International Biodeterioration & Biodegradation 113 (2016) 66–73

Contents lists available at ScienceDirect

International Biodeterioration & Biodegradation

journal homepage: www.elsevier.com/locate/ibiod

Microbial population dynamics and profiling of quorum sensing agents in membrane bioreactor

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ARTICLE INFO

Article history:
Received 19 November 2015
Received in revised form
15 December 2015
Accepted 15 December 2015
Available online 24 December 2015

Keywords:
Membrane bioreactor
Quorum sensing
Acyl homoserine lactones (AHLs)
16S rRNA

ABSTRACT

The biofilm formation on membrane surface in a membrane bioreactor (MBR) leads to membrane biofouling and retards the filtration performance. Researchers have been focused towards investigating the physicochemical fouling control strategies ignoring the microbiological mechanism and agents responsible for biofouling. The study aimed at investigating the signal molecules like *N*-acyl homoserine lactones and dominant bacterial diversity responsible for cell to cell communication, known as quorum sensing which allow them to coordinate gene expression and regulate virulence, leading to formation of mature biofilm. Dominant strains were isolated from the activated sludge of MBR and screened for the ability to produce AHLs as signal molecules, using two biosensors, *Chromobacterium violaceum* CV026 and *Agrobacterium tumefaciens* A136. 16S rRNA sequencing approach was used for identification of dominant bacterial diversity along with QS strains. Among AHLs producing strains, maximum biofilm formation ability was indicated by *Pseudomonas kilonensis* and *Psychrobacter* sp. All bacterial strains showed different biofilm forming tendencies, irrespective of their growth rate, verifies the role of QS agents responsible for biofilm development. The presence of short and medium chain AHLs (C_4-HSL, C_6-HSL and C_8-HSL) was confirmed using HPLC. Production of AHLs and dominance of quorum sensing strains in activated sludge authenticate the key role of these substances in biofilm production and thereby membrane bio-fouling.

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1. Introduction

Membrane bioreactor (MBR) has been accepted as one of the best wastewater treatment technology due to performance efficiency, high load handling and less sludge production (Yeon et al., 2009). However, the widespread application of MBR is severely affected by membrane fouling caused by organic, inorganic or colloidal substances (Amy, 2008; Kim et al., 2012). Among various types of fouling, biofouling caused by formation of biocake (or deposition of microbial flocs) on the membrane surface, has attracted a great deal of attention due to its strong negative effects including, but not limited to, membrane clogging resulting in low treatment efficiency (Lee et al., 2014; Siddiqui et al., 2015).

These biofilms, comprising of polymeric substances, are important for bacterial survival in their social system. Where, these bacteria communicate each other, by a phenomenon known as quorum sensing (QS), using chemical signals or autoinducers (Shrout and Nerenberg, 2012; Xiong and Liu, 2010). Quorum sensing is essential for the development of mature biofilm and can rely on a variety of signal molecules depending upon the type and nature of the bacteria. Among various bacterial communities, signaling of gram-negative bacteria is of utmost importance as they are more dominant in activated sludge than other species. Gram-negative bacteria produce acyl homoserine lactones (AHLs) to communicate each other via LuxI/R system (Costerton et al., 1995). Successive release of these AHLs in the extracellular environment results into the accumulation of the signal molecules within the system. Once the concentration of AHLs reaches upto certain

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http://dx.doi.org/10.1016/j.ibiod.2015.12.014
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threshold level, the phenomenon of QS turned "ON" through binding of AHLs with cytoplasmic LuxR. Consequently, this cell–cell bacterial communication leads to the formation of matured biofilm colonies over the membrane surfaces (Christiaen et al., 2011).

Biocake layer can be developed by a single bacterium, but MBR always consists of a diverse bacterial community belongs to a variety of genera (Yun et al., 2006). MBR systems have been operated on various conditions and each set of conditions can favor a specific group of bacteria which may be different from the bacteria of other similar systems due to environmental factors and nature of inlet. Therefore, investigation and characterization of dominant bacterial community is very crucial prior to the selection of biofouling inhibition strategy for the treatment system. Since last few years, many researchers have focused towards the fouling control strategies by taking QS mechanism into account when developing new systems.

The present study was specifically designed to investigate the microbial diversity and their characteristics in a newly fabricated semi pilot scale MBR system with effective volume of 35 L. This is a well established fact that the bacterial species of a certain MBR cannot be compared and generalized for other MBR systems. Hence, profiling of signal molecules and bacterial communities, present in activated sludge of MBR via 16S rRNA analysis, was carried out to understand the complete picture of agents responsible for biofouling mechanism in MBR, being operated in the environmental conditions of Pakistan. Furthermore, all strains were evaluated in terms of EPS production, types of AHL released and hydrophobicity to authenticate the nature of biofouling bacteria, need to be targeted before selecting QS inhibition strategy.

