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Quorum quenching specific genes screening among indigenous bacteria from full-scale membrane bioreactor and its application for biofouling control in a laboratory-scale membrane bioreactor

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ABSTRACT

Present study isolated novel indigenous quorum quenching (QQ) bacterial species from a full-scale membrane bioreactor (MBR) treating real wastewater and applied in a laboratory-scale MBR for biofouling control. In the first phase, screening of the responsible QQ genes was performed by microbiological and molecular techniques. The QQ activity in the isolated strains was analyzed by biosensor based biochemical analysis. 16S rRNA sequencing yielded nine QQ species, the majority belonging to the genus *Bacillus* sp. and *Enterobacter* sp. Biochemical analysis for the QQ activity indicated higher acyl-homoserine lactones degradation ability by *Pseudomonas* sp. QSP01 (QS – quorum sensing) and *Bacillus cereus* sp. QSP03. In the second phase, the biofouling retardation potential of the *Pseudomonas* sp. QSP01 and *Bacillus cereus* sp. QSP03 was investigated in a laboratory-scale QQ MBR. The working volume of the reactor was 5 L with a 0.5% bead volume. The hollow fiber membrane of surface area 0.1 m^2 was operated at a flux of 15 LMH with a sludge retention time of 20 d and a hydraulic retention time of 4.16 h. Mixed liquor suspended solids of 5–6 g/L was maintained in MBR. The filtration cycle of *Rhodococcus* sp. BH4, QSP01 and QSP03 were found to be 15, 13, and 8 d, respectively.

Keywords: Quorum quenching; Quorum sensing; Biofouling, Acyl-homoserine lactone; Screening methods; Filtration cycle; Membrane bioreactor

1. Introduction

Quorum quenching (QQ) bacterial species inhibit the interspecies quorum sensing (QS) process, the later phenomena play an important role in biofilm formation leading to membrane biofouling [1,2]. QS is a microbial communication system, where bacterial species monitor their own population density through signal molecules or autoinducers [3], including acyl-homoserine lactones (AHLs), oligopeptides and autoinducer-2 (AI-2), etc. Among them, AHLs, comprising of varying chain lengths, have been reported as the most common and abundant signaling system in membrane bioreactors (MBRs) [4,5]. Targeting the signal molecules, via QQ enzymes, produces by-products that are unable to induce QS and might further be utilized as sources of carbon, nitrogen or energy for the QQ population [6]. Moreover, chemicals, like curcumin and (–)–epigallocatechin gallate, have also been reported to inhibit the QS process.

In the recent decade, QQ enzymes and QQ bacterial species, introduced in laboratory-scale MBRs, demonstrated lower membrane biofouling in comparison to non-inoculated MBRs. It opened a new avenue to explore QQ in MBR while the mechanism behind QQ activity remained understudied.

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What had already been established is the role of signal molecules in biofilm formation on the membrane surface in bioreactors. The degradation of AHLs signal molecules by QQ enzymes led to lower extra-cellular polymeric substances (EPS) production and ultimately delayed the biofouling process [1,2,7]. A low understanding of the various factors and kinetics involved had led to the application of QQ majorly in laboratory-scale MBRs only. This biological approach to the biofouling mitigation requires further studies to bring it closer to practical application in full-scale MBR.

Enzymatic degradation of AHLs is reported in numerous bacterial species. *Bacillus* sp. and *Pseudomonas* sp. are most commonly reported to degrade AHLs by lactonases and acylases [8]. While in lab-scale MBRs, *Rhodococcus* sp. BH4 entrapped in alginate media has shown biofouling control ability [2,7]. The three major AHLs degrading enzymes are AHL-acylases, AHL-lactonases and AHL-oxidoreductase [9,10]. These bacteria producing enzymes are potential QQ species while their AHLs degradation ability varies throughout different species. The QQ bacteria with significant AHLs degrading capability under changing hydrodynamic and operational conditions for full-scale MBR is still unknown. Moreover, previous studies have placed less focus on the characteristics of the QQ enzymes produced by indigenous QQ bacterial species in MBR. The QQ genes responsible for the QQ enzyme production, its specific characteristics and the whole mechanisms remain understudied. Furthermore, QQ genes rendering QQ bacteria the ability to degrade AHLs in MBR have rarely been studied. Therefore, the present study attempted to identify the specific genes producing the QQ enzymes in a full-scale MBR and evaluate their application in a laboratory-scale MBR.

AHL degradation based QQ offers varying benefits including minimal or no resource consumption, for example, energy demand. What makes it more attractive is the nominal or no negative impact on treatment efficiency and no production of by-products that pose threat to health or the environment [11]. Therefore, continuous search for novel QQ strain capable of quenching the QS signal molecule remains important. To date, relatively few bacterial strains have been characterized as QQ species. Further study on QQ strategy to control biofouling is required in bringing the technique to real application in full-scale MBRs treating real wastewater.

Therefore, the present study attempted to screen new QQ bacterial species from full-scale MBR and identify the specific QQ genes responsible for the QQ activity in the isolates. Furthermore, it aimed at investigating the role of indigenous QQ strains *Pseudomonas* sp. and *Bacillus* sp. and comparing their performance to the widely studied QQ strain *Rhodococcus* BH4 in a laboratory-scale MBR.

2. Material and methods

2.1. Biosensor strains and growth media

Three biosensor species were used to analyze the QS and QQ activities in bacterial species isolated from the full-scale MBR plant treating real wastewater situated at the National University of Sciences and Technology (NUST), Islamabad, Pakistan. The first strain *Chromobacterium violaceum* CV026, *C. violaceum* via CviI/R AHL QS system regulates violacein production. It responds to C4-6-AHL, the short-chain AHLs producing purple pigment [12]. The strain was aerobically cultured at 37°C in Luria-Bertani (LB) media supplemented with 20 μg/mL kanamycin [4]. The second strain *Agrobacterium tumefaciens* A136 is sensitive to long-chain AHLs (C8-HSL, 3-O-C8-HSL, C10-HSL, C12-HSL, 3-O-C12-HSL and C14-HSL) [13]. The strain was aerobically cultured at 37°C in Luria-Bertani media supplemented with 50 μg/mL spectinomycin and 4.5 μg/mL tetracycline [14]. The third strain used in this study was a QS inhibition selector *Pseudomonas aeruginosa* QSIS2 strain that contains pLasB-SacB1 which encodes killing induced by exogenous AHLs. The strain was cultured in AB media with thiamine (ABT), AB media containing 2.5 mg/L of thiamine, 0.5% glucose, 0.5% casamino acid and 80 μg/mL gentamicin [15].

2.2. QQ bacterial isolation

Bacterial isolation was carried out from sludge samples collected from the full-scale NUST MBR plant. This MBR plant with a total capacity of 50 m^3/d, 2.35 m^3 membrane tank capacity, and 4.8 m^3 bio-tank capacity, treats the domestic wastewater. The operating conditions at the time of isolation were, 3.4 h hydraulic retention time (HRT), 20 d sludge retention time (SRT), 8 g/L mixed liquor suspended solids (MLSS), 2 mg/L dissolved oxygen concentration, 300 mg/L influent chemical oxygen demand (COD) and 7 mg/L effluent COD. Minimal media (MM) with AHLs mixture (3 mM) as sole carbon and nitrogen sources were used [1,10,16] with slight modifications. Briefly, AHLs mixture replaced the use of a single type AHL in the MM, representing all short-chained and long-chained AHLs. The microtubes were incubated at room temperature for 3 d and 100 μL of the aliquot was transferred in a new sterile microtube containing 1 mL fresh MM and 3 mM of all AHLs mixture. After incubation for another 3 d (second cycle), the same procedure was repeated for the third cycle. At the end of the third enrichment cycle, 100 μL cultures were spread and sub-cultured on tryptone soya agar (TSA), LB and nutrient agar media at 37°C for 24–48 h. Morphological characterization and the biochemical analysis aided in the acquisition of different pure cultures which were stored in glycerol stock at –80°C and used for QQ screening.

