mikkelsen et al., 2002; Oh et al., 2013; Ramos et al., 1994). The complex composition of WSS becomes a carbon or microbial sources in bioenergy production. To date, WSS could be used as a substrate or sources of microbial for the production of bioenergy including methane and biohydrogen (Mustapha et al., 2017).

1.6. Lysozyme

Lysozyme is an enzyme which can be utilized not only in digestion but also in immune response. In fact, lysozymes lyse the peptidoglycan wall of bacterial cells by hydrolysing the β-(1,4) glycosidic bond between N-acetylglucosamine and N-acetylmuramic acid in peptidoglycan (Callewaert et al., 2008). Gram-positive bacteria possesses the main peptidoglycan, hence Gram-positive bacteria are sensity to the function of lysozyme but most of the Gram-negative bacteria are not because their outer membrane does not allow the enzyme to enter to the peptidoglycan layer (Biswas et al., 2016). Therefore, Gram-positive bacteria are quickly death when treating lysozyme, while Gram-negative bacteria are not affected. Nowadays, egg white lysozyme is widely used as food preservative and antimicrobial agent (Kandemir et al., 2005; Mecitoğlu et al., 2006).

1.7. Research framework and objectives

1.7.1. Research framework

This study has two approaches: (i) investigate bacterial adaptation for the inhibition of QS in the system of monoculture (*P. aeruginosa* PA14); (ii) investigate how bacterial interaction for the inhibition of QS in the system of microbial consortia (WSS). In both cases, C-30 furanone and AiiM lactonase were used as QSI.

1.7.2. Objectives

Main objectives of the study are:

1. To observe bacterial adaptation in the system of monoculture (*P. aeruginosa* PA14) under the treatment of C-30

2. To understand the bacterial reaction in the addition of AiiM for the inhibition of QS in the system of mono-culture (*P. aeruginosa* PA14)

3. To understand the function of QS system in microbial consortia (WSS) by the treatment of AiiM

4. To confirm bacterial interaction for the inhibition of QS in the system of microbial consortia (WSS)

CHAPTER 2

GENERAL MATERIALS AND METHOD

2.1. Bacteria strains and culture condition

All bacteria strains were incubated at 37°C in Luria-Bertani (LB) medium (10 g/L peptone, 5 g/L yeast extract, 5 g/L NaCl) with the addition of antibiotics if necessary (Table 2.1). Transformation of *E.coli* M15/pREP4-pQE30-AiiM was incubated at 37°C in Terrific Broth (TB) medium (12 g/L peptone, 24 g/L yeast extract, 9.4 g/L K_2HPO_4, 2.2 g/L KH_2PO_4, 4 mL glycerol**)**.

Chemicals were acquired from Sigma Al-rich Co. LLC. (Tokyo, Japan), Wako Pure Chemical Industries, Ltd. (Osaka, Japan), Nacalai Tesque, Inc (Kyoto, Japan), and Chameleon reagent (Osaka, Japan).

Table 2.1. Bacterial strains and primer used in this study

Bacteria strain	**Antibiotic**	**Amount**
P. aeruginosa PA14	-	-
P. aeruginosa PA14 – 53790	Gentamycin	15 µg/mL
P. aeruginosa PA14 transposon mutants	Naladixic acid Tetracycline	20 µg/mL 75 µg/mL
P. aeruginosa PA14 – *mexR*	Tetracycline	75 µg/mL
Escherichia coli S17-1 (λpir)/pUT-miniTn5 luxAB-Tc^R	Tetracycline	10 µg/mL
E. coli M15/pREP4	Kanamycin	50 µg/mL
Chromobacterium violaceum CV026	Kanamycin	50 µg/mL
Agrobacterium tumefaciens NTL4 PZLR4-traG:lacZ	Gentamycin	30 µg/mL

Bacteria strain	Antibiotic	Amount
E. coli M15/pREP4 -pQE30-AiiM	Kanamycin Carbenicillin	50 µg/mL 30 µg/mL
Brevibacillus sp. KH3	-	-
Bacillus subtilis 168	-	-
Lactococcus lactis 12007	-	-
Salmonella typhi 14028	-	-
Methanosarcina acetivorans C2A	-	-

Primers

Arbitrary primer 1	5'-GGCCAGGCCTGCAGATGATGNNNNNNNNNNGTAT-	(Tran et al., 2015)
Arbitrary primer 2	5'-GGCCAGGCCTGCAGATGATG-3'	(Tran et al., 2015)
Internal primer	5'-CTTTCAATTTCCGCTTTCAAGC-3'	(Ramsey and Whitley, 2014)
External primer	5'-GGCCACGCGTCGACTAGTAC-3'	(Ramsey and Whitley, 2014)

2.2. Medium and minimal medium composition

2.2.1. QS minimal medium

QS minimal medium was prepared following the components as Table 2.2. The pH was adjusted at 6.8. After mixed all components, the QS minimal medium was filtered sterilized by 0.22 µm pore filter.

Table 2.2. The composition of QS minimal medium (Maeda et al. 2012)

Chemical	Manufacturer	Amount
Nitrilotriacetic acid	Wako	220 mg/L
Magnesium sulfate heptahydrate	Wako	580 mg/L
Calcium chloride	Wako	67 mg/L

Chemical	Manufacturer	Amount
Iron sulfate heptahydrate	Wako	2 mg/L
Hutner's metal 44	-	1 mL
Seven molybdate hexaammonium tetrahydrate	Wako	0.2 mg/L
Ammonium sulfate	Wako	1 g/L
Potassium dihydrogenphosphate	Wako	0.05 M
Sodium monohydrogenphosphate	Wako	0.05 M
Adenosine	Wako	1 g/L

The composition of Hutner's metal 44 was shown as below (table 2.3)

Table 2.3. The composition of Hutner's metal 44

Chemical	Manufacturer	Amount
Na-EDTA	Wako	250 mg/L
$ZnSO_4$ x $7H_2O$	Wako	1095 mg/L
$FeSO_4$ x $7H_2O$	Wako	500 mg/L
$MnSO_4$ x H_2O	Wako	154 mg/L
$CuSO_4$ x $5H_2O$	Wako	39.2 mg/L
$Co(NO_3)_2$ x $6H_2O$	Wako	24.8 mgL
$Na_2B_4O_7$ x $10H_2O$	Wako	17.7 mg

The EDTA was dissolved and a few drops of concentrated H_2SO_4 were added to retard the precipitation of the heavy metal ions.

2.2.2. M9 minimal medium

The composition of M9 minimal medium was prepared as shown in Table 2.4. This minimal medium was autoclaved at 121 °C for 20 min.

Table 2.4. The composition of M9 minimal medium

Chemical	Manufacturer	Amount
K_2HPO_4	Wako	6 g/L
KH_2PO_4	Wako	3 g/L
$(NH_4)_2SO_4$	Wako	1 g/L
$MgSO_4.7H_2O$	Wako	0.2 g/L

2.2.3. Protein purification and storage buffer

Purification buffer was prepared as follows: 50 mM Tris, 300 mM NaCl, and 10 % glycerol, pH 8. The buffer was autoclaved at 121 °C for 20 min. Then, the buffer was cooled-down at room temperature and mixed with 0.5 mM phenylmethane sulfonyl fluoride (PMSF) (Wako, Osaka, Japan).

Storage buffer was prepared as follows: 20 % glycerol, 50 mM Tris, and 0.1 mM EDTA, pH 7.5. The buffer was autoclaved at 121 °C for 20 min.

2.2.4. BM2-swarming medium (Overhage et al, 2008)

BM2 swarming medium was prepared as follows: 62 mM potassium phosphate, 2 mM $MgSO_4$, 10 μM $FeSO_4$, 0.4 % glucose, 0.1 % casamino acid, and 0.5 % Bacto agar, pH 7.

2.3. WSS sampling and preparation

WSS in this study was collected from Hiagari wastewater treatment plant, Kitakyushu, Japan. WSS was centrifuged at 8000 g in 10 min at 4 °C by TOMY-GRX 250 High Speed Refrigerated Centrifuge and then was washed with distilled water for at least 3 times. The purpose of washing step is to ensure the consistency of sludge sample in the initial conditions.

After washing step, WSS was diluted by distilled water to obtain 10 % (w/w). This concentration had been used for all experiments. Note that, all WSS had been prepared freshly before using for experiments.

2.4. Analytical methods

2.4.1. HPLC analysis

High-performance liquid chromatography (HPLC) is a highly flexible technique, separating components of a liquid mixture according to their different interactions with a stationary phase. In this study, 2 types of column were used, one was used for C-30 furanone and C-56 furanone detection (4.0 x 150 mm Inertisil ODS column, Shimadzu SPD-10AVP instrument, Kyoto, Japan), another was used to detect Bromide ion (L-column ODS, 21 x 150 mm, Shimadzu SPD-10AVP instrument, Kyoto, Japan).

In Inertisil ODS column for detecting C-30 and C-56, the run conditions are as follows: temperature at 25 °C; 20 µL of injection, flow rate at 0.4 µL/min, UV-Vis detector at 285 nm and 50 % acetonitrile as a mobile phase (Maeda et al, 2012).

In L-column ODS for detecting Bromide ion, the run conditions are as follow: 40 °C; 5 µL of injection, flow rate at 0.2 mL/min, UV-Vis detector at 210 nm and mobile phase (5 % acetonitrile with 0.5 mol/L tetra-n-butylammonium phosphate)

2.4.2. Gas measurement

2.4.2.1. Gas chromatography

Gas sample (100 µL) was obtained from the headspace in the vials using a syringe. A GC-3200 gas chromatograph (GL Sciences, Japan) equipped with a thermal conductivity detector was used for the measurement of methane gas produced. Helium as a carrier gas was

used at 100 kPa and current for the detection was set at 100 mA. A molecular Sieve 13X 60/80 mesh column (SUS 2 m x 3 mm I.D (GL Science, Japan)) was used to analyze the methane amount. The oven temperature was set at 40 °C, injector was set at 50 °C and detector temperature were set at 65 °C.

2.4.2.2. Methane standard curve

Methane calibration was performed by using methane pure gases (APHA, 2005). Methane gas was introduced with a syringe at the volume of 10, 20, 30, 40, and 50 µL prior for area detection. Methane standard curve was constructed according to linear response and regression value more than 0.99. The standard curve was drawed by methane amount in µmol (after calculated to standard temperature and pressure) against area. Based on the standard curve, the amount of methane in samples was calculated.

2.4.3. Organic acids analysis

Organic acids was measured based on the description of Shinagawa et al (Shinagawa et al., 1997). Firstly, samples were centrifuged at 13,000 rpm for 1 min to collect the supernatant (microcentrifuge, TOMY, KINTARO-18). Then, the supernatant was filtered using a 0.45 µm membrane syringe filter (Minisart, Sartorius Stedim Biotech, Germany) and used for the measurement of organic acids by HPLC analysis (Shimadzu LC-10AD, Japan). The separation was obtained by two columns at 40 °C, Shim-packed SCR-102H (8.0 mm ID x 300 mm L) column. Mobile phase (5mM p-Toluene sulphonic acid) and buffer (20 mM Bis-Tris, 100 µM EDTA.2Na and 5 mM p- Toluene sulphonic acid) were filtered by a 0.45 µm pore size filter (Lot 10574.38, Thermo Scientific, Nalgene, Japan). The flow rate was 0.8 mL/min. Standard curve of acetic, propionic, succinic, formic and butyric acids were created by

injecting each of organic acid at different concentration to HPLC system. Based on the retention time of each organic acid, the organic acids profile were detected from the samples

2.4.4. pH measurement

A pH meter AQUATwin pH meter was used to measure pH value from samples.

2.4.5. Measuring the turbidity of bacterial cell

Cell turbidity was measured by a UV/VIS spectrophotometer (Jasco V-530) at 600 nm wavelength while the absorbance of 96 well plates samples was measured by a micro-plate reader (Tecan, Wako).

2.5. Microbial community analysis

2.5.1. RNA extraction and cDNA synthesis

RNA extraction and cDNA synthesis from the pellets of WSS and WSS with AiiM or C-30 or lysozyme were conducted following the previous description (Mustapha et al., 2017). The RNeasy kit (Qiagen, Valencia, CA) was used to extract total RNA from the pellets, and the concentration of RNA was determined by NanoDrop ND-1000 spectrophotometer (SCRUM Inc., Japan). These RNA samples were converted to the complementary deoxyribonucleic acid (cDNA) by using the PrimeScript RT Reagent Kits (TAKARA Bio Inc., Shiga, Japan) as the previous description (Mustapha et al., 2017). Briefly, 5 μg of total RNA was mixed with 2 μL of 5x Prime Script buffer, 2 μL of random mers, 0.5 μL of oligo dT primer, 0.5 μL of reverse transcriptase enzyme and distill waster in the total of 10 μL reaction mixture. After that, the mixed samples were incubated at 25 °C for 10 min, 37 °C for 30 min and at 85 °C for 5 s to deactive enzyme. The cDNA concentration was measured by Qubit ssDNA fluorometer (Thermo Fisher Scinetific). The cDNA was used for quantitative real-time PCR (qRT-PCR) and used as a template to analyze microbial communities.

2.5.2. Quantitative real time PCR (qRT-PCR)

Quantitative real time PCR (qRT-PCR) is the technique that developed from PCR technique by using dual-labeled fluorogenic probes to detect PCR products (Heid et al., 1996). Unlike the normal PCR, qRT-PCR provides fast, accurate and reproducible results without requiring post-PCR sample processing (Heid et al., 1996). The measurement is "real-time" because the results are cyclically formed by RT-PCR.

Real-time PCR for the quantification of RNA was conducted by StepOne Real Time PCR System (Applied Biosystem) with probes and primers listed in Table 2.5. For the total 20 μL of the real time PCR mixture, 100 ng of cDNA in 10 μL Taqman Fast Advance master mix (Life Technologies, Cat#444455), 0.72 μL of each primer (25 μM), and 0.34 μL of Taqman probe (11.8 μM) were mixed. Each cDNA samples was evaluated in triplicates. Three steps of PCR amplification were determined by incubating at 50 °C for 2 min and at 95 °C for 20 min to activate polymerase. Next, the 40 cycles of annealing and extension was repeated at 95 °C for 1 s and 60 °C for 20 s, respectively. *Escherichia coli* BW25113 (Yale Coli Genetic Stock Center (USA)) was used for the standard curve of universal bacteria. For the standard curve of archaea, the mixture of *Methanosarcina barkeri* (JCM 10043) and *Methanobacterium formicicum* (JCM 10132) which are obtained from Japan Collection of Microorganism was used. Ultra Clean Microbial DNA Isolation kit (MOBIO, Cat#12224) was used to extract Genomic DNA from each sample and the DNA was amplified in real time PCR with the primer listed in Table 2.5. The intercept and slope values for universal archaea primer and bacterial primer were calculated by plotting CT values against logarithm of template DNA copy number (Nadkarni et al., 2002). The slope and intercept values was indicated in Table 2.6. The copy number was calculated based on the description from Lee et al (Lee et al., 2008).

Table 2.5. Primer and probe sets used in qRT-PCR

Target group	Primer or probes	Sequences (5'-3')	*E. coli* numbering	Product size (bp)	Reference
Universal bacteria	Forward	TCCTACGGGAGGCAGCAGT	331-349	466	(Nadkarni et al., 2002)
	Reverse	GGACTACCAGGGTATCTAAT CCTGTT	772-797		
	Probe	CGTATTACCGCGGCTGCTGG CAC	506-528		
Universal Archaea	Forward	ATTAGATACCCSBGTAGTCC	787-806	273	(Lee et al., 2008)
	Reverse	GCCATGCACCWCCTCT	1044-1059		
	Probe	AGGAATTGGCGGGGGAGCAC	915-934		

Table 2.6. Standard curves to quantify the amount of bacteria and archaea using real-time PCR

Parameter	**Universal bacteria set**	**Universal archaea set**
Linear range (copy μL^{-1})	2.1×10^6 - 2.1×10^{11}	2.5×10^6 – 2.5×10^{11}
Slope	-3.71	-3.55
R^2 of slope	0.994	0.998
Intercept	54.731	53.082
Parameter	**Universal bacteria set**	**Universal archaea set**
Source strains	*Escherichia coli* BW25113	*Methanobacterium formicicum* and *Methanosarcina barkeri*

2.5.3. High-throughput 16S rRNA sequencing and data processing

High-throughput sequencing are known as a low cost and fast technique in analyzing the whole genome sequence from the various samples. High-throughput sequencing could

create multimillion reads leading to a complete genome sequence of the complex microbial communities culture.

The forward primer 341F (5'-CCTACGGGNGGCWGCAG-3') and the reverse primer 785R (5'-GACTACHVGGGTATCTAATCC-3') targeting the V3 and V4 regions were used to amplified 16S rRNA genes (Klindworth et al., 2013). All steps were followed the Illumina protocol to prepare 16S ribosomal RNA gene amplicons for the Illumina Miseq system. The collected data were de-multiplexed and the reads were then classified to different taxonomic levels. The detail procedures were described in previous paper (Mustapha et al., 2018)

The data were analyzed as previously described (Mustapha et al., 2018). Briefly, the de-multiplexed raw paired-end reads sequencing data were analyzed by the LotuS pipeline (Hildebrand et al., 2014), chimera-checking was conducted, and reads were clustered into operational taxonomic units (OTUs). For the α- and β-diversity index of microbial communities, the rarefaction using Quantitative Insights into Microbial Ecology (QIIME) v1.9.0 (Caporaso et al., 2010) was performed and further categorized by the Ribosomal Database Project (RDP) classifier with the Green genes database v13.8 (DeSantis et al., 2006) (80 % of confidence threshold). The PAST (PAleontological STatistics) software was used to calculate the Shannon index (Hammer, 2001). The rarefied OTU tables were displayed as the basis to calculate alpha diversity. The Chao1 richness estimator was used to compute Rarefaction curves. Beta diversity to establish the similarity of bacterial community between samples was analyzed by PCoA.

CHAPTER 3

ABILITY OF *PSEUDOMONAS AERUGINOSA* PA14 IN DEGRADING THE C-30 QUORUM SENSING INHIBITOR

3.1. Introduction

Quorum sensing (QS) is a mechanism for regulating gene expression via signal molecules called autoinducers. Several autoinducers such as acylhomoserine lactones, autoinducer 2, peptide signals were produced by pathogenic bacteria and were utilized during their infection to the host (García-Contreras et al., 2013a). The expression of pathogenic factors and toxins can be controlled by the concentration of these signals. To date, many attempts have been made to inhibit this mechanism, from there, prevent the expression of virulence factors. Antibiotics inhibit the growth of pathogenic bacteria are known as a traditional method for treating infectious diseases caused by these pathogenic bacteria. However, these methods created the pressures for environmental and caused many genetic mutation, which lead to drug resistance (Defoirdt et al., 2010). On the other hand, quorum quenching (QQ) is a method that can disrupt signals from cell without any direct selective pressure, so it is considered that QQ could be difficult to appear resistance. For that reason, the establishment of QQ method has a great potential and is expected as one of the next generation pathogenic treatment methods (Bjarnsholt et al., 2010). However, some literatures mentioned about the ability of bacteria in resistance to QQ. When analyzing the relationship between the receptor and autoinducers in *E. coli*, Kock et al suggested that the pathogenic bacteria might develop resistance to QQ that are based on agonist structure (Koch et al., 2005). Moreover, Defoirdt et al in 2010 suggested that cells can evolve resistance to QQ under minimal media and natural selection could help to spread QQ resistance ability (Defoirdt et al., 2010).

The natural brominated furanone (*5Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(*5H*)-furanone, a secondary metabolite which was extracted from the red alga *Delisea pulchra*, can inhibit both acyl-homoserine lactone-based and autoinducer 2-based QS (Ren et al., 2001). In

which, its derivative - synthetic furanone 4-bromo-5-(bromomethylene)-2(*5H*)-furanone (called C-30) - was well-known as a QQ that can interrupt QS system of the opportunistic pathogens *Pseudomonas aeruginosa*, *Vibrio harveyi* (Defoirdt et al., 2007; Hentzer et al., 2002). Therefore, C-30 is considered as a gold standard of QQ compound (García-Contreras et al., 2013a). However, Maeda et al in 2012 found that *P. aeruginosa* can increase the C-30 furanone resistance ability (Maeda et al., 2012). A mutation in the *mexR* gene in *P. aeruginosa* desmonstrated that it can decreases its susceptibility to C-30 furanone (Maeda et al., 2012). When the mexR gene product acts as an inhibitor for the *mexAB-oprM* operon encoding a multidrug efflux system, the inactivation of *mexR* gene leads to over expression of MexAB-OprM, hence enhancing the furanone efflux. In addition, other studies have illustrated that various multidrug resistant *P. aeruginosa* strains can resist C-30. Moreover, in the presence of the furanone in basal conditions, some of these strains could produce higher amounts of the virulence factors (García-Contreras et al., 2013a; García-Contreras et al., 2013b; García-Contreras et al., 2015). Therefore, additional research on the mechanisms of *P. aeruginosa* to resist to QQ compound is needed.

In this study, *P. aeruginosa* PA14 was treated with a QQ – C-30 furanone in M9 buffer to evaluate the absorption rate of C-30 into cells as well as *P. aeruginosa* PA14 adaptation under QQ treatment. Interestingly, *P. aeruginosa* PA14 can degrade C-30 furanone to 5-(bromomethylene)-2(5H)-furanone (known as C-56), another brominated furanone possess less QQ activity than C-30. This suggested that ways to evolve resistance to QQ compounds are more than one way, and QQ compound degradation is one of the resistance pathways for PA14 to evolve. Finally, our effort was to investigate which gene has responsibility for C-30 degradation by screening the transposon mutants.

3.2. Materials and methods

3.2.1. Bacterial strains, growth conditions and chemicals

Luria-Bertani (LB) medium and LB agar plates containing appropriate antibiotics were used to grow the studied bacterial strains (Table 2.1). *P. aeruginosa* PA14, *P.*

aeruginosa PA14 – 53790, *P. aeruginosa* PA14 *mexR*, *P. aeruginosa* PA14 transposon mutants and *Escherichia coli* S17-1 (λpir)/pUT-miniTn5 luxAB-Tc^R were grown at 37 °C with 120 rpm. The brominated furanone C-30 and C-56 were synthesized and provided by Professor Mariano Martisnez-Vasquez, Mexico. 2(*5H*)-furanone and 3-methyl-2(*5H*)-furanone were purchased from Sigma-Aldrich (St. Louis, MO). Furanones were dissolved in methanol and 50 μM of C-30 were used for all experiments.

3.2.2. Proliferation of PA 14 strain in LB medium and in QS minimal medium in the presence of C-30

Single colony of *P. aeruginosa PA14* was inoculated with 100 mL of LB and incubated overnight at 37 °C, 121 rpm. The cells were washed and resuspended in 1 mL of saline solution (0.85% NaCl). This solution was mixed with 5 mL of LB medium containing C-30 or 5 mL of QS minimal medium containing C-30 (Table 2.2) (Maeda et al., 2012) to get the final turbidity 0.05. Samples were then incubated at 37 °C, 120 rpm. At each time spans, cell growth was determined by measuring turbidity with a UV/VIS spectrophotometer (JASCO V-530, Tokyo, Japan) at 600 nm wavelength.

3.2.2. C-30 degradation by P. aeruginosa PA14

Single colony of *P. aeruginosa* PA14 was inoculated in 100 mL LB medium and incubated overnight at 37 °C, 120 rpm. The cells were washed twice (11,000 rpm, 10 min) and resuspended in 20 mL of M9 minimal medium (Table 2.4) with or without 50 μM C-30 to a turbidity of 1 at 600 nm OD. These solutions were incubated in standing condition at 37 °C for 24 h. At each span of times, 1 mL of supernatant was collected by centrifugation at 13,000 rpm in 1 min and filtered (0.22 μm) to detect C-30 concentration using high-performance liquid chromatography (HPLC) (SHIMADZU, CTO-10AC). Control samples

were performed in M9 minimal medium with 50 μM C-30 and without any bacteria strain. *P. aeruginosa* PA14 – 53790 putative halogenase mutant (Liberati et al., 2006) and *P. aeruginosa* PA14 – *mexR* (Maeda et al., 2012) were also used to compare the C-30 degradation rate with *P. aeruginosa* PA14 wild type.

Identification of P. aeruginosa PA14 –53790 putative halogenase mutant

P. aeruginosa PA14–53790 was taken from the PA14 transposon mutant library (the PA14NR Set) in which each nonessential gene in *P. aeruginosa* PA14 is inactivated by a single transposon insertion (Liberati et al., 2006). To identify whether *P. aeruginosa* PA14–53790 can be a possible mutant for dehalogenase, we designed 2 specific primers (forward primer 3'- TTGTGGTCACTGCCTGGAG-5' and reverse primer 5'-CAGGAAGGAGGAAGTCGGTC-3') to amplify dehalogenase gene in PA14 and PA14-53790 as a PCR product. Briefly, PA14 and PA14-53790 colony were picked up and put into 20 μL dH_2O in 1.5 mL eppendorf tube. These tubes were boiled in water at 100 °C in 5 min and flash centrifuge (Microcentrifuge, TOMY, KINTARO-18). 1 μL of supernatant was taken as DNA template and put in a PCR tube with other PCR reagents (15 μL Quick taq (Toyobo, Japan), 0.5 μL of 20 mM F primer, 0.5 μL of 20 mM R primer, 13 μL dH2O). PCR reaction was done following the conditions of 30 cycles (Pre-denature: 94 °C, 2 min; Denature: 94 °C, 15 s; Anneal: 52 °C (based on Tm of primer), 1 min; Extension: 68 °C, 1 min; Final extension 68 °C, 5 min; Hold at 4 °C). PCR products were then run on 0.8 % agarose gel to confirm the nucleotide size.

3.2.3. Identification of C-30 metabolite

The experiment was conducted as above description to identify the C-30 metabolite. After 2 h of incubation in M9 minimal medium with and without C-30, 20 mL of solutions

were centrifuged at 11,000 rpm in 10 min to collect supernatants (TOMY-GRX 250 High Speed Refrigerated Centrifuge). These supernatants were filtered (0.22 μm) and used to extract C-30 metabolite by mixing to the same volume of dichloromethane. The mixer then was separated into 2 layers, one was aqueous layer and another was organic layer. The aqueous layer was checked for the remaining C-30 metabolite by HPLC analysis, the organic layer was used to identify C-30 metabolite by Gas chromatography–mass spectrometry (GC-MS) (AGLIENT, QP 2010 plus).

GC-MS conditions: GC-MS analysis was performed using an J&W DB-5 ms (5 % phenyl-95 % methylsilicone) fused silica capillary column, 30 m × 0.25 mm I.D., 0.25 μm film. Firstly, the column oven temperature was increased to 40 °C for 2 min, then reach to 310 °C, at a rate increase of 8 °C/min, and held for 5 min. The temperatures of the injector port and transfer line were set at 250 °C and 300 °C, respectively. The carrier gas (helium) linear velocity was 40 cm/s. The total run time was 12 min. Mass spectra were collected by scanning from 33 amu (Atomic Mass Units) to 600 amu at 0.3 s intervals.

3.2.4. Detection of C-56 furanone concentration and bromide ion concentration

To calculate the concentration of C-56 furanone presence in *P. aeruginosa* PA14 supernatant treated with C-30 at different times of incubation, standard curve of C-56 was determined by HPLC analysis. Briefly, the synthetic C-56 was dissolved in acetonitrile to obtain a final concentration of 50 mM. 1 mL of sterilized water was added in the centrifuge tube (1.5 mL) together with 1 μL of acetonitrile as a control or 1 μL of C-56 solution (50 mM) as a C-56 sample. C-56-acetonitrile solution (50 mM) was diluted with acetone to 50 μM for GC – MS analysis.

For measuring bromide ion concentration, L-column was used with UV-Vis detector at 210 nm and 5 % acetonitrile with 0.5 mol/L tetra-n-butylammonium phosphate as a mobile phase. Standard curve of bromide ion was determined using bromide ion standard solution (Chameleon reagent, Osaka, Japan).

3.2.5. Swarming assay

Swarming motility assays were performed by using BM2-swarming medium after incubating at 24 h (Overhage et al., 2008). Briefly, 1 μL of *P. aeruginosa* PA14 overnight culture was inoculated on swarm agar plates with and without the studied furanones; these plates were incubated at 37 °C, and swarming motility was observed after 24 h.

3.2.6. Random transposon mutagenesis

Transposon mutagenesis was performed with a slight modification according to the previous report (Ueda and Wood, 2009). *P. aeruginosa* PA14 and *E. coli* S17-1 (λpir)/pUT-miniTn5 luxAB-Tc^R were streaked from a −70 °C stock vial on a LB agar with or without antibiotic (10 μg/mL tetracycline), respectively and incubated overnight (16 h). A single colony of *P. aeruginosa* PA14 and *E. coli* S17-1 (λpir)/pUT-miniTn5 luxAB-Tc^R was inoculated in a test-tube containing 5 mL of LB medium with or LB (10 μg/mL tetracycline)), respectively and incubated overnight at 37 °C, 120 rpm. 1 mL of *P. aeruginosa* PA14 was taken and incubated at 42 °C for 2 h to deactivate the endogenous DNase and then centrifuged at 15,000 rpm for 2 min. 1 mL of *E. coli* S17-1 (λpir)/pUT-miniTn5 luxAB-Tc^R was taken and centrifuged as the same conditions with *P. aeruginosa* PA14. Both strains were washed 2 times with 1 mL LB and kept pellets. 100 μL of LB was added to *E. coli* S17-1 (λpir)/pUT-miniTn5 luxAB-Tc^R pellet and mixed with *P. aeruginosa* PA14 pellet. This mixture was spread on LB plate and incubated within 18 h, at 30 °C. The next day, bacteria from LB agar was washed with 1 mL LB and plated 10-50 μL onto LB agar plate containing 20 μg/mL

naladixic acid (*E. coli* cells is killed by this antibiotic while *P. aeruginosa* can resist to this antibiotic) and 75 µg/mL tetracycline (to identify *P. aeruginosa* transposon mutants). The plates were incubated overnight at 37 °C. The transformants were picked up and used for screening.

3.2.7. Isolation of mutants less ability to degrade C-30

The transposon mutants were used to screening the mutant that have less ability in degrading C-30. Firstly, the mutants were inoculated in 1 mL LB medium (20 µg/mL naladixic acid and 75 µg/mL tetracycline) and incubated at 37 °C, overnight. The overnight culture was washed with saline 2 times and added 1 mL saline with 50 µM C-30, then incubated in 4 h. The control samples were done with *P. aeruginosa* PA14 and without any bacteria. After that, the incubated samples were centrifuge at 13,000 rpm in 5 min. Then, 4 µL of bromophenol blue (pH indicator) was added into 100 µL of supernatant. Candidates were collected by observing the color of supernatant from mutants and control samples. These candidates then were used to check the ability in degrading C-30 by HPLC analysis as previous description (Section 3.2.2)

3.2.8. Biofilm assay

Biofilm assays were conducted as previous description from Pratt and Kolter (Pratt et al., 1998) with some modifications. The overnight cultured of PA14 strain and F12-3 mutant were diluted to an optical density (OD) of 0.05 at 600 nm in LB medium with and without C-30 (50 µM). Subsequently, 200 µL of PA14 or F12-3 with or without C-30 were added into 96 well flat bottom assay plate (Costar, Corning NY) and 200 µL of LB medium without bacteria was used as a negative control. The 96-well plate was incubated at 37 °C within 24 h under static conditions. Crystal violet (0.1%) was used to stain the formed biofilm on 96 well plate and the biofilm formation was quantified as previous described (Westas et al., 2014).

Firstly, the absorbance of cell growth was measured at 620 nm using a microplate reader (Tecan, Waco). A paper towel was used to dry plate by tapping on it after the plates were washed three times with dH_2O. Crystal violet (300 µL) was added to the plate and incubated for 20 min at room temperature. Next step, the solution was poured off plate and the plate was washed three times with water. Finally, 300 µL of 95 % ethanol was added to plate and the plate was incubated for 30 min. The plate was mixed for 50s and measure the absorbance by using a microplate reader (Tecan, Waco) at 540 nm.

