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Reduction of biofouling using vanillin as a quorum sensing inhibitory agent in membrane bioreactors for wastewater treatment

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Abstract. Membrane biofouling impedes wide application of membrane bioreactor (MBR) for wastewater treatment. Recently, quorum sensing (QS) mechanisms are accounted for one of major mechanisms in biofouling of MBRs. In this study, vanillin was applied to investigate reduction of biofouling in MBRs. MBR sludge was analyzed to contain QS signal molecules by cross-feeding biosensor assay and HPLC. In addition, the inhibitory activity of vanillin against bacterial quorum sensing was verified using an indicator strain CV026. The vanillin doses greater than 125 mg/L to 100 mL of MBR sludge showed 25% reduction of biofilm formed on the membrane surfaces. Two MBRs, i.e., a typical MBR as a control and an MBR with vanillin, were operated. The TMP increases of the control MBR were more rapid compared to those of the MBR with the vanillin dose of 250 mg/L. The treatment efficiencies of the two MBRs on organic removal and MLSS were maintained relatively constant. Extracellular polymeric substance concentrations measured at the end of the MBR operation were 173 mg/g biocake for the control MBR and 119 mg/g biocake for the MBR with vanillin. Vanillin shows great potential as an anti-biofouling agent for MBRs without any interference on microbial activity for wastewater treatment.

Keywords: vanillin; biofouling; quorum sensing; EPS; MBR

1. Introduction

Application of membrane bioreactors for wastewater treatment has been markedly expanded during recent decades. These bioreactors gained much attention due to their great effluent concentration, small foot print, and possibility of automation of operation. However, a major concern is membrane fouling that impedes membrane operation by substantial reduction of water production (Chaize and Huyard 1991). Membrane fouling has been caused by various ways: precipitation of inorganics, adsorption or formation of concentration polarization of organic matters, formation of cake layers by particulates and biofilm formation by microbial growth (Nguyen *et al*. 2012, Cui and Choo 2014**)**. Biofouling results from biofilm formation by microbial communities of surface-attached cells embedded in a self-produced extracellular polymeric

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substance.

Several control strategies are applied to prevent biofouling in MBRs including extensive pretreatment of a feed solution, development of fouling resistant membrane materials and establishment of efficient cleaning strategies. However, all these methods by physical and chemical approaches have not been succeeded to prevent biofilm formation on MBRs. Recently, Xiong and Liu (2010) mentioned that biological methods for mitigation of microbial attachment would be an attractive alternative for controlling biofouling.

One of the biological approaches on biofouling control is quorum sensing (QS) inhibition. Quorum sensing is a mechanism of cell-to-cell communication to detect their cell density, which regulates bacterial group behaviors such as biofilm formation (Hentzer and Givskov 2003). Gram-negative bacteria produce small molecules called N-acyl homoserine lactone (AHL) autoinducers as signal compounds for the cell-to-cell communication. When a concentration of AHLs in the surrounding environment reaches a threshold level, it combined with a receptor protein and activates a transcription of target genes to induce group behaviors such as colonization of microorganisms. The biofouling from the biofilm built up on the near membrane surface was also proposed to be controlled by quorum sensing of signals in the microbial communities (Yeon *et al*. 2009). Therefore, manipulation of AHL concentrations in MBRs could be a novel way to prevent the biofilm formation.

There are three possible ways to control of the AHLs concentrations; blockage of AHL synthesis, breaking AHL down before AHL reaches to receptors, and finally interference with signal receptors. For instance, several researchers applied AHL-acylase, which can destruct the acyl chain structure in the AHL type quorum sensing signal molecules, to reduce biofouling in membrane processes including MBR, nanofiltration and reverse osmosis membrane (Kim *et al*. 2011, Yeon *et al*. 2009, Paul *et al*. 2009). Another approach by Oh *et al*. (2012) used quorum quenching bacteria, which have an enzyme (*Rhodococcus sp*. BH4) to degrade the signal compounds and showed some reduction in biofouling of a submerged MBR.

Naturally occurred compounds having quorum sensing inhibition effects were investigated substantially by scientists in medical fields. For example, halogenated furanone compounds were extracted from the red marine alga Delisea pulchra and proven to have capability of inhibition of quorum sensing. Givskov *et al*. (1996) suggested that furanone have ability to regulate AHL producing systems thus harmful activities by the number of plant and animal pathogens. However, since these furanone compounds showed limitation to use for human due to their composition with halogen elements, search for natural products with quorum sensing inhibition (QSI) has been expanded tremendously in recent decades. Extract from garlic (Bjarnsholt *et al*. 2005), vanilla planifolia from tropical medicinal plants (Choo *et al*. 2006), extract of Piper betle plant leaves (Siddiqui *et al*. 2012), plant extract ursolic acid (Ren *et al*. 2005), and cranberry polyphenol (Yamanaka *et al*. 2007) have all shown various extents of anti-bacterial and/or anti-biofilm properties.

In recent studies, vanillin (4-hydroxy-3-methoxy benzaldehyde) was proposed to be a biofouling control agent in membrane systems applied for water treatment (Ponnusamy *et al*. 2009, Kappachery *et al*. 2010). Vanillin is the main constituents of vanilla beans and is frequently used for food ingredient such as ice cream and chocolate. The authors reported that concentrations ranging from 0.063 to 0.25 mg/mL reduced the biofilm formation by Aeromonas hydrophila on polystyrene surface without impeding the growth of planktonic cells. The strain, *A. hydrophila*, isolated from a fouled reverse osmosis membrane was used as a model microorganism for AHL-mediated biofouling occurrences in the system. The fact that most of studies use a model

single strain for experiments restricts application of QSI compounds to wastewater treatment due to complexity of microbial community. To date, little study has been conducted on QSI effects of vanillin on heterogeneous microbial systems such as MBRs for wastewater treatment.