2.3. Analysis of AHL based QS activity

Well-diffusion assay was used for screening QS activity in gram-negative species using two biosensor species, CV026 and A136 [17]. All the plates were incubated at 28°C for 48 h. The diameter of colonies, producing purple or blue coloration, was measured in centimeters. The parallel streak method was also performed for further verification of AHLs producing ability of the isolated bacterial species [18]. Test strain was streaked on LB agar plates beside biosensor strains. The colored test strain colonies were noted as positive or negative, based on visual observation.

2.4. Analysis of AHL-degradation activity in consortia and isolates

The QSIS2 assay was performed according to the method reported [10,15]. Initially, spent media from the three

enrichment cycles during sample preparation were filtered through a 0.22 μm filter and diluted by 1:100 using distilled water. One mL of the diluted media was added in a vial containing 3 mL QSIS2 culture, incubated for 24 h and analyzed in a spectrophotometer (DR2010, HACH, USA). Sterile non-inoculated MM in QSIS2 culture media served as the negative control.

Well-diffusion and QSIS2 assay were used for the detection of QQ activity in the isolates. Briefly, 50 μL CV026 liquid culture was added to 5 mL LB agar cooled down to below 50°C, supplemented by a short-chain AHL (5 μM C6HSL) and kanamycin. It was poured on the surface of LB agar plates. Upon solidification in sterile conditions, 5 mm diameter wells were made in the agar with a sterile pipette. The wells were labeled and filled with 50 μL test isolate's liquid culture. In the case of A136 indicator plates, 50 μL A136 liquid culture was used and supplemented with a long-chain AHL (5 μM C10HSL), *x*-gal and the relevant antibiotics. Sterile LB broth was added as a negative control. All the indicator plates were incubated for 3 d at 28°C. The plates were observed for the inhibition zone in a colored background.

Moreover, the QSIS2 assay was performed for individual isolates, after obtaining 24 h of liquid cultures in LB broth [10,15]. Briefly, each test strain was inoculated in a mixture containing 800 nM of each C4HSL and 3OC12HSL. After incubation for 24 h at 37°C, all samples were filtered, sterilized, and supplemented with 24 h fresh QSIS2 liquid culture in ABT media. Non-inoculated LB broth with distilled water and 800 nM of each C4HSL and 3OC12HSL mixture was served as negative and positive controls, respectively. All vials were incubated for another 24 h at 37°C. Optical density at 600 nm was measured and the percent remaining AHLs were determined indirectly based on the decrease in QSIS2 growth equation relative to the positive (100% AHLs remaining) and negative control (0% AHLs).

2.5. Analysis of QQ enzyme activity and location

The heat stability of QQ molecules and QQ enzyme location was predicted via biosensor based biochemical analysis [10]. For heat treatment, 24 h cell cultures in LB broth were filtered and sterilized at 121°C for 20 min. For another 24 h, 1 mL of this sterile supernatant was incubated with 3 mL QSIS2 culture at 37°C supplemented with 800 nM each of C4HSL and 3OC12HSL. Further methods were similar for the QSIS2 assay for isolates. Further elucidation of QQ activity was carried out by bio-agar assay as per the methods reported [11]. *Rhodococcus* BH4 was added as a known QQ reference species [1]. The plates were incubated for 48 h at 28°C. The colonies in the colored background were observed for inhibition zone patterns and diameters. Heat stability was determined to evaluate the sensitivity of QQ enzyme to temperature. High heat stability indicates lower denaturation of proteins at high temperature.

2.6. Bacterial identification

All strains were amplified using universal primers, 785F/907R and 27F/1492R, via polymerase chain reaction (PCR) and gel electrophoresis. Whereas, sequencing analysis was conducted using the services of Macrogen (Seoul, South Korea). The raw sequences were processed in BioEdit (version 7.2.5) and verified online via NCBI gene bank (http://www.ncbi.nlm.nih.gov). After acquiring the accession numbers, MEGA 7 (version 10.0) software was used for the phylogenetic analysis.

2.7. Identification of QQ genes

DNA extraction of QQ strains was performed via Genomic DNA Mini Kit (PureLink, USA). Specific primers for QQ genes, encoding AHL acylases and AHL lactonase, were designed using Primer3Plus software [19] and synthesized by Operon, EU. The PCR (Thermocycler Extra Gene 9600) reaction mixture, conditions and cycling program for a specific primer were optimized (Table 1). Resultant amplicons were studied by gel documentation.

2.8. QQ beads in laboratory-scale MBR

Isolated QQ strains were studied in a laboratory-scale MBR. In MBR, EPS are formed which allows them to bind cell to cell and cumulatively results in membrane biofouling [20]. With the production of AHL hydrolyzing enzymes, lower EPS can be anticipated as signal molecules are degraded and bacterial communication is retarded [21]. This prolongs the membrane run time, extending the life of the membrane [22].

Table 1
Specific primers for QQ genes, composition of reaction mixture and cycling programs for PCR reactions

Primer	Sequence (5′ to 3′)	Reaction mixture	PCR cycling program
PvdQ-for	GTTCTGCACGAAGTCCCTG	1 μL, DNA; 2 μL each, forward	60 s of aneal (30 cycles) at 62.3°C, 60 s
PvdQ-Rev	GCTGTTGGGTTCGATGATG	and reverse primers; 32.5 μL, nuclease-free water	of extending at 60.2°C (30 cycles), 5 min of post-extension at 60.2°C (1 cycle)
AiiA-for	GATGGCCTGGAGAATGAC	0.5 μL, DNA; 2 μL each, forward	60 s of aneal (30 cycles) at 59.9°C, 60 s
AiiA-Rev	GCGTGTAGGGTATGAGCC	and reverse primers; 1 μL $MgCl_2$; 32 μL, nuclease-free water	of extending at 62.2°C (30 cycles), 5 min of post-extension at 62.2°C (1 cycle)
QuiP-for	GTCGGCCAGGTAATAGAGC	0.5 μL, DNA; 2 μL each, forward	60 s of aneal (30 cycles) at 62.3°C, 60 s
QuiP-Rev	GCTACCGTCCGGAATACTG	and reverse primers; 1 μL $MgCl_2$; 32 μL, nuclease-free water	of extending at 62.3°C (30 cycles), 5 min of post-extension at 62.3°C (1 cycle)

The working volume of the reactor was 5 L with a 0.5% bead volume as shown in Fig. 1. The surface of the membrane (Hollow fiber, Econity, USA) was 0.1 m^2. Specification of the membrane is given (Table 2). The membrane was operated at a flux of 15 LMH using a peristaltic pump (BT300-2J, Longer Pump, China). The HRT was kept at 4.16 h. The membrane was operated at a filtration mode of 8 min of filtration and 2 min of relaxation without backwashing until the transmembrane pressure (TMP) reached 30 kPa. TMP was monitored using data logging TMP meter (840086, Sper Scientific, USA).

2.9. QQ beads preparation

Alginate beads of 7 mg QQ bacteria/g sodium alginate were prepared by dripping mixture into 4% calcium chloride solution and left for 2 h, followed by coating with a 10% polysulfone solution. Finally, the prepared beads were solidified by storing at 4°C in distilled water for 24 h [1].