3.2.9. ***Identification of insertion site***

The candidate mutant was incubated in LB medium containing 20 µg/mL naladixic acid and 75 µg/mL tetracycline at 37 °C, overnight. Cell pellets was collected and extracted DNA genome using UltraCleanTM Microbial DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA). A two-round arbitrary PCR with some modifications was performed to amplify the miniTn5-*luxAB* flanking region (Tran et al., 2015). The external, internal primers and arbitrary (1, 2) are listed in Table 2.1. [15-17]. LuxAB-inside primer and Arb1 primer were used for first PCR. Reaction conditions were: 94 °C for 5 min; 6 cycles of 94 °C for 30 s, 30 °C for 30 s, 72 °C for 1.5 min; 30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1.5 min; and 72 °C for 5 min. 100 ng of the first round PCR product was used as a template for the second PCR. The Arb2 primer (5 -GGCCAGGCCTGCAGATGATG-3) and the luxAB-outside primer (5 -CGATGGTGAGTTGTTCAAAATC-3) were used for the second PCR with the following conditions: 95 °C for 1 min; 30 cycles of 95 °C for 30 s, 65 °C for 30 s, and 72 °C for 1.5 min; and 72 °C for 4 min. All samples were held at 4 °C . The PCR2 product were run on 0.8 % agarose gel at 100 V for 25 min. A single band with around 500 base pairs was subjected for gel cutting. Gel extraction was done using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). The extracted DNA fragment was used for

the DNA sequencing. Sequence identity was characterized using a basic local alignment search tool (BLAST) which is provided by NCBI (http://www.ncbi.nlm.nih.gov/) (Wheeler and Bhagwat 2007) and the sequenced *P. aeruginosa* PA14 genome (http://www.pseudomonas.com).

3.2.10. Statistical analysis

Means were calculated from at least triplicate data (n = 3). The Student's t test (GraphPad software) was used to compare means and standard deviations and evaluated at a significance level of $p < 0.05$.

3.3. Results and discussion

3.3.1. Confirmation of QSI function from brominated furanone C-30 against P. aeruginosa PA14

Growth of PA14 strain in LB medium and QS minimal medium, both with 50 μM of C-30 were compared to determine the function of C-30 in inhibiting QS system (Fig. 3.1). In the LB medium, the presence of C-30 did not affect on the growth of *P. aeruginosa* PA14 (Fig. 3.1a), whereas in the QS minimal medium with C-30, *P. aeruginosa* PA14 grew slower than that in QS minimal medium (Fig. 3.1b). C-30 had been reported to interrupt QS by interacting with transcriptional regulators that responsible for the QS products (Defoirdt et al., 2007), and it does not affected the bacterial growth in rich medium (Hentzer et al., 2002; Ren et al., 2005). Our result showed a similar trend, in which the growth of PA14 strain in LB medium with and without C-30 was not significantly different. In QS minimal medium, *P. aeruginosa* PA14 can utilize adenosine as a carbon source by producing nucleoside degrading enzyme (Nuh). Since the expression of Nuh is controlled by the transcription regulator LasR of the *las* system and C-30 is a QSI, Nuh enzyme will not produce in the addition of C-30 in QS minimal medium. As a result, the growth of *P. aeruginosa* PA14 was inhibited in QS

minimal medium in the presence of C-30 as previous reports (Hentzer et al., 2002; Maeda et al., 2012).

3.3.2. P. aeruginosa PA14 uptake C-30 and convert C-30 to C-30 metabolite

The QSI function of C-30 has been explained by the competition with QS signals to bind to the transcriptional regulators (Defoirdt et al., 2007). This means that C-30 can be uptake into the cells and undergo the interactions inside the cell. In this study, our aim is to understand on the PA14 reaction towards C-30 under slow growth conditions. Therefore, PA14 strain was incubated in M9 minimal medium with C-30 to check the absorption rate of C-30 in PA14. Using HPLC analysis, the peak of C-30 appeared in both samples (control without PA14 and with PA14) with the retention time of 12.9 min and decreased by the time (Fig. 3.2). However, the reduction rate of two samples was different (Fig. 3.3). PA14 strain was rapidly uptake the C-30, therefore C-30 was disappeared within 6 h while C-30 in M9 minimal medium was remained until 24 h. The reduction of C- 30 in M9 minimal medium has demonstrated that it can be hydrolyzed under normal conditions.

Interestingly, as the C-30 decreased in the sample containing PA14 strain, the HPLC results also showed another peak. This peak appeared at the retention time of 7.6 min and increased proportionally to C-30 decreased. Note that, this peak did not appear in M9 minimal medium with C-30 only (Fig. 3.2) or in M9 minimal medium with PA14 only. Based on the appearance of new peak in the sample incubated with PA14 and C-30, we hypothesized that the C-30 can be converted to C-30 metabolite by PA14 or a new product was produced in the reaction of PA14 with C-30. As for the next step, we tried to identify the product of this peak by extracting it from the samples.

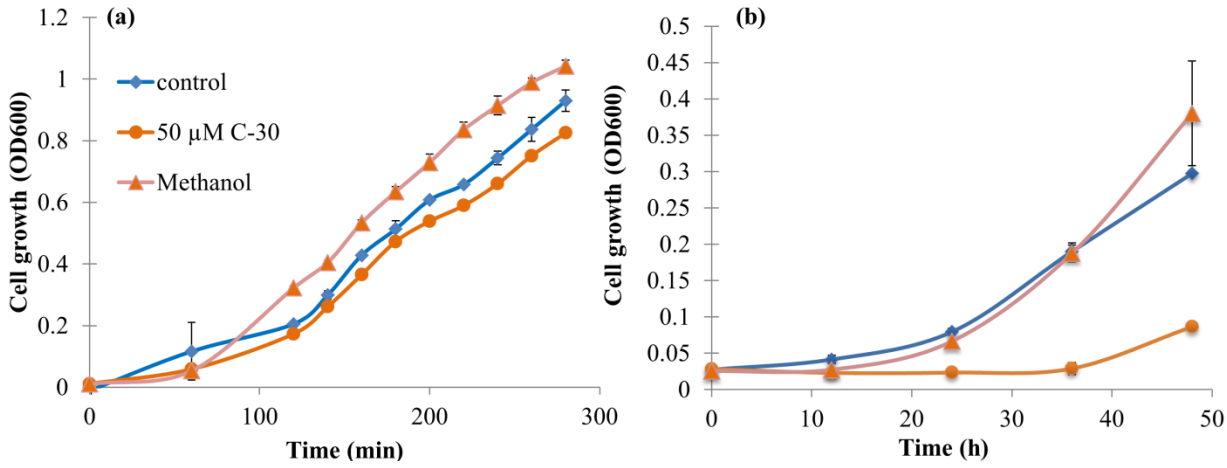

Figure 3.1. Growth of *Pseudomonas aeruginosa* PA14 under the treatment of C-30. (a) In LB medium, the growth of PA14 strain was measured within 300 min. (b) In QS minimal medium, the growth of PA14 strain was measured within 48 h. Control sample (blue diamonds) represent PA14 strain only, 50 µM of C-30 (orange cycles) represent PA14 strain with C-30, methanol (orange triangles) represent to PA14 with methanol (same volume with C-30 volume). Error bars indicate standard errors (n = 3).

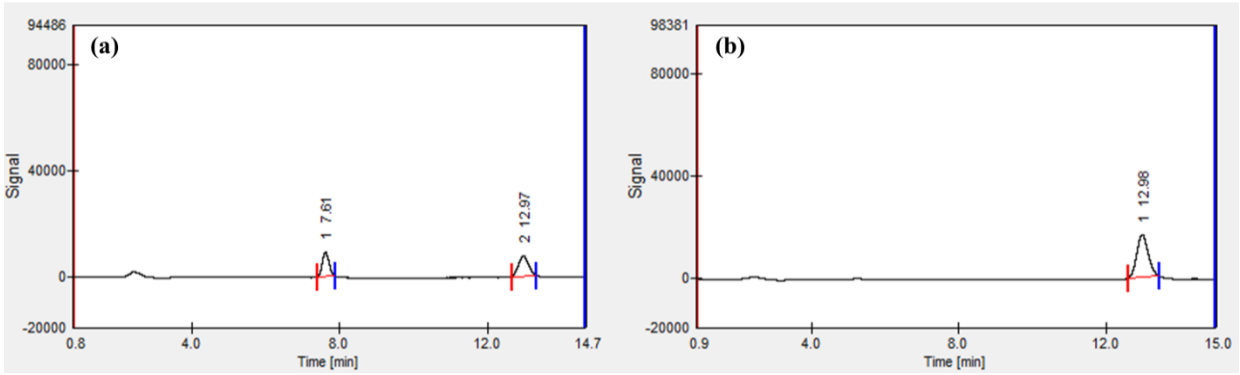

Figure 3.2. Detection of C-30 peak and C-30 metabolite peak by HPLC analysis after 3 h of incubation. (a) Sample is PA14 in M9 minimal medium containing C-30, there were two peaks, one has retention time at 7.6 min (C-30 metabolite) and another peak has retention time at 12.9 min (C-30). (b) Sample is M9 minimal medium containing C-30, only one peak appear at the retention time 12.9 min.

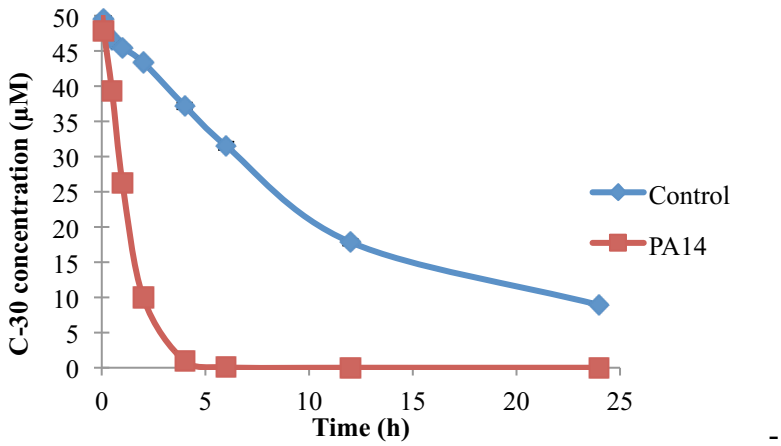

Figure 3.3. C-30 reduction in M9 minimal medium containing C-30 with and without PA14 within 24 h of incubation. Blue line with diamond points is control of C-30 without PA14; red line with square points is C-30 with PA14. Error bars indicate standard errors (n = 3)

3.3.3. C-30 metabolite identified as C-56 furanone

It was confirmed by HPLC analysis that C-30 was transformed into another substance by PA14 strain. In this section, we tried to extract the substance and attempted to determine the structure of the substance using various instrumental analyzes (GC-MS and HPLC). The PA14 strain was incubated with C-30 in M9 minimal medium for 6 h (the period of time was enough for C-30 to disappear), and the metabolite was extracted using dichloromethane from the cultured supernatant. The remaining aqueous layer was kept for HPLC analysis to confirm whether it was completely extracted. Since the peak of the metabolite was not detected, it was confirmed that the metabolite contained in the culture supernatant was completely extracted into dichloromethane (data not shown). The organic layer was then analyzed by GC-MS. The results of GC-MS analysis of the metabolite were shown in Figure 3.4. GC analysis result showed the retention time for C-30 metabolite is 13.3 min (Fig. 3.4a). For MS analysis of metabolites, several fragment peaks were appeared. In which, a pair of molecular weight was seen (Fig. 3.4b), this characteristic of substances is belong to

compounds containing one bromide ion in the structure. There are 2 isotopes of bromine atomic existing in environment which has atomic weights of 79 and 81 (Meija et al., 2016). When bromide ion was released out from the compound, the molecular weight of the compound is reduced as 79 or 81 (same weight of bromine atomic weight). In addition, the molecular weight of the detected metabolite was around 174 (Fig. 3.4b). Furthermore, the molecular weight of C-30 was 253, if one bromine atomic is released from C-30 (in case the bromine atomic weight is 79 or 81), the molecular weight of new compound will be 172 or 174. Therefore, C-30 can be converted into another compound which contains one out of two bromines in the structure due to metabolic conversion. The structures of C-30 metabolite were predicted as in Figure 3.5.

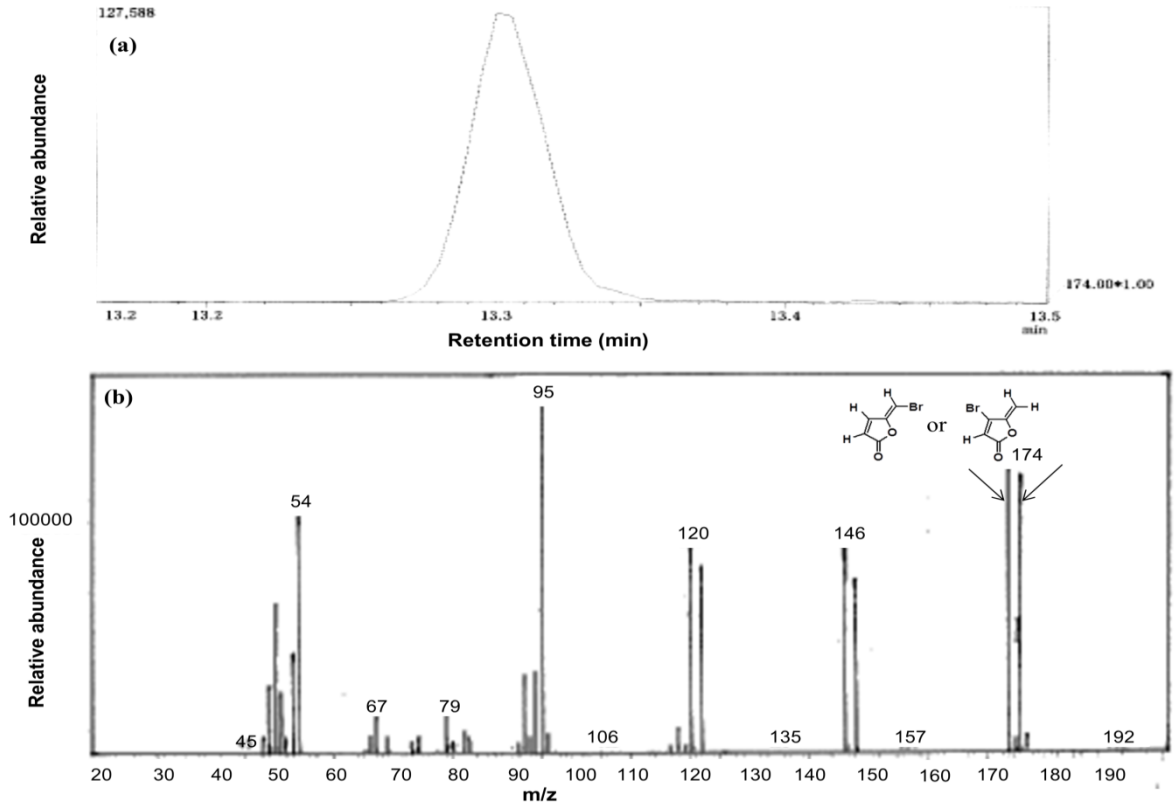

Figure 3.4. GC-MS analysis of C-30 metabolite obtained from organic layer. (a) GC analysis showed one peak appeared at the retention time of 13.3 min. (b) The mass fractions of C-30 metabolite showed many fragments, in which, a pair of molecular weight

was appeared in similar position (black arrows) indicated C-30 metabolite with one bromide ion in structure.

Two structures of C-30 metabolites are predicted based on GC-MS results (Fig. 3.5). However, further analysis to determine the exact structure of the C-30 metabolite was limited. Furthermore, among these compounds, a compound called C-56 furanone had been synthesized (Fig. 3.5a). Therefore, we attempted to confirm whether C-30 metabolite in our study has the same structure as C-56 by comparing the HPLC and GC-MS results of C-56 and C-30 metabolite. For HPLC analysis, the peak of C-56 had the retention time at 7.6 min (data not shown). As expected, the retention time of C-56 peak was similar to the peak of C-30 metabolite. Besides, the results of the GC-MS analysis for C-56 also confirmed to have the same result as C-30 metabolite (Fig. 3.6). Based on the data collected from HPLC analysis and GC-MS analysis (Table 3.1), we concluded that the C-30 metabolite in the incubation of PA14 with C30 in M9 minimal medium was C-56. As a result, the PA14 strain possesses the ability to degrade C-30 to C-56.

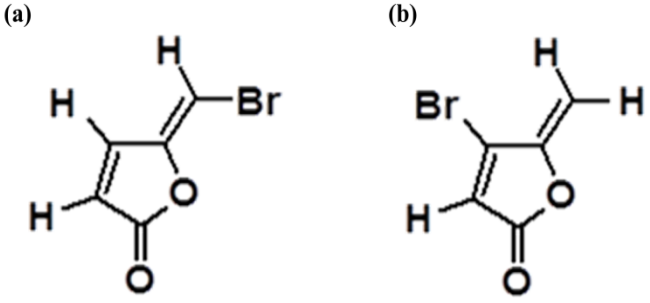

Figure 3.5. Predicted structure of the C-30 metabolite from MS analysis. The structures contain one bromine atomic with two different positions. (a) Structure with bromine at C6; (b) Structure with bromine at C4.

Table 3.1. Results of HPLC and GC-MS analysis of C-30 metabolite and C-56

	C-30 metabolite	C-56
HPLC: RT[min]	7.6	7.6
GC: RT[min]	13.300	13.330
MS: m/z value	54, 95, 120, 146, 174	54, 95, 120, 146, 174

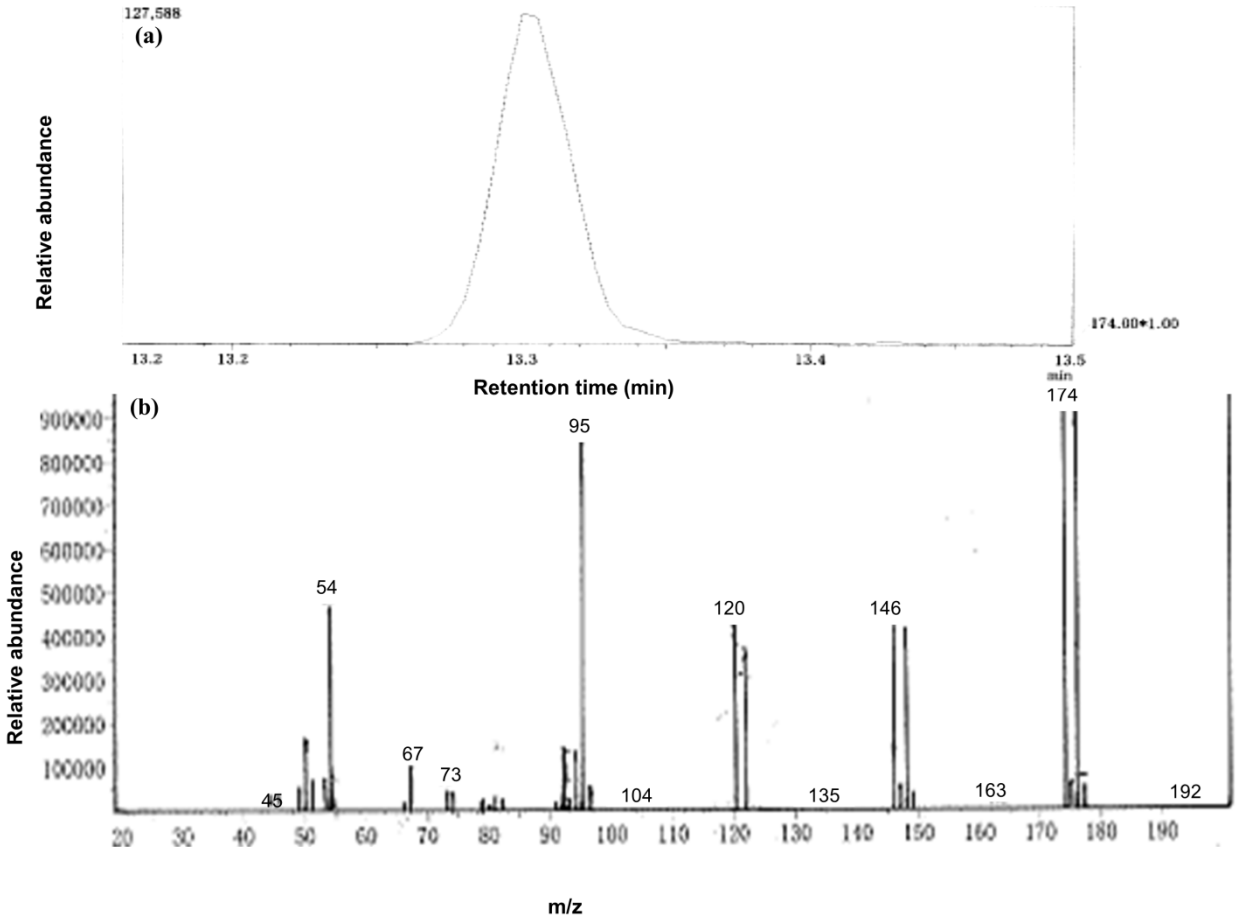

Figure 3.6. GC-MS analysis of C-56. (a) GC analysis of C-56 showed one peak appeared at the retention time of 13.3 min. (b) MS analysis of C-56 showed the similar mass fragments with C-30 metabolite.

C-56 was recognized as QS inhibitor in the treatment to QS system from *P. aeruginosa* PAO1 (Wu et al., 2004). To understand the reason for the transformation of C-30 to C-56 of PA14 strain as well as the QS inhibitory effect of C-56 to PA14 strain, we conducted the experiment using C-56 in the QS minimal medium and incubated with PA14 (all steps were the same as C-30 in QS minimal medium with PA14). Figure 3.7 showed the

effect of C-56 and C-30 on the growth of PA14 strain in QS minimal medium. The C-56 inhibited the growth of PA14 in QS minimal medium. Therefore, it demonstrated the same function of C-56 as C-30 in inhibiting QS system in the PA14 strain. However, within 24 h of incubation, the growth of PA14 in C-56 was slightly higher than that in C-30 (Fig. 3.7). This is due to the stronger QQ effect of C-30 to *P. aeruginosa* than QQ effect of C-56 (Wu et al., 2004). In addition, C-56 was incubated with PA14 in LB medium to check the toxicity effect. The result showed that C-56 did not affect to the growth of PA14 in LB medium (data not shown). Consequently, PA14 may become resistant to C-30 by converting C-30 to C-56 to reduce the QS inhibitory effect of C-30 on them.

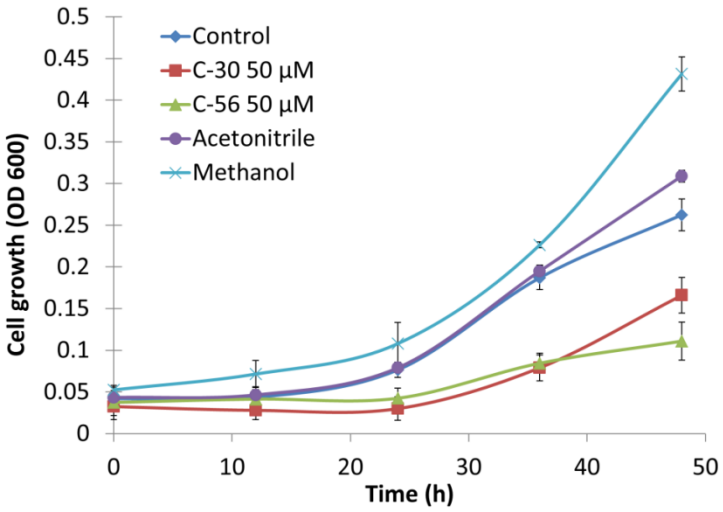

Figure 3.7. Growth curves of *P. aeruginosa* PA14 strain in the presence and absence of C-56 and C-30 in minimal medium containing 0.1 % adenosine. Control sample (blue diamonds) represents PA14 strain only, 50 µM of C-30 (red squares) represent PA14 strain with C-30, 50 µM of C-56 (green triangles) represent PA14 strain with C-56, acetonitrile (violet cycles) represent PA14 with acetonitrile (same volume presented in C-56 solution), and methanol (blue x) represents PA14 with methanol (same volume with C-30 volume). Error bars indicate standard errors (n = 3).

3.3.4. C-56 concentration and bromide ion concentration released from PA14 – C-30 incubation

The correlation of the amount of C-30 degradation, the amount of C-56 and bromide ion released were evaluated, C-56 concentration and bromide ion concentration were measured in the same time points for measuring C-30 concentration in M9 buffer with PA14 (Fig. 3.8). During the reduction of C-30 with PA14 strain, the concentration of C-56 was increased. C-56 concentration rapidly increased and achieved the highest concentration at 4 h. This speed was consistent with the reduced speed of C-30 during the incubation with PA14. Besides the appearance of C-56, the incubation of PA14 with C-30 also released bromide ion into M9 buffer. At 24 h, the concentration of bromide ion in the sample was about 100 μM (Fig. 3.8), twofold higher than the concentration of C-30 (50 μM). Note that, the C-30 possesses two bromine atoms in its structure, which means a C-30 can release two bromide ions. This demonstrated that the bromide ions had been completely released from C-30 and C-56 after 24 h of incubation.

3.3.5. Evaluation of QQ function from C-30 analogs against P. aeruginosa PA14

From the result of figure 4, we hypothesize that PA14 can continue to degrade C-56 after the degradation of C-30 and the final product will not possess bromide ion in the structure. We tried to synthetic the final compound, however, it was quickly degraded under normal condition. Therefore, two commercial compounds have the structure similar to C-30 and C-56 but without the bromide ion, 2(*5H*)-furanone and 3-methyl-2(*5H*)-furanone, were used to check QQ function by incubated with PA14 in adenosine minimal medium and swarming assay.

In adenosine minimal medium, both 2(*5H*)-furanone and 3-methyl-2(*5H*)-furanone did not effect to the growth of PA14, meanwhile C-30 inhibited PA14 growth (Fig. 3.9a). Swarming assay also confirmed the lack of QQ function of 2(*5H*)-furanone and 3-methyl-2(*5H*)-furanone at the 50 μM of concentration (Fig. 3.9b).

As expected, the non-brominated furanone did not show QQ activity with PA14 when treating as the same concentration with C-30 (50 μM). The function of the bromine atom in furanone compound was investigated by comparing QQ activity of brominated furanones and non-brominated furanone through the treatment with *Salmonella* (Janssens et al., 2008). From this study, Janssen and co-worker found that several brominated furanones have QQ activity while non-brominated furanone did not show QQ function. This evidence demonstrated the ability of PA14 to reduce the QQ function of C-30 by releasing two bromide ions out of one C-30 structure.

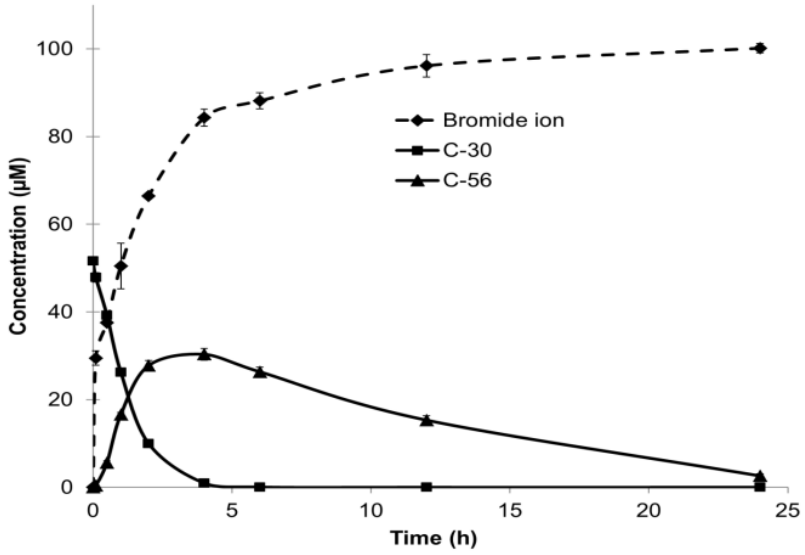

Figure 3.8. The correlation of the amount of C-30 degradation, the amount of C-56 and bromide ion released in the incubation of PA14 with C-30 in M9 buffer. Error bars indicate standard errors (n = 3).

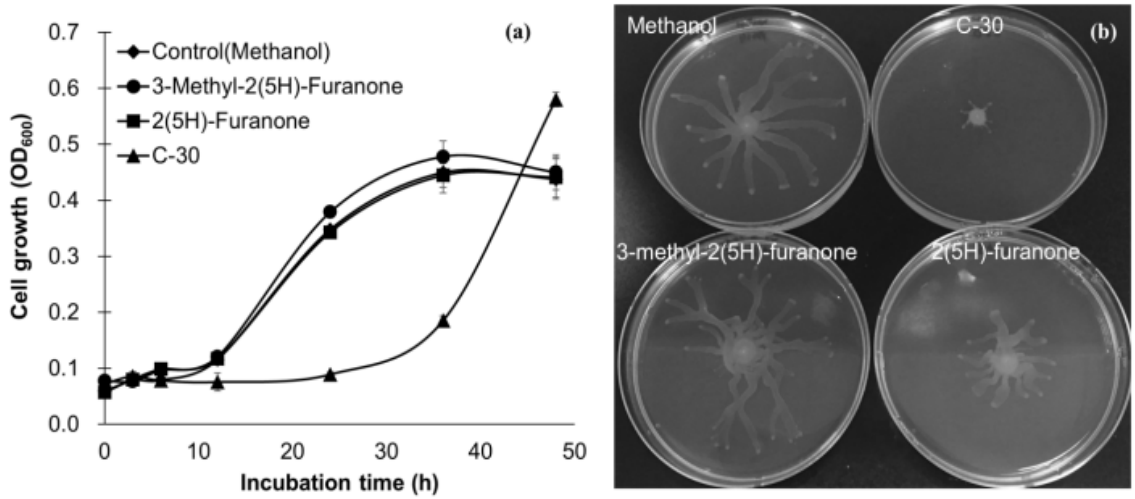

Figure 3.9. Quorum quenching function of C-30 and non-brominated furanones. (a) PA14 grew in minimal medium containing 0.1% adenosine. (b) Swarming assay. All furanones were used at 50 μM of concentration. Error bars indicate standard errors (n = 3)

3.3.6. Ability of PA14 haloacid dehalogenase and PA14-mexR in degrading C-30

In this section, we have attempted to find the genes responsible to degrade C-30 to C-56 as well as obtaining for the mutants that could not degrade C-30 to C-56. First mutant that we considered was dehalogenase mutant. *P. aeruginosa* PA14-53790 haloacid dehalogenase mutant was created by transposon mutagenesis (Liberati et al., 2006), in which a transposon MAR2xT7 was inserted inside dehalogenase gene position to disrupt dehalogenase function. As a result, the length of dehalogenase transposon mutant gene was increased. To identify whether *P. aeruginosa* PA14-53790 can be a possible mutant for dehalogenase gene, the length of dehalogenase gene from PA14 was compared with the length of dehalogenase gene mutant from PA14-53790 (*Section 3.2.2*). Base on NCBI data (https://www.ncbi.nlm.nih.gov/gene/4382803), the sequences of haloacid dehalogenase of PA14 is 701 bp. The result showed that the length of dehalogenase transposon mutant gene was longer than the length of haloacid dehalogenase gene (Fig. 3.10). Therefore, *P. aeruginosa* PA14-53790 is a mutant for dehalogenase gene.