The purpose of this study was to demonstrate whether vanillin addition could reduce the membrane biofouling in MBRs for wastewater treatment. The existence of QS activity in submerged membrane bioreactor was verified. The QS inhibitory activity of vanillin was evaluated at several vanillin doses. Additionally, two laboratory-scale MBRs were installed to investigate effects of continuous feeding of vanillin on MBR performance and fouling.

2. Materials and methods

2.1 Chemicals

The pure grade vanillin (4-hydroxy-3-methoxybenzaldehyde, molecular weight, 152.15) was purchased from Sigma-Aldrich (St. Louis, MO, USA). A Henry's law constant for vanillin in water at 25°C is 2.15×10^{-9} m^3 • atm/mol, which indicates that vanillin is nonvolatile in experiments at room temperature. HPLC grade ethyl acetate, methanol, and acetonitrile were purchased from Fisher Scientific (Fairlawn, NJ, USA). 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), kanamycin sulfate, spectinomycin and tetracycline were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dehydrated culture media including Luria-Bertani (LB) broth was procured from BD-Difco (Franklin Lakes, NJ, USA). All the chemicals used were of highest analytical grade.

2.2 Standard N-acyl homoserine lactones

Standard AHLs including N-butanoyl-L-homoserine lactone (C4-HSL), N-hexanoyl-L-homoserine lactone (C6-HSL), N-octanoyl-L-homoserine lactone (C8-HSL), 3-oxo-octanoyl-L-homoserine lactone (3-oxo-C8-HSL) and N-decanoyl-L-homoserine (C10-HSL) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.3 Quorum sensing reporter's strains

Chromobacterium violaceum CV026 (Mini-Tn5 mutant of ATCC31532) and *Agrobacterium tumefaciens* A136 which are deficient in AHLs production were used as an indicator for AHL detection. *C. violaceum* 026 can produce purple pigment violacein when C4-HSL, C6-HSL, C8-HSL, and 3-oxo-C4~C8 exogenous AHLs were present (McClean *et al*. 1997). *A. tumefaciens* A136 can produce a blue color from hydrolysis of 5-bromo-4-chloro-3-indolyl-*β*-D-galactopyranoside (X-gal) by the *β*-galactosidase activity, in response to C8-HSL, 3-oxo-C8-HSL, and C10-HSL exogenous AHLs molecule. (Choo *et al*. 2006, Yeon *et al*. 2009, Ravn *et al*. 2001).

2.4 Synthetic wastewater

The composition of synthetic wastewater was followed by the work of Oh *et al*. (2012). Chemical oxygen demand (COD) concentration of the wastewater was 500~600 mg/L as follows (mg/L): glucose, 400; yeast extract, 14; bactopeptone, 115; $(NH_4)_2SO_4$, 104.8; KH_2PO_4, 21.75; $MgSO_4$, 32; $FeCl_3$, 0.075; $CaCl_2$, 2.525; $MnSO_4$, 2.65; and $NaHCO_3$, 255.5. The synthetic wastewater was fed to two MBR reactors and also used to investigate vanillin effects of QS

inhibition on MBR sludge.

2.5 Detection of AHLs from MBR sludge

2.5.1 Extraction of AHLs from MBR sludge

AHLs were extracted from the MBR sludge operated in this laboratory followed by the method of Oh *et al*. (2012) with minor modification. 200 mL of sludge was centrifuged at 9000 rpm for 10 min at 4°C and supernatant was collected. Equal volumes of the supernatant and acidified ethyl acetate (0.1% acetic acid) were mixed. The mixture was then vortexed at 250 rpm for 2 h on a shaker (KMC-8480S, Vision Scientific Co., Ltd., Korea) at 25°C. After shaking, the upper layer was separated into a glass petri dish and evaporated in a fume hood. The residues of sample were suspended in 1 mL of acetonitrile for further experiments.

2.5.2 Detection of sludge AHLs by agar plate cross-feeding bioassay

The LB agar plates (1.2% agar) for *C. violaceum* CV026 and for *A. tumefaciens* A136 were prepared. The plates for *A. tumefaciens* A136 were arranged with spectinomycin (50 μg/ml), tetracycline (4.5 μg/ml) and X-gal (80 μg/ml). A loopful of extracted sample was streaked on the both agar plates with approximately 1 cm away from biosensor strains. After incubation for overnight at 28°C the presence of AHLs was observed with the development of purple and blue coloration along the line of indicator strains (Lade *et al*. 2014).

2.5.3 Detection of sludge AHLs HPLC analysis

The AHLs extract from MBR sludge were analyzed with an Agilent 1200 HPLC system (Agilent, Santa Clara, CA, USA) equipped with a ZORBAX Eclipse XDB-C18 column. The above mentioned five standard AHLs (Section 2.2) were prepared in acetonitrile to make 500 μg/mL of a stock solution. Each of the standard AHLs was further diluted in acetonitrile to obtain 50 μg/mL of individual and one mL of the standard AHLs were mixed together. The resulting AHLs mixture, crude sludge extract and acetonitrile as blank were injected into a column at a flow rate of 0.25 mL/min. The HPLC conditions and instrument parameters as previously described includes an isocratic profile of acetonitrile /water (35:65, v/v) for five min, followed by a linear gradient from 35% to 95% acetonitrile in water over 33 min. A subsequent linear gradient from 95% to 35% acetonitrile in water over 2 min, and an isocratic profile of methanol/water (35:65, v/v) for five min were applied for flushing the column for the following run (Kim *et al*. 2013). The detection was conducted with a Diode-array detector at 210 nm of wavelength.