2. Materials and methods

2.1. Experimental setup

A semi pilot scale membrane bioreactor with a total working volume of 35 L equipped with a immersible hollow fiber membrane (Memstar, China) with an average pore size of 0.1 μm and a surface area of 0.7 m^2 was used. The flux of permeate was maintained at 15 $L/m^2/h$ with a corresponding 4 h hydraulic retention time (HRT). Solids retention time (SRT) was maintained at 20 d with the biomass concentration ranging from 10 to 11 g/L. The membrane modules were equipped with an assembly for the coarse bubbling whereas, an additional aerator was used to assure thorough mixing to avoid dead zone at the bottom of the reactor. Synthetic wastewater was used as a substrate for the microbes with a COD:N:P as 100:10:2. Detailed recipe of the synthetic wastewater was similar to that reported by Maqbool et al. (2015).

2.2. Procedure for extraction and detection of AHLs

AHLs were extracted as per method described by Kim et al. (2012). Briefly, activated sludge sample (20 mL) was centrifuged at 4000 rpm for 15 min to remove large flocs. The supernatant and ethyl acetate was mixed in a ratio of 1:1. The mixture was vortexed at 120 rpm for 2 h, and the organic layer was collected using a separatory funnel. Cell debris were removed by centrifugation at 4000 rpm for 10 min. The supernatant was then dried in a rotary evaporator at 30 °C and residue was dissolved in 300 μl of methanol. *N*-octanoyl-*DL*-homoserine (C_8-HSL), *N*-hexanoyl-*DL*-homoserine (C_6-HSL) and *N*-butanoyl-*DL*-homoserine (C_4-HSL), purchased from Sigma–Aldrich, were used as standards. These standards were dissolved in methanol to obtain 1 mg/mL stock solution. Working solutions were prepared by mixing 20 μl of stock solution with 980 μl of methanol. Analysis were performed using a water/methanol (35:65) as mobile phase, UV detector along with column C18 (Gemini) was used for HPLC (Perkin Elmer) analysis. AHL standards and samples were injected HPLC system at a flow rate of 0.25 mL/min.

2.3. Isolation of dominant bacterial diversity

Activated sludge samples were collected from semi pilot scale MBR set up. The dominant microbial consortia were isolated by serial dilution of samples up to 10^{-9}. Each dilution (0.1 mL) was plated on different media including nutrient, Luria Bertani (LB) and tryptone soy agar and incubated at 37 °C for 24–48 h. Overall, thirty morphologically distinct colonies were isolated and confirmed through 16S rRNA analysis using polymerase chain reaction (PCR, thermocycler 9600).

2.3.1. Selection of quorum sensing bacteria

Among all isolated strains, screening of quorum sensing bacteria responsible for biofouling behavior was carried out using genetically modified organisms, *Chromobacterium violaceum* CV026 and *Agrobacterium tumefaciens* A136 (received from Seoul National University, South Korea), as biosensors.

2.3.2. Bioassay for quorum sensing bacteria

Bioassay, consisting of an indicating agar plate and bacteria to be tested, was carried out as per method described by Lade et al. (2014). A fresh culture, of CV026 or A136 and LB agar were mixed in a ratio of 1:9 to prepare indicating agar plate. The indicating agar plates for CV026 was supplemented with kanamycin (20 μg/mL), and spectinomycin (50 μg/mL) and tetracycline (4.5 μg/mL) were added along with X-gal for A136 bioassay, respectively. All the strains in the presence of CV026 and A136 produced purple or blue pigmentation were considered as QS or biofouling bacteria.

2.3.3. 16S rRNA sequencing analysis

DNA extraction of selected strains was performed using Direct DNA extraction kit (Norgen, Canada). All strains, including general bacterial population as well as QS bacteria, were amplified using universal primers, as listed in Table 1, through PCR and gel electrophoresis. After gel purification, sequencing analysis were conducted using services of Macrogen (Seoul, South Korea). Results obtained after sequencing were verified online using NCBI gene bank, http://www.ncbi.nlm.nih.gov/. After obtaining accession numbers, Eztaxon and MEGA 6 softwares were used to generate a phylogenetic tree.

2.4. Analysis for the biofilm formation

Biofilm formation ability of selected strains was observed using Microtiter plate assay. Briefly, wells of the micro plate were filled with LB broth for 1 h to perform conditioning at room temperature. Wells were then emptied and bacterial suspension along with LB agar was added to each well at ratio of 1:100 μL. Plates were sealed with para film and incubated at 28 °C for 24, 48 and 72 h under static condition. A classical crystal violet assay was used to quantify the biofilm (Lade et al., 2014). Amount of crystal violet absorbed by biofilm was extracted with 200 μl ethanol (95%), per well for 1 h,

Table 1
Primers used in the study.

Primer	Sequence (5′→3′)	Target genes (bp)	Reference
H^+	GAGTTTGATCCTGGCTCAG	16S rRNA (19)	Cheong et al., 2013
E^-	AGAAAGGAGGTGATCCAGCC	16S rRNA (20)	
518F	CCAGCAGCCGCGGTAATACG	16S rRNA (20)	Lade et al., 2014
800R	TACCAGGGTATCTAATCC	16S rRNA (18)	

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