When C-30 was converted to C-56, one bromide ion was released. We hypothesized that PA14 could produce one kind of halogenase enzyme to cut the bromide ion from C-30. Therefore, PA14-53790 was chosen to check the C-30 degradation ability. The experiment was conducted with PA14 wild type and PA14 – 53790 in M9 minimal medium with C-30. The results were shown in Figure 3.11

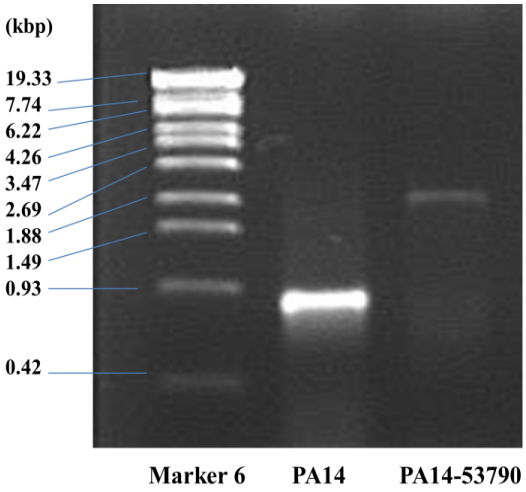

Figure 3.10. Gel electrophoresis for dehalogenase gene of PA14. The band of dehalogenase gene in PA14 sample was 702 bp (0.7 kbp), while the band of dehalogenase mutant gene in PA14-53790 appeared at 1880 bp (1.8 kbp)

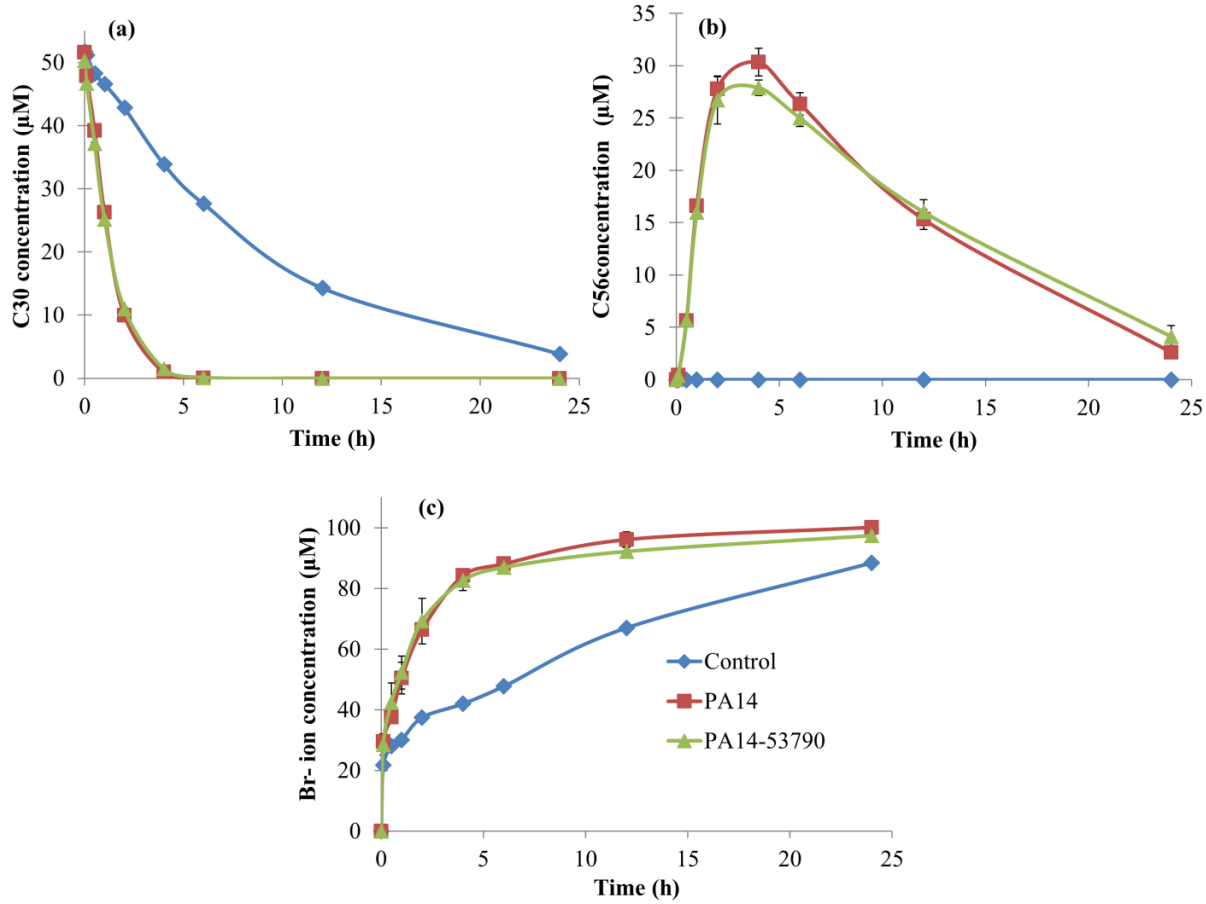

Figure 3.11. Degradation of C-30 compound (50 µM) in M9 minimal medium with PA14 mutant; (a) C-30 concentration, (b) C-56 concentration and (c) bromide ion concentration in M9 minimal medium with C-30 50 µM. Control sample (blue diamonds) represent M9 with C-30 only, PA14 (red squares) represent PA14 strain in M9 with C-30, PA14-53790 (green triangles) represent PA14 haloacid dehalogenase mutant in M9 with C-30. Error bars indicate standard errors (n = 3).

C-30 was immediately reduced in both samples containing PA14 and PA14-53790 (Fig. 3.11a). Consequently, the C-56 was produced with the similar concentration in PA14 wild type and in PA14-53790 samples (Fig. 3.11b). The increasing of bromide ion concentration in PA14-53790 samples had the same tendency with that in PA14 wild type (Fig. 3.11c). Therefore, dehalogenase gene possessed by PA14 strain was not involved in the metabolic conversion of C-30.

Another mutant that we considered was PA14-*mexR*. PA14-*mexR* had been reported to be resistance to C-30 by enhancing efflux of C-30 after its uptake into the cell (Maeda et al., 2012). To investigate whether the C-30 efflux ability can reduce the C-30 degradation rate in PA14 or not, PA14-*mexR* was incubated in M9 with C-30. Figure 3.12 showed that the degradation rate of C-30 in PA14-*mexR* was slower than in PA14 wild type. However, *mexR* mutant still converted C-30 to C-56 and the highest C-56 production had the same amount with the highest C-56 production in PA14 wild type. The C-56 in *mexR* mutant was then slowly decreased due to the efflux pump that can increase the efflux activity. Consequently, *mexR* mutant had less ability in reducing C-30 because this mutant efflux the C-30 out of the cell.

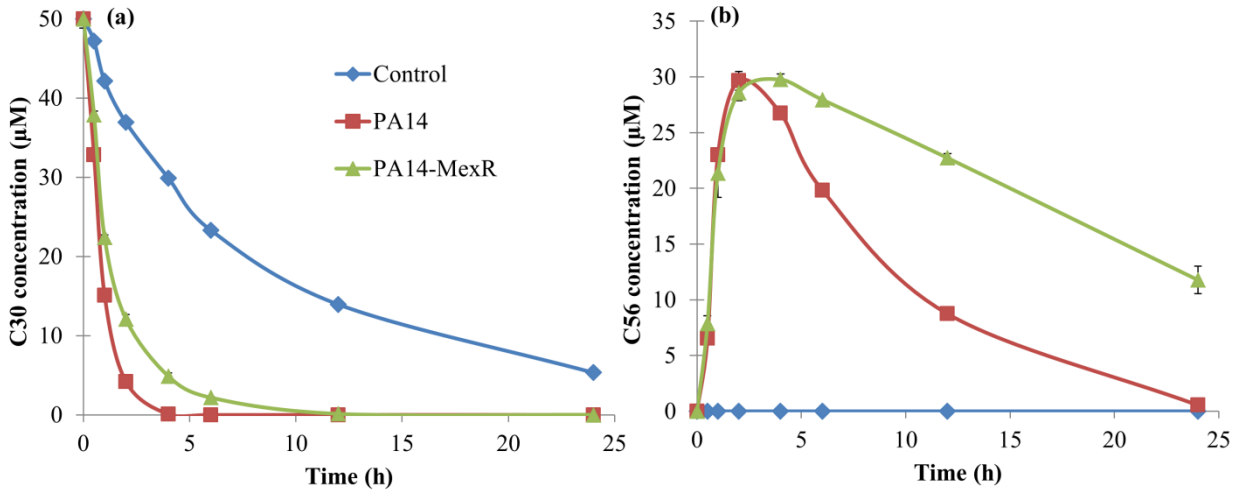

Figure 3.12. Degradation of C-30 compound (50 µM) in M9 minimal medium with PA14 mutant (MexR); (a) C30 concentration and (b) C56 concentration. Control sample (blue diamonds) represent M9 with C-30 only, PA14 (red squares) represent PA14 strain in M9 with C-30, PA14-*mexR* (green triangles) represent PA14-*mexR* in M9 with C-30. Error bars indicate standard errors (n = 3).

Two above mutant candidates showed the same function with PA14 wild type in converting C-30 to C-56. Therefore, we need to find other mutants that do not degrade C-30 or at least with minimum degradation and these mutants can be resisted to C-30 as well as C-

56. For that reason, random transposon mutagenesis was performed to collect the targeted mutants. We had been successfully collected 954 mutants. These mutants were utilized to check the ability in degrading C-30.

3.3.7. Identify the PA14 mutant less ability in degrading C-30

We need to find the screening method to check 954 transposon mutants with the less ability in degrading C-30. For that reason, we focused on the pH variation during 24 h of the incubation of PA14 strain with C-30 in M9 minimal medium. However, the pH remained at 6.8 for all samples with and without PA14 (Fig. 3.13). Then, we tried to use saline solution with C-30 in the incubation with and without PA14. Interestingly, the pH of the samples with PA14 immediately reduced within 4 h compare to the samples without PA14 (Fig. 3.13). After 24 h and 48 h, the pH of all samples in saline solution was decreased to pH 5. This indicated that the C-30 was hydrolyzed and made reduction in pH in the saline solution. Besides, pH value was remained at pH 6.8 in PA14 incubated in saline solution without C-30 during 24 h (data not shown). This result demonstrated that PA14 had reacted with C-30 which contributed to the immediate reduction of pH. Our strategy was isolating the mutants which do not react with C-30, therefore will not reduce the pH within 4 h of incubation.

Next step, we used pH indicator to check the variation of color in the C-30-saline solution with and without PA14 at 4 h of incubation. Bromophenol blue (BPB) is the pH indicator which can change the color when mix with the solution of different pH (Henari et al., 2010). It will be changed to blue color when mix with basic solution and changed to yellow color when mix with acidic solution. Figure 3.14a showed the color changes when BPB was added to the samples. The blue color appeared in saline with C-30 samples which represented high pH (pH was 6.4), while the yellow color appeared in PA14 with C-30-saline samples which represented low pH (pH was 5.2) (Fig. 3.14a). From this result, we continued to check

all mutants that incubated with C-30-saline solution within 4 h with pH indicator and observed the change of color. The candidate was chosen when it had the same color with C-30-saline solution (blue color) (Fig. 3.14b).

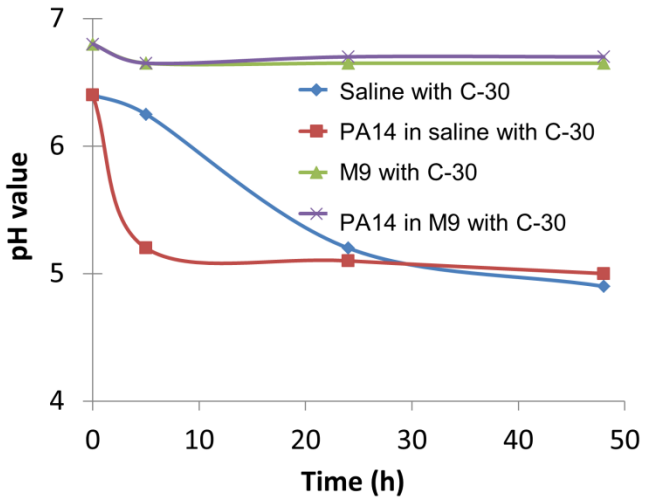

Figure 3.13. pH variation of C-30 degradation in saline solution and M9 minimal medium with or without PA14 strain. Blue diamonds represent C-30 in saline solution, red squares represent C-30 in saline solution with PA14 strain, green triangles represent C-30 in M9 minimal medium, and violet (x) represent C-30 in M9 minimal medium with PA14 strain. Error bars indicate standard errors (n = 3).

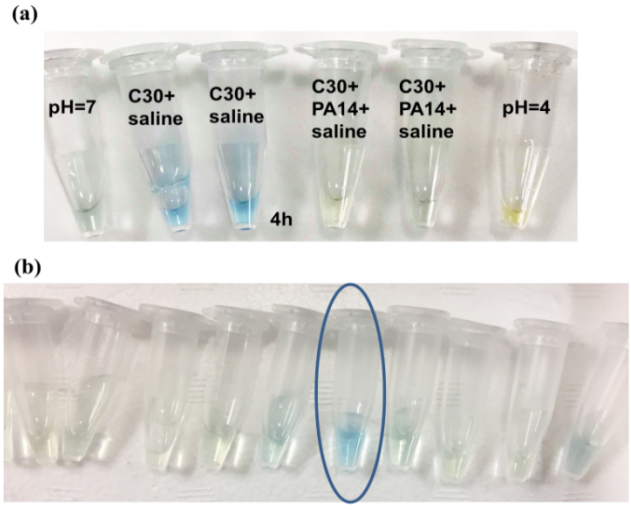

Figure 3.14. The change of color under the treatment of bromophenol blue-pH indicator. (a) Saline solution containing C-30 with and without PA14 under the treatment of

BPB. (b) Saline solution containing C-30 with mutants under the treatment of BPB, blue cycle indicated the candidate which was chosen.

From the mutants, we chose 26 mutants which had the blue color when react with BPB. These mutants were then confirmed on their ability in degrading C-30 by HPLC analysis (C-30 concentration, C-56 concentration and bromide ion concentration). All mutants degraded C-30 to C-56, however, the amount of C-56 appeared as well as the amount of C-30 disappeared in each of the mutant was different (Fig. 3.15). This result indicated that the function was regulated by the complex genes instead of single gene. Indeed, transposon mutants interrupting or modifying the function related to the degradation of C-30 or C-56 were created. This mutation possesses one modified gene, which is inserted by transposon Tn5.

The best candidate named F12-3 was chosen based on the highest concentration of C-30 together with the lowest concentration of C-56 and bromide ion concentration (Fig. 3.15 and fig. 3.16). This mutant was used to compare the C-30 degradation rate, the C-56 production and the concentration of bromide ion with PA14 wild type within 24 h of incubation in saline solution (Fig. 3.17).

F12-3 mutant showed less C-30 reduction when compared with PA14 within 8 h. Then, the concentration of C-30 was similar for both strains at the end of incubation. The amount of C-56 in F12-3 samples was significantly lower than that in PA14 samples during 8 h, however, it was increased at 24 h while C-56 in PA14 samples was decreased. The increasing of C-56 in F13-2 samples may be due to the reduction of C-56 absorption, so the C-56 remained in the saline solution. Similarly, bromide ion concentration of F12-3 samples was lower than PA14 samples (Fig. 3.17).

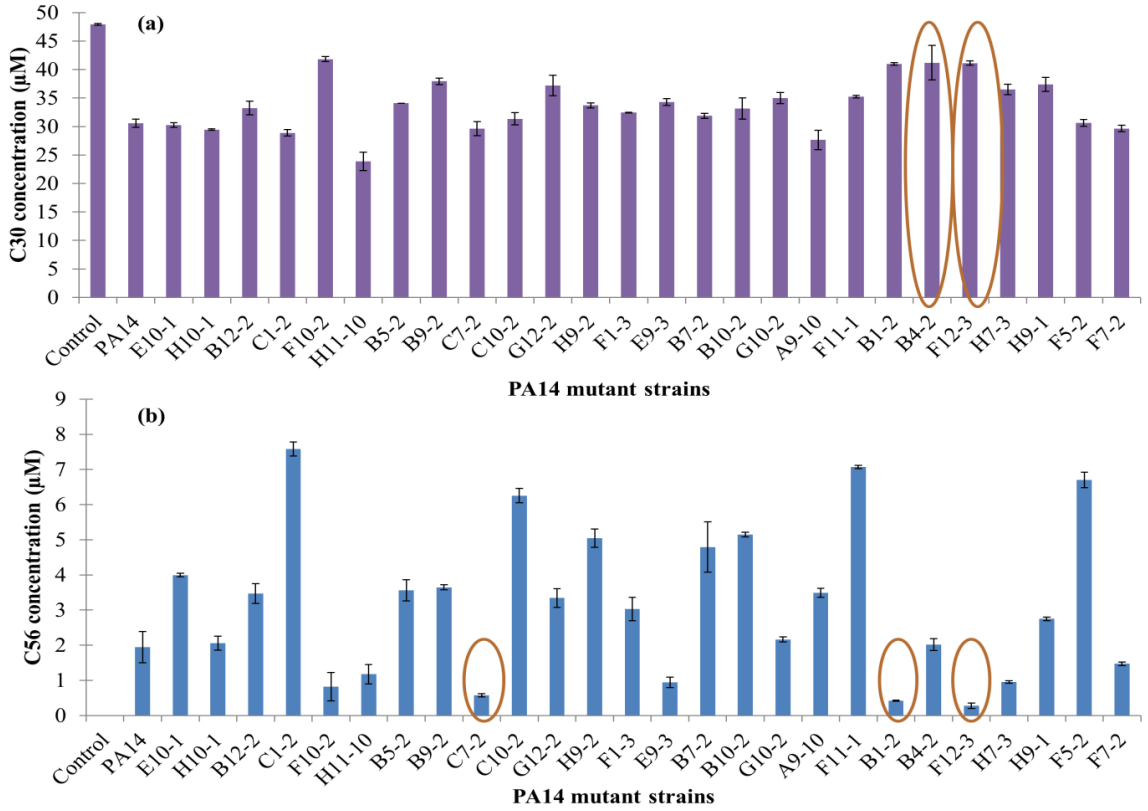

Figure 3.15. Selection of transposon mutant candidates based on C-30 and C-56 concentration; (a) C-30 concentration and (b) C-56 concentration in saline solution. The brown cycle indicated the most expected candidates. Error bars indicate standard errors (n = 3).

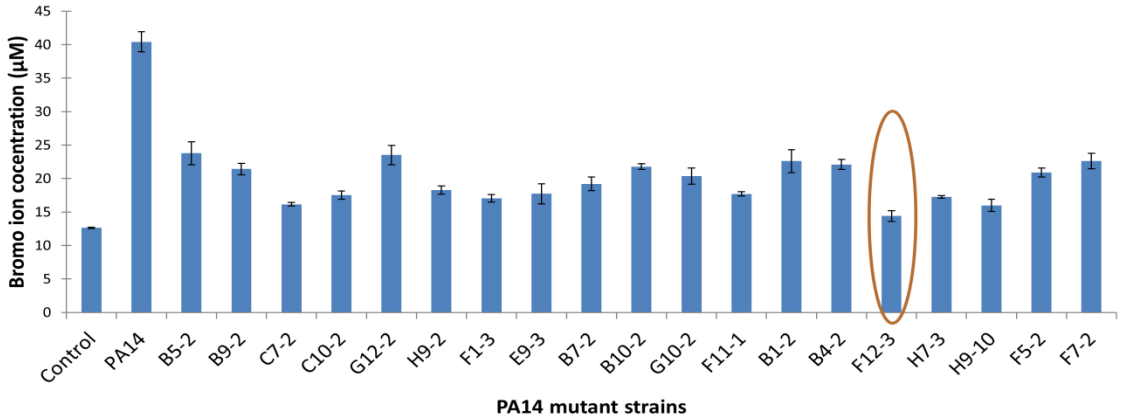

Figure 3.16. Bromide concentration of transposon mutant incubated in saline with C-30 at 1 h. The brown cycle indicated the most expected candidate compare to other candidates. Error bars indicate standard errors (n = 3).

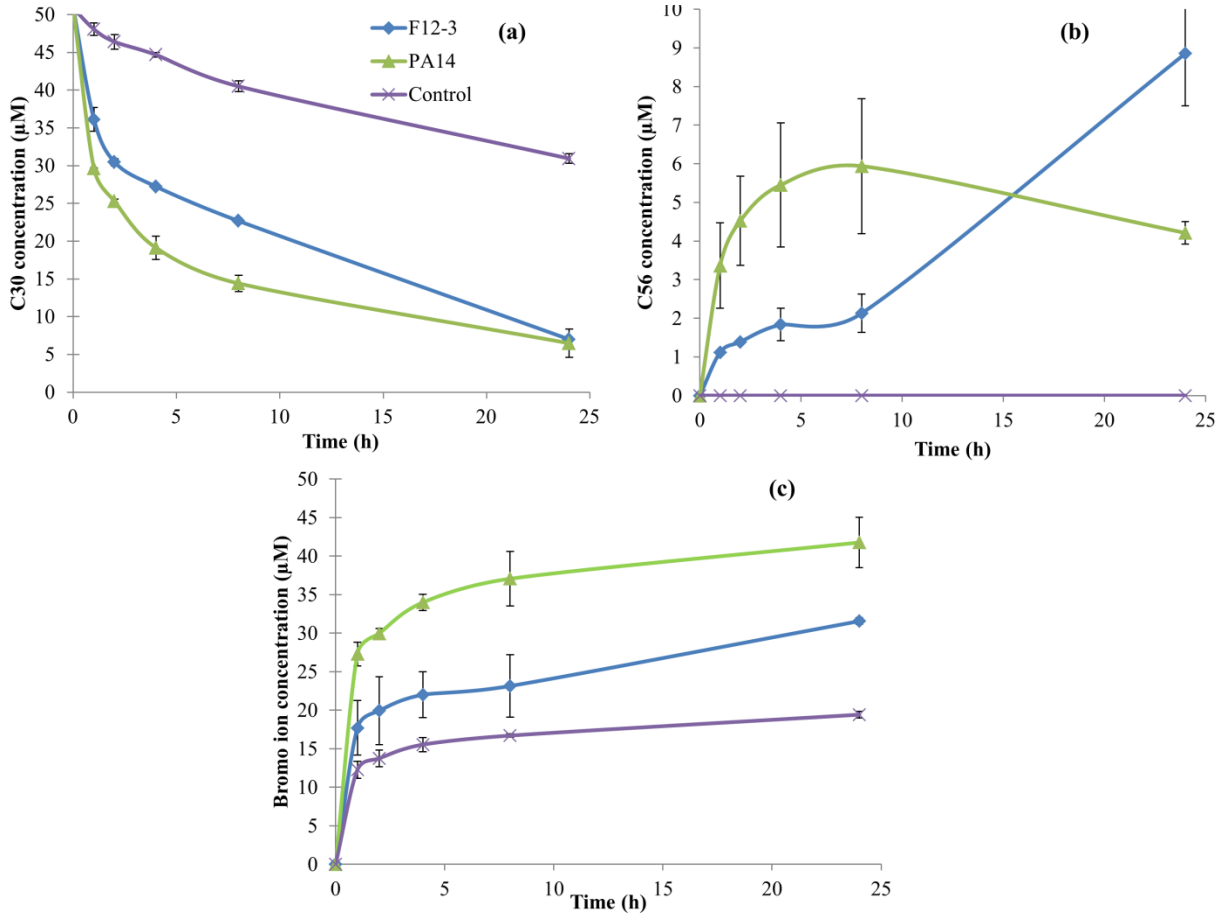

Figure 3.17. Degradation of C-30 compound (50 µM) in saline solution for selected transposon mutant F12-3 (a) C-30 concentration, (b) C-56 concentration, and (c) bromide ion concentration. Control sample (violet x) represent saline with C-30 only, PA14 (green triangle) represent PA14 strain in saline with C-30, F12-3 (blue diamond) represent F12-3 mutant strain in saline with C-30. Error bars indicate standard errors (n = 3).

3.3.8. Biofilm formation in F12-3 mutant defends C-30 uptake into cells

In this section, we confirmed the viability of F12-3 mutant in saline solution with C-30 to ensure that the C-30 does not have any toxicity to F12-3. F12-3 was incubated in saline solution with and without C-30 for 24 h, after that, F12-3 was spread on LB plate with antibiotics (Table 2.1) and incubated overnight. The number of colonies was counted on the next day. There was no significant difference in the number of colonies in both samples (Fig. 3.18). Hence, C-30 did not have toxicity to F12-3.

Biofilm formation analysis was also tested to investigate the QSI effect of C-30 to F12-3 and PA14 in LB medium. Biofilm formation of F12-3 strain was increased in the addition of C-30 compare to PA14 strain (Fig. 3.19). The process of bacterial biofilm formation is related to the QS systems (Parsek et al., 2005). Other studies found that the C-30 furanone and C-56 furanone can inhibit biofilm formation in *P. aeruginosa* (Hentzer et al., 2002; Maeda et al., 2012). Similarly, in our study, only PA14 biofilm formation was inhibited during the treatment with C-30. In contrast, the biofilm formation of F12-3 was not affected when treated with C-30, and even slightly increased the biofilm formation. This result indicated that the C-30 did not inhibit QS system in F12-3 mutant. In fact, Anguige et al. (2005) found that the critical concentration of anti-QS drugs increases exponentially with the biofilm thickness. Therefore, QQ resistance in biofilm could be performed by limiting the permeability of the QQ drugs when the extracellular matrix components which can separate the QQ agent are over produced (García-Contreras et al., 2013).

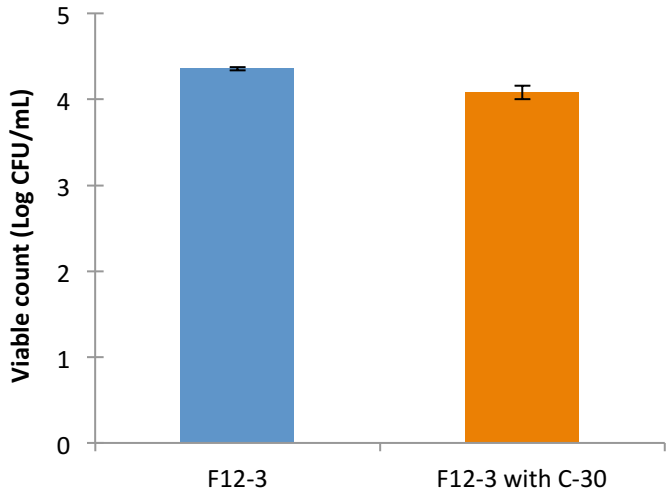

Figure 3.18. The number of F12-3 cells under the treatment of C-30. Error bars indicate standard errors (n = 3).

Based on this evidence, we can assume that biofilm formation could be the factor of F12-3 showed the slow degradation of C-30 as well as the increasing of C-56 by the time.

Firstly, the increasing of biofilm prevented the C-30 uptake into the F12-3 cells, resulting in higher concentration of C-30 in F12-3 than in PA14 (Fig. 3.17a). However, C-30 still could be converted to C-56 in a low amount (Fig. 3.17b) which demonstrated that the small amount of C-30 has been uptake into the cells. Secondly, when C-30 was converted to C-56 inside the cell, C-56 was efflux outside the cell. Then, this compound was prevented to be uptake into the cell because of the high biofilm formation. Therefore, C-56 was accumulated in the supernatant or outside of the cell (Fig. 3.17c).

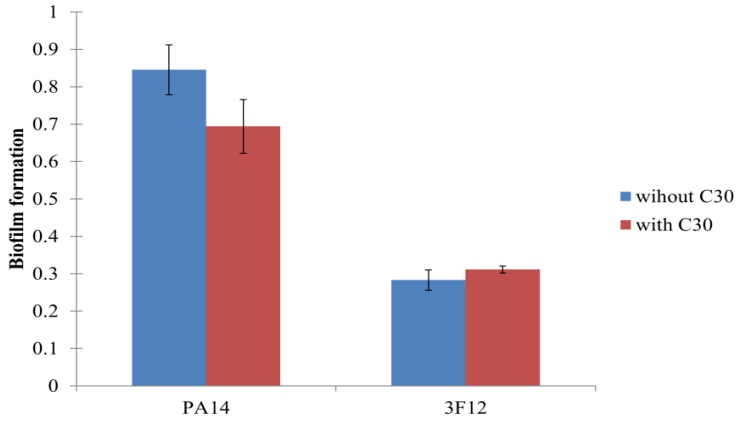

Figure 3.19. Biofilm formation of F12-3 and PA14 in LB medium with the addition of C-30. Biofilm formation of F12-3 and PA14 in LB medium with the addition of C-30. Initial OD was set at 0.05 at 600 nm and then incubated at 37 °C for 24 h. Error bars indicate standard errors (n = 3).

3.3.9. Identification of gene responsible for C-30 degradation ability

F12-3 strain was identified as a mutant has less ability in degrading C-30 to C-56. Therefore, we isolated genomic DNA of F12-3, conducted the arbitrary PCR and sent for sequencing the PCR product. After getting the sequences, we did the BLASTN to confirm the gene. The gene that we identified is *crc* (catabolite repression control protein). Crc is a translational repressor of mRNAs, which encoding the ability of uptake and breakdown of

different carbon sources (Milojevic et al., 2013). Based on Crc's function, if the cells rely C-30 as a carbon source, it can uptake and degrade C-30. Therefore, when *crc* is inactived by Tn5*luxAB* transposon, it may affect to the uptake and breakdown carbon sources, including C-30.

3.4. Conclusion

In conclusion, PA14 has been demonstrated to have the function in degrading C-30 to C-56. This ability is one of the evolve strategies of PA14 strain in the resistance to QSI, especially to C-30. The transformation of C-30 to C-56 is considered the transformation from the stronger QSI to the weaker QSI. Moreover, PA14 continued to degrade C-56 to non-bromine compound which was not possess QQ function. This disrupts the QSI efficiency towards PA14. Besides, the halogenase gene was not related to the C-30 degradation function of PA14. In addition, there was no mutant had been found to lose the ability in degrading C-30, which indicated that this function is regulated by the complex genes instead of single gene. The isolated PA14 mutant (F12-3) was proved to have less function in degrading the C-30 and less ability in uptake the C-56 into the cell. *crc* is the gene that is deactivated in 3F12, may responsible for the less ability in degrading C-30.

CHAPTER 4

ADAPTATION OF QUORUM SENSING SYSTEM IN *PSEUDOMONAS AERUGINOSA* PA14 UNDER THE TREATMENT OF AIIM LACTONASE QUORUM QUENCHING

4.1. Introduction

Pseudomonas aeruginosa is a Gram-negative bacteria which causes many human infection and makes serious health problems. This kind of bacteria is related to disease associated infections in the world (around 10 %) (García-Contreras et al., 2016). Like many other bacteria, *P. aeruginosa* use molecular signals to communicate, called as quorum sensing (QS). This QS system controls the production of its virulence factors which regulates the expression of virulence factors when bacteria have reached a high population density, therefore increasing their occasion to overtake the host defenses (Castillo-Juárez et al., 2015). QS in *P. aeruginosa* is regulated by four different systems: Las, Rhl, Pqs, and Iqs (Lee et al., 2015). The first system is the *las* system which is activeated based on the production an AHL signal: N-(3-oxododecanoyl)-homoserine lactone (3-oxo-C_{12}-HSL). The *las* system controls the expression of elastase and Las protease as well as activates the other QS systems (Lee et al., 2015). The synthesis of N-(butanoyl)-L-homoserine lactone (C_4-HSL) is controlled by *rhl* system, this signal interacts with the RhlR receptor and activates target gene promoters. The *las* and/or *rhl* systems regulate virulence factors production, for examples, elastase, alkaline protease, pyocyanin, exotoxinA, lectines and rhamnolipids (Hirakawa et al., 2013).