2.6 Evaluation of QS inhibitory activity of vanillin against bacterial quorum sensing

C. violaceum CV026 was enriched overnight and inoculated in three Erlenmeyer flasks containing LB alone, LB supplemented with N-hexanoyl homoserine lactone (C6-HSL), and LB supplemented with C6-HSL and 0.25 mg/ml of vanillin as shown in Table 1. The flasks were incubated at 30°C for 24 hours in a shaking incubator with stirring at 150 rpm (Choo *et al*. 2006). 0.5 mL of culture from each flask was mixed with 0.5 mL of dimethyl sulfoxide (DMSO). The mixture was centrifuged at 10,000 rpm for 3 minutes. Each 0.2 mL of the supernatant was added into the 96-well plate (SPL, Korea). The absorbance at 595 nm was measured using IMark microplate reader (BioRad, Japan). Three replicates for each condition were prepared and evaluated for the absorbance.

Table 1 Composition of media for investigation on QS inhibitory activity of vanillin against bacterial quorum sensing using C. violaceum CV026 cultures

Material	Flask			Final concentration
	(1)	(2)	(3)	
LB broth (mL)	8.8	8.8	8.8	-
CV026 (mL)	0.1	0.1	0.1	-
C6-HSL (mL)	-	0.1	0.1	5 μM
Vanillin (mL)	-	-	1	1.65 mM (0.25 mg/L)
Acetonitrile (mL)	1.1	1	-	-
Total (mL)		10		

2.7 Effects of vanillin doses on biofilm reduction

Five Erlenmeyer flasks with 100 ml of the MBR sludge were prepared. A mixed liquor suspended solids (MLSS) concentration of the MBR sludge was approximately 5,000 mg/L. Vanillin was added to the flasks to make concentrations of 0, 62.5, 125, 250, 375 mg/L, respectively. One Erlenmeyer flask without the sludge was also prepared as a blank control. Polyvinylidene fluoride (PVDF) hollow fiber membranes (Zee-Weed 500, GE-Zenon, USA) were sterilized under ultraviolet light for 4 hours in a clean bench. The sterilized fiber membranes were cut into 48 cm length with an effective area of 28 cm^2. The fiber membranes were inserted in each flask. Lastly, the synthetic wastewater was added to be 200 mL of total volume. The flasks were then incubated at 30°C for 24 hours in a shaker at 150 rpm to form biofilm on the membrane fibers. After the incubation, the fibers were taken out to measure EPS concentrations of the biofilm.

2.8 MBR operation

Two MBRs with 3.0 L working volume were installed as shown in Fig. 1. Activated sludge was taken from a wastewater treatment plant (Sihwa, Korea) and acclimated into the synthetic wastewater. The MBRs were operated at 8 hours of hydraulic retention time (HRT) and 30 days of sludge retention time (SRT). MLSS in the both reactors were maintained within the range of 5,500-6,000 mg/L before addition of vanillin. A specified dose, i.e., 250 mg/L of vanillin was added to one of the MBRs. The PVDF hollow fiber membrane with a pore size of 0.04 μm were inserted in the middle of the both MBR reactors. The effective area of the module was 155 cm^2. The MBRs were operated under the same flux of 25 $L/m^2/h$ and sterilized air was supplied by a compressor at a flow rate of 2 L/min during operation. The membrane fouling was monitored by increases in transmembrane pressure (TMP) using a pressure gauge meter. The TMP rises were recorded to a computer through a Labview software (National Instruments, Hungary). When TMP rose up to 40 kPa, the filtration was stopped. A portion of the membrane with a surface area of 38.75 cm^2 was taken out for further experiments for biofilm characterization.

2.9 Extracellular polymeric substances measurement

The biocake from membrane surfaces was extracted to measure EPS concentrations by the thermal method (Ma *et al*. 2006). The selected fibers were rinsed with 30 mL of 0.9% NaCl