2.10. Wastewater feed composition

Synthetic wastewater feed was prepared chemically for the study [23]. It was prepared in a batch before each membrane run and stored at 4°C. Wastewater was added into the influent storage tank periodically. The influent wastewater characteristic for glucose. Ammonium chloride, sodium bicarbonate, calcium carbonate, potassium dihydrogen phosphate, magnesium sulfate, ferric chloride, nickel chloride, zinc chloride and cobalt chloride were 500, 191, 100, 4.87, 23.85, 4.87, 0.5, 0.05, 0.05, and 0.05 mg/L, respectively.

2.11. Activated sludge characteristics

Activated sludge was taken from the full-scale MBR and acclimatized to synthetic wastewater. SRT was kept at 20 d to keep the sludge healthy. Eventually, the MLSS was stabilized between 5–6 g/L under ambient conditions.

2.12. Analytical methods

Soluble microbial products (SMP) were measured by centrifuging MLSS at 5,000 rpm to remove the soluble fraction while EPS was measured for both loosely and tightly bound EPS using cation exchange resins (CER). Concentrations for both SMP and EPS were measured in terms of carbohydrates and proteins by Dubois and Lowry methods, respectively.

3. Results and discussion

3.1. AHL degrading bacterial diversity in MBR

Based on the distinct morphology and biochemical characteristics, a total of 17 isolates were screened out using AHLs mixture as the sole carbon and nitrogen source. Briefly, pure colonies were streaked on nutrients, TSA and LB agar plates in triplets and the colony characteristics were visually observed based on the form, elevation and margin. To further distinguish among the acquired bacterial colonies, biochemical analysis including gram-testing, oxidase test and catalase test was conducted. Among these, 47% and 53% were comprised of gram-positive and gram-negative bacteria species, respectively; whereas reported gram-negative species were found to be the most abundant (75%) among bacteria isolated from environmental samples by the same isolation methodology [10]. The present study indicated that gram-positive species were also equally able to use AHL as a sole carbon source. The most active 9 QQ species were identified through 16S rRNA sequencing (Table 3). *Bacillus* sp. have been characterized previously as QQ species and their QQ activity has been demonstrated in

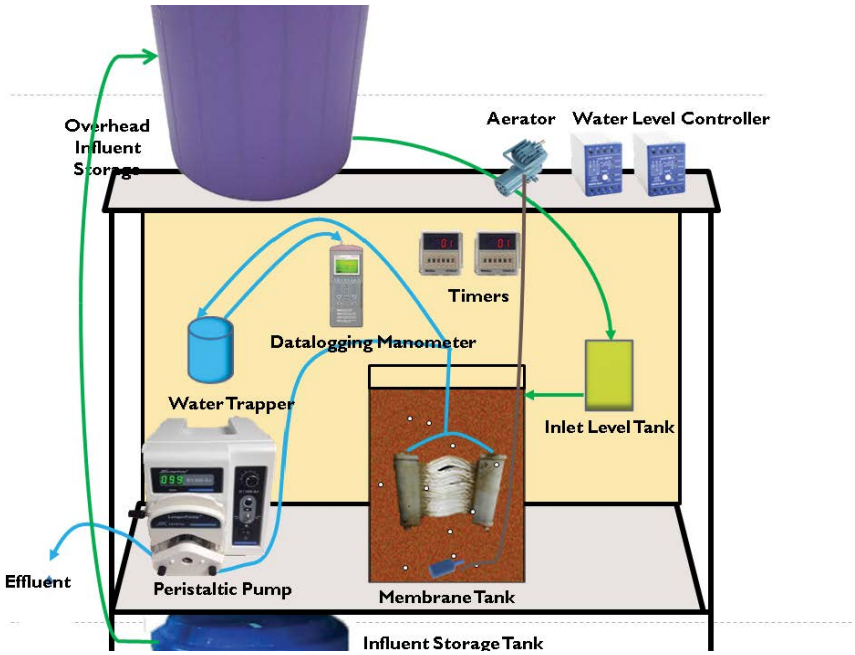

Fig. 1. Laboratory-scale MBR setup with QQ beads.

laboratory-scale MBR [24,25,34,35]. Moreover, indigenous strains *Corynebacterium* sp. QSP02, *Kocuria* sp. QSP05, and *Brucella* sp. QSP09 were reported for the first time for their ability to survive on AHLs as a carbon source. Previously, *Enterobacter* and *Pseudomonas* sp. have been detected in laboratory-scale MBR [7], owing to their ability to withstand harsh conditions and their prevalence in wastewater as appeared in full-scale MBR as well.

Among the isolated strains classified into 7 different genera, species belonging to the genus *Bacillus* and *Enterobacter* sp. appeared in abundance. The evolutionary relationship of taxa (Fig. 2) between species isolated in the present study (with blue dots) and QQ strains reported from previously reported laboratory-scale MBR [26] gave further insight into the QQ bacterial profiles of the two MBRs of different scales. Very few similarities were observed among isolates from full-scale vs. laboratory-scale MBRs. The change in physical conditions, operating conditions, and wastewater composition in both MBRs favor different taxa, as indicated by the different profiles of laboratory-scale and full-scale MBRs under similar ambient environment.

Table 2
Hollow fiber membrane specifications

Specification	Range
Membrane chemistry	High density polyethylene
Membrane type	Hollow fiber
Membrane pore type	Slit pore and asymmetric structure
Membrane pore size	0.4 μm
Membrane outer/inner diameter	0.65/0.41 mm
Average operating flux	12.5–20 LMH
Chlorine resistance	1,000,000 ppm h
Operating pressure	0.05–0.6 bar
Allowable pH range	2–13
Operating temperature range (°C)	5–40

3.2. AHLs degradation via QQ consortium

QSIS2 assay for consortia gave an insight into the joint QQ activity of unknown bacterial species in the samples. It was assumed until the third enrichment cycle, bacterial species were able to use AHLs as sole C and N sources enabling them to survive. The results (Fig. 3) indicate a 40%–85% decrease in QSIS2 strain's growth compare to blank lacking AHLs (100% QSIS2 growth) after the first enrichment cycle. During this stage, most probably due to mixed QS and QQ species, the QS bacterial species may have contributed to the decrease in biosensor strain's growth through AHLs production.

The QSIS2 growth was further lowered (5%–24%) in the second enrichment sample and it appeared to be the lowest level (2%–12%) in the third enrichment cycle. It may be inferred that almost all AHLs in the sample had been degraded via QQ consortium.

3.3. AHLs based QS profile of isolated bacterial species

Gram-negative bacterial species play a dominant role in the AHL production while previous studies have shown the simultaneous potential of QS and QQ in some bacterial species [27–29]. Therefore, AHL based QS analyses of the gram-negative isolates were carried out by CV026 and A136 biosensor strains. The lowest QS activity was indicated by *Pseudomonas* sp. QS01 strain while *Brucella* sp. QS09 indicated the highest QS activity. Short-chain AHLs production was more prominent than the long-chain AHLs on the biosensor containing indicator plates (Fig. 4).