Based on the importance function of QS in the expression of virulence from bacterial pathogen, quorum quenching (QQ) is considered as a strategy proposed to inhibit virulence by disrupting QS systems without inhibiting the development of bacterial pathogens. QQ compound, such as brominated furanones, can inhibit bacterial virulence by compete the

binding site with QS molecules. For example, C-30 furanone has also been reported as a QS disruptor and decrease the virulence of *P. aeruginosa* (Defoirdt et al., 2007; Hentzer et al., 2002). QQ enzymes such as lactonase or acylase was known to degrade AHL signals (Rajesh et al., 2014; Wang et al., 2010). Among these, AiiM lactonase identified from *Microbacterium testaceum* StLB037 (Wang et al., 2010), was considered based on its ability in degrading both short- and long-chain AHLs.

However, Maeda et al in 2012 reported that C-30 furanone can be resisted by *P. aeruginosa* (Maeda et al., 2012). When C-30 was added to inhibit the QS pathways, *P. aeruginosa* growth on adenosine minimal medium (which requires active QS to grow) was decreased, and from the screening thoughout four sequential dilutions, the resistant cells from transposon mutant appeared. The C-30 resistant strains became resistant because of the greater efflux of the C-30 compound than the wild type (Maeda et al., 2012). In addition, C-30 resistance is also common in multidrug resistant strains (Maeda et al., 2012). Therefore, strains in both the laboratory and in the clinic showed the ability to evolve resistance to QQ. Furthermore, some of theories about the way of bacteria can collect the resistance to QQ was given (García-Contreras et al., 2013a; Kalia et al., 2014). In which, Kalia et al mentioned about the potential of bacterial in resisting to both QQ compound and QQ enzyme (Kalia et al., 2014). QQ enzyme is a protein enzyme, whereas *P. aeruginosa* produces several kind of proteases such as the LasA protease, the LasB protease (known as elastase) and alkaline protease (Venturi, 2005). Therefore, we reasonably believe that *P. aeruginosa* can evolve to become resistant to QQ enzyme or at least has the adaptation during the treatment with QQ enzyme.

In this study, *aiiM* gene derived from *M. testaceum* StLB037 (Wang et al., 2010) was cloned and the AiiM enzyme was purified and utilized to evaluate the adaptation of *P. aeruginosa* PA14 in QS minimal medium as well as in rich medium. The reason to use *P.*

aeruginosa PA14 because it is more virulent than *P. aeruginosa* PAO1 and one report demonstrated the resistance of *P. aeruginosa* PA14 to QQ compound (Maeda et al., 2012). Our results revealed that *P. aeruginosa* PA14 can utilize AiiM as a nutrient for their growth under the lack of nutrient in minimal medium. This discovery raised the apprehension about the resistance to AiiM by the mutant in some environments where the lack of nutrient may appear. To prove this statement, the clinical isolates were utilized to test the resistance ability to AiiM. As expected, 2 clinical isolates with high protease activity showed the resistance to AiiM by increasing the biofilm formation, meanwhile other clinical isolates with low protease activity were reduced the biofilm formation under the treatment of AiiM.

4.2. Materials and methods

4.2.1. Bacterial strains and growth conditions

All of studied bacteria were cultured on Luria-Bertani (LB) agar with or without antibiotics (Table 2.1). *P. aeruginosa* PA14 and *E. coli* M15/pREP4 were grown at 37 °C with 120 rpm. *Chromobacterium violaceum* CV026 and *Agrobacterium tumefaciens* NTL4 PZLR4-traG:lacZ were grown at 30 °C with 250 rpm.

4.2.2. Cloning, expression, and purification of AiiM lactonase

The plasmid (*aiiM* was cloned into the pGEM-T easy cloning vector) was kindly provided by Dr. Tomohiro Morohoshi, Utsunomiya University (Wang et al., 2010a). The *aiiM* gene (AB513359) (DDBJ/EMBL/GenBank accession number) in the plasmid was amplified by polymerase chain reaction (PCR) using T100™ Thermal Cycler (BIO-RAD) with the following two primers: forward primer 5'-AAGGTACCTTGATCCTCGCCCACGACGTGT - 3' with *Kpn*I restriction site (underlined)

and reverse primer 3' – AAAGCAGGCGACTTGCGTGTTCGAAGGCGA - 5' with *Hind*III restriction site (underlined). The PCR product (765 bp) was digested with *Kpn*I and *Hind*III and purified from an agarose gel by using the QIAquick® Gel extraction kit (Qiagen, Japan). The digested fragment was cloned into the pQE30 cloning vector (Qiagen, Japan) which was also digested with *Kpn*I and *Hind*III. The recombinant plasmid (pQE30-AiiM) with a histidine tag was transformed into *E. coli* M15/pREP4 by electroporation, and the transformants were selected on LB agar plates containing 50 µg/mL kanamycin and 30 µg/mL carbenicillin. The positive clones were confirmed by colony PCR and DNA sequencing.

For the expression and purification of AiiM lactonase, a positive clone harboring pQE30-AiiM was inoculated into 100 mL Terrific Broth (TB) medium containing 50 µg/mL kanamycin and 30 µg/mL carbenicillin and incubated at 37 °C with 120 rpm overnight. The overnight cultured was transferred to a new TB medium (1 L) with the above antibiotics and incubated to a turbidity of 0.4 at 600 nm. Then, 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Nacalai tesque, Kyoto, Japan) was added and the culture was incubated for 7 h at 37 °C for the expression of the target protein. After the incubation, cell pellets were harvested by centrifugation at 6000 rpm for 10 min at 4 °C and resuspended in purification buffer. The cell suspension was sonicated for 30 s at 100 % amplitude with 30 s cooling on ice (6 times) and centrifuged at 10000 × g for 40 min at 4 °C to remove cell debris. The supernatant obtained was mixed with the Ni-NTA resin and incubated on ice for 1 h with inverting every 5 min and then was transferred to a purification column. The purification buffer solutions with different concentrations of imidazole (10 mM and 20 mM) were used to wash the column and finally the target protein, AiiM, was eluted with 150 mM of imidazole. The AiiM protein was dialyzed overnight at 4 °C with gentle stirring against at least 500 mL of purification buffer (no imidazole). The dialysis tubing was transferred to 250 mL of storage buffer, dialyzed as above for at least 8 h and the dialysis was repeated with new

storage buffer for overnight. The AiiM protein was recovered from the dialysis tubing and stored at –20 °C. The presence of the purified protein was confirmed by SDS-PAGE using GE Healthcare miniVE Vertical Electrophoresis System (GE Healthcare 80-6418-77). The final concentration of the protein was measured using a Lowry assay.

4.2.3. Colony PCR and DNA sequencing

To confirm whether pQE30-aiiM was transformed into *E.coli* M15/pREP4, PCR colony method was used to check the presence of *aiiM* gene in the transformed strains. Two primers were used for PCR reaction, forward primer 5' - AGATGTCATGATCCTCGCCC – 3' and reverse primer 3' - GTTCAGCGGACGAAAGCC - 5'. Random colonies appeared on the LB-Car-Km agar plates were picked up and put into 20 µL dH_2O in 1.5 mL eppendorf tube. These tubes were boiled in water at 100 °C in 5 min and flash centrifuge. 1 µL of supernatant was taken as DNA template and put in a PCR tube with other PCR reagents (15 µL Quick taq (Toyobo, Japan), 0.5 µL of 20 mM F primer, 0.5 µL of 20 mM R primer, 13 µL dH2O). PCR reaction was done following the conditions of 30 cycles (Pre-denature: 94 °C, 2 min; Denature: 94 °C, 15 s; Anneal: 52 °C (based on Tm of primer), 1 min; Extension: 68 °C, 1 min; Final extension 68 °C, 5 min; Hold at 4 °C). PCR products were then run on 0.8 % agarose gel to confirm the nucleotide size as well as for the DNA sequencing. A basic local alignment search tool (BLAST) from NCBI (http://www.ncbi.nlm.nih.gov/) was used to identify DNA sequencing belonged to specific gene.

4.2.4. Confirmation of quorum quenching activity by the AHL lactonase

The AHL-degradative activity of purified AiiM AHL-lactonase was tested using biosensor strains, *C. violaceum* CV026 for short-chain AHLs (McClean et al., 1997), and *A. tumefaciens* NTL4 PZLR4-traG:lacZ, for long-chain AHLs (Schaber et al., 2004). The

analysis of short-chain AHLs was carried out by using C_6-HSL and C_8-HSL with or without the treatment of AiiM. Briefly, the overnight culture *C. violaceum* CV026 (100 µL) was inoculated into 100 mL of LB agar medium heated to 45 ºC and immediately poured and solidified into each petri dish (20 mL). Sterile filter paper (8 mm diameter) with or without 10 µL of purified AiiM was put on the surface of the LB agar along with *C. violaceum* CV026, then 10 µL of 100 µM C_6-HSL or C_8-HSL was added and the plate incubated for 24 h at 30 °C. AiiM AHL-lactonase activity was evaluated by observing the color area surrounding the filter paper. The production of a violet pigment was compared in the presence of short-chain AHLs with or without the AiiM enzyme. For the assay of long chain AHLs, *P. aeruginosa* PA14 was used as a tester strain capable of producing a long chain AHL, 3oxo-C_{12}-HSL. *P. aeruginosa* PA14 was grown in LB medium at 37 °C for 16 h. Supernatants were collected by the centrifugation at 13,000 rpm for 5 min and either immediately utilized in the assay or stored at −20 °C. *A. tumefaciens* NTL4 PZLR4-traG:lacZ NTL4 was grown overnight at 30 °C in LB broth with 180 rpm shaking. The pre-culture was inoculated to a new LB medium (5 mL) and grown to 0.1 cell turbidity at 600 nm. Supernatants (400 µL) were mixed with 10 µL purified AiiM or 10 µL sterile distilled water (as a negative control) and incubated at 37 °C for 4 h and 24 h. After that, each mixture was inoculated into a medium containing the pre-culture and incubated for 24 h at 30 °C. The cell pellet was harvested from the 24 h cultures, washed with sterile saline solution 0.1 % NaCl, and centrifuged again at 13,000 rpm for 1.5 min at room temperature. Then the pellets were collected and dissolved with 500 µL of saline solution. Solubilized pellets were mixed with permeabilization solution and incubated at 37 °C with 180 rpm shaking for 30 min. Then 600 µL of substrate solution was added to the incubated solutions and incubated for 30 min at the same condition. Finally, 700 µL stop solution was added to the incubated solutions. The absorbance was measured at 420 nm and 550 nm for each of incubated solutions. 3oxo-C_{12}-

HSL level was detected using wavelengths Abs_{420}, $Abs_{550,}$ and Abs_{600} for calculating β-galactosidase activity (Schaber et al., 2004).

Quorum quenching activity of AiiM was evaluated by assaying QS phenotypes of *P. aeruginosa* PA14 such as pyoverdine production, pyocyanin production, and swarming ability as previously described (Maeda et al., 2012). Briefly, in the pyoverdine assay, overnight cultures of *P. aeruginosa* PA14 were inoculated into succinate minimal medium (Ren et al., 2005) with or without 1 µL purified AiiM and incubated for 7 h. The supernatant (diluted 10 times) was measured at 370 nm to determine the amount of pyoverdine. To determine the production of pyocyanin, 5 mL of *P. aeruginosa* PA14 with or without 1 µL AiiM lactonase were grown in LB medium for 9 h, then extracted with 3 mL chloroform and re-extracted with 1 mL 0.2 M HCl. The absorbance of each extracted sample was measured at 520 nm. The concentration of pyocyanin was calculated by multiplying the value of absorbance OD_{520} by 17.072 (molar extinction coefficient ε) (Essar et al., 1990). Swarming motility assays were performed by using BM2-swarming medium after incubating at 24 h (Overhage et al., 2008). Briefly, 1 µL of *P. aeruginosa* PA14 overnight culture with or without 1 µL purified AiiM was inoculated on swarm agar plates as grown in LB medium; these plates were incubated at 37 °C, and swarming motility was observed after 24 h.

4.2.5. The growth of P. aeruginosa PA14 in the addition of AiiM in LB medium and in QS minimal medium

A single colony of *P. aeruginosa* PA14 was inoculated to 100 mL LB medium and incubated overnight at 37 °C, 120 rpm. This overnight culture then was inoculated to 50 mL LB medium with and without AiiM (1 µg/mL) to get the initial turbidity of 0.05 and incubated at 37 °C, 121 rpm for 24 h. Each time spans, 1 mL of samples were taken and used

to measure the absorbance at OD600 wavelength to determine the growth of PA14. The same experiment was conducted with PA14 using QS minimal medium with and without AiiM.

4.2.6. Protease activity analysis

PA14 and its clinical strains were cultured in LB medium at 37 °C, 121 rpm for overnight. 1 mL overnight cultures were centrifuge at 13,000 rpm in 1 min to collect supernatant. 200 µL supernatants were added into new eppendorf tubes (1.5 mL), 200 µL LB medium was used as a control. These supernatants were incubated at 37 °C for 5 min, then were mixed with 200 µL of 1 % casein. The reaction was conducted at 37 °C for 15 min. 400 µL of 0.44 M trichloroacetic acid was added to the mix solution and continued incubated in 30 min at room temperature. Samples were then centrifuged at 13,000 rpm for 10 min. 200 µL supernatants were mixed with 1 mL of 0.4 M Na_2CO_3 and 200 µL of 1M phenol. This mixture was incubated at 37 °C for 30 min. The absorbance was measured at OD 660 nm.

4.2.7. Biofilm formation assay

Biofilm assay was conducted base on the methods of Pratt and Kolter (Pratt et al., 1998) with some modifications. The overnight cultured of clinical strains were diluted to an optical density (OD) of 0.05 at 600 nm in LB medium with and without AiiM (5 µg/mL and 10 µg/mL). Subsequently, 200 µL clinical strains with or without AiiM were added into 96 well flat bottom assay plate (Costar, Corning NY) and 200 µL LB medium without bacteria was used as a negative control. The plate was incubated at 37 °C within 24 h under static conditions. Crystal violet was used to stain the formed biofilm on 96 well plate and the biofilm formation was quantified as previous described (Westas et al., 2014). Firstly, the absorbance of cell growth was measured at 620 nm using ELISA microplate reader (Tecan, Waco). Plates was then washed 3 times with dH_2O and dried by tapping on a paper towel.

Three hundred μL of 0.1 % crystal violet was added to each well of the 96 well plate and this plate was incubated for 20 min at room temperature. Next step, the solution was poured off plate and the plate was washed three times with water. Finally, 300 μL of 95 % ethanol was added to plate and the plate was incubated for 30 min. The plate was shaked for 50 s to measure the absorbance at 540 nm by using an microplate reader (Tecan, Wako).

4.2.8. Statistical analysis

Means were calculated from at least triplicate data (n = 3). Means and standard deviations was evaluated by using the Student's t test (GraphPad software) at a significance level of $p < 0.05$.

4.3. Results and discussion

4.3.1. Cloning AiiM lactonase

An AHL lactonase gene, *aiiM* was cloned into the expression plasmid pQE30 (Fig. 4.1) so that the AiiM protein could be expressed and purified. The resulted plasmid (pQE30-*aiiM*) was confirmed by colony PCR and DNA sequencing. The expected size of PCR product should be 765 bp, include forward primer, *aiiM* gene and reverse primer (Fig. 4.2). Figure 4.2 showed that only control samples did not have any band. Meanwhile, in all random colonies, the expected band appeared at around 0.76 kbp. Otherwise, the result from DNA sequencing also showed PCR product sequences belong to *aiiM* gene (data not shown). It means we were successful in cloning the *aiiM* gene.

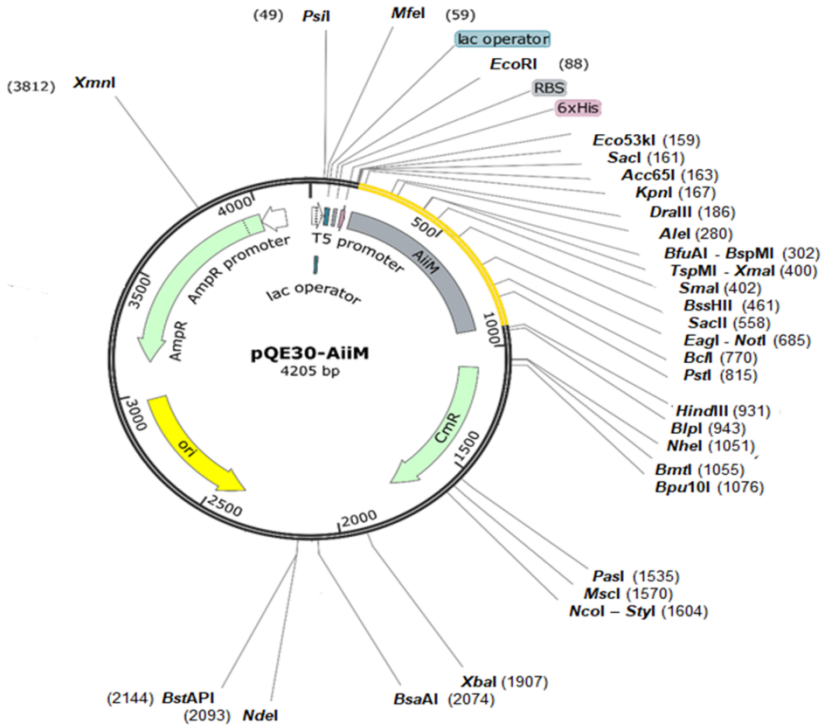

Figure 4.1. Schematic diagram of plasmid pQE30-*aiiM*. The *aiiM* was cloned downstream of the T5 promoter. The total size of pQE30-*aiiM* was 4205 bp.

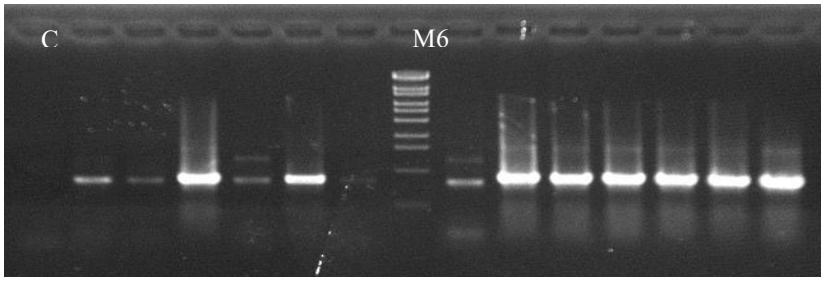

Figure 4.2. Confirmation of *aiiM* gene in cloned product by colony PCR method. Lane 1 indicate as C is the *E.coli* M15/pREP4; M6 was marker 6; Others band were random colonies.

After confirmed the present of *aiiM* gene, we purified the AiiM protein via the histidine-tag and affinity chromatography and AiiM had a molecular weight of about 29.68 kDa (Fig. 4.3). One study by Wang et al (2010) (Wang et al., 2010a), *aiiM* was cloned into the pGEM-T easy cloning vector, then *aiiM* was continued to be cloned into pMAL-c2X (a cloning vector to make maltose binding protein (MBP) fusions) and purified as MBP fusion. In this study, we cloned the aiiM gene into pQE30 so that we can purify AiiM protein as a

His-tag protein. Therefore, we need to prove whether the newly cloned AiiM protein is absolutely functional.

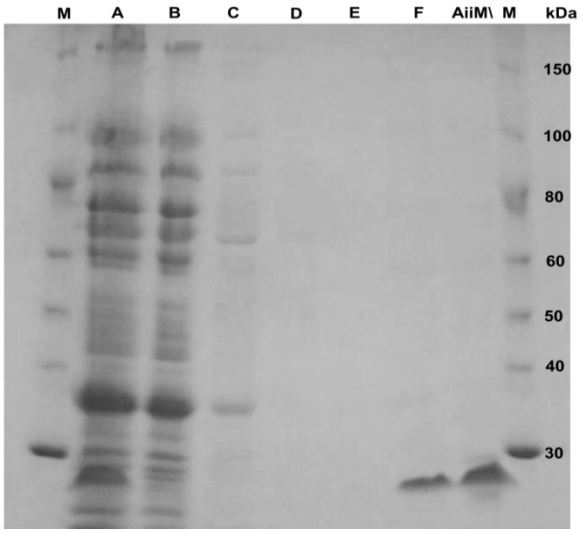

Figure 4.3. Protein purification as evaluated via SDS-PAGE. M: Marker; A: cell lysate; B: Flow through (after binding to Ni-NTA resin); C: after 1st washing with wash buffer + 10 mM imidazole; D: after 2nd washing with wash buffer + 20 mM imidazole; E: after 3rd washing with wash buffer + 20 mM imidazole; F: Eluate - after elute with wash buffer + 150 mM; AiiM: purified AiiM after lysis.

4.3.2. AiiM characterization

AiiM function was evaluated via the AHL-degrading ability. Here, we used biosensor *C. violaceum* CV026 for short-chain AHLs and *A. tumefaciens* NTL4 PZLR4-traG:lacZ for long-chain AHLs (3-oxo-C_{12}-HSL from *P. aeruginosa* PA14) (Schaber et al., 2004). According to McClean et al. (1997), in the assay for short-chain AHLs, biosensor *C. violaceum* CV026 produced purple pigment violacein in the presence of C_6-HSL and C_8-HSL. However, in this study, the purple color disappeared after the treatment with AiiM (Fig. 4.4a). This indicated that C_6-HSL and C_8-HSL did not keep the original structure under the effect of AiiM, hence these compound could not induce the production of violacein in *C. violaceum* CV026. The same result was obtained when AiiM was added to the mixture of AHL signals

(C_6-HSL, C_8-HSL, 3-oxo-C_6HSL, and 3-oxo-C_8HSL, each 100 μM) (data not shown). In addition, the amount of 3-oxo-C_{12}-HSL produced from *P. aeruginosa* PA14 was significantly reduced after treatment with AiiM at 4 h and 24 h of incubation (Fig. 4.4b). These results demonstrate that the AiiM used in this study actively degrades AHL signals.

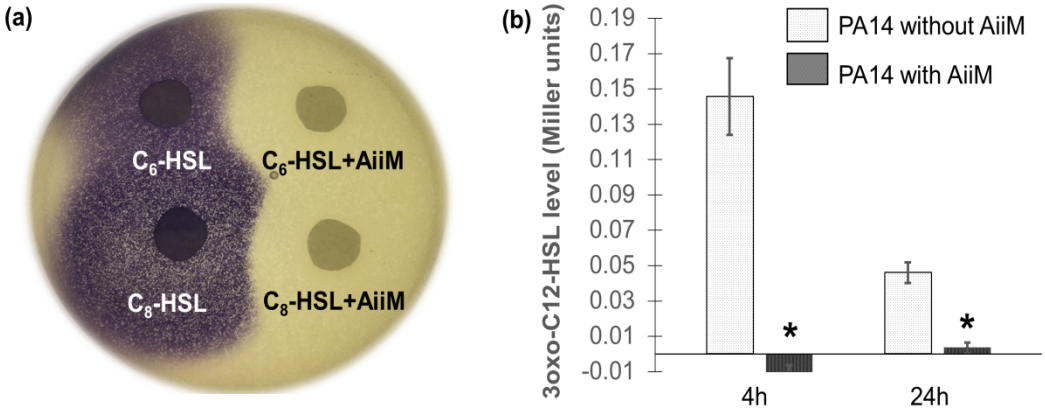

Figure 4.4. HSL degradation ability of AiiM AHL lactonase. (a) For *Chromobacterium violaceum* CV026 in the presence of C_6-HSL and C_8-HSL, the purple color indicates HSL and the yellow color indicates the degraded HSL. (b) The level of 3oxo-C_{12}-HSL from *P. aeruginosa* PA14, which was treated with or without AiiM for 4 h and 24 h assayed via the biosensor *A. tumefaciens* NTL4 PZLR4-*traG*::*lacZ*. Error bars indicate standard errors (n = 3). * Indicate the significant difference in the addition of AiiM.

4.3.3. The effect of AiiM to P. aeruginosa PA14 QS system

The QQ effect of AiiM protein on *P. aeruginosa* PA14 was evaluated to see the production of QS-related factors (Fig. 4.5). In *P. aeruginosa*, the amount of virulence factors production including swarming, pyoverdine (PVD), pyocyanin (PCN), are generally thought to act in concert with the production of QS signals. Therefore, in this experiment, we checked 3 QS-related factors; swarming ability, pyocyanin, and pyoverdine. Swarming abiltity was tested based on the form of flagella-dependent movement on semi-solid agar surfaces such as BM2 (Harshey, 2003). To test the swarming ability, PA14 was grown on BM2-swarming medium with and without AiiM. PA14 swarming ability was inhibited in the presence of

AiiM (Fig. 4.5a). *P. aeruginosa* produce PCN as a secondary metabolite and use QS system to regulate the synthesis of PCN (Lau et al., 2004). In the addition of AiiM, the amount of PCN in PA14 was significantly reduced (Fig. 4.5b). Otherwise, *P. aeruginosa* produce PVD to uptake iron, this function is essential for the production of virulence of *P. aeruginosa* (Meyer et al., 1996). PVD amount was also reduced in the addition of AiiM (Fig. 4.5c). Consequently, three virulence factors were inhibited under the treatment of AiiM, this result demonstrated that AiiM had the function to inhibit QS system of PA14 as well as reduce virulence factor of PA14.

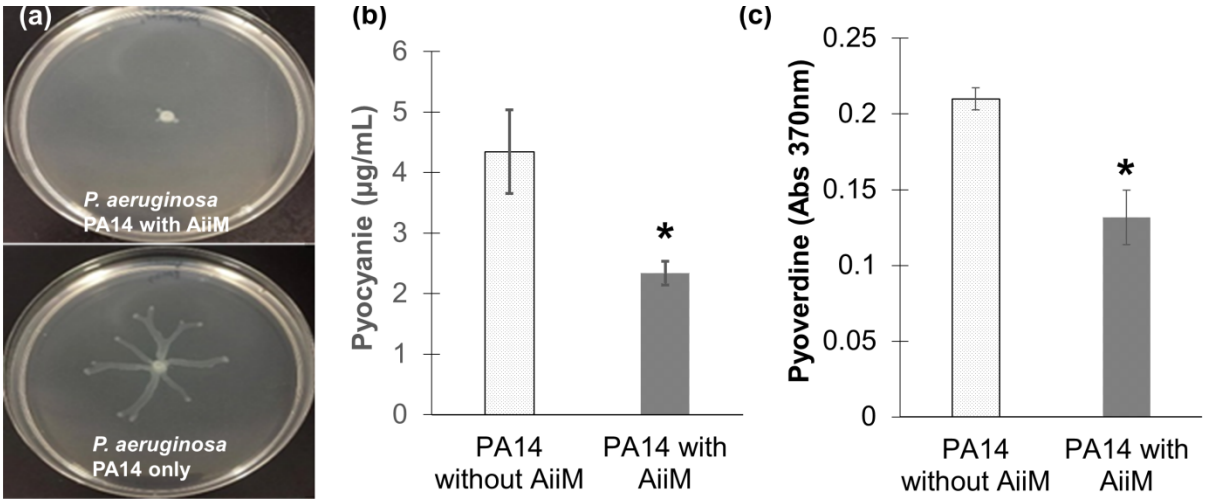

Figure 4.5. The inhibitory effect of QS on *P. aeruginosa* PA14 by the AHL lactonase. (a) The swarming of *P. aeruginosa* PA14 was assayed after 24 h with AiiM (top) and without AiiM (bottom). (b) and (c) The concentration of pyocyanin and pyoverdine in *P. aeruginosa* PA14 with and without AiiM, respectively. Error bars indicate standard errors (n = 3). * Indicate the significant difference in the addition of AiiM.

4.3.4. The growth of P. aeruginosa PA14 in the addition of AiiM in LB medium and in QS minimal medium

The growth of PA14 with the addition of AiiM in LB medium was performed to evaluate the toxicity of AiiM on PA14. The result showed that AiiM did not inhibit the growth of PA14, moreover it slightly induced the growth from 12 h of incubation (Fig. 4.6).

This result demonstrated that the QSI function of AiiM only affected on the QS system and inhibit the factor regulated by QS system rather than inhibit the growth of bacteria (Wang et al., 2010a). However, the remarkable finding was the faster growth of PA14 under AiiM treatment. In fact, *P. aeruginosa* produces several proteases such as alkaline protease, the LasA protease and elastase (Venturi, 2005). Bacteria can digest protein and this protein can be used as nutrition to promote growth (Diggle et al., 2006). In this case, AiiM, a protein enzyme, could be considered as a nutrient for PA14. Therefore, PA14 could produce protease enzyme to hydrolyze AiiM.

P. aeruginosa can grow in QS minimal medium when the QS system become active (Heurlier et al., 2005). Under the treatment of QSI (C-30 furanone), QS and growth of PA14 were inhibited (Maeda et al., 2012). Besides, the growth of *P. aeruginosa* PAO1 *lasI* mutant was reported to be inhibited in QS minimal medium (Mund et al., 2017). In fact, *lasI* mutant did not produce HSL signals, and Mund et al. (2017) demonstrated that the HSL signals were related to the function of adenosine in QS minimal medium. Hence, the degradation of HSL signals by QQ enzyme can be expected to inhibit the growth of PA14 in QS minimal medium. In our study, we want to confirm whether AiiM – a kind of QQ enzyme - can inhibit the growth of PA14 in QS minimal medium. Therefore, an experiment was conducted using 1 μg/mL of AiiM in QS minimal medium to check the survival of PA14. In the growth of PA14 with and without AiiM within 36 h, there was no significant difference among samples (Fig. 4.6b). AiiM slightly inhibited PA14 growth during 36 h, and then PA14 increased rapidly while PA14 in QS minimal medium without AiiM started to reduce. This result was similar to the result of PA14 growth in LB with AiiM. However, in QS minimal medium, adenosine is the only nutrient source for PA14. To utilize this nutrient, PA14 must produce nucleoside hydrolase which is regulated by QS system. But, this QS system was inactivated by AiiM. This indicated that PA14 unable to produce nucleoside hydrolase which lead to

non-utilization of adenosine. However, our result showed that PA14 can grow in QS minimal medium with AiiM. There are two possible ways to explain this phenomenon. Firstly, Huang et al. (2003) found that *P. aeruginosa* PAO1 can degrade 3-oxo-C12-HSL and the metabolite can be used as carbon and nitrogen sources by its acylase quorum quenching (Huang et al., 2003). In our case, AiiM can degrade 3-oxo-C12-HSL, hence PA14 may use the degraded 3-oxo-C12-HSL as a carbon source instead of adenosine to survive. Secondly, PA14 can use another source to grow, and AiiM could be a potential source for PA14. From the above result, we found that AiiM can inhibit QS system of PA14, and in contrast PA14 can utilize AiiM to grow. Or in another way, there was an interaction that occurred between PA14 and AiiM. In this case, PA14 can produce protease enzyme to hydrolyze the AiiM. Therefore, we hypothesized that PA14 could evolved to resist the AiiM by increasing the protease activity. To confirm this theory, clinical isolates were used to test the protease activity because clinical isolates were known to possess many potential in resistance to QSI (García-Contreras et al., 2013b). The clinical isolate had highest protease activity was chosen to test the resistance to AiiM.