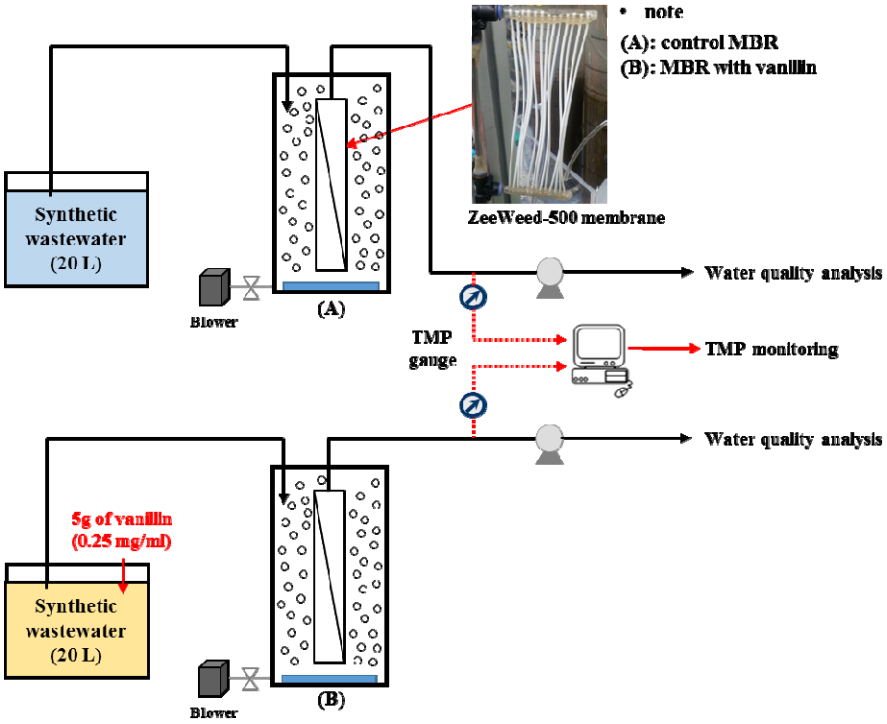


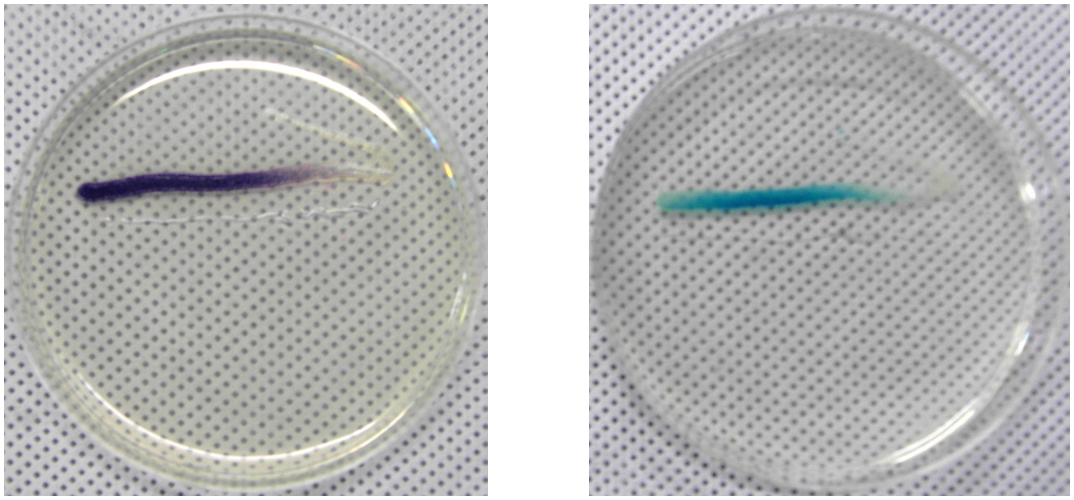
Fig. 1 Schematic diagram of the membrane bioreactors

solution and moved into conical tubes with 15 mL of 0.9% NaCl solution. The fibers from MBR operations were soaked into 30 mL of 0.9% NaCl instead of 15 mL. The fibers were cut into approximately 1-2 cm length of pieces in the NaCl solution. The solution with chopped membrane fibers were agitated with a vortex mixer for 10 minutes and sonicated for 60 minutes. After the fiber was removed from the tube, the solution was centrifuged at 5,000 rpm for 25 minutes to collect the detached biofilm. The supernatant was discarded and 0.9% NaCl solution was filled up to maintain the same volume. The resuspended solution was treated by heat as follows: the solution was moved to an evaporating dish, heated at 100°C for one hour, and cooled down in a desiccator for one hour. The heat-treated solution was centrifuged at 5,000 rpm for 25 minutes and the supernatant was filtered with 0.45 μm. The filtered supernatant containing extracted EPS was analyzed for polysaccharide and protein contents. The TOC analyzer (SIEVERS 5310C, GE) was used to determine amounts of polysaccharides. The protein in EPS was analyzed using Protein Assay Kit (BR500, Bio-Rad, CA, USA) followed by Kappachery *et al*. (2010). MLSS and chemical oxygen demand (COD) were determined according to Standard Methods.

3. Results and discussion

3.1 Identification of AHLs from MBR sludge extract

The identification and characterization of AHLs of the MBR sludge were needed prior to applying a QS inhibitor to control biofouling in MBR operation. The cross-feeding agar plate



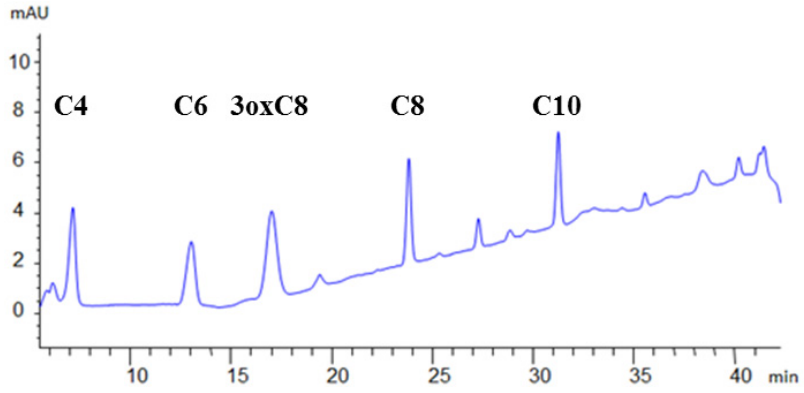
(a) (b)

Fig. 2 Agar plate cross-feeding assay for dection of AHLs in MBR sludge extract by: (a) *C. violaceum* CV026; and (b) *A. tumefaciens* A136