Previously, high AHL production by *Pseudomonas kilonensis* and *Psychrobacter* sp. was observed among isolates from laboratory-scale MBR [26]. It was also reported that *P. aeruginosa* PAO1 produced N-butanoyl-L-homoserine

Table 3
16S rRNA sequence identity of isolated QQ strains, their QQ activity potential and probable enzyme location

Isolate code	Closest 16S rRNA identity	Accession no.	QS activity		QQ activity			Probable QQ enzyme location
			Well-diffusion	Parallel streak	Well diffusion	Bioagar assay	QSIS2 assay	
QSP01	*Pseudomonas aeruginosa* VSS6/99%	KY576793	+	+	+++	++	+++	Exoenzyme
QSP02	*Corynebacterium striatum* 1954BRRJ/94%	KY576794	++	+	+	+	+	Unrecognizable
QSP03	*Bacillus cereus* PSMRRAAGRI15/99%	KY576795	+	++	+++	++	++	Endoenzyme
QSP04	*Enterobacter cloacae* B3/100%	KY576796	++	++	+	+	++	Exoenzyme
QSP05	*Kocuria flava* HO-9041/99%	KY576797	+	++	+	++	++	Exoenzyme
QSP06	*Lysinibacillus* sp. BAB-4376/99%	KY576798	+	+	+	+	++	Exoenzyme
QSP08	*Enterobacter cloacae* APSAC 03/99%	KY576799	+++	++	+	+++	++	Exoenzyme
QSP09	*Brucella suis* AAg01/99%	KY576800	+++	+	+	+	++	Endoenzyme
QSP10	*Bacillus subtilis* LG4	KY576801	+	+	++	++	+	Endoenzyme

+ Low activity; ++ Medium; +++ High activity

lactone and was positive for QS production [30]. While *Pseudomonas* sp. QSP01 in the present study indicated minimal AHLs production (Fig. 4).

3.4. AHLs degradation ability of isolated bacterial species

The intensity of pigment produced or inhibited on a CV026 indicator plate may correspond to QS as well as QQ ability [30]. Well diffusion assay, supplemented with C6HSL and C10HSL, indicated *Pseudomonas* and *Bacillus* sp. as strong degraders (Fig. 5) than *Rhodococcus* sp. BH4 [11] and *Delftia* sp. [26].

Enterobacter cloacae QSP04, *Pseudomonas* sp. QSP01, and *Lysinibacillus* sp. QSP06 showed higher QQ activity as compared to the other strains. All strains showed some degree of QQ ability (Fig. 6). *Bacillus* sp. QSP10 degraded approximately half, that is, 800 nM of the total supplemented AHLs of 1,600 nM in 24 h, evaluated from % remaining AHLs given in Fig. 6.

It is also reported the QQ activity in *Bacillus* sp. and *Pseudomonas* sp., the three most active species including *Pseudomonas* sp. QSP01, *Bacillus cereus* QSP03 and *Bacillus subtilis* QSP10 were selected out of 9 strains for the identification of responsible QQ genes [8].

3.5. Characteristics of QQ molecules produced by isolated bacterial species

It is reported that thermal resistance and thermophilicity in the microbial lactonases family of thermophilic archaea [31]. Later, Christiaen et al. [10] also identified heat-stable QS signal molecules mainly in genera *Arthrobacter*, *Bacillus*, *Pseudomonas*, and *Delftia* species.

Various QQ enzyme's characteristics and nature impact the overall QQ activity of bacterial species. In the present study, most of the species indicated varying heat stability. The QQ molecules, responsible for AHLs degradation, appeared to retain or recover their activity under room temperature after heat sterilizations at 121°C for 20 min (Fig. 7).

QQ enzyme activity of *Pseudomonas* sp. via biofilter assay and observed a ring formation around the inhibition zone, usually formed by exoenzymes [11]. Among the known QQ

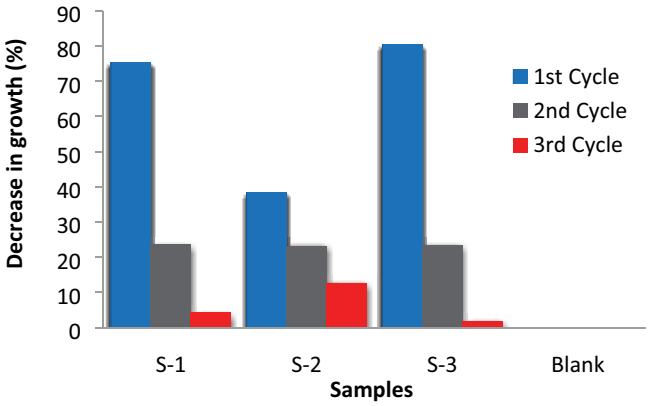

Fig. 3. The decrease in QSIS2 biosensor strain growth induced by AHLs in three coded samples: sample 1 (S-1), sample 2 (S-2) and sample 3 (S-3).

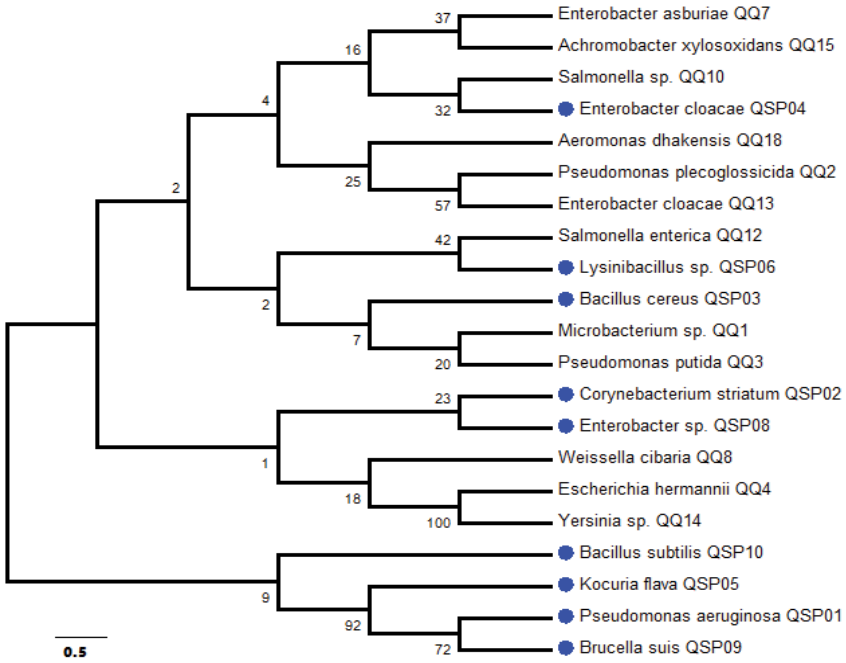

Fig. 2. Evolutionary relationships of QQ bacterial isolates from laboratory-scale vs. full-scale MBRs at NUST, Islamabad (Neighbor-Joining method, MEGA 7).

molecules of low weight that were capable of degrading enzymes, lactonase is an endoenzyme while acylase is an

exoenzyme. Based on these indicators, most of the isolates in the present study indicated the exoenzyme production, except *Bacillus cereus* QSP03, *Brucella suis* QSP09, and *Bacillus subtilis* QSP10 (Table 3).

Whereas, a white ring appeared around the colony of *Pseudomonas* sp. QSP01 strain. *Pseudomonas* sp. also indicated the production of extra-cellular QQ enzymes in a study conducted by Kim et al. [32]. Therefore, it was assumed as an indication of extra-cellular activity in *Pseudomonas* sp. QSP01. The white ring was not shown by reference species, *Rhodococcus* sp. BH4 [20]. Further, *Bacillus cereus* QSP03 and *Bacillus subtilis* QSP10 indicated the presence of endoenzymes, which could be lactonase.

3.6. QQ specific genes in the isolated QQ species

Various known species of *Pseudomonas* genus were reported for acylase enzyme production [11,29,33]. Two acylase producer genes, *PvdQ* (1411 bp) and *QuiP* (572 bp) were selected for the study [26,34]. Whereas, *AiiA* gene, responsible for lactonase production, was also opted [35].