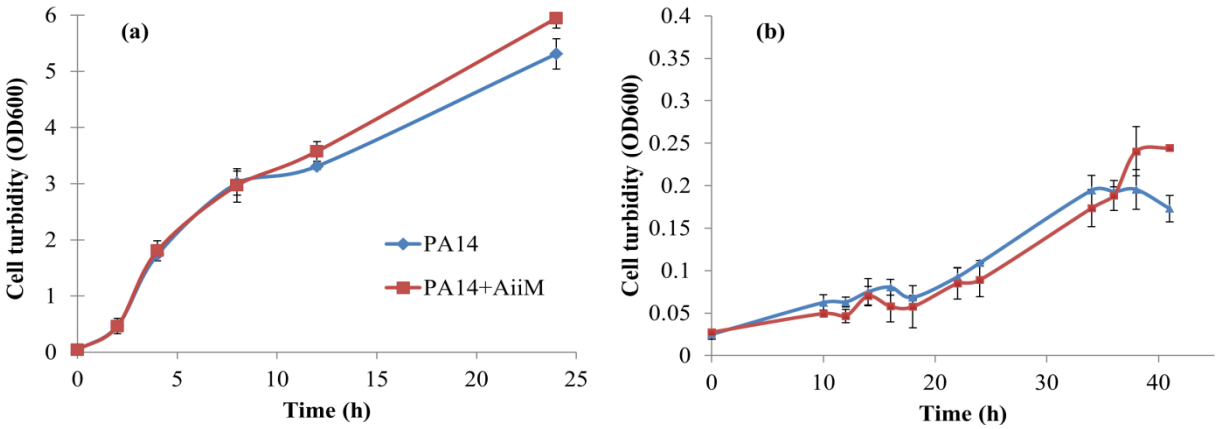

Figure 4.6. The growth of PA14 in LB medium (a) and in QS minimal medium (b) with and without AiiM. Error bars indicate standard errors (n = 3).

4.3.5. Protease activity of P. aeruginosa clinical isolates

In this section, we performed the experiment to measure protease activity from 16 *P. aeruginosa* clinical isolates (these strains were kindly provided by Dr. Rodolfo García Contreras, UNAM University, Mexico). Figure 4.7 showed the differential production of protease activity from 17 strains including PA14 strain. Among them, 3 clinical strains (INP57M, INP58M and INP42, respectively) had higher protease activity than PA14 strain, while the other strains were lower. *P. aeruginosa* proteases were considered an important virulence factors which damage the host (Ołdak et al., 2005). Additionally, some clinical isolates can resisted to QQ (including C-30 and 5-FU) and increased their alkaline protease activity (García-Contreras et al., 2013b). Therefore, we concluded that the clinical strains can be resistant to AHL lactonase, namely AiiM lactonase. Notably, AiiM is a protein enzyme and the clinical strain possesses high protease activity. This protease was expected to degrade AiiM, leading to reduction in the function of AiiM in degrading HSL signals. Consequently, two clinical strains (INP57M and INP58M) which had highest protease activity were chosen for testing the resistance with AiiM.

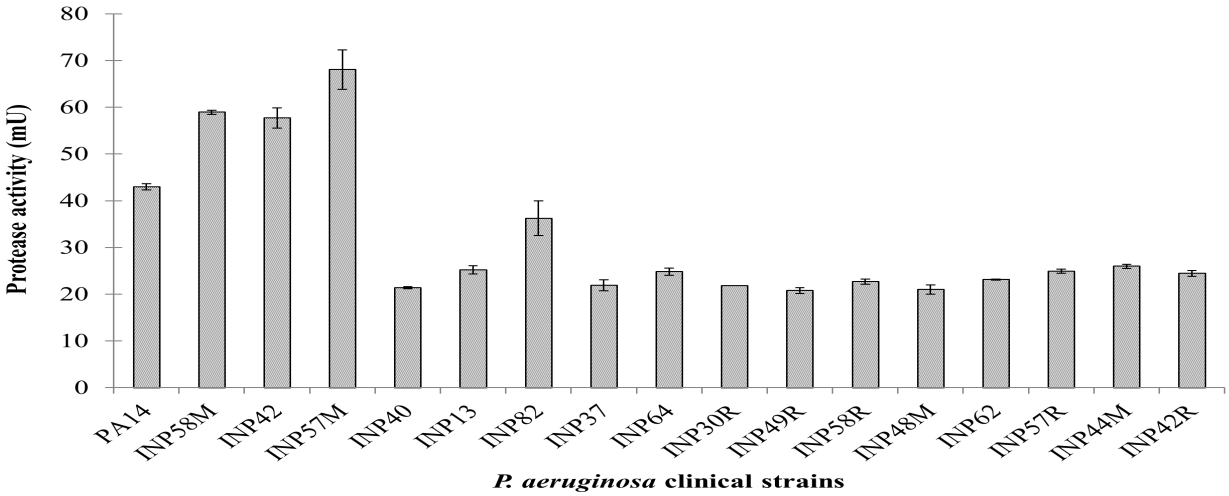

Figure 4.7. Protease activity of *P. aeruginosa* isolated clinical strains. Error bars indicate standard errors (n = 3).

4.3.6. The growth of P. aeruginosa clinical isolates in LB medium with the addition of AiiM

Two clinical strains (INP57M and INP58M) were used to check their growth in LB medium with and without AiiM (1 µg/mL). There were not inhibited in the growth of INP57M and INP58M in the addition of AiiM (Fig. 4.8). Furthermore, after 8 hours of incubation, the growth of both strains in LB medium with AiiM was higher than that in LB medium. This result demonstrated that AiiM can be used as a nutrient for the growth of INP57M and INP58M.

In the next step, AiiM function was evaluated by biofilm formation for INP57M and INP58M in LB medium with and without AiiM. With the addition of AiiM in LB medium, the biofilm formation of INP57M and INP58M were higher than without AiiM (Fig. 4.9). Especially, the biofilm formation of INP58M was increased when AiiM concentration increased. However, AiiM is one kind of lactonase enzyme, the QQ enzyme can inhibit biofilm formation by disrupting the QS signals (Rajesh et al., 2014). Our result showed that QS system in INP57M and INP58M were not affected by AiiM treatment. Furthermore, we conducted the experiment to compare the biofilm formation under AiiM treatment of two clinical strains (INP57M and INP58M) which possess high protease activity with one clinical strain (INP40) (Fig. 4.10) and one mutants (LasB) which possess low protease activity. The result showed that the biofilm formation of the strain which had low protease activity was inhibited, meanwhile the strain which had high protease activity was not affected under the treatment of AiiM. Consequently, INP57M and INP58M can become resistant to AiiM.

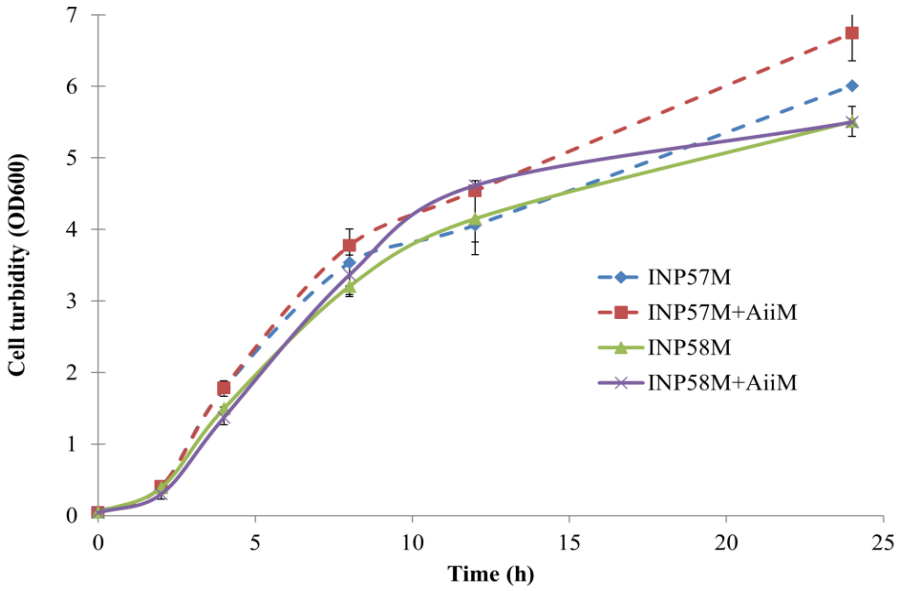

Figure 4.8. The growth of INP57M and INP58M in LB medium with and without AiiM. Dash line represented the growth of INP57M strain with AiiM (red square) and without AiiM (blue diamond). Solid line represented the growth of INP58M strain with AiiM (violet x) and without AiiM (green triangle). Error bars indicate standard errors (n = 3).

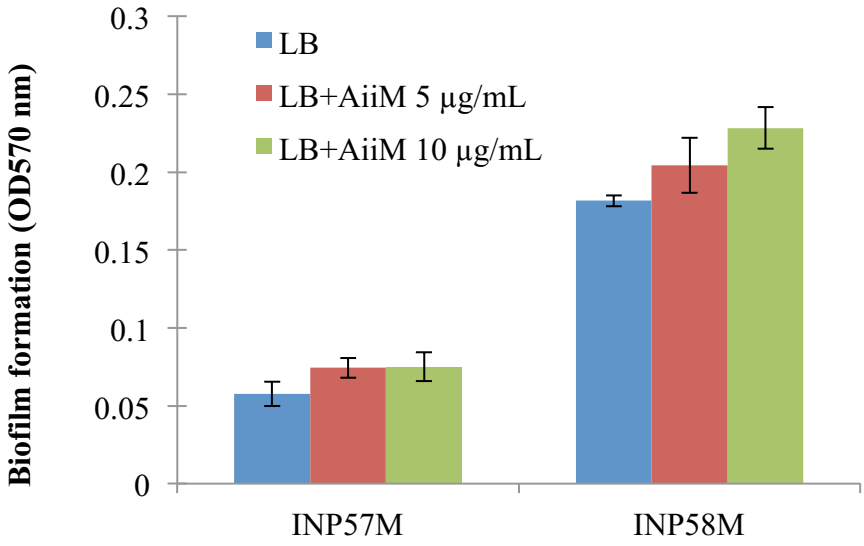

Figure 4.9. Biofilm formation of INP57M and INP58M in LB medium with the addition of AiiM (5 µg/L and 10 µg/mL). The first OD of each samples were 0.05 at 600 nm. Error bars indicate standard errors (n = 3).

4.3.7. The growth of P. aeruginosa clinical isolates in minimal medium with the addition of AiiM

The above results demonstrated that PA14 and two clinical strains (INP57M and INP58M) can increase the resistance to AiiM, and AiiM can be a sole carbon source for PA14.

To confirm that hypothesis, we incubated PA14, INP57M and INP58M in minimal medium without any carbon source and added AiiM with the same concentration of BSA (5 µg/mL) which enough for the strains can grow. PA14 was incubated with different concentration of BSA in minimal medium to check the minimum concentration of BSA for the growth of PA14 (data not shown). Figure 4.11 showed that with the addition of AiiM (5 µg/mL), all 3 strains grew, meanwhile all of them could not grow in minimal medium. This result revealed that PA14, INP57M and INP58M can utilize AiiM as a sole carbon source for their growth. Consequenlty, the QQ function of AiiM to these strains can be reduced.

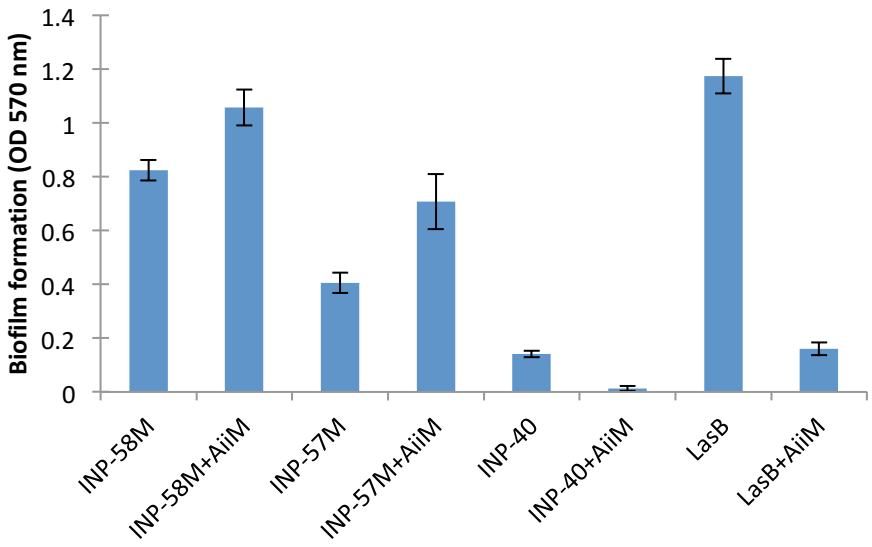

Figure 4.10. Biofilm formation of clinical strains with and without AiiM (5 µg/L). Error bars indicate standard errors (n = 3).

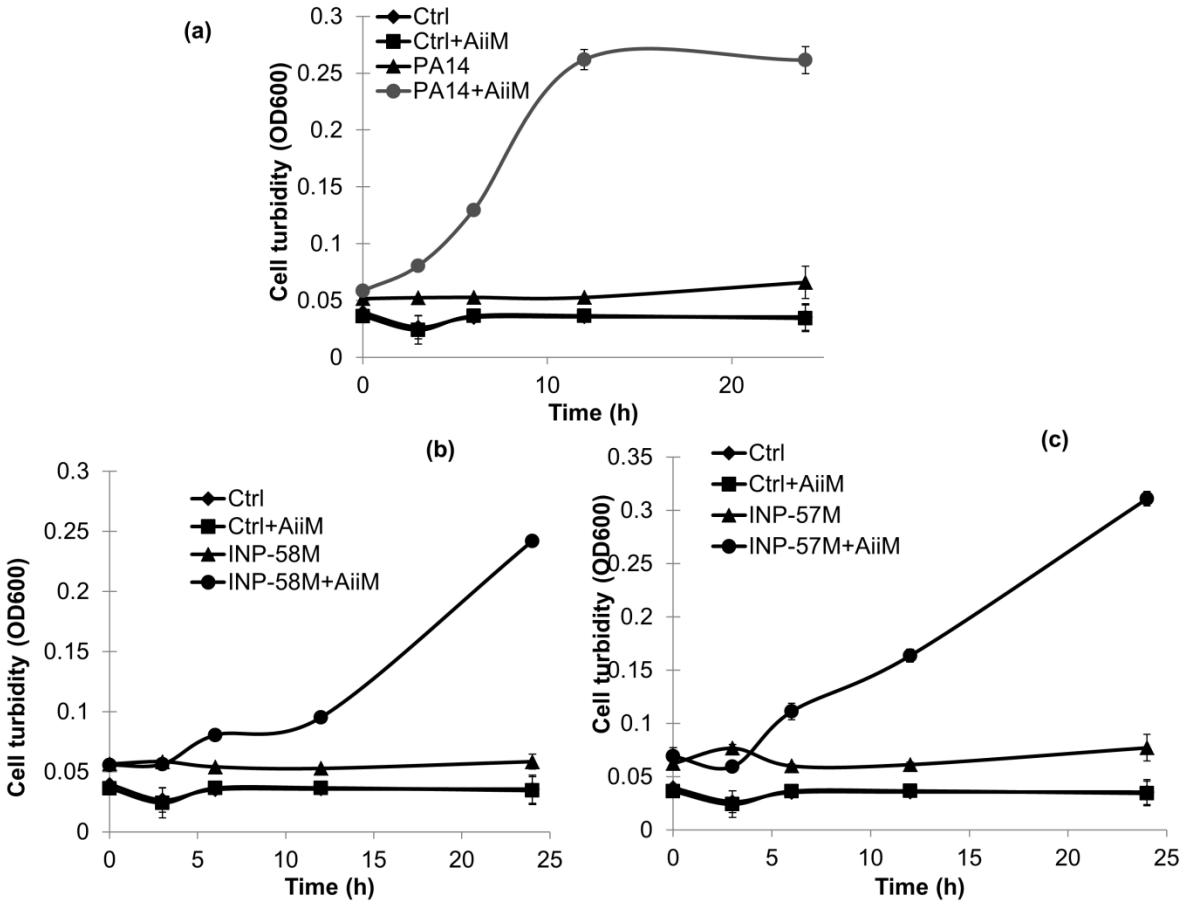

Figure 4.11. The growth of *P. aeruginosa* PA14 and clinical strains in minimal medium with and without AiiM (5 µg/mL). Error bars indicate standard errors (n = 3)

4.4. Conclusion

In this study, we were successful cloned AiiM and proved the QSI function of AiiM in PA14 strains. We also confirmed that PA14 can utilize AiiM as a nutrient for their growth under the lack of nutrient in environment. This discovery raised the apprehension about the resistance to AiiM by the mutant in environment where the lack of nutrient may appear. To prove this statement, the clinical isolates were utilized to test the resistant ability to AiiM. As expected, 2 clinical isolates with high protease activity showed the resistance to AiiM. Consequently, although AiiM is a QQ enzyme, PA14 can adapt the AiiM and use it as a source for growth.

CHAPTER 5

EFFECT OF AIIM LACTONASE IN METHANE PRODUCTION DURING ANAEROBIC DIGESTION PROCESS

5.1. Introduction

Waste sewage sludge (WSS) containing a large population of microbes that exist in biofilms, flocs, and granules can be produced under biological wastewater treatments (Mikkelsen et al., 2002; Oh et al., 2013; Ramos et al., 1994). These microbial consortia, including Gram-negative and Gram-positive bacteria, was considered to degrade a variety of waste compounds (Dhall et al., 2012; El-Bestawy et al., 2005). As quorum sensing (QS) plays an important role in many bacteria interactions via its regulation to bacterial behaviors, QS could contribute to the interactions between bacterial species in WSS. Many QS signals are species specific, in a part of the Gram-negative bacteria, AHL signals can be used for enhancing the bacterial functions (Dubuis et al. 2007; Duerkop et al. 2009). On the other hand, Gram-positive bacteria use oligopeptide to control the bacterial virulence and activity (Sturme et al., 2002). In addition, Gram-negative bacterial activity can be regulated by AHLs (Huang et al., 2016) and variety of AHL molecules could be presence in microbial consortia (Scott et al., 2016). Quorum quenching (QQ) was all processes that can disturb QS (Dong et al. 2001). All of steps of QS pathway including synthesis, accumulation, diffusion can be affected by QQ factors because of its diversity in nature (degradation enzymes, chemical compounds), in mode of action (QS degradation, competition) and targets (Grandclément et al., 2015b). QQ enzymes were known to inactive QS signals, while QQ compound were known to disrupt QS pathway. The effect of QQ enzymes on the microbial communities as well as the relationship between QQ bacteria and QS bacteria in some of biological wastewater treatment systems was reported in recent studies (Cheong et al., 2013; Oh et al.,

2013). *Pseudomonas* sp. 1A1, a QQ bacterium, was used to inhibit biofouling in a membrane bioreactor in the wastewater treatment (Cheong et al., 2013). Through the QQ acylase enzyme of *Pseudomonas* sp. 1A, the membrane biofouling in MBR was reduced (Cheong et al. 2013). Another QQ strains *Rhodococcus* sp. BH4 had been reported to degrade a broad range of AHLs, thereofore inhibiting biofouling found in a wastewater treatment MBR (Oh et al. 2013). Also, the inactivation of QS of bacteria in membrane biocake by AHL acylase on nanofiltration membranes inhibit biofouling in wastewater treatment (Kim et al., 2011). In addition, when investigating the relationship between QS and QQ activities in floccular and granular sludge, Tan and co-workers found that a high QQ activity is found in the floccular sludge and this QQ activity reduced during the transformation from the floccular biomass into the granular sludge (Tan et al., 2015). The above studies demonstrate that the balance between QS and QQ influences the performance of each bacterial fermentation and explains the presence of AHL signals in environmental samples that contain QQ bacteria possessing AHL-degrading ability (Cheong et al., 2013; Mayer et al., 2015; Oh et al., 2013; Tan et al., 2015).

Anaerobic digestion of WSS is considered as one of the critical approaches to produce methane together with the reduction of WSS amount. Hydrolysis, acidogenesis, acetogenesis, and methanogenesis are four stages in the anaerobic digestion of WSS. In the hydrolysis stage, hydrolytic bacteria produce extracellular enzymes (e.g. protease, amylase, lipase and cellulase) to degrade large molecules (e.g. protein, carbohydrate, and lipid) into small molecules such as amino acids, sugars, and fatty acids. These small molecules are converted into volatile fatty acid (e.g. propionate, pyruvate, acetate, formate, so on.), alcohol, carbon dioxide and hydrogen in the acidogenesis and acetogenesis stages. Then, in the final stage, methanogenic archaea and bacteria utilize acetate and hydrogen to produce methane (Appels et al., 2008; Ziemiński et al., 2012). Therefore, a complex microbial community in WSS have

a critical role in methane production. Moreover, microorganism can be a key for accelerating or suppressing of methane fermentation in WSS (Mustapha et al., 2018; Mustapha et al., 2017). Among the microbes in WSS, bacteria including Gram-negative bacteria and Gram-positive bacteria may have a key role in the digestion of WSS under anaerobic condition. On the other hand, in the biological wastewater treatments including the anaerobic digestion process, one report indicated that Gram-negative bacteria are dominant in microbial community (Huang et al., 2016); hence, inactivation of the system of QS in the Gram-negative bacteria can be a reasonable approach to check the role of QS in Gram-negative to methane production. Hence, the effect of AHL degradation to QS system of Gram-negative bacteria was evaluated by adding AHL lactonase, AiiM, into WSS in the methane fermentation. Our results indicate that the growth of Gram-negative or Gram-positive bacteria was inhibited based on the differences in the production of antibiotic molecules when adding AiiM lactonase; therefore, leading to inhibition of methane production.

5.2. Materials and Methods

5.2.1. Waste sewage sludge source and preparation

WSS was taken from the Hiagari wastewater treatment plant in Kitakyushu City, Japan. WSS was centrifuged and washed with distilled water at 8000 g in 10 min at 4 °C by TOMY-GRX 250 High Speed Refrigerated Centrifuge. Washing step was done at least three times to make a constant WSS concentration. After washing step, WSS was diluted by distill water to get 10 % (w/w) for further studies.

5.2.2. Bacterial strains and growth conditions

LB agar and LB medium including appropriate antibiotics (Table 2.1) were used to culture all the studied bacteria. *Chromobacterium violaceum* CV026 and *Agrobacterium tumefaciens* NTL4 PZLR4-traG:lacZ were cultured at 30 °C with 250 rpm in LB containing 50 μg/mL kanamycin or 30 μg/mL gentamycin, respectively. Gram-negative bacteria such as *Salmonella typhi* 14028, *C. violaceum* CV026, and *A. tumefaciens* NTL4 PZLR4-traG:lacZ and Gram-positive bacteria such as *Brevibacillus* sp. KH3, *Bacillus subtilis* 168, and *Lactococcus lactis* 12007 were used to investigate the bactericidal activities by antibiotic factors produced by microbes in the WSS with or without AiiM. The growth and methane production function of *Methanosarcina acetivorans* C2A in the addition of AiiM was investigated in HSYE-methanol medium as previously reported (McAnulty et al., 2017).

5.2.3. Methane production at the different concentrations of AiiM

Different concentration of AiiM (0, 0.6, 1.2 or 2.4 μg/mL) was added into WSS to 30 mL of total volume in each of 66-mL vial. To facilitate an anaerobic condition, nitrogen gas was introduced into the vial which were sealed with a rubber stopper and aluminum cap before. The vial was then incubated within 10 days at 37 ºC with 120 rpm. Each experiment was carried out at least in triplicate. During 10 days of incubation, 100 μL of headspace gas of each sample was injected into GC-3200 gas chromatograph (GL Sciences, Japan) to detect the amount of methane gas released from WSS with and without AiiM. The effects of protein (BSA) and peptide signals (QS signals from Gram-positive bacteria) degradation (peptidase) on methane production were checkecd by adding BSA (2.4 μg/mL) and peptidase (2.4 μg/mL) into the WSS. Otherwise, purification buffer with 0.05 mM PMSF as the same amount with AiiM (highest concentration) was also introduced into 30 mL WSS to investigate the effect of dilute solution to methane production.

5.2.4. Other analytical methods

The supernatant from each sample was collected by centrifuging at 13,000 rpm for 7 min and was filtered by a 0.2 μm pore membrane filter. Then, the supernatant was utilized to measure pH using a compact pH meter (AS ONE, AS-211, Japan) and to check organic acids profile using a high performance liquid chromatography (Shimadzu LC-10AD) as previously described (Mohd Yusoff et al., 2012). Besides, the supernatant was used to detect the amount of the soluble protein concentration using the Lowry method with a standard protein (bovine serum albumin (BSA)) (Lowry et al., 1951) and to measure protease activity as described previously (Maeda et al., 2011). One unit of protease activity was analyzed as the amount of tyrosine (μmol) produced from casein by 1 mg of enzyme in one minute. Each experiment was performed at least in triplicate. The samples of WSS with or without AiiM, which were incubated for 2 days, were used for the Gram staining procedures. Briefly, 2 mL of WSS was taken out of vial and filtered with a 5 μm membrane filter paper (Toyo Roshi Kaisha, Ltd, Japan) to collect the microbial cells for the Gram staining. First, 10 μL of filtered sample was spread onto a glass slide using a pipette tip; the glass slide was fixed under the fire and was stained by using a Gram Stain kit (BD, USA). A microscope (Olympus, BX51, Japan) was used at a 100 × magnification to observe the cells and to count the number of Gram-positive bacteria and Gram-negative bacteria (5 random positions were chosen).

5.2.5. Methane production in the addition of AiiM with pH controlling and effect of AiiM on Methanosarcina acetivorans

WSS (30 mL) with and without 2.4 μg/mL of AiiM was prepared under the same conditions in the section "Methane production at the different concentrations of AiiM" in Materials and methods. On the second day of anaerobic incubation, pH value in all vials was adjusted to 7 by NaOH. Methane production was measured until day 10.

M. acetivorans (0.6 mL) was inoculated into fresh HSYE-methanol medium (60 mL) in a vial (100 mL). In addition, AiiM solution (dissolved in autoclaved deaerated distilled water) was added to the vial (final concentration 2.4 μg/mL). For the control sample, autoclaved deaerated distilled water was used. The growth of *M. acetivorans* with or without the addition of AiiM was evaluated by measuring cell turbidity at 600 nm for 10 days. In addition, methane was also measured as mentioned above.

5.2.6. RNA extraction, cDNA synthesis, high-throughput 16S rRNA sequencing and data analyzing

Pellets of WSS with and without AiiM were collected to extract RNA and synthesis cDNA synthesis according to the previous report (Mustapha et al., 2017) (Chapter 2). High-throughput 16S rRNA sequencing and data analyses were conducted as described previously (Chapter 2).

5.2.7. Bactericidal test

Solid-phase extraction (SPE) method with a slight modification was used to extracted organic compounds from WSS samples with or without the addition of AiiM as previous description (Chau et al. 2017). Briefly, for sample preparation, 1 L of WSS samples was incubated in anaerobic condition for 2 days and centrifuged at 10,000 × g for 10 min at 4 ºC to collect supernatant. The supernatants were filtered by a Whatman filter paper and re-filtered by a 0.45 μm pore size filter paper (Nihon Millipore, Yonezawa, Japan). SPE cartridges were conditioned by passing 10 mL acetone, 10 mL of methanol, and 20 mL of purified water at a flow rate of 5 mL/min. Then, the filtered samples were passed through SPE cartridges (Oasis HLB Plus, Nihon Waters, Tokyo, Japan). These cartridges were then dried by flowing for 60 min, and were eluted with 5 mL of methanol. The final elution

solvents (SPE solutions) were concentrated to 0.9 mL with N_2 stream and kept at −20 ºC for further study.

The organic compound in SPE solutions were checked the antibiotic function by using a disk-agar diffusion method. Briefly, bacteria was cultured for overnight and then 100 μL of overnight cultured was inoculated into 100 mL of LB agar medium heated at 45 ºC to collect agar disk (20 mL) including bacteria for further study. Next step, two filter papers (8 mm diameter) were put on the surface of each agar plate and 20 μL of each SPE solution (WSS or WSS with AiiM) were put onto the filter paper. These agar plates were incubated for 24 h to evaluate the antibiotic function by seeing a clear zone of growth inhibition around filter paper.

The growth of strains which were tested by disk-agar diffusion method were continue to test the bactericidal effect with the SPE solutions in a quantitative assay. Firstly, *C. violaceum* CV026 (as a Gram-negative bacteria) and *B. subtilis* 168 (as a Gram-positive bacteria) were cultured overnight in LB with 120 prm at at 30 ºC or 37 ºC, respectively. Each overnight culture (5 mL) was transferred to new test tubes and mixed with 1 % (v/v) SPE solution. Otherwise, 5 mL of overnight culture was also used as a control by mixing with sterile distilled water as the same volume with 1 % SPE solution. The mixture was incubated at 30 ºC (*C. violaceum* CV026) or 37 ºC (*B. subtilis* 168) with 120 rpm for 30 h. At 0 h, 2 h, 6 h, 24 h, and 30 h, 0.1 mL of the sample was collected and diluted with a sterilized saline solution. An serial dilution of each bacteria was spread on LB agar plates and incubated for 24 h at 30 ºC (*C. violaceum* CV026) or 37 ºC (*B. subtilis* 168). After that, the number of colonies of each condition was count to compare and evaluate the quantitative bactericidal activity.

5.2.8. Statistical analysis

Means were calculated from at least triplicate data (n = 3). To compare means and standard deviations, the Student's t test (GraphPad software) was used and evaluated at a significance level of $p < 0.05$.

5.3. Results and discussion

5.3.1. Effect of AiiM lactonase on methane production

In this study, a QQ enzyme – AiiM lactonase - was utilized to investigate the effect of QS system in Gram-negative bacteria on methane production. Different concentrations of AiiM (0, 0.6, 1.2, and 2.4 µg/mL) were used to evaluate the influence of AiiM on anaerobic digestion through the production of methane. The result from figure 5.1 showed that methane production using WSS was remarkably reduced in the presence of AiiM (Fig. 5.1). Higher amount of AiiM indicated a high inhibitory effect of methane production; thus, AHL signaling increases methane production.

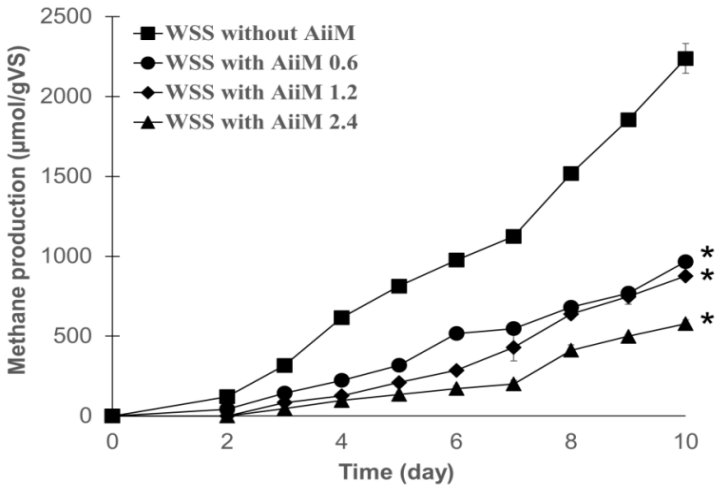

Figure 5.1. Methane production from WSSwith the addition of different concentration of AiiM. WSS without AiiM (*dark squares*), AiiM at 0.6 µg/mL (*dark circles*), at 1.2 µg/mL (*dark diamond*) and at 2.4 µg/mL (*dark triangle*) are shown. *Error bars* indicate standard errors (n = 3). * Indicate the significant difference in the presence of AiiM

0.5 mM of PMSF (a protease inhibitor) was added to WSS in anaerobic digestion to check the function in methane production because this reagent was used for AiiM purification.

The result showed 0.5 mM of PMSF did not affect to methane production (Fig. 5.2a). BSA as a negative control or peptidase were mixed to WSS at the same concentration as AiiM (2.4 μg/mL) to see the function of protein as well as autoinducer oligopeptides during the anaerobic digestion. Figure 5.3b showed that BSA and peptidase with the same concentration of AiiM did not affect to methane production. This result indicated that protein and oligopeptides did not inhibit the production of methane. Therefore, methane production using WSS was inhibited by the AiiM lactonase.