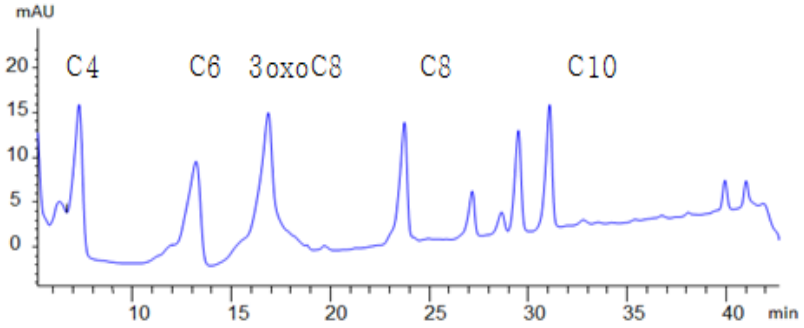
assay with C. *violaceum* CV026 and A. *tumefaciens* A136 was used to identify the presence of AHLs in the MBR sludge. The cross-feeding assay was selected due to an advantage of rapid detection of AHLs without identifying the exact types of AHLs. The results from the assay revealed violet color by the reporter strain CV026 and blue color by the reporter strain A136 as shown in Fig. 2. It has been known that the reporter strain CV026 indicates the presence of short and medium-chain AHLs and the reporter strain A136 does the presence of medium and long-chain AHLs. Therefore, this coloration of the MBR sludge confirmed the presence of wide range of AHLs in the MBR sludge (Lade *et al*. 2014).

Separation and detection of individual AHLs of MBR sludge extracts were performed with HPLC analysis. The sludge AHLs were recognized by comparing retention times of peaks with those of respective standard AHLs. The retention times of the five standard AHLs were 7.116 for C4-HSL, 13.024 for C6-HSL, 17.018 for 3-oxo-C8-HSL, 23.817 for C8-HSL, 31.264 for C10-HSL (Fig. 3(a)). The peak at a retention time of 27.260 was for acetonitirile solvent. The MBR sludge extract showed five peaks with retention times similar to the standard AHLs. The appearance of three more peaks compared to the standard AHLs suggest the presence of unidentified AHLs or microbial products from metabolic activity in the MBR sludge (Lade *et al*. 2014).

Yeon *et al*. (2009) also reported that at least three different AHLs were present at the biocake detached from fouled PVDF hollow fibers in an MBR. The authors used a thin layer chromatography to detect AHLs by comparing the ratio of the distance moved by AHLs to that moved by the solvent front. The authors found three blue colors with the MBR extract and the strongest blue color at a spot by C8-HSL indicating the AHL is the most dominant AHL in the biocake. A work by Siddiqui *et al*. (2012) also confirmed the three different AHLs from biocake detached from polysulfone hollow fibers. The authors stated that the main AHLs produced in biocake in the MBR were C6-HSL and C8-HSL and the dominant AHLs could be different by variation in operating conditions and metabolic activities of the microbial communities.



(a)



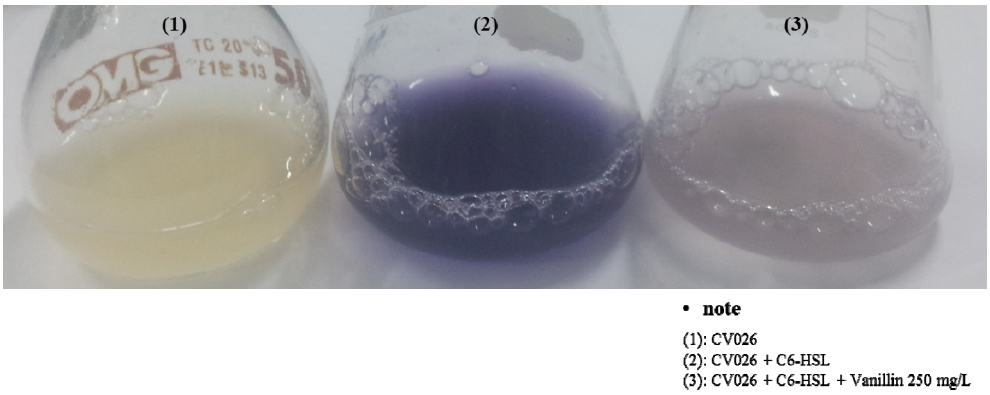
(b)

Fig. 3 HPLC results: (a) Peaks of standard AHLs; and (b) Peaks of AHLs extracted from the MBR sludge

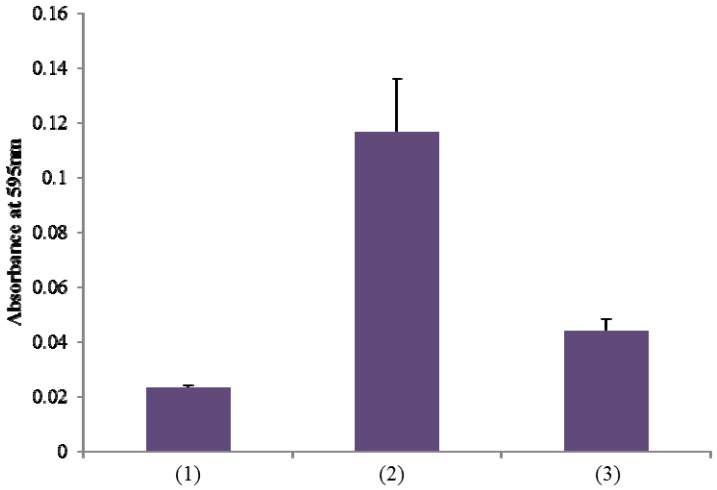
From the cross-feeding assay result and the HPLC analyses in this research, it can be concluded that the activated sludge contains QS signal compounds and it may involve in the active microbial attachment on the MBR surfaces.