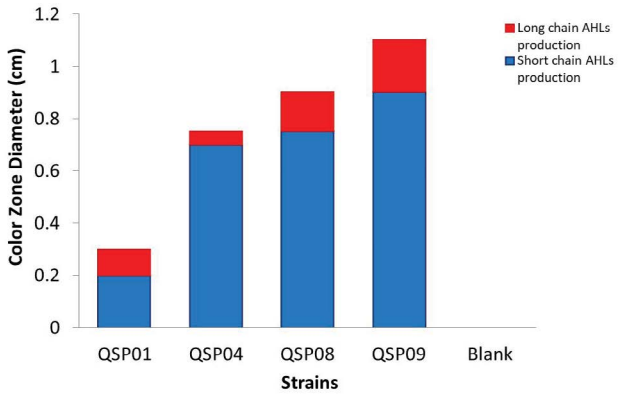

Fig. 4. QS in the gram-negative bacterial species for short and long-chain AHLs.

Pseudomonas sp. QSP01 amplified with two acylase specific primers yielded products corresponding to QQ acylase base pair size reported in the literature [26,34] (Figs. 8a–c).

While, *Bacillus cereus* QSP03 and *Bacillus subtilis* QSP10 amplified with lactonase specific primers yielded products corresponding to QQ lactonase base pair size (Figs. 9a and b). Therefore, it can be inferred that QSP01, QSP03 and QSP10 might have significant potential for quenching as compared to other isolates due to production of QQ acylase and QQ lactonase.

3.7. Effluent water quality

Effluent water quality was measured in terms of biological oxygen demand (BOD), COD, total phosphorous (TP) and total nitrogen (TN). The results are shown in Table 4.

While the highest removal efficiency was observed in conventional MBR in terms of water quality parameters, the removal efficiency of QQ-MBR was not compromised and still exhibited excellent effluent quality as reported in the previous study [36]. Among QQ-MBRs, better removal efficiencies were reported in *Rhodococcus* sp. BH4 where it was 90.3%, 92.8%, 68.2%, and 70.1% in terms of BOD, COD, TP, and TN, respectively.

3.8. Fouling mitigation with laboratory-scale QQ-MBR

The indigenous QQ strains *Pseudomonas* sp. QSP01 and *Bacillus* sp. QSP03 identified in the full-scale MBR plant, having the ability of QQ as well as QS, were encapsulated in PVDF alginate beads and their effects were studied individually in a laboratory-scale MBR along with the comparison with *Rhodococcus* sp. BH4, vacant beads and conventional MBR. The performance of these QQ strains was evaluated in terms of SMP and EPS as reported in Table 5. There were two replicates taken under each MBR condition during the study.

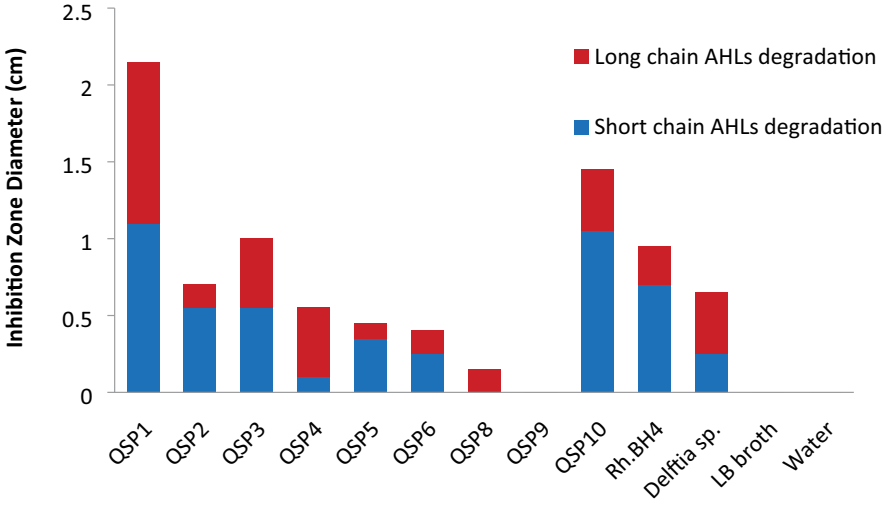

Fig. 5. Combined QQ activity in the isolated bacterial species for short and long-chain AHLs.

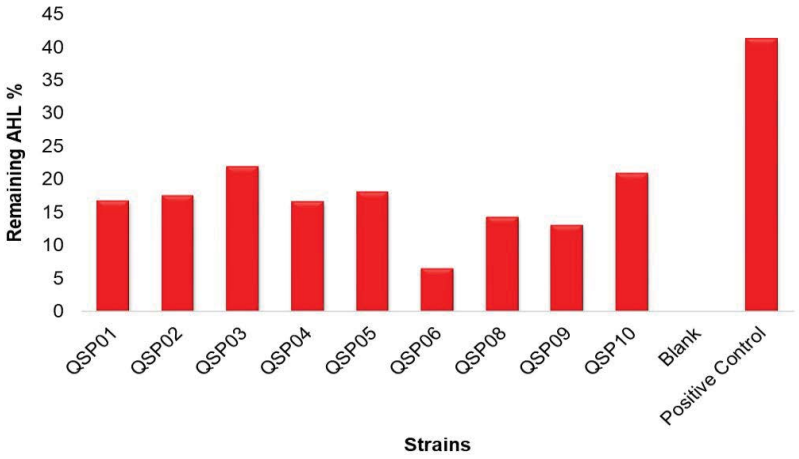

Fig. 6. Residual AHL level relative to positive and negative control is considered 100% and 0% AHL residual, respectively.

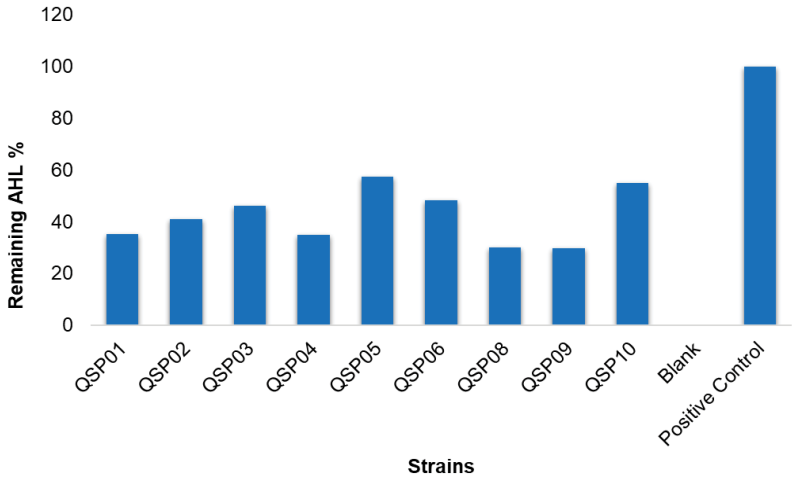

Fig. 7. QQ activity was studied in QSIS2 assay after heat and filter sterilization of isolates.

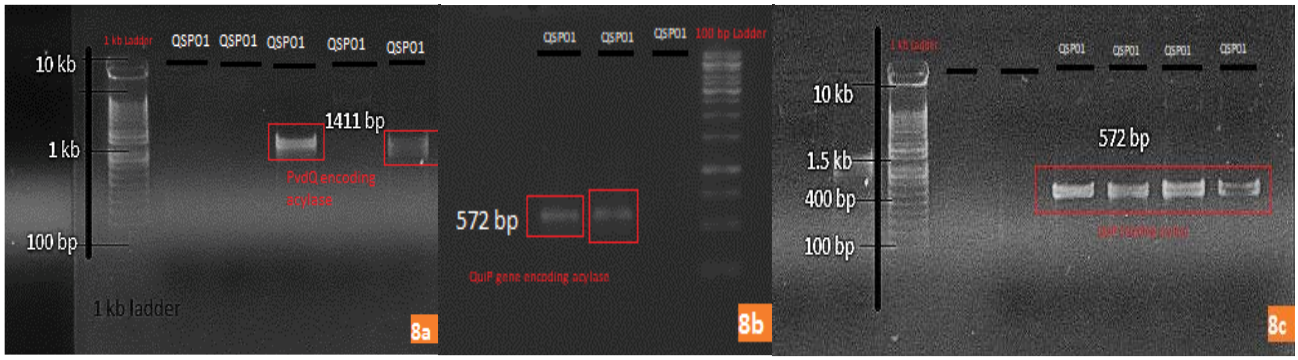

Fig. 8. Gel documentation of, (a) *PvdQ* gene size of 1411 bp detected in *Pseudomonas* sp. QSP01, (b) *QuiP* genes responsible for QQ enzyme production in strain *Pseudomonas* sp. QSP01 with 100 bp ladder and (c) *QuiP* genes in *Pseudomonas* sp. QSP01 cross-verified by using a 1 kb ladder.