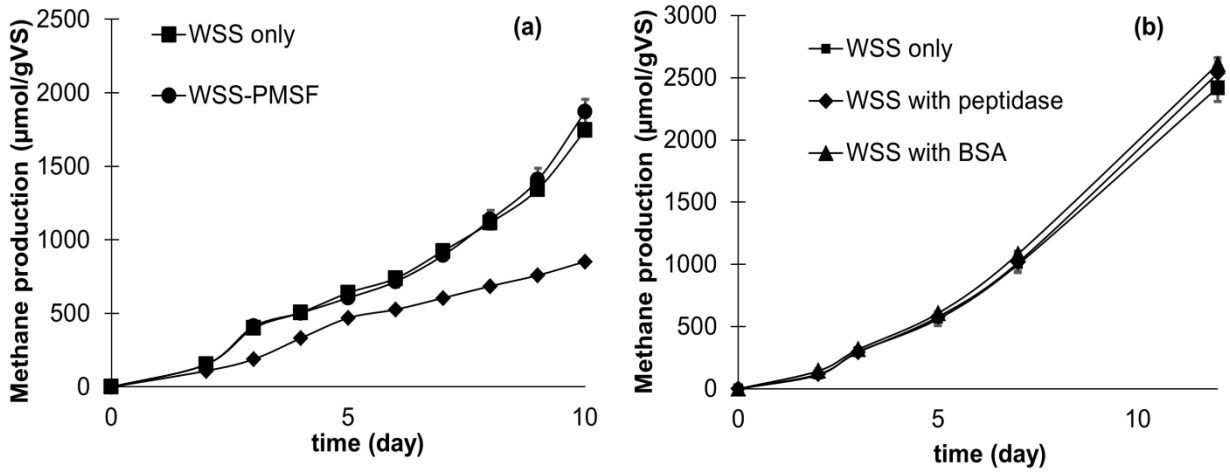

Figure 5.2. Production of methane in WSS after PMSF, peptidase, and BSA treatments. (a) Methane production in WSS with PMSF. (b) Methane production in WSS with BSA or peptidase. Error bars indicate standard errors (n = 3)

5.3.2. The addition of AiiM lactonase changes bacterial activities during methane fermentation

There are four stage in methane fermentation, including hydrolysis, acidogenesis/acetogenesis, and methanogenesis. As previous results, AiiM lactonase inhibited methane production, therefore, each of stage was investigated to understand the reason. In hydrolysis stage, the large molecules such as protein can be converted to the small molecules, hence, to find the different point in WSS with or without AiiM, protease activity and the soluble protein concentration were evaluated at day 2 and day 4. Although the protease activity in WSS with AiiM was a little higher than WSS without AiiM, we found that

the addition of AiiM to WSS did not create the significant difference between WSS with or without AiiM (Fig. 5.3). Therefore, the presence of AiiM did not affect to the hydrolysis process.

For acidogenesis and acetogenesis stage, the samples of WSS with or without AiiM were collected at day 2, 4, 7 and 10 to compare pH value and the production of organic acids. At day 2, the pH of WSS with AiiM was decreased quickly, from 6.85 to 5.6 in WSS with 0.6 µg/mL AiiM, from 6.9 to 5.2 in WSS with 1.2 µg/mL AiiM and from 6.9 to 4.8 in WSS with 2.4 µg/mL AiiM (Fig. 5.4a). After that, pH value of WSS with all concentrations seemed steady until 7 days and then increased slightly to 6.15, 5.45 and 4.9 in WSS with 0.6 µg/mL AiiM, WSS with 1.2 µg/mL AiiM and WSS with 2.4 µg/mL AiiM, respectively. Meanwhile, pH value in WSS without AiiM at 0 day was 6.8 and was raised to 7.5 after 7 days of incubation. As a result, the pH value during the methane fermentation was decreased by the influence of AHL lactonase. The low pH in anaerobic digestion could inhibit methane production because methanogenesis stage is inhibited by the production of organic acids (a kind of volatile fatty acids). This inhibition is stronger when pH value is reduced (Venkiteshwaran et al., 2015). Otherwise, the methane production decrease when pH is lower than 6.3 (Lay et al., 1998). Consequently, pH reduction is the reason for the inhibition of methane production with the addition of AiiM.

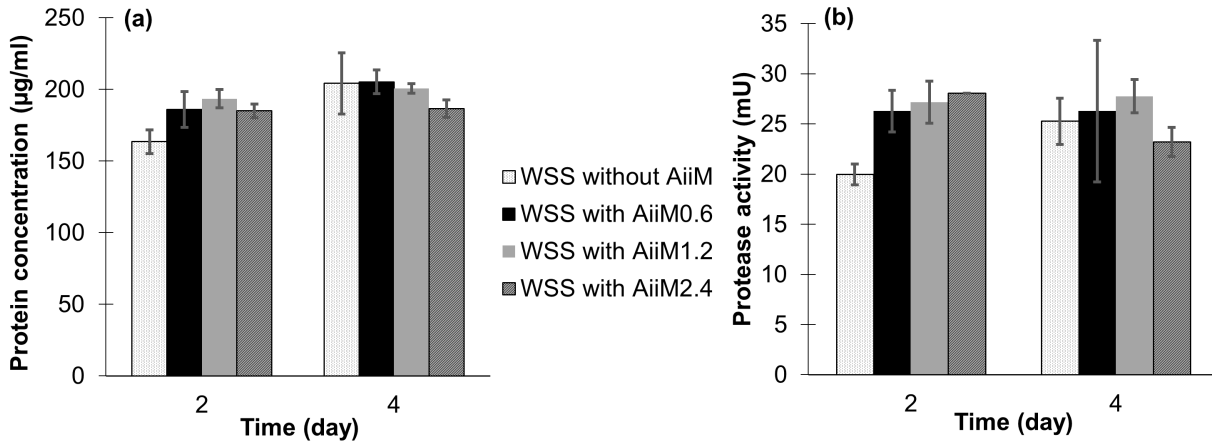

Figure 5.3. Protein concentration and protease activity in WSS with or without AiiM. Purified AiiM was added at 0.6 µg/mL, 1.2 µg/mL, and 2.4 µg/mL. Error bars indicate standard errors (n = 3)

The lower of pH is synonymous with the higher of organic acid production; therefore, the acetic acid, formic acid and propionic acid were found in all samples in the presence or the absence of AiiM. However, the pattern of each organic acid detected in WSS control and WSS with AiiM was different. The amount of propionic acid was higher in the WSS control than that in WSS containing AiiM from day 2 to day 10 of anaerobic digestion (Fig. 5.4b). In opposite way, acetic acid and formic acid were detected at a higher concentration in the WSS with AiiM than the WSS control (Fig. 5.4c, 4d). In which, the concentration of acetic acid at day 2 in WSS-AiiM was higher remarkably than the WSS control. Besides, the amount of formic acid in the WSS-AiiM was initially lower and then was higher than that in the WSS control at the end stage of the anaerobic digestion. Meanwhile, its concentration in the WSS control samples was quickly consumed during the fermentation. The higher amount of organic acids, particularly acetic acid and formic acid which is known as an important substrate for methane production (Ahring et al., 1995) and for hydrogen production (Barbosa et al., 2001; Kurokawa et al., 2005) could be the factor of drastically reduction in pH for WSS with AiiM. The accumulation of acetic acid and formic acid may be because of the reduced consumption of the two organic acids due to the low pH. Moreover, methane production

could be inhibited by formic acid (Ungerfeld, 2015). Also, the concentration of acetic acid increases, lead to inhibits the breakdown of propionic acid (Fukuzaki et al., 1990). Therefore, the small amount of propionic acid detected in WSS with the AiiM could be because of the inhibition in producing propionic acid. In fact, AHL lactonase (namely, AiiM) can catalyze the cleavage of AHL lactone ring in AHL signals (Grandclément et al., 2015a); hence, the inactivation of AHL-regulated QS sytems in the Gram-negative bacteria may be lead to the variation in the production of organic acids.

In methanogenesis stage, archaea acts an important function in producing methane from organic acids and hydrogen, in which, *M. acetivorans* C2A was known as one of archaea which can produce methane. Thus, we checked the effect of AiiM to methane production function of *M. acetivorans* C2A. As a result, AiiM did not inhibit methane production ability of *M. acetivorans* C2A. Otherwise, we also tested the production of methane in WSS with AiiM in case pH was remained at 7.0. As a result, by controlling pH, the methane production in WSS with AiiM was restored to the same amount of methane in WSS control (Fig. 55a). Therefore, the main reason for the less methane production finding in the presence of AiiM was because of the difference of producing organic acids; especially, base on the low pH through the fermentation.

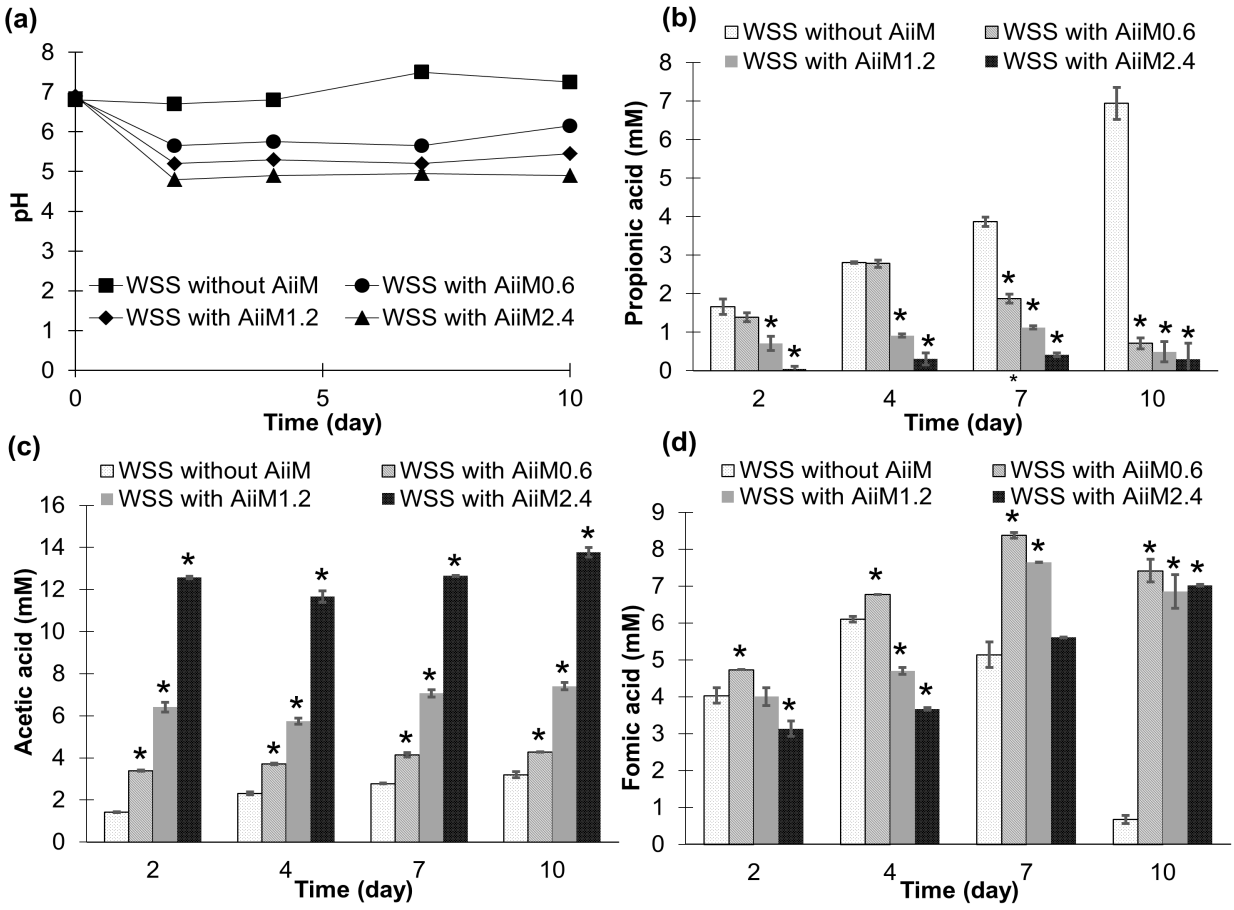

Figure 5.4. Profile of pH value and organic acids during the methane fermentation using WSS in the presence or the absence of AiiM. During the fermentation process, the pH value (a) and organic acids including (b) propionic acid, (c) acetic acid, and (d) formic acid were monitored Error bars indicate standard errors (n = 3). * Indicate the significant difference in the addition of AiiM

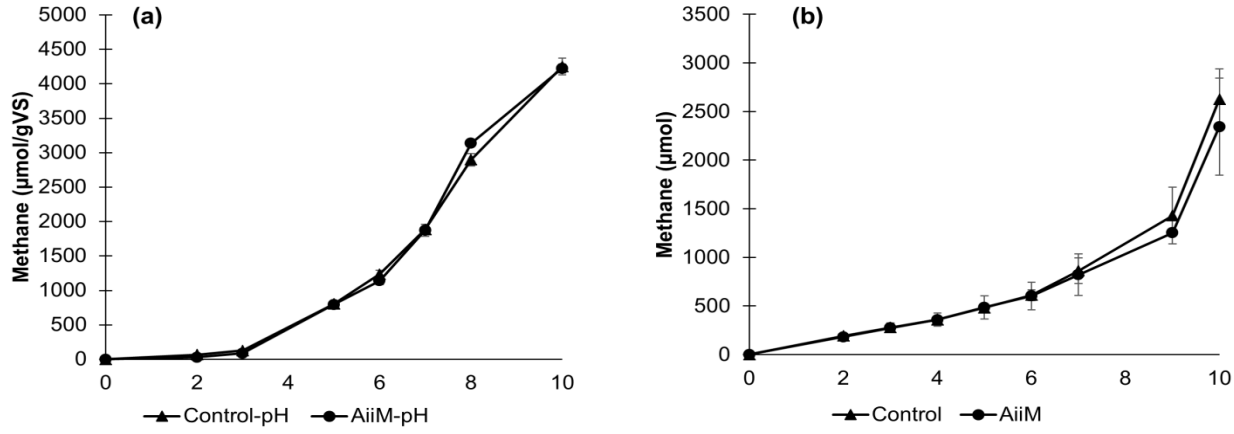

Figure 5.5. Methane production in WSS (a) and in *M. acetivorans* (b). (a) Methane production in WSS with or without AiiM under the pH control. (b) Methane production in *M. acetivorans* with or without AiiM. Error bars indicate standard errors (n = 3).

5.3.4. Richness and diversity of microbial communities in the presence of AiiM lactonase

WSS with the highest amount of AiiM (2.4 μg/mL) and WSS control were used to compare the richness and diversity in microbial communities. The richness of bacterial community were indicated by operational taxonomic units (OTUs) and the Chao1 index. Table 5.1 showed the OTUs and the Chao1 value in the WSS without AiiM were higher than that in the WSS with AiiM at the same time (2 days and 10 days) of the incubation process. However, the Shanon index, estimates the diversity of the microbial population, indicated the opposite trend as the OTUs and Chao1 index. Namely, in 2 days and 10 days, the Shanon index of WSS control was lower than that in WSS with AiiM. As a result, the presence of AiiM in WSS during the anaerobic digestion contributed the microbial population diversity.

Table 5.1. Analyses of operational taxonomic units (OTUs) and alpha diversity (Chao1 and Shannon index) for the bacterial community in WSS with or without AiiM (2.4 μg/mL)

		OTUs[a]	Chao1[a]	Shannon index[a]
WSS	0 day	1779.1	2223.9	3.548
	2 days	1794.8	2231.9	3.452
	10 days	1849.7	2183.3	3.014
WSS-AiiM	2 days	1688.7	1975.8	3.515
	10 days	1714.7	1985.6	3.203

[a] Values were defined using a dissimilarity level of 0.03.

5.3.5. Dynamics of bacterial population and the variation of Gram-negative and Gram-positive bacteria in the addition of AiiM lactonase

RNA templates of the WSS with or without AiiM (2.4 μg/mL) were analyzed at day 2 and day 10 of methane fermentation to evaluate the variation in the taxonomy levels and compare the dynamics of microbial community as well as the percentage of Gram-positive bacteria and Gram-negative bacteria. In this study, phylum level was utilized to evaluated the

composition of bacterial community. Table 5.2 and figure 5.6 revealed seven dominant phyla, *Firmicutes, Bacteroidetes*, *Proteobacteria, Plantomycetes, Nitrospirae, Spirochaetes,* and *Actinobacteria* in total 13 phyla which presented in both WSS control and WSS with AiiM. At day 2 of anaerobic digestion, the proportions of *Firmicutes, Actinobacteria*, *and Bacteroidetes* in WSS control were lower than those in the WSS-AiiM. Especially, the percentage of *Firmicutes* phylum significantly increased in WSS with AiiM (16 ± 7 % in WSS-AiiM and 4 ± 2 % in WSS, Table 5.2). Meanwhile, the ten phyla residues had lower percentages in WSS with AiiM than those in the WSS without AiiM. In total 13 phyla presented in WSS and WSS-AiiM, only 2 phyla (*Firmicutes and Actinobacteria*) were belonged to Gram-positive bacteria and both of them possessed higher percentage in WSS with the addition of AiiM than in WSS only (Table 5.2) although a slight reduction of *Actinobacteria* percentage was detected in WSS with AiiM after 10 days. Besides, 11 phyla were belonged to Gram-negative bacteria, in which only *Bacteroidetes* had the lower percentage in WSS only. To confirm the proportion of Gram-positive bacteria in WSS when adding AiiM higher than WSS only in the second day of methane fermentation as showed data in table 5.2 and figure 5.6, we conducted the Gram staining experiment with WSS control and WSS with AiiM. Figure 5.7 showed that the number of Gram-positive bacteria in the WSS-AiiM were higher than in the WSS control. Meanwhile, a lower number of Gram-negative bacteria was observed in WSS with AiiM.

Table 5.2. The abundance ratio of Gram-negative and Gram-positive bacteria in the phyla level in WSS and WSS-AiiM (2.4 μg/mL) after 2 days and 10 days. The data represent the mean ± SD. * Mostly Gram-negative; **: only the *Veillonellaceae* family is Gram-negative

Phylum	Percentage of phylum abundance (%)				
	WSS-2d	**WSS-10d**	**WSS-AiiM-2d**	**WSS-AiiM-10d**	**Gram staining**
Proteobacteria	44 ± 8	34 ± 4	37 ± 8	32 ± 9	Gram-negative
Bacteroidetes	17 ± 3	21 ± 3	21 ± 1	25 ± 4	Gram-negative
Planctomycetes	7 ± 3	3 ± 2	4.8 ± 0.3	3 ± 1	Gram-negative
Nitrospirae	6.9 ± 0.7	1.05 ± 0.07	6 ± 3	0.9 ± 0.6	Gram-negative
Chloroflexi	5.8 ± 0.6	6.0 ± 0.2	4.7 ± 0.8	6.0 ± 0.4	Gram-negative*
Spirochaetes	4.2 ± 0.3	13.1 ± 0.6	1.4 ± 0.4	4 ± 2	Gram-negative
Verrucomicrobia	3.1 ± 0.8	1.3 ± 0.4	2.5 ± 0.2	0.9 ± 0.3	Gram-negative
Acidobacteria	1.0 ± 0.0	0.2 ± 0.0	0.9 ± 0.2	0.45 ± 0.07	Gram-negative
Elusimicrobia	0.5 ± 0.0	0.4 ± 0.2	0.35 ± 0.07	0.3 ± 0.0	Gram-negative
Cyanobacteria	0.4 ± 0.1	0.2 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	Gram-negative*
Chlorobi	0.4 ± 0.1	1.4 ± 0.7	0.5 ± 0.4	0.9 ± 0.6	Gram-negative
Firmicutes	4 ± 2	5.5 ± 0.0	16 ± 7	17 ± 7	Gram-positive**
Actinobacteria	1.7 ± 0.4	6.3 ± 0.9	1.9 ± 0.7	5 ± 1	Gram-positive
Total Gram-negative	89.9 ± 0.3	82.1 ± 0.0	79 ± 8	74 ± 8	
Total Gram-positive	6.0 ± 0.2	11.8 ± 0.9	17.6 ± 0.7	22 ± 8	

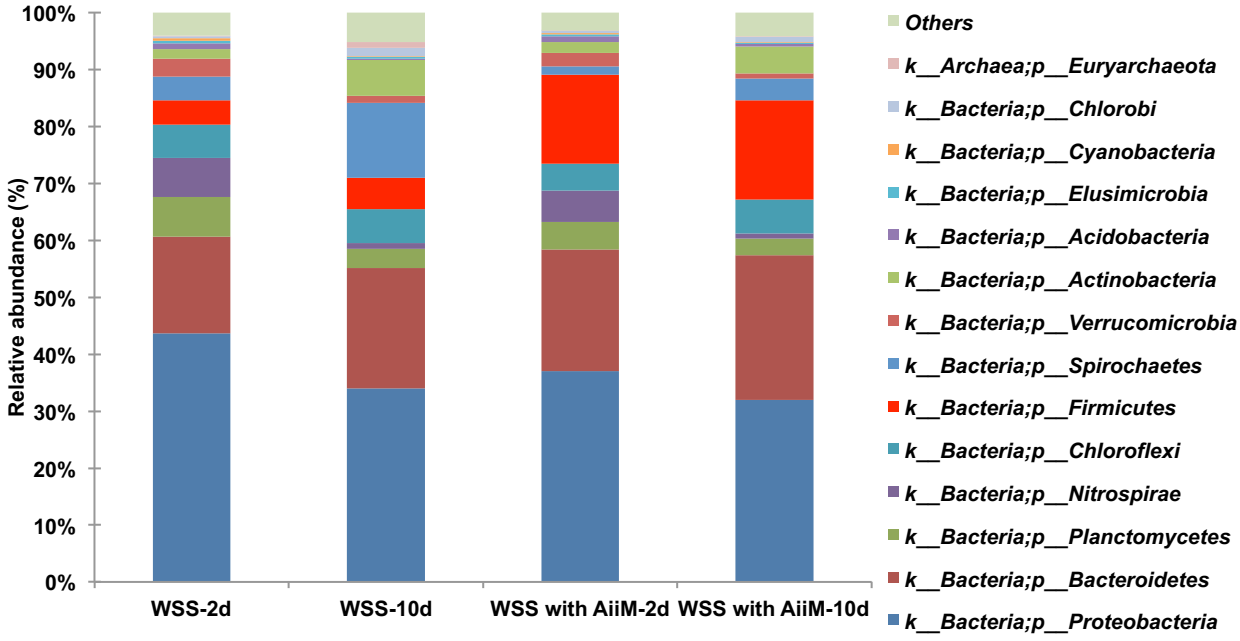

Figure 5.6. Relative abundance of the dominant microbial communities categorized according to the phylum present in WSS with or without the addition of AiiM (2.4 µg/mL). Results were derived from high-throughput 16S rRNA sequencing. Minor phyla present at less than 0.5 % are indicated as "Others."

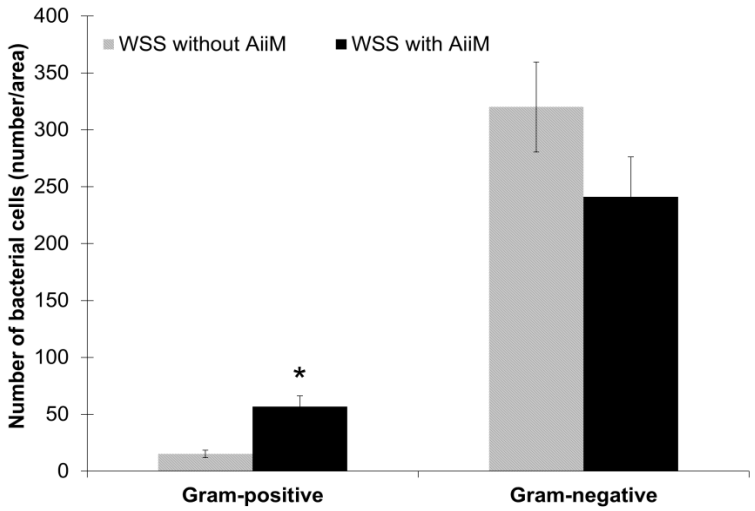

Figure 5.7. The number of Gram-negative and Gram-positive bacteria in WSS with or without AiiM. AiiM was mixed with the final concentration of 2.4 µg/mL. Samples were taken after two days of incubation Error bars indicate standard errors (n = 3). * Indicates a significant difference in Gram-positive bacteria after the addition of AiiM.

The above results indicated the change of the bacterial community in the addition of the AHL-lactonase (AiiM). Since the AHL lactonase has the function in degrading AHL signals from Gram-negative bacteria without any antimicrobial effect, it was considered that the bacterial community should be no different with or without the AHL; therefore, the change in the WSS microbial community is an interesting point of this study. Besides, the percentage of phylum *Firmicutes* and phylum *Bacteroidetes* increase quickly in the addition of AiiM. In which, both of them act an important role in digesting substrate from WSS (Regueiro et al., 2012). Furthermore, the percentage of *Actinobacteria* had increased in the WSS with AiiM at day 2 indicating the rise of the microbial cellulolytic activity (Lynd et al., 2002). From the above results, we hypothesize that the inactivation of AHL-based QS systems for the Gram-negative bacteria by the AHL lactonase may change the production of bactericidal factors in the Gram-negative bacteria; therefore, the effect of the Gram-negative bacteria in the WSS should be decreased and lead to the reduction of percentage of Gram-negative bacteria in microbial community. In natural environment, due to the limitation of the nutrients, many microorganisms can produce the molecules, namely, bactericidal factors or antimicrobial moleculars which are used to inhibit or kill the competitors; this competitions occur among the microorganisms living in the same community and plays an important role in ecological fitness (Clardy et al., 2009; Yan et al., 2018). In many kinds of the Gram-negative and Gram-positive bacteria, QS systems regulate the synthesis of these antimicrobial molecules as a secondary metabolite; in another way, QS is a mechanism that bacteria use to compete with the others (Dubuis et al., 2007; Duerkop et al., 2009). Once a QQ enzyme degrades AHL signals, the Gram-negative bacteria could not produce these factors as a secondary metabolite and therefore their growth could be inhibited by the bactericidal factors produced from Gram-positive bacteria, which are not affected by QQ enzyme. In our study, AiiM was used as a factor to degrade AHL signals, therefore, it was necessary to analyze

AHL signals between WSS control and WSS with AiiM during methane production. Samples from day 2, day 4 and day 10 of methane fermentation were used to detect AHL signals. However, AHL signals were not detected in both samples during analyzed process (data not shown). According to Hu (2017), they can detected AHL signals from week 2 of incubation in sequencing batch biofilm reactor (Hu et al., 2017). Therefore, until 10 days of incubation, AHL signals were not produced enough to analyze. In addition, AHLs is easily converted to the new compound in aqueous environment. Hence, we focused on the proportion of Gram-negative and Gram-positive bacteria to confirm the above supposition. Our Miseq data and the Gram staining result revealed that the Gram-negative bacteria dominated the microbial community in WSS control of anaerobic digestion, with approximate 89.9 % in 2 day and 82.1 % in 10 day of incubation (Fig. 5.6; Fig. 5.7; Table 5.2). The Gram-positive bacteria such as *Firmicutes* and *Actinobacteria*, which contributed for anaerobic digestion, were also presence in WSS control but with the low percentage (Table 5.2). The proportion of Gram-positive bacteria were increased quickly at day 2, encompass the phylum *Firmicutes* and *Actinobacteria*. Meanwhile, the Gram-negative bacteria proportion from WSS with AiiM was decreased, with approximate 79.25 % in 2 days and 73.7 % in 10 days of incubation (Table 5.2). Normally, *Bacteroidetes, Chloroflexi, Firmicutes*, and *Proteobacteria* are phyla which are belonging to the acidogenic bacteria (Venkiteshwaran et al., 2015), thereby they are the predominant bacteria at acidogenesis stage of WSS control in AD process (Fig. 5.6, Table 5.2). However, the percentage of *Cloroflexi* phylum and *Proteobacteria* phylum in the addition of AiiM to WSS were decreased compare to WSS control. Notably, two of the phyla are belong to Gram-negative bacteria. This data contributed for the effect of AiiM to Gram-negative bacteria. Otherwise, although *Bacteroidetes* is Gram-negative bacteria, *Bacteroidetes* was increased in the presence of AiiM in WSS (Table 5.2). Some of research demonstrated the function of species in producing QQ enzyme from *Bacteroidetes* (Mayer et al., 2015;

Romero et al., 2010). Moreover, many genus from *Bacteroidetes* were reported to use QS system via Autoinducer-2 (AI-2) – another kind of quorum sensing molecules (Antunes et al., 2005; Peixoto et al., 2014). These AI-2 QS molecules are different from AHL signals. Therefore, AiiM lactonase could not affect to *Bacteroidetes* via degrade AHL signals

5.3.6. AiiM lactonase is a factor making the transformation of secondary metabolites from WSS

AiiM lactonase is a QQ enzyme that can only affect to QS system without any bactericidal effect and the above results desmonstrated that bacterial dynamics were drastically changed by adding AiiM lactonase when compared to the control. Therefore, we hypothesized that the presence or the absence of AiiM lactonase may change the production of antimicrobial compounds because this enzyme can inactivate the AHL-based QS system. Hence, antimicrobial molecules (organic compounds) in the WSS-contorl or WSS-AiiM were extracted using the SPE system and each SPE fraction was used to test a bactericidal effect by using some bacterial strains: *C. violaceum* CV026, *S. typhi* 14028 and *A. tumefaciens* NTL4 PZLR4-traG:lacZ as the representative Gram-negative bacterial strains and *B. subtilis* 168, *Brevibacillus* sp. KH3, and *L. lactis* 12007as the presentative Gram-positive strains. Interestingly, the SPE fraction from the WSS control revealed a noteworthy ability to prevent the growth of Gram-positive strains, *B. subtilis* 168, *Brevibacillus* sp. KH3, and *L. lactis* 12007 (Fig. 5.8a, 5.8b, 5.8c) although it did not influence the growth of Gram-negative strains, *C. violaceum* CV026, *S. typhi* 14028, and *A. tumefaciens* NTL4 PZLR4-traG:lacZ (Fig. 5.8d, 5.8e, 5.8f). In contrast, the SPE solution from the WSS with the addition of AiiM inhibited the growth of Gram-negative bacteria but did not have any effect to the Gram-positive bacteria. Meanwhile, methanol as a solvent to dissolve organic compound did not affect to bacterial growth represented to no inhibition halo (data not shown). Moreover, when checked

the antibiotic ability of the extracted solution to Gram-positive anaerobic bacteria (*Lactobacillus lactis*, *Streptococcus suis*) isolated from WSS, we found the growth *Lactobacillus lactis* was inhibited by WSS extracted solution, while *Streptococcus suis* did not have any inhibition (data not shown).

B. subtilis 168 (Gram-posittive bacteria) and *C. violaceum* CV026 (Gram-negative bacteria) were continued to use for quantitative assay in the addition of SPE-WSS extracted or SPE-WSS-AiiM extracted within 30 h of incubation (Fig. 5.8g, 5.8h). The number of surviving Gram-positive bacteria *B. subtilis* decreased quickly in the presence of 1 % SPE-WSS within 2h of incubation. In contrast, 1 % of SPE-WSS-AiiM did not effect to the growth of *B. subtilis* (Fig. 7g). Especially, the turbidity of *B. subtilis* 168 became clear after 24 h of incubation with 1 % SPE-WSS control (Fig. 5.9). This phenomenon indicated *B. subitlis* cell can be lysed under the treatment of SPE-WSS. In contrast, 1 % of SPE-WSS-AiiM did not influence the growth of *B. subtilis* 168. As mention before, AHL signals could be converted to AHL metabolite. In which, tetramic acid, a product from the AHL hydrolysis process, is an antibiotic compound which only kill Gram-positive bacteria (by cell lysis) (Kaufmann et al., 2005). Otherwise, the results revealed that the number of surviving *C. violaceum* CV026 reduced in the incubation with both concentrations of SPE-WSS-AiiM (0.5 % or 1 % v/v), meanwhile no bactericidal effect was observed in the presence of 1 % SPE-WSS (Fig. 7h). In addition, under the treatment of SPE-WSS-AiiM, the growth of *C. violaceum* CV026 was inhibited but unlike the case of *B. subtilis* 168 under the treatment of SPE-WSS. Consequently, it demonstrated the presence of AHL signals in WSS control rather than in WSS-AiiM.