3.2 Inhibitory activity of vanillin against bacterial quorum sensing

The proposed biofouling control compound, vanillin was tested to broth of C. *violaceum* CV026 as follows: the first flask contained only CV026 broth, the second flask contained CV026 broth with C6-HSL, and the last flask contained CV026, C6-HSL, and 250 mg/L of vanillin. The activity of CV026 was verified by purple coloration after 24 hours of incubation. Photos of the three flasks were taken as shown in Fig. 4(a). The flask with only CV026 broth was used as a negative control. The broth maintained the active growth phase indicated by turbid yellowish color. The flask with the signal compounds showed an extensive blue color (i.e., 0.12 of absorbance at 595 nm of wavelength) due to expression of *LuXR* homologue, *CviR* (which controls production of violacein) by acceptance of C6-HSL in the broth (McClean *et al*. 1997). Lastly, considerable drop in purple coloration was observed by bare eyes in the flask with vanillin. To quantify the difference in color intensity, purple pigment violacein was extracted and measured by a microplate reader as shown in Fig. 4(b). The violacein intensity was corresponded to the observation by bare



(a)



(b)

Fig. 4 Inhibition of quorum sensing of CV026 by vanillin: (a) photographic images of color in three flasks; (b) absorbance of violacein at 595 nm

eyes. The development of purple coloration by violacein production was reduced by 62% by adding vanillin to the broth. Three replicates for each condition were experimented. The values in Fig. 4(b) are mean of the three replicates and vertical bars represent the standard deviations.

3.3 Effects of vanillin doses on reduction of biofilm formation

Effects of vanillin addition on biofilm formation by MBR sludge on the PVDF hollow fibers were investigated at five different doses of vanillin as shown in Fig. 5. Compared to the flask with no vanillin addition, the amounts of protein in the biocake were reduced at the three doses, except at the vanillin dose of 62.5 mg/L. The greatest reduction in protein contents was occurred at the dose of 125 mg/L. The amounts of protein increased to 385 mg/cm^2 at the highest dose of 375 mg/L although the amount was still smaller than the control case with no vanillin, i.e., 475 mg/cm^2. The total organic carbon concentration in the biocake of the control case was 350 mg/cm^2 and the

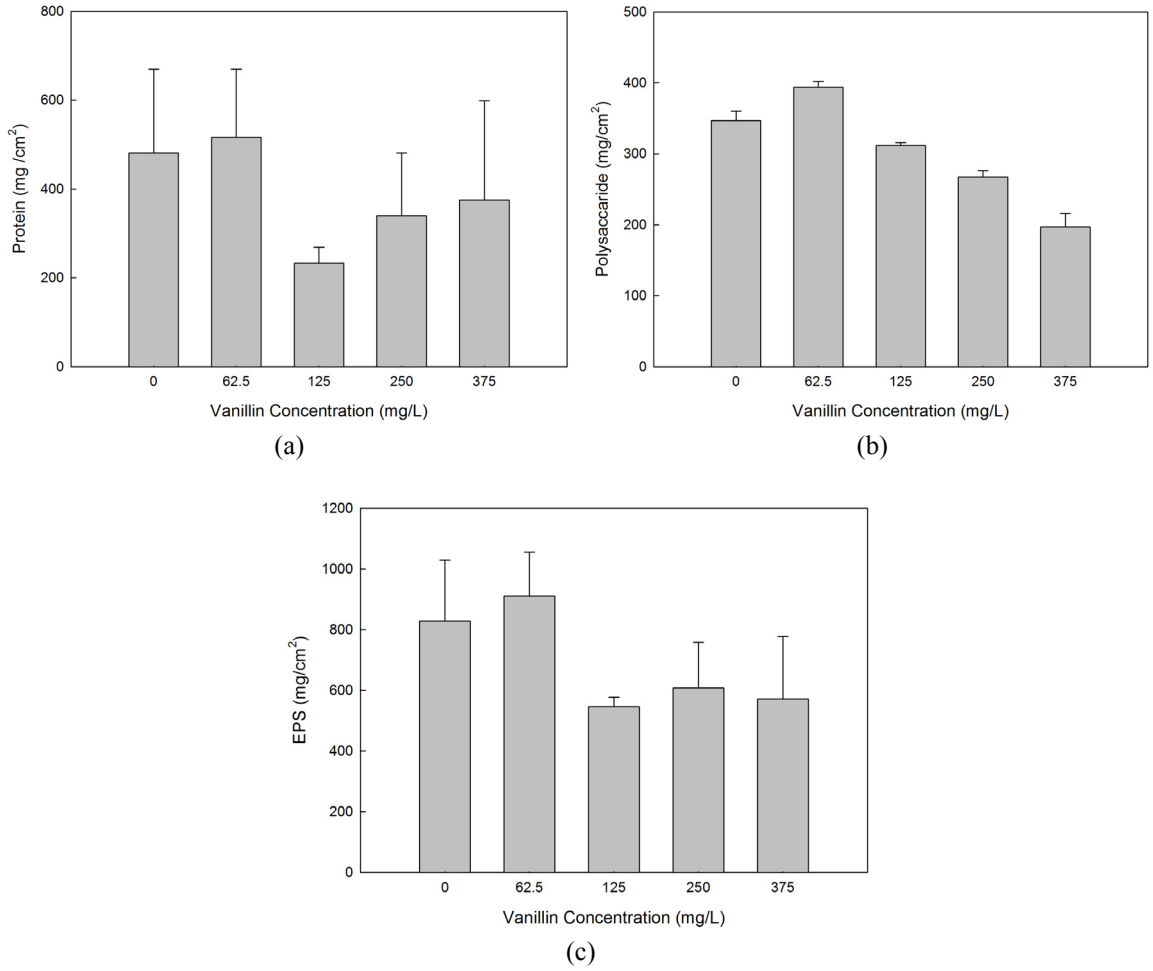


Fig. 5 Amounts of EPS in the biofilm formed on PVDF hollow fibers at different vanillin concentrations: (a) protein; (b) polysaccharide; (c) total EPS. Values are means of three replicates and vertical bars represent the standard deviation