Table 4
Effluent quality and removal efficiencies in conventional, vacant, and QQ MBRs

MBR run	Unit	BOD	COD	TP	TN
Conventional MBR	mg/L	5.8 ± 0.14	10.3 ± 4.88	15.8 ± 0.66	14.3 ± 0.79
	%	96.1 ± 0.09	97.9 ± 0.97	80.3 ± 0.83	79.6 ± 1.13
Vacant MBR	mg/L	9.8 ± 0.23	14.2 ± 4.99	16.9 ± 0.05	14.9 ± 1.98
	%	6.5 ± 0.15	97.1 ± 0.99	78.9 ± 0.06	78.7 ± 2.82
Pseudomonas sp. QSP01	mg/L	11.7 ± 1.46	19.5 ± 9.17	22.4 ± 1.41	22.9 ± 0.79
	%	92.2 ± 0.97	96.1 ± 1.83	72.0 ± 1.76	67.3 ± 1.13
Bacillus sp. QSP03	mg/L	8.5 ± 0.21	12.9 ± 0.0	17.8 ± 0.15	23.5 ± 1.58
	%	94.3 ± 0.14	97.4 ± 0.0	77.7 ± 0.18	66.4 ± 2.26
Rhodococcus sp. BH4	mg/L	17.6 ± 4.33	32.9 ± 4.12	25.5 ± 0.25	20.9 ± 3.96
	%	88.3 ± 2.88	93.4 ± 0.82	68.2 ± 0.31	70.2 ± 5.66

Table 5
Performance of QQ strains in lab-scale MBR

Parameter	Conventional MBR	Vacant MBR	*Pseudomonas* sp. (QSP01)	*Bacillus* sp. (QSP03)	*Rhodococcus* BH4
SMP, mg/L	197.1 ± 27.6	170.2 ± 23.4	138.3 ± 20.0	154.8 ± 20.6	104.3 ± 16.3
EPS, mg/g	146.9 ± 18.5	130.6 ± 18.4	82.7 ± 16.4	93.8 ± 18.9	67.9 ± 16.5

EPS decreased in *Rhodococcus* sp. BH4 and *Pseudomonas* sp. QSP01 runs by 47% and 30%, respectively and SMP decreased by 54% and 44% over conventional MBR respectively, a trend observed in an earlier study [2]. A more profound decrease in SMP by *Rhodococcus* sp. BH4 led to a longer membrane run as compared to other QQ species as shown in Fig. 10.

TMP trends shown in Fig. 10 depict that *Rhodococcus* sp. BH4 was still the most suitable QQ strain lasting 15 d in MBR while indigenous QQ strains *Pseudomonas* sp. QSP01 exhibited a slightly shorter membrane run time of 13 d followed by *Bacillus* sp. QSP03 with a runtime of 8 d. A longer and stable run in *Pseudomonas* sp. QSP01 and *Rhodococcus* sp. BH4 can be caused due to a 36% and 48% decrease in EPS which delayed the formation of biofilm on the membrane surface. Conventional and vacant MBR showed a membrane runtime of only 5 and 6 d, respectively.

3.9. Discussion

In this study, Proteobacteria phyla dominated the QQ bacterial species isolated from full-scale MBR. This is consistent with previous findings of Proteobacteria as the most abundant phyla in MBR [37–40]. The biological profile, especially QQ bacterial screening in full-scale MBR had not been extensively studied so far. It is hard to generalize bacterial species abundance based on a few studies. Upon comparison of the QQ bacterial profile of full-scale MBR in the present study with QQ bacterial profile of laboratory-scale MBR reported previously [26], minor similarities were observed, although the involved methodologies were similar. The contributing factors to the difference require further studies, including impacts due to the physical conditions, operating conditions, wastewater composition and scale of the MBR.

Furthermore, the dual nature of a single species, that is QS as well as QQ by the same genus demands a deeper understanding of the underlying mechanism involved in AHLs production and degradation. This study indicated that isolated bacterial species can exhibit both tendencies of QS and QQ. QQ tendency in few species was higher than the QS tendency of the same species, indicated by QQ and QS analysis conducted in parallel qualitatively. But it does not completely represent the environment in a full-scale MBR, for which application in laboratory-scale MBR will verify the dominant role of bacterial species in QS and QQ. Therefore, the QQ study of bacterial species shall be supplemented with QS study, keeping in view the dual nature of bacterial species, for practical application for biofouling control in MBR.

QQ enzymes have been identified previously for their ability to degrade biofilm produced by disease-causing bacteria in humans, animals and plants, as a potential alternative to antibiotics [35]. In MBRs, AHLs degradation can also be attributed to QQ enzymes, including AHL-acylase and AHL-lactonase produced by *PvdQ*, *QuiP*, and *AiiA* genes. These genes or QQ enzymes might be responsible for membrane biofouling retardation observed in previous studies. In a study conducted by Cheong et al. [20], they observed lower membrane biofouling in a laboratory-scale MBR inoculated with *Pseudomonas* sp. 1A1 QQ bacteria. Also, these bacterial species may be used for biofouling control in MBR. Screening results from this study yielded nine QQ species, the majority belonging to the genus *Bacillus* sp. and *Enterobacter* sp. The AHLs degrading ability of *Pseudomonas* sp. and *Bacillus* sp. surpassed the others. The gel documentation of PCR products indicated gene products of base-pair sizes corresponding to AHL-acylase producing *PvdQ* and *QuiP* genes in *Pseudomonas* sp. QSP01 and lactonase producing *AiiA* gene in *Bacillus cereus* QSP03 and *Bacillus subtilis*

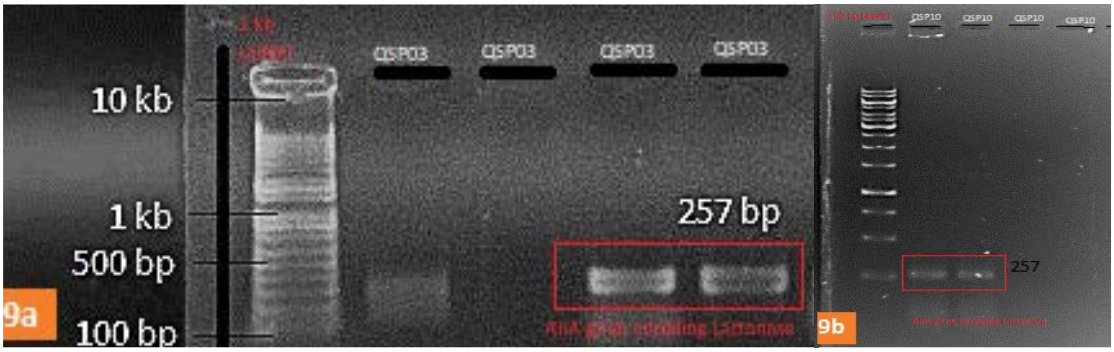

Fig. 9. (a): *AiiA* gene responsible for QQ enzyme production in *Bacillus cereus* QSP03 and (b) *AiiA* gene in *Bacillus subtilis* QS10.