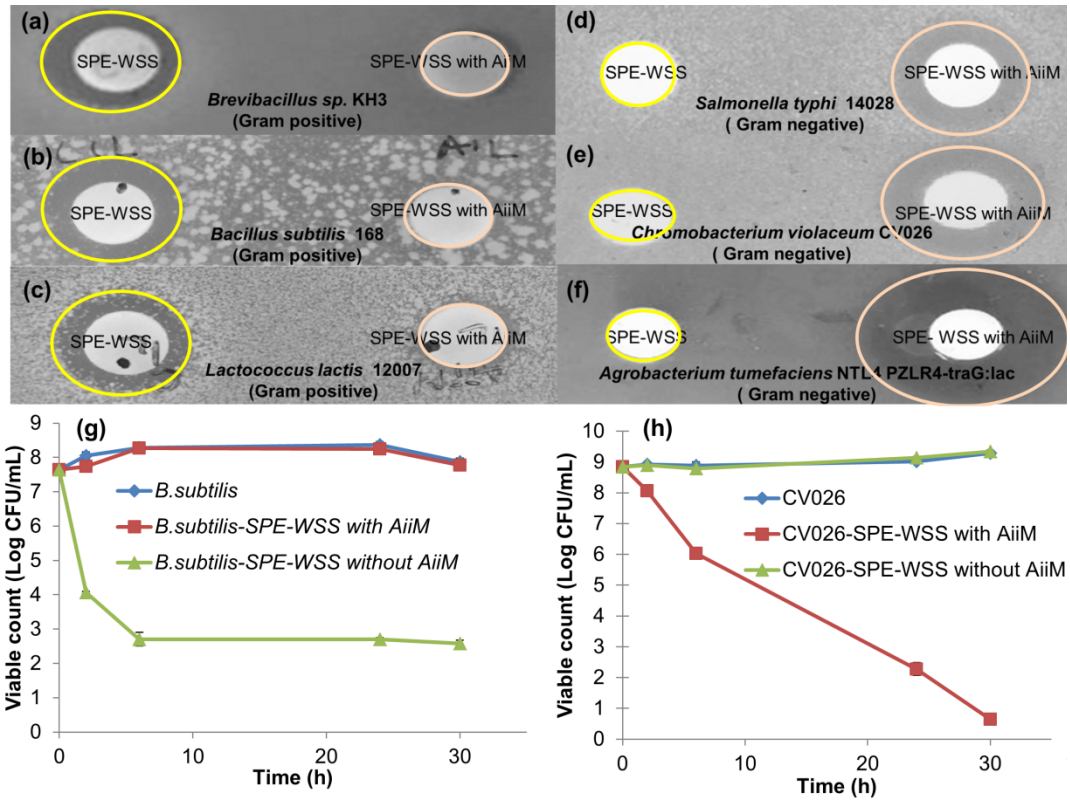

Figure 5.8. Antibiotic ability from WSS and WSS-AiiM by SPE extraction. Zone of inhibition against *Brevibacillus* sp. KH3 (a); *Bacillus subtilis* 168 (b) and *Lactococcus lactis* (c) from WSS. Zone of inhibition against *S. typhi* 14028 (d); *Chromobacterium violaceum* CV026 (e); *Agrobacterium tumefaciens* NTL4 PZLR4-traG:lacZ from WSS-AiiM (f). The survival of *B. subtilis* 168 (g) and *C. violaceum* CV026 (h) with SPE-WSS (1 % v/v) and SPE-WSS-AiiM (1 % v/v). *Error bars* indicate standard errors (n = 3)

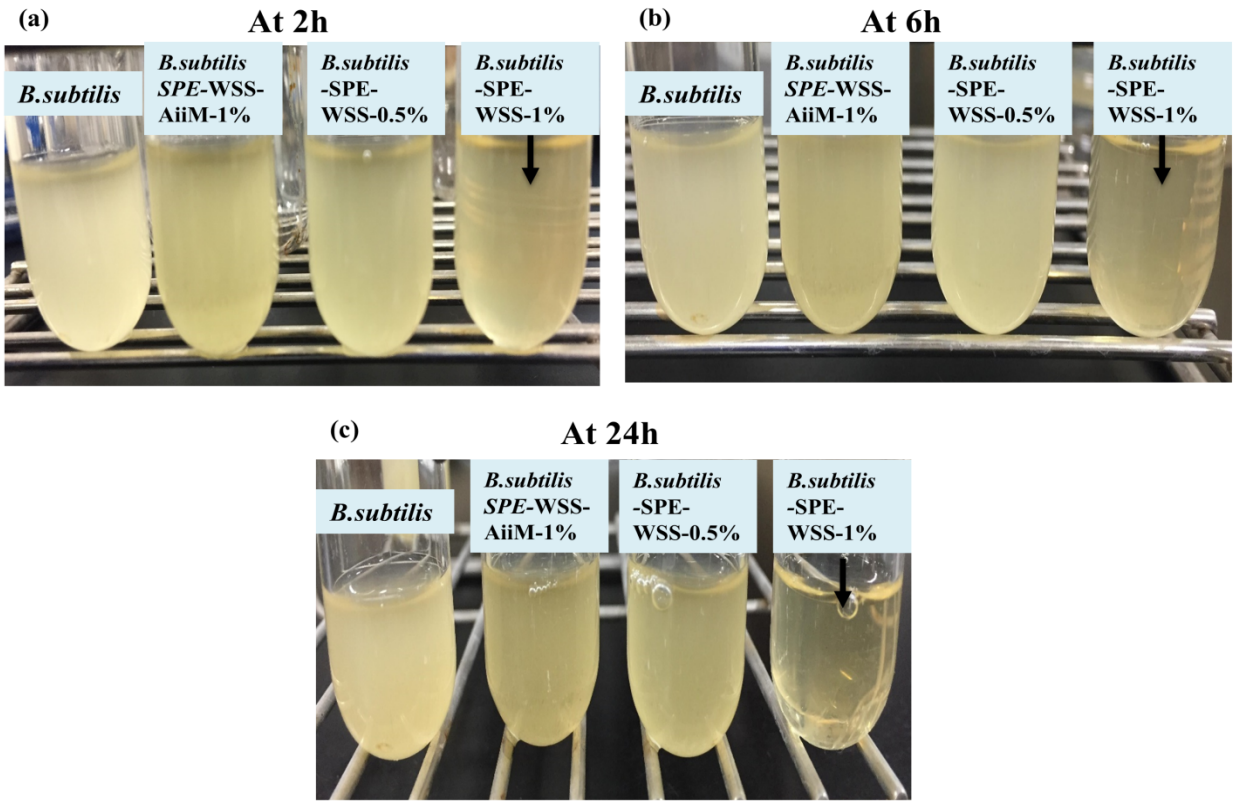

Figure 5.9. The effect of SPE extracted solution in the survival of *Bacillus subtilis*. At 2 h (a), the turbidity of all medium containing *B. subtilis* was the same. At 4 h (b) and 24 h (c), the turbidity of *B. subtilis* sample with 1 % SPE-WSS (black arrow) became clearer.

In WSS, there are many types of antibiotic molecules can be produced by microorganism. It is difficult to analyse each of antibiotic compound presented in WSS; hence, the identification of antimicrobial molecules should be further studied. The result of the bacterial community data showed that two Gram-negative phyla, *Proteobacteria and Chloroflexi*, are decreased in WSS with AiiM. Therefore, the bactericidal factors produced by these two phyla without the presence of AiiM may inhibit the Gram-positive bacteria. The synthesis of antimicrobial secondary metabolites of some of bacteria which are regulated by QS system inhibit other competitors including bacteria, fungi, protozoa, and nematodes (Dubuis et al., 2007; Duerkop et al., 2009). For instance, some of research demonstrated the function of species in producing QQ enzyme from *Bacteroidetes* (Mayer et al., 2015; Romero et al., 2010). Moreover, many genus from *Bacteroidetes* were reported to use QS system via

autoinducer-2 (AI-2) – another kind of quorum sensing molecules (Antunes et al., 2005; Peixoto et al., 2014). These AI-2 QS molecules are different from AHL signals. AiiM lactonase could not affect to *Bacteroidetes* via degrade AHL signals. Therefore, the bactericidal factors of *Bacteroidetes* could prevent Gram-negative bacteria utilizing AHL-based QS. Besides, *Firmicutes* produce various bacteriocins to inhibit Gram-negative bacteria (Ananou et al., 2005; Smaoui et al., 2010) and *Actinobacteria* are a resource for antibiotics and quorum quenching compounds against both Gram-negative bacteria and Gram-positive bacteria (Barka et al., 2016; Undabarrena et al., 2016). Therefore, some of the Gram-negative bacteria could be inactived under the presence of antimicrobial activities from the two phyla, *Firmicutes* and *Actinobacteria.*

Finally, the effect of AiiM to anaerobic digestion process was demonstrated in figure 5.10. The mechanism is proposed like this: Firstly, under normal condition, Gram-negative bacteria predominate in WSS control during methane fermentation and created virulence factor via the regulation of QS mechanism to inhibit the growth of Gram-positive bacteria. In this case, the pH always stable and the value was approximately 6.8 to 8. This pH range was consistent with the growth of Archaea. Consequently, the high percentage of Archaea produced the high methane amount. Secondly, when AiiM was added to WSS, AiiM could degraded HSL signals from Gram-negative bacteria, therefore, Gram-negative bacteria could not produce bactericidal factor via the regulation of QS mechanism - activated by the high density of HSL signals. Hence, Gram-positive bacteria became stronger than Gram-negative bacteria in the survival competition, they started to produce their bactericidal factors against Gram-negative bacteria as well as produced the secondary metabolites like organic acids. Namely, *Firmicutes* phylum increased quickly in the presence of AiiM and produced more organic acid (acetic acid). As a result, the pH was decreased and inhibited the growth of Archaea. The low percentage of Archaea produced the low methane amount.

5.4. Conclusion

In conclusion, QQ enzyme AiiM lactonase, which degrade lactone ring of acyl homoserine lactone (AHL) signals from Gram-negative bacteria, reduced the QS-regulated factors of Gram-negative bacteria in WSS and lead to inhibit the methane production. Due to the different antibiotic factors produced in the addition of AiiM, namely, some of Gram-negative bacteria were killed by adding SPE fraction from WSS with AiiM while the growth of some of Gram-positive bacteria were reduced in the presence of SPE fraction from WSS without AiiM, it demonstrated that AiiM influences the change of bacterial community. Therefore, QS signals among Gram-negative bacteria in a microbial consortia is very important for the production of methane.

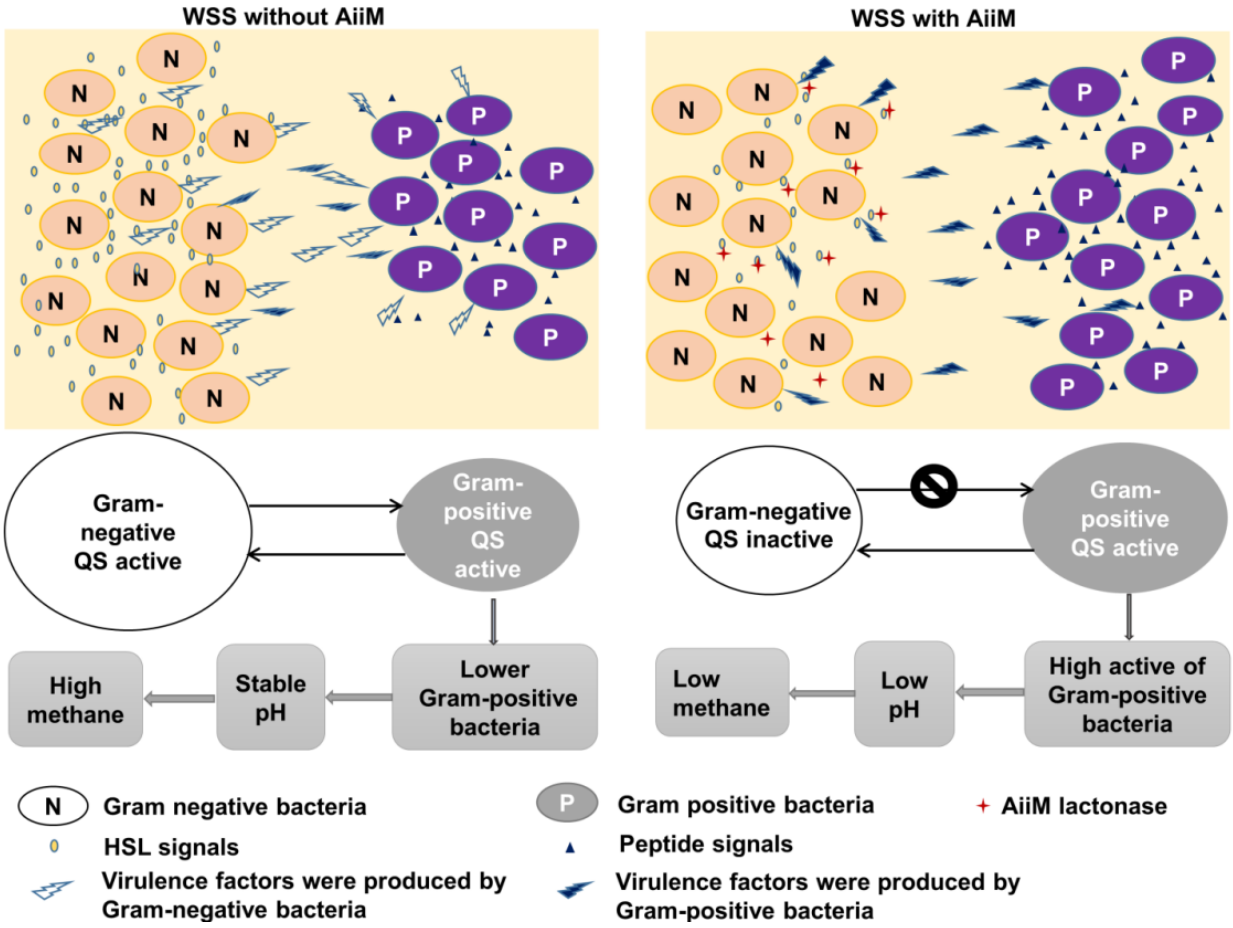

Figure 5.10. The proposed model for microbial community interaction from WSS in anaerobic digestion process with and without the addition of AiiM. In WSS

without AiiM or in normal condition, Gram-negative bacteria hole the predominant in anaerobic digestion process and produced bactericidal factors to inhibit the growth of Gram-positive bacteria, in this case, pH value is suitable for methane fermentation process. As a result, methane production was induced. In the addition of AiiM, AiiM degraded HSL signals produced by Gram-negative, therefore the competition of Gram-negative decrease meanwhile the proportion of Gram-positive, especially *Firmicutes* which were known as acid producing bacteria, increase. Since then, pH decrease and lead to the inhibition of methane production.

CHAPTER 6

EFFECT OF C-30 AND LYSOZYME IN METHANE PRODUCTION DURING ANAEROBIC DIGESTION PROCESS

6.1. Introduction

Many bacterial populations can control their population density and regulate the expression of their gene via QS system (Fuqua et al., 1994). QS signals are utilized to coordinate and synchronize several interactions in different environments, such as microorganisms interactions or host–microbe interactions (Grandclément et al., 2015a). Under biological wastewater treatment systems, waste sewage sludge (WSS) which contains a huge number of microbes that exist in granules, biofilms and flocs was produced (Mikkelsen et al., 2002; Oh et al., 2013; Ramos et al., 1994). The microorganism in WSS including Gram-negative bacteria and Gram-positive bacteria can degrade the large amount of compounds during anaerobic digestion. Therefore, it was considered that there are many interactions via QS systems between Gram-negative bacteria and Gram-positive bacteria in WSS.

Methane production is a bioenergy source which can be produced during anaerobic digestion of WSS to reduce the amount of WSS. Besides, methane production is a microbiological process (Segers, 1998). Moreover, when comparing the difference of active bacterial communities in different WSS samples, Mustapha et al found that a key microorganism could be become an accelerator or a suppressor in the methane fermentation (Mustapha et al., 2018; Mustapha et al., 2017). The results revealed a complicated bacterial interaction and showed the need to understand the bacterial network for enhanced methane production. Among the microorganisms in WSS, bacteria may act an important function in WSS degradation and consists of mainly Gram-negative and Gram-positive bacteria. QS

systems among Gram-negative bacteria and Gram-positive bacteria should be active when WSS is a bacterial community. In a part of the Gram-negative bacteria, AHL signals can be used for activating the bacterial functions (Dubuis et al., 2007; Duerkop et al., 2009). On the other hand, Gram-positive bacteria regulate the bacterial virulence and activity via oligopeptides (Sturme et al. 2002). In addition, some of Gram-negative and Gram-positive bacteria can share AI-2 signal as a common quorum sensing system used for interspecies communication (Papenfort et al., 2016). The interaction between Gram-negative and Gram-positive bacteria may be important for the performance of methane fermentation. Therefore, the motivation of this study was to understand the effect of QS system and the power balance between the Gram-negative and the Gram-positive bacteria in the methane fermentation. In this study, two approaches were investigated. One is the effect of brominated furanone QSI, C-30, by which QS system via AHLs can be inactivated during the methane fermentation. Another approach is lysozyme, a Gram-positive bacteria killing compound that was utilized to evaluate its function in methane fermentation of WSS. Our results demonstrated that C-30 furanone inhibits methane production by reducing the pH together with increasing the percentage of Gram-positive bacteria. In contrast, lysozyme enhanced methane production by reducing the percentage of Gram-positive bacteria.

6.2. Materials and Methods

6.2.1. Waste sewage sludge source and preparation

WSS was sampling from the Hiagari wastewater treatment plant in Kitakyushu City, Japan. WSS was centrifuged at 8000 g in 10 min at 4 °C by TOMY-GRX 250 High Speed Refrigerated Centrifuge and then was washed with distilled water for at least 3 times. The purpose of washing step was mentioned above.. After washing step, WSS was adjusted by distill water to get 10 % (w/w) for further studies.

6.2.2. Methane production in the presence of C-30 or lysozyme

WSS (30 mL) containing 500 μM C-30 or 150 μL ethanol 98 % (same volume to dissolve 500 μM C-30) was prepared in 66-mL vials. Rubber stopper and aluminum cap was used to seal vials and nitrogen gas was added into these vials (2 min) to create the anaerobic condition. Control WSS without any addition was prepared as the same way. These vials were then incubated with 120 rpm at 37 ºC in 10 days. Each experiment was performed at least in triplicate. 100 μL of headspace gas from each vial was collected during the fermentation and injected to GC-3200 gas chromatograph (GL Sciences, Japan) to measure methane gas as previously described (Mohd Yasin et al., 2015).

For methane production in the addition of lysozyme, WSS (30 mL) containing, 0.1, 0.5 or 1 % lyzosyme was prepared in a 66-mL vial. Control WSS without any addition was prepared similarly. Then the same procedure as methane production with C-30 was conducted. Additionally, casein (1 %) was supplied to the WSS to check the effects of protein on methane production. In addition, another experiment was conducted using 0.1 % of lysozyme and without lysozyme in the anaerobic incubation within 7 days, then 1 % lysozyme was added to the samples to see the effect Gram-positive bacteria at later stage of anaerobic digestion process.

6.2.3. Other analytical methods

pH was determined using a compact pH meter (AS ONE, AS-211, Japan). Gram staining was conducted by using WSS control, WSS-lysozyme or WSS-C-30 which were incubated in anaerobic condition for 2 days or 10 days (as described in methane production preparation). After 2 days or 10 days, 2 mL of samples were collected and filtered by a 5 μm pore size filter paper (Toyo Roshi Kaisha, Ltd, Japan) to collect only the microbial cells. These microbial cells were stained by the Gram staining method. First, 10 μL of each sample

was transferred and gently spread onto a glass slide; the samples were fixed on glass slide by heat fixation and stained with a Gram Stain kit (BD, USA). After that, the glass slides contained samples were observed at a 100 × magnification by a microscope (Olympus, BX51, Japan), and the number of Gram-positive bacteria and Gram-negative bacteria were counted (5 random positions were chosen). Each experiment was performed at least in triplicate.

6.2.4. Total RNA extraction, cDNA synthesis and quantitative RT-PCR

RNA extraction and cDNA synthesis from the pellets of WSS, WSS with ethanol and WSS with C-30 or control WSS and WSS with 1 % lysozyme was conducted as described previously (Mustapha et al., 2017) (Chapter 2). High-throughput 16S rRNA sequencing and data analyzing were conducted as described previously for WSS with and without C-30 (Chapter 2).

6.2.5. The survival of anaerobic bacteria in WSS with C-30 during anaerobic incubation

The number of anaerobic bacteria was performed by using the samples of WSS control, WSS-C-30 which were incubated anaerobically for 2 days or 10 days (as described in methane production preparation). After 2 days and 10 days, 0.1 mL of samples were taken, diluted and spread on LB agar plate. LB agar plates were incubated at 37 °C, in anaerobic condition. The survival of anaerobic bacteria in WSS with and without C-30 was determined by counting the number of colonies from incubated LB agar plates. Each experiment was conducted at least in triplicate.

6.2.6. Confirm the function of lysozyme in killing Gram positive bacteria

Two strains of bacteria, *E.coli* BW25113 which belong to Gram negative bacteria and *B. subtilis* 168 which belong to Gram positive bacteria were chosen to test the effect of

lysozyme. These two strains were incubated in LB medium at 37 °C, 121 rpm for overnight. These overnight cultures were then inoculated in 50 mL LB medium with and without 0.1 % lysozyme to get the initial turbidity 0.05 and incubated at 37 °C, 121 rpm for 24 h. Each time spans, 1 mL of samples were taken and used to measure the absorbance at OD600 wavelength to determine the growth of two strains. Each experiment was conducted at least in triplicate.

6.2.7. Statistical analysis

Means were collected from at least triplicate data (n = 3). The statistical analysis was performed by the Student's t test (GraphPad software) at a significance level of $p < 0.05$.

6.3. Results and discussion

6.3.1. The effect of C-30 furanone in methane production during anaerobic digestion

6.3.1.1. Methane production in the addition of C-30

By adding AiiM lactonase, an enzyme catalyzes the lactone ring breakdown of AHL signals, the effect of AHL-regulated QS of Gram-negative bacteria on methane fermentation was analyzed. The result showed that when AHL-regulatedd QS of the Gram-negative bacteria was inactivated, methane production was also inhibited (Chapter 5). C-30 furanone was known as a QQ compound, which has the same function as AiiM lactonase in disrupting the QS signal from Gram-negative bacteria (Hirakawa et al., 2013). However, the way to interrupt QS system of Gram-negative by C-30 was different from AiiM. AiiM degrade HSL signals outside of bacteria cell while C-30 compete the binding site with HSL signals (Costas et al., 2015; Wang et al., 2010a, 2010b). Therefore, C-30 should be checked as another kind of QSI to QS of bacteria in methane production of WSS. C-30 at 500 μM was used to check the effect of AiiM on methane production. Similar to AiiM lactonase, methane production

using WSS was significantly inhibited in the presence of C-30 (Fig. 6.1). Within 6 days of incubation, methane was not produced in WSS with C-30 while WSS with ethanol had higher amount of methane than WSS only. It means that C-30 was the factor inhibited methane production. Or in another way, the interruption of QS system of bacteria led to the inhibition of methane production.

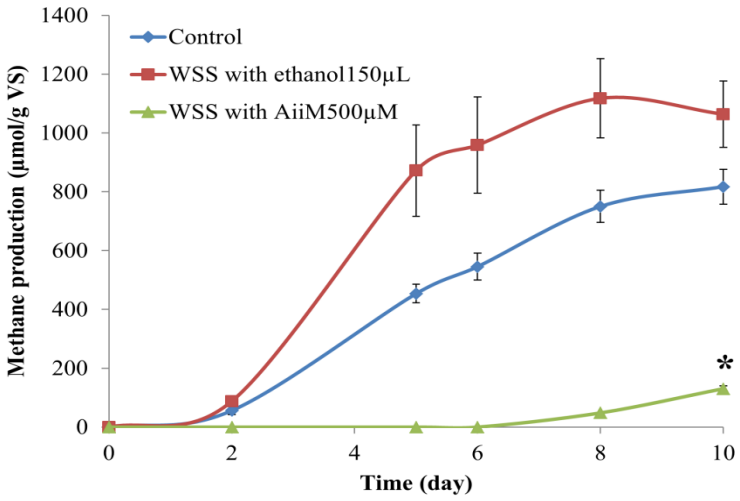

Figure 6.1. Impact of C-30 furanone on methane fermentation using waste sewage sludge. WSS control (*blue diamond*), ethanol at 150 µl (*red square*), and C-30 at 500 µM (*green triangle*) are shown. *Error bars* indicate standard errors (n = 3). * Indicate the significant difference in the presence of C-30

6.3.1.2. pH variation in the presence of C-30 in WSS

As previous results, pH reduction was the main reason to inhibit methane production in WSS with AiiM (Chapter 5). In this study, we want to confirm whether the addition of C-30 can reduce the pH in anaerobic digestion like the addition of AiiM or not. pH value was measured at day 0, day 2 and day 10 of anaerobic incubation. At day 0, all pH values of all samples were similar (around pH 6.5). However, at day 2, only WSS control was slightly decreased to pH 6.3. Meanwhile, the pH of WSS with ethanol and WSS with C-30 were significantly decreased to 5 (Fig. 6.2). Based on a report from Lay et al in 1998, the optimum of anaerobic digestion could be established when pH values are around 6.6-7.8 and the

production of methane is inhibited at less than pH 6.3 (Lay et al., 1998). Also, since organic acids can inhibit methanogenesis stage during the anaerobic digestion due to the low pH, methane fermentation was reduced and this inhibition is stronger at a lower pH value (Venkiteshwaran et al., 2015). Therefore, methane production of WSS with ethanol should be inhibited rather than increased. However, other results showed ethanol is a substrate for methane production because it can be converted to acetate – main substrate for methane production (Schink et al., 1985; Yang et al., 2015). Besides, acetate had been reported to increase in the addition of AiiM to WSS (Chapter 5). Therefore, the main reason for inhibiting methane is not only pH but also other factors that can combine with pH. As a result of Chapter 5, Gram-positive bacteria increased with the presence of AiiM lead to pH reduction. Here, we tried to confirm the increasing of Gram-positive bacteria in the addition of C-30.

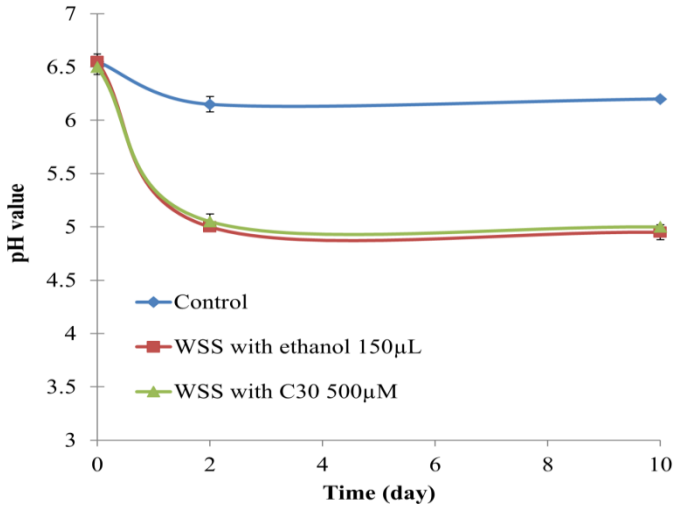

Figure 6.2. pH variation in WSS with and without C-30.

6.3.1.3. The proportion of Gram-negative bacteria and Gram-positive bacteria in WSS with or without C-30

The taxonomy levels, the dynamics of bacterial community as well as the proportion of Gram-negative and Gram-positive bacteria were compared by analyzing the RNA templates of WSS with or without C-30 at day 10 of anaerobic incubation. The composition of bacterial community was displayed at a phylum level and *Proteobacteria, Bacteroidetes, Firmicutes, Chloroflexi, Spirochaetes,* and *Actinobacteria* were the six dominant phyla in the 12 phyla analyzed (Figure 6.3). In which, *Firmicutes* and *Actinobacteria* belong to Gram positive bacteria. The phylum *Firmicutes* was significant increased in the WSS with C-30 samples, while the percentage of *Actinobacteria* in WSS was similar to WSS with C-30 (Figure 6.3). Besides, Gram staining was performed on day 10 of anaerobic incubation. As expected, the proportion of Gram-positive bacteria was increased in the sample with the supplement of C-30. Meanwhile, a lower number of Gram-negative bacteria was observed in WSS with C-30. WSS control and WSS with ethanol had the similar percentage of Gram-positive bacteria and Gram-negative bacteria (Fig. 6.4). Under the treatment of QQ, QS system of Gram-negative bacteria was inactivated. Otherwise, QS systems in Gram-negative bacteria and Gram-positive bacteria can control the production bactericidal factors (Dubuis et al., 2007; Duerkop et al., 2009). In chapter 5, we proved that the production of bactericidal factor in Gram-negative bacteria could be changed when the AHL-regulated QS systems of the Gram-negative bacteria were inactivated by the AHL lactonase; thereby, the proportion of the Gram-negative bacteria in the WSS were reduced. This conclusion is also suitable with WSS under the treatment of C-30, once the QS system was interrupted by C-30, Gram-negative bacteria could not produce bactericidal agents; thus, the effect of the Gram-negative bacteria in the microbial community is reduced. In fact, microorganisms have many pathways to create bactericidal elements which kill or inhibit the competitors. Otherwise, furanone is not only

inhibit AI-1 system (QS system in Gram-negative bacteria) but also inhibit AI-2 system which was used in both Gram-negative and Gram-positive bacteria (Ren et al., 2005). The effect of C-30 to methane production seems stronger than AiiM lactonase because it can inhibit two types of QS system. According to our results, percentage of Gram-positive bacteria was higher than Gram-negative bacteria. This is due to most of Gram-positive bacteria use peptide-based signaling systems (Montgomery et al., 2013) which is not affected by C-30. As a result, the percentage of Gram-negative bacteria was decreased meanwhile Gram-positive bacteria was increased in WSS with C-30.

As mentioned before, the main reason for inhibiting methane is not only pH, but also other factors that can be combined together. In addition, in this section, we found that Gram-positive bacteria increased in the presence of C-30. Consequently, the other factor was the increasing of Gram-positive associated with a reduction in pH to inhibit methane production in WSS.