concentration was increased slightly at the dose of 62.5 mg/L and then decreased gradually to 195 mg/cm^2 at the highest dose of 375 mg/L. Since the protein concentration was in general greater than polysaccharide concentration, therefore, the total extracellular polymeric substance concentration had the greatest reduction at the dose of 125 mg/L. Ponnusamy *et al*. (2009) investigated several vanillin doses (i.e., 62.5, 125, 250 mg/L) to *Aeromonas hydrophila* in 96-well microtiter plates and found that the biofilm formation was reduced gradually to the dose of 250 mg/L. The reduction was reached to 46.3% at the highest dose. However, in this experiment with MBR sludge, the vanillin dose of 62.5 mg/L did not show any reduction. Furthermore, the reduction was observed approximately 25% at the dose higher than 125 mg/L. The results revealed that vanillin addition in the MBR sludge showed some effects on biofilm reduction and the MBR sludge with numerous microbes had more resistance to vanillin inhibitory activity than the single strain *A*. *hydrophilla*.

Table 2 Water quality of two MBRs

Parameter	Control MBR	MBR with vanillin
pH	6.9 ~ 7.5	6.5 ~ 7.5
DO (mg O_2/L)	3.4 ~ 4.2	3.4 ~ 4.3
MLSS (mg/L)	6000	6000 ~ 8000
COD influent (mg/L)	500	980 ~ 1030
COD permeate (mg/L)	16	0 ~ 18

3.4 Effects of vanillin addition on MBR performance

Two MBRs were installed to investigate effects of vanillin addition on the TMP rises of microfiltration and removal efficiency on organic matter in wastewater. The major water quality parameters in the MBRs were summarized in Table 2 and the TMP variations were shown in Fig. 6 with changes in mixed liquor suspended solids (MLSS) concentrations.

The chemical oxygen demand removal efficiency was 96.8% for the control MBR and over 99% for the MBR with vanillin (namely, the second MBR). The COD values in permeate were kept under 20 mg/L which means that removal of organic matter was substantial in the both reactors. The addition of vanillin did not have any impact on the biodegradation of the organic matter, which is an important property of QS inhibitors in MBR systems for wastewater treatment (Oh *et al*. 2012).

The increase in the influent COD of the second MBR was due to the carbon content in the vanillin. The molecular formula of vanillin is $C_8H_8O_3$ and this phenolic aldehyde has a theoretical COD value of 1.9 mg O_2 /mg vanillin. Since 250 mg/L of vanillin was added to the reactor, the

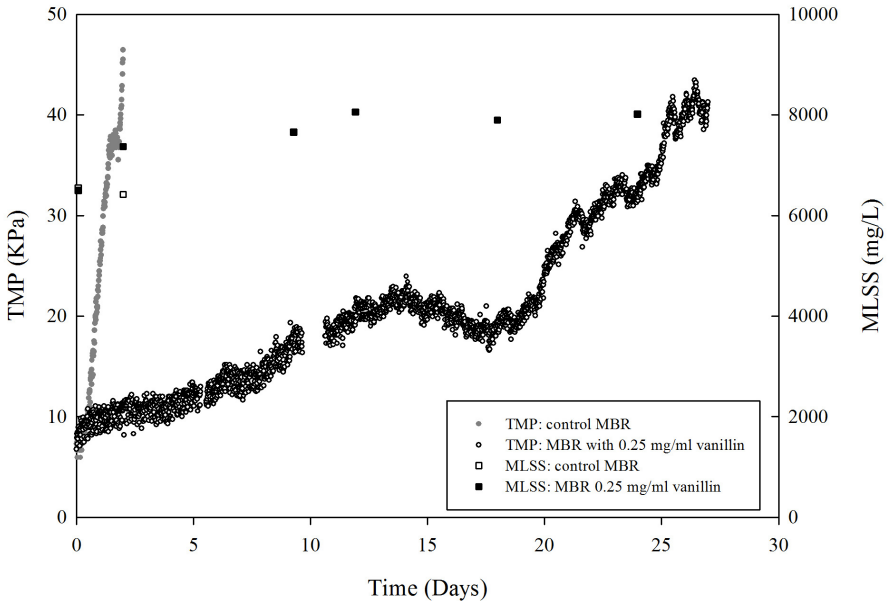


Fig. 6 Variation of TMP of the control MBR and the MBR with vanillin

theoretical influent COD value should be 975 mg/L, which was similar to the measured COD value in the influent. Due to the increased influent COD and the similar effluent COD to the control MBR, the removal efficiency in the second MBR was greater than the control MBR.

The control MBR maintained the mixed liquor suspended solid concentration (MLSS) at 6000 mg/L. The MLSS of the MBR with vanillin was 6,000 mg/L at the start up and was increased to 8,000 mg/L over the operation period of 27 days. As discussed in the previous paragraph, the addition of vanillin was increased influent COD values, which definitely yielded to increases in the MLSS of the reactor. In usual, biofouling has been corresponded to the MLSS concentrations. The higher MLSS has obtained, the greater fouling by biofilm has been found. Yigit *et al*. (2008) reported that the permeate flux was reduced to 50% of the initial flux when the MLSS was increased from 4,600 mg/L to 12,600 mg/L during the operation. In addition, other research by Trussell *et al*. (2006) concluded that aggravation of fouling became five times greater compared to the increase in organic loading in a pilot-scale submerged MBR. However, the authors also emphasized that increase of fouling was related to more EPS or SMP concentrations than MLSS concentrations.

