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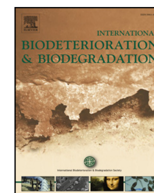

Control of sludge microbial biofilm by novel quorum quenching bacteria *Pseudomonas nitroreducens* JYQ3 and *Pseudomonas* JYQ4 encapsulated sodium alginate - Magnetic iron nanocomposites

Jaskiran Kaur, K.N. Yogalakshmi*

Centre for Environmental Sciences and Technology, School of Environment and Earth Sciences, Central University of Punjab, Bathinda, 151001, India

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ABSTRACT

Biofouling control via quorum quenching (QQ) approach is becoming popular among other fouling mitigation strategies. In the present study, the mitigation of membrane biofouling problem via disruption of cell-to-cell communication was studied by immobilizing QQ bacteria in sodium alginate magnetic nanocomposite beads. Two QQ bacteria viz. *Pseudomonas nitroreducens* JYQ3 and *Pseudomonas* JYQ4 isolated from dairy waste activated sludge was immobilized individually into the beads. Scanning electron microscopy (SEM) analysis revealed the successful immobilization of QQ bacteria into the alginate nanoparticle beads. The Confocal laser scanning microscopy (CLSM) results provided evidence of biofouling controlled activities of *Pseudomonas nitroreducens* JYQ3 and *Pseudomonas* JYQ4 immobilized nanocomposite (IMN) beads. Further, the membranes incubated with *Pseudomonas nitroreducens* JYQ3 and *Pseudomonas* JYQ4 IMN beads showed better flux (19% and 22% higher, respectively) than the control. Hence, the results revealed that the QQ bacteria IMN beads would be an efficient approach for controlling the biofilm developed on the cellulose acetate membrane surface.

1. Introduction

Fouling is one of the serious problems faced by membrane processes. Operationally, it is defined as a cutback in water transport per unit area of membrane (flux), caused by the substance or substances in the feed water that accumulates on or within the membrane (Paul and Abanmy, 1990). Biofouling contributed a significant portion in the fouling mechanism. Biofouling is exemplified by the formation of biofilm or accretion of biological material such as extracellular polymeric substances (EPS) on the membrane surface. Flux decline, increased transmembrane pressure (TMP), high energy consumption and loss of membrane shelf life are the effects of biofouling that interrupts the widespread application of membranes thereby posing a major problem in the environmental sectors (Wisniewski and Grasmick, 1998; Gander et al., 2000; Meyer, 2003). Quorum sensing (QS), a cross talk system utilized by bacteria, regulates the biofilm formation via secretion of various signaling molecules such as acyl homoserine lactone (*N*-butanoylhomoserine lactone, *N*-hexanoylhomoserine lactone, 3-oxo-C10-HSL, *N*-(3-oxododecanoyl)-homoserine lactone) and autoinducing peptides (Vuong et al., 2003; Parsek and Greenberg, 2005; Waheed et al., 2015). To control biofouling, an array of conventional and advanced methods has been studied in the past few years (Prado et al., 2017). But, none of these approaches are efficient in preventing the biofilm growth as it is an innate phenomenon for bacteria to attach to the membrane surface. However, disruption of these QS signals, a phenomenon termed as quorum quenching (QQ), would minimize the hostile effects of membrane biofouling.

Targeting the signaling molecules through enzymatic degradation has received great attention in recent times. QQ bacteria coexist with QS bacteria and possess an inherent ability to disrupt the signaling molecules (Chan et al., 2011; Christiaen et al., 2011). Enzymes such as lactonases and acylases encoded by QQ bacteria are few among the popularly reported enzymes to carry out the AHL inactivation (Lin et al., 2003; Kalia et al., 2011). Several studies have been reported to mitigate membrane biofouling through enzymatic AHL inactivation in membrane based wastewater treatment processes such as membrane bioreactors (Yeon et al., 2009; Kim et al., 2011; Lee et al., 2014). Immobilized QQ enzyme acylase is reported to alleviate the biofilm maturation of *Pseudomonas aeruginosa* PAO1 on polyvinylidene fluoride (PVDF) membrane surface thereby enhancing the filtration performance through prevention of membrane fouling (Lee et al., 2014). However, fouling control through enzymatic degradation suffers from certain problems such as the cost of production and purification.

To overcome the above mentioned problems, research focus has

* Corresponding author.
E-mail addresses: yogalakshmi25@gmail.com, yogalakshmi@cup.edu.in (K.N. Yogalakshmi).

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been shifted towards the application of intact bacterial cell immobilized carriers for antifouling studies. In view of this, Oh et al. (2012) encapsulated QQ bacteria *Rhodococcus* sp. in the membrane vessel and observed biofouling inhibition in the membrane bioreactor (MBR). The entrapped bacteria was successful in degrading only those signaling molecules that diffused into the vessel, thus restricting the QQ activity. This limitation was successfully embarked upon by entrapping the *Rhodococcus* sp. BH4 into the microstructural pores of the alginate beads. The entrapped species showed a reduction in extracellular polymeric substances (EPS) generation by microbial cells of the biofilm (Kim et al., 2013). The stability of the alginate beads was increased by coating with polymers poly(vinylidene) fluoride (PVDF), polyethersulfone (PES) and polysulfone (Kim et al., 2015).