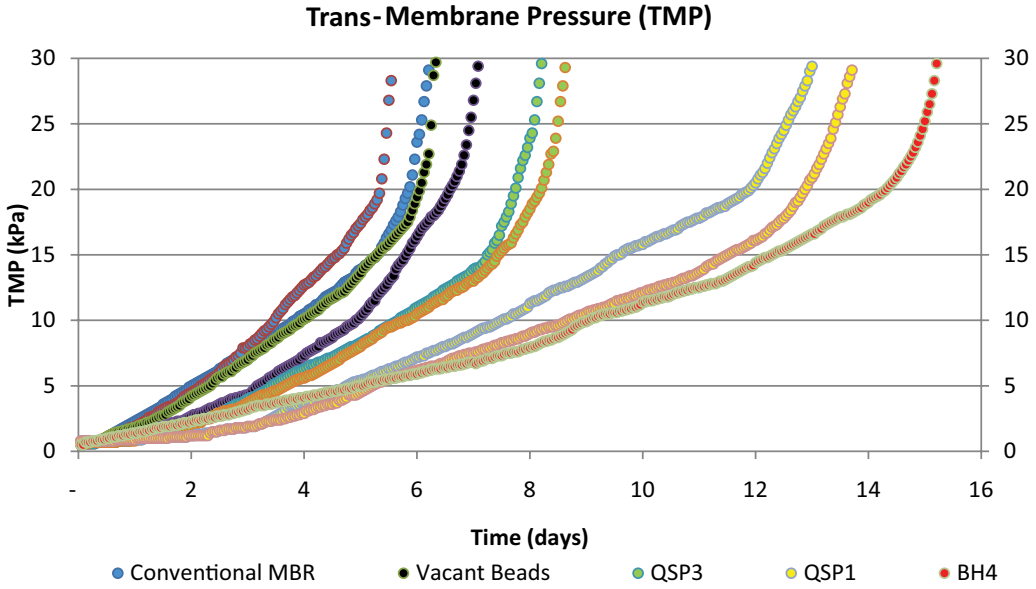

Fig. 10. Transmembrane pressure (TMP of conventional MBR and MBRs with vacant, QSP1, QSP3 and *Rhodococcus* sp. BH4 embedded beads.

QSP10 strains. Therefore, these genes verify the production of QQ enzymes with the ability of biofouling retardation in MBR.

It was observed that MBR containing *Pseudomonas* sp. QSP01 beads were able to last 13 d as compared to 8 d of *Bacillus* sp. QSP3. Both were able to perform significantly well in comparison to conventional MBR and vacant beads filtration runs which lasted only 5 and 6 d, respectively. The physical scouring effect of vacant beads increased the membrane run time by 1 d over conventional MBR. Replicate runs were performed in each case to ensure the reliability of the results. However, the most suitable QQ strain was still found to be *Rhodococcus* sp. BH4 where membrane filtration lasted 15 d. There was a delay in TMP rise observed in QQ-MBR inoculated with *Pseudomonas* sp. and *Rhodococcus* sp. BH4 embedded beads in comparison to conventional and vacant MBR due to low SMP/EPS and consequently, impeding biofilm growth on the membrane surface.

4. Conclusions

The factors contributing to the QQ bacterial profile in the full-scale MBR include physical conditions, operating conditions, wastewater composition and scale of the MBR. The indigenous strains, *Pseudomonas* sp. QSP01 and *Bacillus* sp. QSP03 identified to have more chances of survival in the pilot and full-scale MBR since it naturally exists in the local wastewater. This study indicated that isolated species have both tendencies of QS and QQ. QQ tendency in few species was higher than the QS tendency of the same species. Such QQ species embedded beads in MBR translated in decreasing the membrane biofouling potential and increasing membrane run time.

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References

[1] S.-R. Kim, H.-S. Oh, S.-J. Jo, K.-M. Yeon, C.-H. Lee, D.-J. Lim, C.-H. Lee, J.-K. Lee, Biofouling control with bead-entrapped quorum quenching bacteria in membrane bioreactors: physical and biological effects, Environ. Sci. Technol., 47 (2013) 836–842.

[2] T. Maqbool, S.J. Khan, H. Waheed, C.-H. Lee, I. Hashmi, H. Iqbal, Membrane biofouling retardation and improved sludge characteristics using quorum quenching bacteria in submerged membrane bioreactor, J. Membr. Sci., 483 (2015) 75–83.

[3] S. Schauder, B.L. Bassler, The languages of bacteria, Genes Dev., 15 (2001) 1468–1480.

[4] D. Anbazhagan, M. Mansor, G.O.S. Yan, M.Y.M. Yusof, H. Hassan, S.D. Sekaran, Detection of quorum sensing signal molecules and identification of an autoinducer synthase gene among biofilm forming clinical isolates of *Acinetobacter* spp., PLoS One, 7 (2012) e36696.

[5] A. Camilli, B.L. Bassler, Bacterial small-molecule signaling pathways, Science, 311 (2006) 1113–1116.

[6] N. Amara, B.P. Krom, G.F. Kaufmann, M.M. Meijler, Macromolecular inhibition of quorum sensing: enzymes, antibodies, and beyond, Chem. Rev., 111 (2011) 195–208.

[7] H. Waheed, S. Pervez, I. Hashmi, S.J. Khan, S.-R. Kim, High-performing antifouling bacterial consortium for submerged membrane bioreactor treating synthetic wastewater, Int. J. Environ. Sci. Technol., 15 (2018) 395–404.

[8] K. Morohoshi, A.M. Goodwin, M. Ohbayashi, S.J. Ono, Autoimmunity in retinal degeneration: autoimmune retinopathy and age-related macular degeneration, J. Autoimmunity, 33 (2009) 247–254.

[9] S. Uroz, S.R. Chhabra, M. Cámara, P. Williams, P. Oger, Y. Dessaux, *N*-Acylhomoserine lactone quorum-sensing molecules are modified and degraded by *Rhodococcus erythropolis* W2 by both amidolytic and novel oxidoreductase activities, Microbiology, 151 (2005) 3313–3322.

[10] S.E.A. Christiaen, G. Brackman, H.J. Nelis, T. Coenye, Isolation and identification of quorum quenching bacteria from environmental samples, J. Microbiol. Methods, 87 (2011) 213–219.

[11] W.-S. Cheong, C.-H. Lee, Y.-H. Moon, H.-S. Oh, S.-R. Kim, S.H. Lee, C.-H. Lee, J.-K. Lee, Isolation and identification of indigenous quorum quenching bacteria, *Pseudomonas* sp. 1A1, for biofouling control in MBR, Ind. Eng. Chem. Res., 52 (2013) 10554–10560.

[12] K.H. McClean, M.K. Winson, L. Fish, A. Taylor, S.R. Chhabra, M. Camara, M. Daykin, J.H. Lamb, S. Swift, B.W. Bycroft, G.S.A.B. Stewart, P. Williams, Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of N-acylhomoserine lactones, Microbiology, 143 (1997) 3703–3711.

[13] B. Watson, T.C. Currier, M.P. Gordon, M.D. Chilton, E.W. Nester, Plasmid required for virulence of *Agrobacterium tumefaciens*, J. Bacteriol., 123 (1975) 255–264.