The TMP variations of the two MBRs were recorded to the point where the TMP reached to a critical TMP, i.e., 40 kPa, which was given by the manufacturer as a condition for backwash. The control MBR was operated only 3 days and reached to 40 kPa. However, the MBR with vanillin has taken 27 days to stop the operation. It clearly showed that vanillin addition gave a positive effect to maintain TMP by reduction of biofouling on the membrane surface, even though the MLSS of the second MBR was higher than the control MBR. The research by Oh *et al*. (2012) used quorum quenching bacteria to reduce membrane fouling in MBRs. They observed half a day of delay of the TMP rise by adding quorum quenching bacteria compared to the control MBR with the same permeate flux of 20 L/m^2/hr. Siddiqui *et al*. (2012) also investigated whether addition of Piper betle extract had effects on a TMP rising pattern in MBRs and found that the MBR with the Piper betle extract showed slower increases in the TMP than the control MBR.

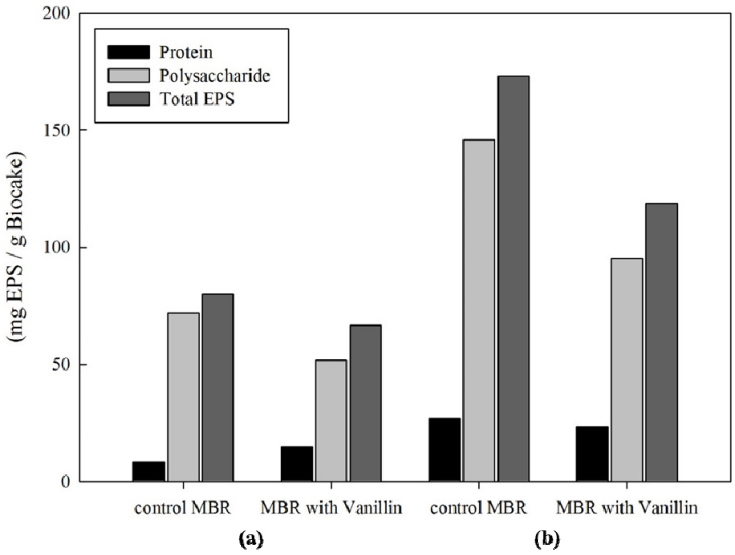


Fig. 7 Total EPS concentrations as sum of protein and polysaccharide in: (a) suspended sludge; and (b) biocake on the membrane surfaces

After the TMP rose to 40 kPa, membrane filters were taken out to evaluate amount of biofilm on the membrane surfaces. The amount of biofilm was evaluated with total extracellular polymeric substance (EPS) as a sum of protein and polysaccharide concentrations. In addition, suspended sludge in the reactors was also sampled to measure EPS concentrations. As shown in Fig. 7, the EPS concentrations were always greater in biocake than in suspended sludge regardless of vanillin addition. It has been already known that the attached microorganisms have produced more EPS than microbes in suspension (Liao *et al*. 2001).

The EPS concentrations in the suspended sludge, the MBR with vanillin showed greater protein concentration but lower polysaccharide concentration compared to the control MBR. Thus, the total EPS concentration in the MBR with vanillin was 16% smaller than the control MBR. Interestingly, both protein and polysaccharide contents in biocake were substantially lower in the MBR with vanillin than in the control MBR, therefore the total EPS in biocake of the MBR with vanillin was reduced to 31% of that in the control MBR. These results clearly tell that vanillin addition has reduced the EPS concentration, which positively related to the alleviation of biofouling in the MBR with vanillin although the total MLSS was greater in the control MBR as shown in Fig. 6. In addition, among the EPS, the polysaccharide concentrations were five times greater than protein, which means that the polysaccharide concentrations had great influence on biofouling in MBRs. Mukai *et al*. (1997) were also reported that the membrane permeate flux was seriously reduced in MBRs when the ratio of polysaccharide to protein was great. The composition of EPS, rather than the quantity of EPS was recognized as a governing factor of sludge properties (Liao *et al*. 2001). Therefore, the vanillin addition could be prospected to affect microbial physiology due to the difference in microbial community in the MBRs.

4. Conclusions

A compound inhibitory to quorum sensing, vanillin was investigated as a biofouling reducing agent of membrane bioreactors for wastewater treatment. The conclusions of this research as follows:

- The MBR sludge for this research was confirmed to contain QS signal molecules from the cross-feeding assay and the HPLC analysis, which indicates that the active microbial attachment on the MBR surfaces was involved by quorum sensing.
- The addition of vanillin was clearly reduced quorum sensing in CV026 by 62% reduction in terms of development of purple coloration.
- The dose of vanillin to MBR sludge was varied from 62.5 mg/L to 375 mg/L to evaluate biofilm formation in hollow fiber membranes. The vanillin doses more than 125 mg/L reduced biofilm formation on soaked membranes in the MBR sludge.
- Vanillin addition at the dose of 250 mg/L gave a positive effect to maintain TMP in the MBR by reduction of biofouling on the membrane surface, even though the MLSS concentration was higher at the MBR with vanillin than the control MBR. The reduction of biofouling was due to the reduced amount of EPS concentrations in the MBR with vanillin.

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