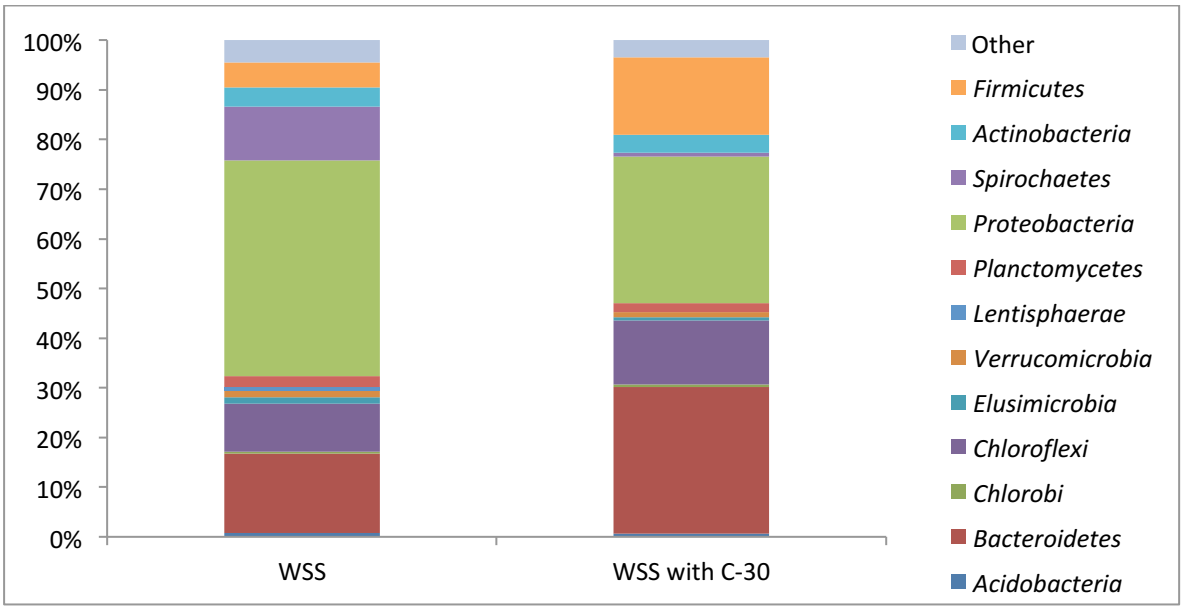

Figure 6.3. Relative abundance of the dominant microbial communities categorized according to the phylum present in WSS with or without the addition of C-

30 (50 µM). Results were derived from high-throughput 16S rRNA sequencing. Minor phyla present at less than 0.5 % are indicated as "Others."

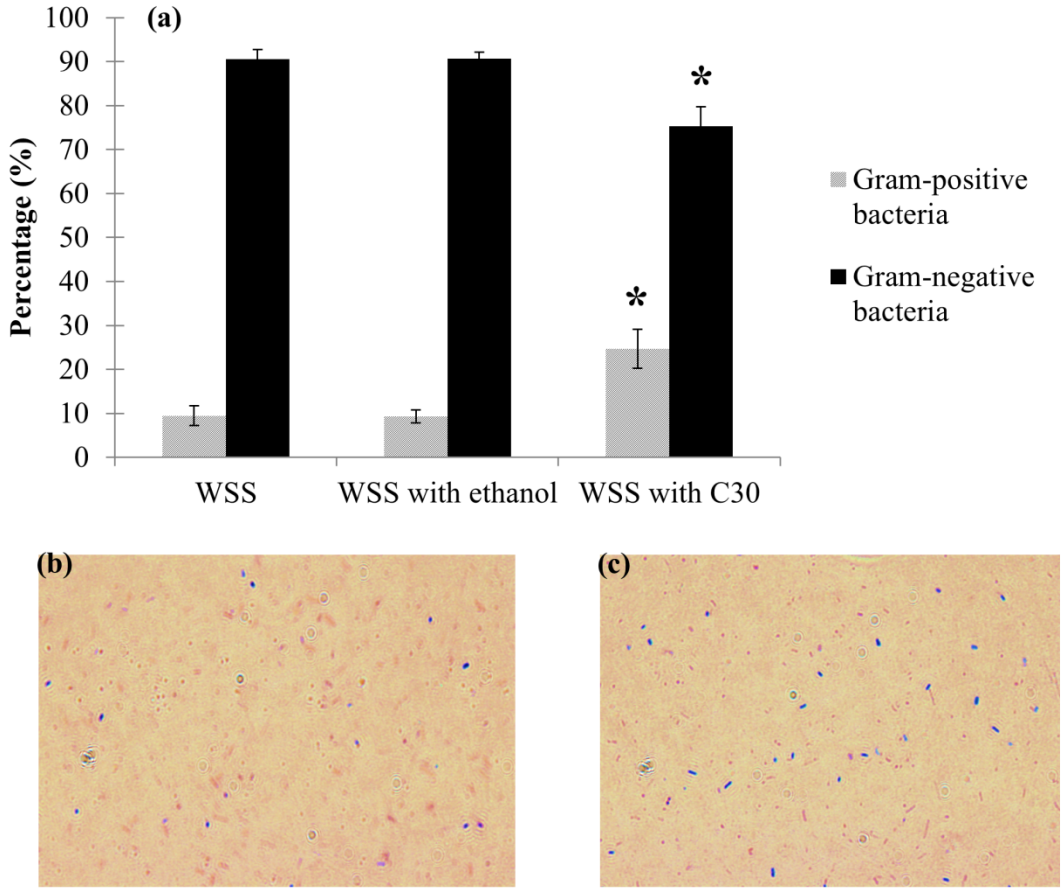

Figure 6.4. The proportion of Gram-negative and Gram-positive bacteria in WSS with or without C-30 at day 10 of anaerobic incubation. (a) Percentage of Gram-negative and Gram-positive bacteria in WSS control, WSS with ethanol and WSS with C-30. (b) Gram staining of WSS with ethanol, (c) Gram staining of WSS with C-30. Samples were observed under microscopy at 100x. *Error bars* indicate standard errors (n = 3). * Indicate the significant difference in the presence of C-30

6.3.1.4. The survival of bacteria in WSS with C-30 during anaerobic incubation

C-30 interrupts QS system of Gram-negative bacteria rather than kill them (Wu et al., 2004). By adding C-30 to WSS in anaerobic condition, we found that C-30 can inhibit methane production. Methane production is a microbiological process (Segers, 1998). Therefore, the reduction of the total bacteria number is one of the reasons that inhibit methane

production. To make sure C-30 does not act as a toxic compound to bacteria, we conducted an experiment to count the number of bacteria in WSS with and without C-30 at day 2 and day 10 of the anaerobic process. Figure 6.5 showed that the number of anaerobic bacteria did not affect by the addition of C-30 to WSS. Even the number of bacteria was slightly increased at day 10 of incubation in WSS with C-30. Besides, bacterial activities and archaea activities at day 10 of incubation of WSS with and without C-30 were checked using RT-PCR analysis. The total activity of archaea in the WSS control was higher than WSS in the presence C-30 demonstrating the higher methane production in WSS (Fig. 6.6). Meanwhile, there was no difference in bacterial activities between WSS and WSS with C-30. Therefore, the inhibition of methane production was not because of the toxicity of C-30 to bacteria.

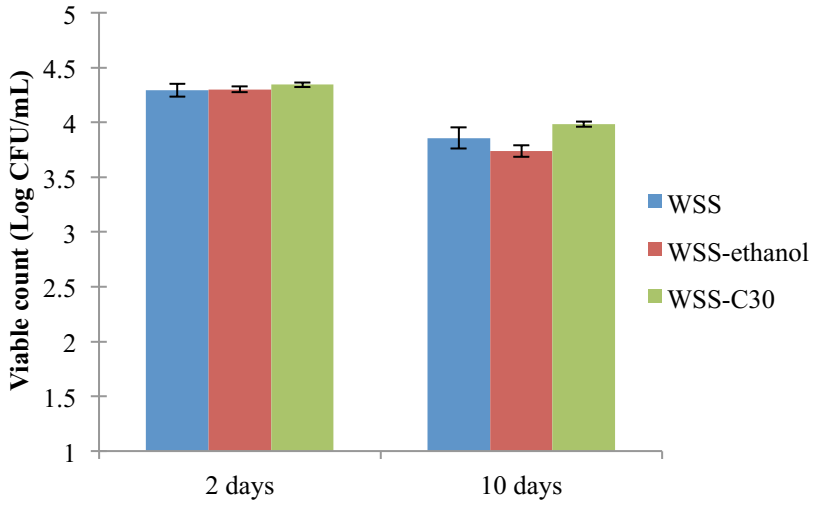

Figure 6.5. The survival of anaerobic bacteria in WSS with and without C-30. *Error bars* indicate standard errors (n = 3)

Similar to the AiiM, the inhibition of methane production in the presence of C-30 demonstrated that QS system of Gram-negative bacteria play an important role in contributing methane production.

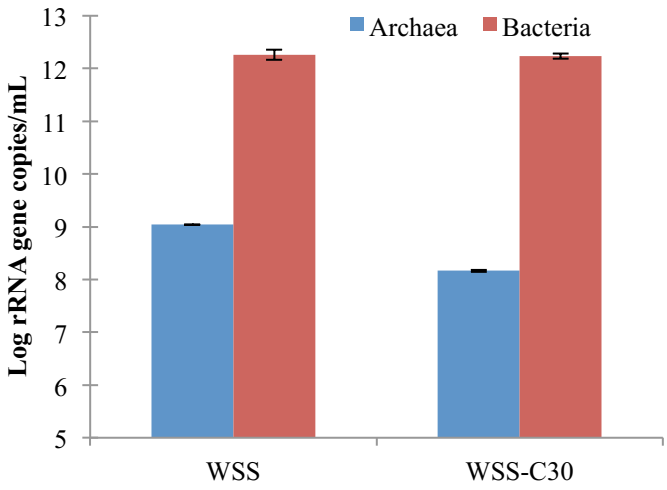

Figure 6.6. Bacterial and archaeal activities in WSS with and without C-30. *Error bars* indicate standard errors (n = 3)

6.3.2. The effect of lysozyme in methane production during anaerobic digestion

In previous section, we have reported the importance function of Gram-negative bacteria in methane production process. In this section, we tried to investigate the role of Gram-positive bacteria in methane production during anaerobic digestion by using lysozyme – Gram-positive bacteria killer.

6.3.2.1. Lysozyme activity in the incubation with Gram-negative bacteria and Gram-positive bacteria

Lysozyme was known as an antimicrobial protein that can kill Gram-positive bacteria (Nash et al., 2006). To confirm lysozyme activity, we conducted the experiment using 0.1 % lysozyme in the incubation of *E. coli* BW25113 (represent as Gram-negative bacteria) and *B. subtilis* 168 (represent as Gram-positive bacteria). After 20 h of incubation, only *B. subtilis* 168 with lysozyme did not grow. Meanwhile, the growth of *E. coli* BW25113 with 0.1 % lysozyme was faster than *E. coli* BW25113 only during 10 h of incubation (Fig. 6.7). Gram-negative have a double lipid bilayer which separated by peptidoglycan and periplasm while

the peptidoglycan surround to the single lipid bilayer of Gram-positive bacteria (Kashef et al., 2017). Lysozyme degrades peptidoglycan membrane in the bacterial cell wall of Gram-positive bacteria leading to its rapid death, but does not kill Gram-negative bacteria (Nash et al., 2006). Our result confirmed the activity of lysozyme in killing only Gram-positive bacteria and enhancing the growth of Gram-negative bacteria. Therefore, it was expected that lysozyme only inhibited Gram-positive bacteria in bacterial consortia such as in WSS bacterial community during anaerobic digestion.

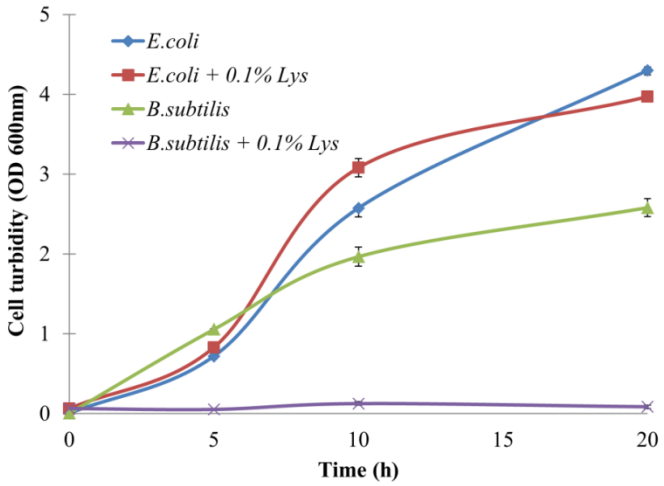

Figure 6.7. Bacteria growth in LB medium with and without 0.1% lysozyme. *E. coli* BW25113 (*blue diamond*), *E. coli* BW25113 with 0.1 % lysozyme *(red square)*, *B. subtilis* 168 *(green triangle)*, *B. subtilis* 168 with 0.1 % lysozyme *(violet x)* are shown. *Error bars* indicate standard errors (n = 3).

6.3.2.2. Methane production in the presence of lysozyme

Different concentrations of lysozyme (0.1 %; 0.5 % and 1 %) were used to check the effect of lysozyme on methane production. The addition of lysozyme has increased methane production from day 4 to day 10 of anaerobic incubation, specifically higher concentration of lysozyme enhanced higher methane amount (Fig. 6.8a). At day 2, the methane amount of WSS with 1 % lysozyme was lower than others (control, 0.1 % and 0.5 % lysozyme),

however, from day 4, it increased rapidly and achieved the highest methane amount compared to others. This indicated that with the high concentration of lysozyme, Gram-negative bacteria could not grow and affected to the earlier stage of anaerobic digestion process (Fig. 6.8a). Casein (negative control) was also added to WSS at the same concentration as lysozyme (1%, highest concentration) to see the effect of protein during the methane fermentation. Protein was considered as a nutrient source for methane fermentation (Kovács et al., 2015). As a result, the methane fermentation using casein was increased within 8 days of anaerobic incubation, however it had the similar amount of methane as control on day 10 (Fig. 6.8b). Meanwhile, methane production with the addition of 1 % of lysozyme was increased significantly from day 4 to day 10 of incubation. To test whether the high concentration of lysozyme or the non-active Gram-positive bacteria can inhibit methane production at later process, we added 1 % of lysozyme to WSS with 0.1 % lysozyme sample on the 7th day of the incubation process, the control was added the same amount of dH_2O used to dissolve lysozyme. The result showed that lysozyme did not inhibit methane production in later stages of anaerobic digestion (Fig. 6.9). This showed that Gram-positive bacteria did not affect on methane production in the later stage of anaerobic digestion. Taken together, methane production using WSS was induced by the addition of lysozyme.

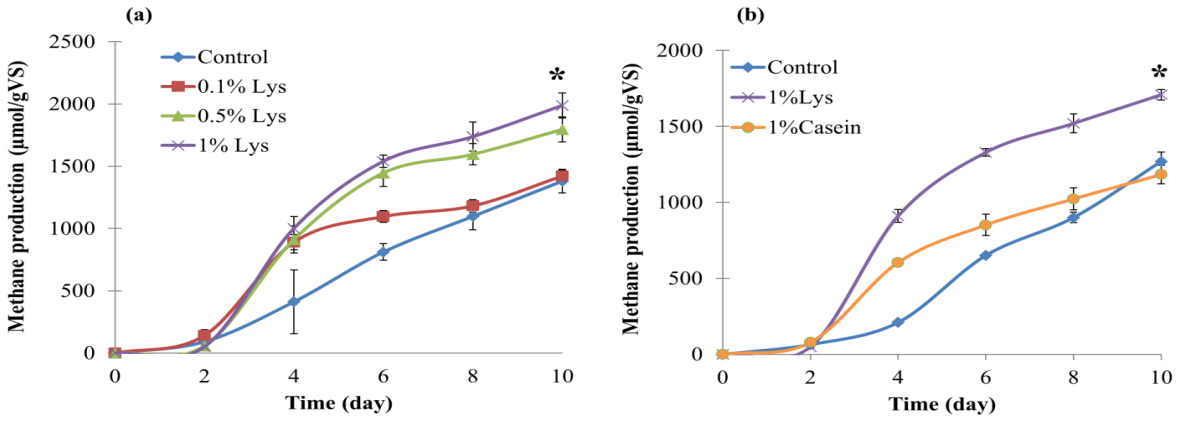

Figure 6.8. Methane production by lysozyme and casein using waste sewage sludge by; (a) Different concentrations of lysozyme. WSS without lysozyme - control (*blue*

diamond), 0.1 % lysozyme (*red square*), 0.5 % (*green triangle*) and 1 % lysozyme (*violet x*) are shown. (b) by another protein substrate. Control (*blue diamond*), 1 % casein (orange cycle) and 1 % lysozyme (*violet x*) are shown. *Error bars* indicate standard errors (n = 3). * Indicate the significant difference in the presence of lysozyme

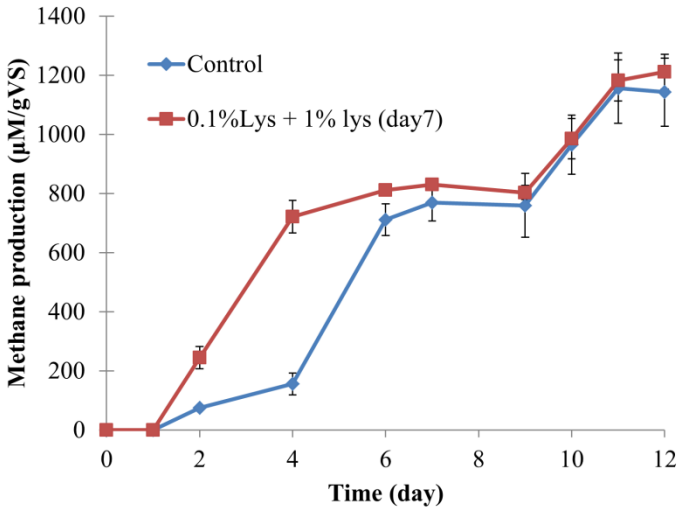

Figure 6.9. Impact of second addition of lysozyme on methane fermentation using waste sewage sludge at 7 days of incubation. Control and WSS with 0.1 % of lysozyme were anaerobically incubated until day 7, then 1 % of lysozyme was added to WSS in 0.1 % lysozyme sample. *Error bars* indicate standard errors (n = 3).

6.3.2.3. Bacterial activities in WSS with lysozyme

To evaluate bacterial activities in WSS with lysozyme, three factors including pH value, percentage of Gram-positive bacteria and activities of bacteria and archaea were checked. pH value of WSS with and without lysozyme was measured at 2, 4, 7 and 10 days. As a result, pH was increased in the presence of lysozyme. The pH of WSS with 0.5 % and 1 % lysozyme was higher than 0.1 % lysozyme (Fig. 6.10). According to Lay (1998), anaerobic digestion occurs optimally at a pH range of 6.6–7.8 (Lay et al., 1998). Besides, increasing pH increased the efficiency of methane production (Ye et al., 2012). Consequently, the increased of pH was a reason that methane production was enhanced in WSS with lysozyme.

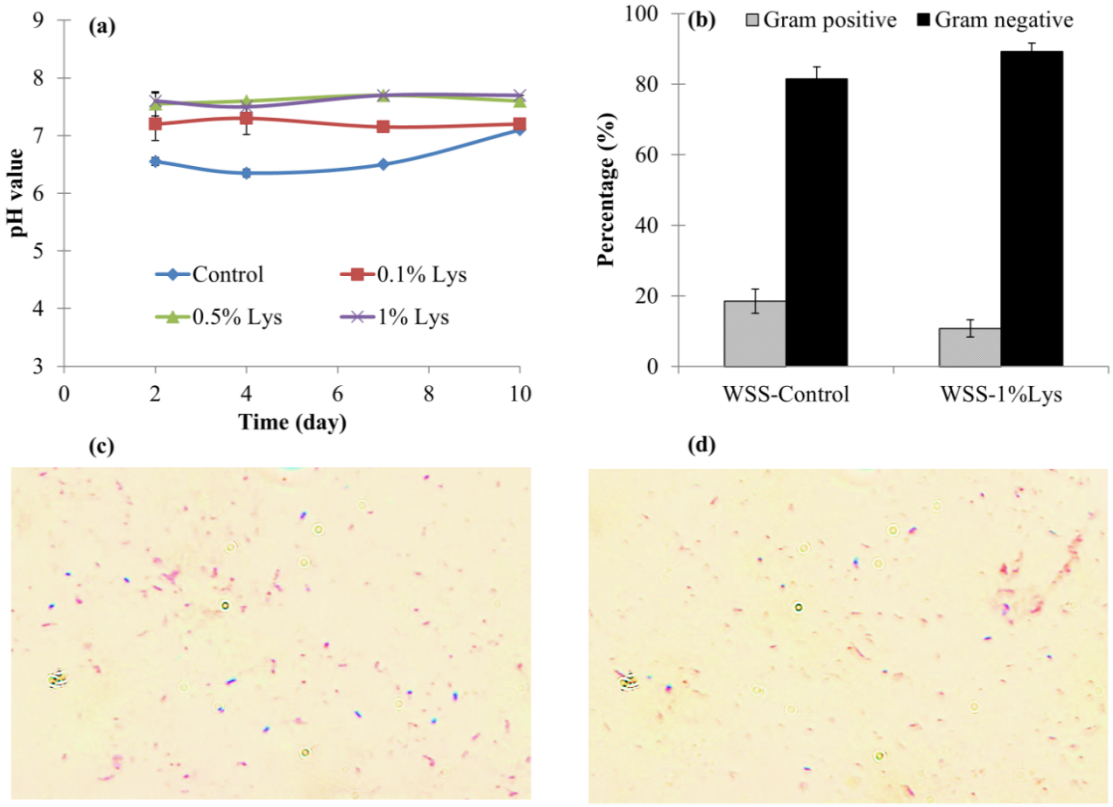

Figure 6.10. pH variation and proportion of Gram-positive bacteria in WSS with lysozyme. (a) pH variation; (b) Percentage of Gram-positive bacteria and Gram-negative bacteria in WSS with and without lysozyme; (c) Gram staining for WSS with and without lysozyme (d). Samples were observed under microscopy at 100x. *Error bars* indicate standard errors (n = 3)

To confirm the activity of lysozyme in bacterial community of WSS in anaerobic digestion, Gram staining for WSS with and without lysozyme (1 %) was conducted at day 4 of incubation. As expected, the percentage of Gram-positive bacteria was lower in WSS with lysozyme than in WSS without lysozyme (Fig. 6.10b, c, d). This result demonstrated the function of lysozyme in killing Gram-positive bacteria in bacterial consortia. Therefore, we could evaluate the function of Gram-positive bacteria in anaerobic digestion by adding lysozyme to inhibit their growth.

The activities of bacteria and archaea in the methane fermentation were evaluated at day 4 and day 10 of incubation. The total activity of archaea in the WSS control was lower

than WSS in the presence of lysozyme at day 4 (Fig. 6.11a). The result shows a similar trend with the profile of methane production as the control WSS had the lowest methane production (Fig. 6.8a). However, at day 10, the total activity of archaea in the WSS control was higher than WSS in the presence of lysozyme. Besides, the total activity of bacteria was similar in both samples at day 4 and day 10 of incubation (Fig. 6.11b). As a result, lysozyme did not inhibit the activity of archaea as well as the activity of bacteria (Gram-negative bacteria) during anaerobic incubation process.

Based on these above results that we collected from the experiments of WSS in the presence of lysozyme during anaerobic digestion, the knowledge about the functions of Gram-positive in anaerobic digestion was clearer. Firstly, as compared to the Gram-negative bacteria, Gram-positive bacteria showed less function in anaerobic digestion. Although at day 2 of fermentation (earlier process), the inhibition of Gram-positive decelerated the methane production (Fig. 6.8). The growth inhibition of Gram-positive bacteria did not affect on the methane production in later process of anaerobic incubation (Fig. 6.9).

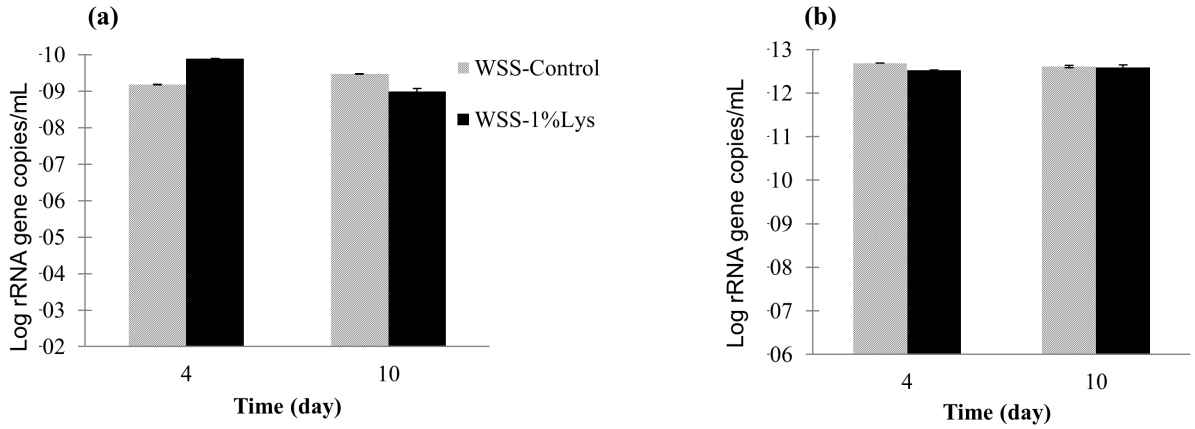

Figure 6.11. Quantity of active archaeal (a) and bacterial (b) populations in WSS in the presence of 1% lysozyme. Error bars indicate standard errors (n = 3)

6.4. Conclusion

By adding C-30 furanone which is the factor that affecting on the QS system of Gram-negative bacteria and some Gram-positive bacteria and adding lysozyme as a factor that inhibiting the growth of Gram-positive bacteria in WSS during anaerobic digestion, we evaluated the roles of QS system as well as the roles of Gram-negative bacteria and Gram-positive bacteria in methane production process. Firstly, Gram-negative bacteria showed the important functions due to the inhibition of methane production when QS system of Gram-negative was inhibited by C-30 furanone. Otherwise, the universal QS system also inhibited by C-30 demonstrated the strong inhibition of methane production. Therefore, QS system acts an importance function in methane production process. Secondly, the survival of Gram-positive bacteria slightly affected on the methane production in early stage of anaerobic digestion, however the later process was not affected by the survival of Gram-positive bacteria. Besides, the increase of methane in WSS with lysozyme is related to the increase of pH which demonstrates the increasing in producing organic acids.

CHAPTER 7

CONCLUDING REMAKS AND SUGGESTION FOR FUTURE

Bacterial adaptation in inhibiting the QS system of monoculture (*P. aeruginosa* PA14) and bacterial consortia (WSS) were two investigation approaches throughout this study. Using two types of QSI, C-30 furanone and AiiM lactonase, we demonstrated that *P. aeruginosa* PA14 can evolve the resistance to both of them in a monoculture system. Otherwise, the addition of QSI to WSS showed the dynamic in bacterial community which demonstrated the importance function of QS system in the interaction among bacterial consortia during anaerobic digestion. Furthermore, the contribution of Gram-negative and Gram-positive bacteria in methane production was investigated.

For the first study, it was found *P. aeruginosa* PA14 can convert C-30 to C-56 and then convert C-56 to furanone compound without bromine atom in the structure. The transformation is considered to disrupt the QQ effect to PA14. In addition, there was no transposon mutant had been found to lose the ability in degrading C-30, which indicated that this function is regulated by the complex genes instead of single gene. The isolated PA14 mutant (F12-3) was proved to have less function in degrading the C-30 and less ability in uptake the C-56 into the cell. The gene that we identified is *crc* (catabolite repression control protein). This gene could be related to the C-30 degradation ability of PA14. This discovery demonstrated the evolution in resistant of PA14 to QQ compound which possess halogen atom in structure. Therefore, further study need to find QQ compound that cannot be degraded by PA14.

For the second study, *P. aeruginosa* PA14 can grow in QS minimal medium with the treatment of AiiM lactonase. This demonstrated the adaptation of *P. aeruginosa* PA14 in utilizing AiiM lactonase as a carbon source for its growth. This discovery raised the

apprehension about the resistance to AiiM by the mutant in environment where the lack of nutrient may appear. To prove this statement, the clinical isolates were utilized to test the resistant ability to AiiM. As expected, 2 clinical isolates with high protease activity showed the resistance to AiiM. Consequently, although AiiM is a QQ enzyme, PA14 can adapt the AiiM and use it as a source for growth. This study demonstrated that QQ enzyme is not the potential way for treating bacteria since it is utilized by bacteria. Therefore, further study need to find the way to inhibit the ability of PA14 in utilizing AiiM.

The results from third study showed that QQ enzyme AiiM lactonase, which degrade lactone ring of acyl homoserine lactone (AHL) signals from Gram-negative bacteria, reduced the QS-regulated factors of Gram-negative bacteria in WSS and lead to inhibit the methane production. Due to the different antibiotic factors produced in the addition of AiiM, namely, some of Gram-negative bacteria were killed by adding SPE fraction from WSS with AiiM while the growth of some of Gram-positive bacteria were reduced in the presence of SPE fraction from WSS without AiiM, it demonstrated that AiiM influences the change of bacterial community. Therefore, QS signals among Gram-negative bacteria in a microbial consortia is very important for the production of methane. The antimicrobial agents produced from WSS with and without AiiM should be further analyzed to determine what kind of the compounds as well as the bacteria that it belongs to. Otherwise, AiiM need to add to other kind of bacterial consortia to investigate the interaction among bacteria under the inhibiting of QS system.

The forth study showed that by C-30 furanone which is the factor that affecting on the QS system of Gram-negative bacteria and some of Gram-positive bacteria and adding lysozyme as a factor that inhibiting the growth of Gram-positive bacteria in WSS during anaerobic digestion, the roles of QS system as well as the roles of Gram-negative bacteria and Gram-positive bacteria in methane production process were confirmed. Firstly, Gram-

negative bacteria showed the important functions due to the inhibition of methane production when QS system of Gram-negative was inhibited by C-30 furanone. Otherwise, the universal QS system also inhibited by C-30 demonstrated the strong inhibition of methane production. Therefore, QS system acts an important function in methane production process. Secondly, the survival of Gram-positive bacteria slightly affected on the methane production in early stage of anaerobic digestion, however the later process was not affected by the survival of Gram-positive bacteria. Moreover, the inhibition of Gram-postive bacteria by lysozyme enhanced the production of methane. Therefore, another compound (commercial and cheaper than lysozyme) can be utilized to control Gram-positive bacteria in anaerobic digestion process. Others bacterial consortia source, for example, gut samples can be utilized with and without QSI to determine the effect of QSI to the bacterial community in that source.

Consequently, this study contributes to the understanding of bacterial adaptation to QSI in monoculture and bacterial consortia. Therefore, further study need to conduct to more understand about how single strain of bacteria can adapt to QSI and increase their evolve resistance. In addition, the bacterial interaction via QS systems should be further study to determine the effect of QS system to bacteria community. This understanding helps for further applications in treating bacterial pathogen and in environmental treating process.

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PUBLICATION AND CONFERENCE ATTENDED

Publication

Nguyen, P. D. T., Mustapha, N. A., Kadokami, K., Garcia-Contreras, R., Wood, T. K., & Maeda, T. (2018). Quorum sensing between Gram-negative bacteria responsible for methane production in a complex waste sewage sludge consortium. *Applied microbiology and biotechnology*, 1-11. (IF 3.34)

Nguyen, P. D. T., Kadokami, K., Garcia-Contreras, R., Wood, T. K., & Maeda, T. *Pseudomonas aeruginosa* PA14 increase the ability in C-30 furanone resistance by degrading C-30 furanone to C-56 furanone. *ACS journal* (to be submitted)

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1. Phuong Thi Dong Nguyen, Takahiro Hashimoto, Toshinari Maeda. Understanding a Bacterial Strategy to Degrade Quorum Sensing Inhibitors, Furanone compounds C30 and C56 by *Pseudomonas aeruginosa* PA14. The 10th Japan-Korea joint symposium on the biomicrosensing, Kitakyushu, Japan (2017/11/20-21)

2. Phuong Thi Dong Nguyen, Nurul Asyifah Mustapha, Toshinari Maeda. Effect of AiiM-lactonase on methane production using waste sewage sludge. The 52th annual meeting in the Japan Society on Water Environment, Hokkaido, Japan (2018/3/15-17)

3. Phuong Thi Dong Nguyen, Nurul Asyifah Mustapha, Toshinari Maeda. Quorum sensing between Gram-negative bacteria responsible for methane production in complex waste

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