[14] K.H. Tang, Y.H. Zhang, M. Yu, X.C. Shi, T. Coenye, P. Bossier, X.-H. Zhang, Evaluation of a new high-throughput method for identifying quorum quenching bacteria, Sci. Rep., 3 (2013), https://doi.org/10.1038/srep02935.

[15] T.B. Rasmussen, T. Bjarnsholt, M.E. Skindersoe, M. Hentzer, P. Kristoffersen, M. Köte, J. Nielsen, L. Eberl, M. Givskov, Screening for quorum-sensing inhibitors (QSI) by use of a novel genetic system, the QSI selector, J. Bacteriol., 187 (2005) 1799–1814.

[16] H. Lade, D. Paul, J.H. Kweon, Combined effects of curcumin and (–)–epigallocatechin gallate on inhibition of N-acylhomoserine lactone-mediated biofilm formation in wastewater bacteria from membrane bioreactor, J. Microbiol. Biotechnol., 25 (2015) 1908–1919.

[17] H. Lade, D. Paul, J.H. Kweon, Quorum quenching mediated approaches for control of membrane biofouling, Int. J. Biol. Sci., 10 (2014) 550–565.

[18] N.G. Heatley, A simple plate method for multiple tests of the antibacterial activity of many bacteria against other bacterial strains, Microbiology, 1 (1947) 168–170.

[19] A. Untergasser, H. Nijveen, X.Y. Rao, T. Bisseling, R. Geurts, J.A.M. Leunissen, Primer3Plus, an enhanced web interface to Primer3, Nucleic Acids Res., 35 (2007) W71–W74.

[20] W.-S. Cheong, S.-R. Kim, H.-S. Oh, S.H. Lee, K.-M. Yeon, C.-H. Lee, J.-K. Lee, Design of quorum quenching microbial vessel to enhance cell viability for biofouling control in membrane bioreactor, J. Microbiol. Biotechnol., 24 (2014) 97–105.

[21] J.R. Liu, D. Nguyen, M. Paice, Aerobic granule formation in a sequencing batch reactor treating newsprint effluent under low phosphate conditions, Water Sci. Technol., 62 (2010) 2571–2578.

[22] H.-S. Oh, K.-M. Yeon, C.-S. Yang, S.-R. Kim, C.-H. Lee, S.Y. Park, J.Y. Han, J.-K. Lee, Control of membrane biofouling in MBR for wastewater treatment by quorum quenching bacteria encapsulated in microporous membrane, Environ. Sci. Technol., 46 (2012) 4877–4884.

[23] J. Wu, C.D. He, Effect of cyclic aeration on fouling in submerged membrane bioreactor for wastewater treatment, Water Res., 46 (2012) 3507–3515.

[24] J. Kaur, K.N. Yogalakshmi, Efficacy of quorum quenching bacteria *Bacillus cereus* 1306 encapsulated sodium alginate-magnetic iron nanocomposites in controlling quorum sensing mediated biofilm formation, Environ. We Int. J. Sci. Technol., 12 (2017) 29–36.

[25] F.F. Xu, C. Zhao, C.H. Lee, W.Z. Wang, Q.Y. Xu, Anti-biofouling performance of an immobilized indigenous quorum quenching bacterium *Bacillus cereus* HG10 and its influence on the microbial community in a bioreactor, Int. J. Environ. Res. Public Health, 16 (2019) 3777.

[26] H. Waheed, I. Hashmi, S.J. Khan, S.R. Kim, M. Arshad, H. Nasir, Microbial population dynamics and profiling of quorum sensing agents in membrane bioreactor, Int. Biodeterior. Biodegrad., 113 (2016) 66–73.

[27] K.-M. Yeon, C.-H. Lee, J.B. Kim, Magnetic enzyme carrier for effective biofouling control in the membrane bioreactor based on enzymatic quorum quenching, Environ. Sci. Technol., 43 (2009) 7403–7409.

[28] K.-G. Chan, S. Atkinson, K. Mathee, C.-K. Sam, S.R. Chhabra, M. Cámara, C.-L. Koh, P. Williams, Characterization of *N*-acylhomoserine lactone-degrading bacteria associated with the *Zingiber officinale* (ginger) rhizosphere: co-existence of quorum quenching and quorum sensing in *Acinetobacter* and *Burkholderia*, BMC Microbiol., 11 (2011), https://doi.org/10.1186/1471-2180-11-51.

[29] T.-M. Chong, C.-L. Koh, C.-K. Sam, Y.-M. Choo, W.-F. Yin, K.-G. Chan, Characterization of quorum sensing and quorum quenching soil bacteria isolated from Malaysian tropical montane forest, Sensors, 12 (2012) 4846–4859.

[30] L. Zhang, P.J. Murphy, A. Kerr, M.E. Tate, Agrobacterium conjugation and gene regulation by N-acyl-L-homoserine lactones, Nature, 362 (1993) 446–448.

[31] L. Mandrich, L. Merone, G. Manco, Hyperthermophilic phosphotriesterases/lactonases for the environment and human health, Environ. Technol., 31 (2010) 1115–1127.

[32] S.-R. Kim, K.-B. Lee, J.-E. Kim, Y.-J. Won, K.-M. Yeon, C.-H. Lee, D.-J. Lim, Macroencapsulation of quorum quenching bacteria by polymeric membrane layer and its application to MBR for biofouling control, J. Membr. Sci., 473 (2015) 109–117.

[33] C.F. Sio, L.G. Otten, R.H. Cool, S.P. Diggle, P.G. Braun, R. Bos, M. Daykin, M. Cámara, P. Williams, W.J. Quax, Quorum quenching by an *N*-acyl-homoserine lactone acylase from *Pseudomonas aeruginosa* PAO1, Infect. Immun., 74 (2006) 1673–1682.

[34] M. Bokhove, P.N. Jimenez, W.J. Quax, B.W. Dijkstra, The quorum-quenching *N*-acyl homoserine lactone acylase *PvdQ* is an Ntn-hydrolase with an unusual substrate-binding pocket, 9/24/2020 10:16:56 AM, 107 (2010) 686–691.

[35] C. Reimmann, N. Ginet, L. Michel, C. Keel, P. Michaux, V. Krishnapillai, M. Zala, K. Heurlier, K. Triandafillu, H. Harms, G. Défago, D. Haas, Genetically programmed autoinducer destruction reduces virulence gene expression and swarming motility in *Pseudomonas aeruginosa* PAO1, Microbiology, 148 (2002) 923–932.
[36] N.A. Weerasekara, K.-H. Choo, C.-H. Lee, Biofouling control: bacterial quorum quenching vs. chlorination in membrane bioreactors, Water Res., 103 (2016) 293–301.
[37] Z. Ahmed, J.W. Cho, B.-R. Lim, K.-G. Song, K.-H. Ahn, Effects of sludge retention time on membrane fouling and microbial community structure in a membrane bioreactor, J. Membr. Sci., 287 (2007) 211–218.
[38] M.W. Falk, K.-G. Song, M.G. Matiasek, S. Wuertz, Microbial community dynamics in replicate membrane bioreactors – natural reproducible fluctuations, Water Res., 43 (2009) 842–852.
[39] C.-Y. Wan, H. De Wever, L. Diels, C. Thoeye, J.-B. Liang, L.-N. Huang, Biodiversity and population dynamics of microorganisms in a full-scale membrane bioreactor for municipal wastewater treatment, Water Res., 45 (2011) 1129–1138.
[40] S.J. Jo, H. Kwon, S.-Y. Jeong, S.H. Lee, H.-S. Oh, T. Yi, C.-H. Lee, T.G. Kim, Effects of quorum quenching on the microbial community of biofilm in an anoxic/oxic MBR for wastewater treatment, J. Microbiol. Biotechnol., 26 (2016) 1593–1604.