The present work was commenced with the objective to explore the potential of dairy waste activated sludge (WAS) QQ bacteria *Pseudomonas nitroreducens* JYQ3 and *Pseudomonas* JYQ4 immobilized on magnetic nanocomposites (IMN) carriers in degrading signaling molecules N-hexanoyl homoserine lactone (C6-HSL). Special focus was given to investigate and compared the QQ potential of different QQ bacteria IMN beads against QS mediated biofilm formed by sludge bacteria on cellulose acetate membrane. The magnetic iron nanoparticles were used as a carrier of nanoparticles that would allow the magnetic separation of IMN beads from the mixture of bacteria in the sludge sample during the experiments. To the best of our knowledge, so far no study has reported the QQ potential of *Pseudomonas nitroreducens* JYQ3 IMN beads and their ability to control membrane biofouling. The results obtained in the study showed successful control of biofilm formation on cellulose acetate membrane. Hence, the present study intends to provide a breakthrough for controlling membrane biofouling using QQ bacteria IMN beads in MBR.

2. Materials and methods

2.1. Bacterial strain and culture conditions

Two native QQ bacteria namely *Pseudomonas nitroreducens* JYQ3 (KP340459) and *Pseudomonas* JYQ4 (KU555415) isolated from the waste activated sludge (WAS) of dairy industry wastewater (Verka milk plant, Bathinda, India) were used in the study (Kaur and Yogalakshmi 2018). For the isolation and enrichment of QQ bacteria, KG medium given by Kok-Gan was used (Chan et al., 2009). Signaling molecule C6-HSL was added into the liquid KG medium as the solitary source of carbon and nitrogen. The isolates were cultured in Luria Bertani (LB) broth overnight at 28 °C and 220 rpm.

2.2. Synthesis and characterization of magnetic iron nanoparticles

Magnetic iron nanoparticles were synthesized by co-precipitation method (Kouassi et al., 2005). Salts of ferrous and ferric chloride (Fe^{2+} and Fe^{3+} ratio = 1:2) of 0.25 M concentration were dissolved in distilled water and precipitated chemically at room temperature (25 °C) with 3M NaOH at pH 10. The precipitate was heated (80 °C) for 35 min under continuous shaking. The Fe_3O_4 particles were separated using a magnet (20 megaoersted strength) and washed four times in distilled water and several times with ethanol. The particles were then dried in a vacuum oven at 70 °C. The synthesized magnetic iron nanoparticles were examined for morphology and size using Transmission electron microscope (TEM; Model TECNAI G^2 2S-TWIN) and Scanning electron microscope (SEM; Merlin Compact 6073). The magnetic strength of the nanoparticles was quantified using Vibrating Sample Magnetometer (VSM; Lakeshore Model 7410).

2.3. Immobilization of QQ bacteria in IMN beads

The QQ bacteria were immobilized in sodium alginate magnetic iron nanocomposites as per the method described by Ivanova et al. (2011). The reason behind using sodium alginate/magnetic iron nanocomposites as carrier material for immobilization was attributed to its low cost and non-toxic nature. The cultures of individual QQ bacteria *Pseudomonas nitroreducens* JYQ3 and *Pseudomonas* JYQ4 maintained at 28 °C on LB agar medium (50 mL) were harvested at the early stage of their exponential growth phase by centrifuging at 9400 rpm, 4 °C for 10 min. The QQ bacterial pellets were washed twice with sterile distilled water. After washing, about 1g wet cells were finally mixed with 2% sterilized sodium alginate (20 mL) and 0.5 g of magnetic iron nanoparticles. The mixture was then dropped into the ice cold solution of 2% calcium chloride (w/v) via a silicone tubing (ID 3.5 mm/OD 7 mm) using a peristaltic pump (Masterflex, Cole Parmer). Gel beads formed were allowed to harden for 2 h at room temperature (25 °C) and then stored at 4 °C for 12 h. The hardened wet beads were filtered, washed with distilled water and used for antifouling studies. The average size of the beads ranged from 4 mm to 5 mm.

2.4. Characterization of QQ bacteria IMN beads

The immobilization of QQ bacteria in the nanocomposites was confirmed by SEM analysis. The sample preparation for SEM analysis was done according to the procedure given by Chen et al. (2012). The blank and QQ bacteria IMN beads were washed three times with distilled water and fixed in 3% glutaraldehyde for 2 h at room temperature. The fixed beads were washed with distilled water to remove residual glutaraldehyde and dehydrated with series of ethanol gradient (30%, 50%, 70%, 90% and 100%). At last, all the samples were freeze dried and coated with gold for SEM analysis.

2.5. QQ potential of IMN beads

The QQ ability of *Pseudomonas nitroreducens* JYQ3 and *Pseudomonas* JYQ4 IMN beads was determined through degradation of C6-HSL. Gas chromatography-Mass spectrometry (GC-MS) analysis was carried out for determining the degradation ability of QQ bacteria IMN beads using protocol suggested by Kim et al. (2013). 25 mg l^{-1} of C6-HSL was added to 50 mM Tris HCl buffer (30 mL) in four different conical flasks designated as A_0, A_1, A_2 and A_3. About 10 beads of *Pseudomonas nitroreducens* JYQ3, *Pseudomonas* JYQ4 IMN beads were added to the flasks A_2 and A_3 and incubated at 28 °C and 150 rpm. Flasks A_0 (without any beads) and A_1 (blank nanoparticle beads) were operated as control and incubated along with the flasks A_2 and A_3. Every hour about 1 mL of sample was withdrawn from each flask up to 6 h and extracted with 1 mL ethyl acetate. The mixture was shaken vigorously for 30 s on vortex mixer and two phases were allowed to separate. The top layer of the extract was collected and the whole extraction process was repeated three times. The extract was analyzed for C6-HSL degradation compounds as per the protocol suggested by Rani et al. (2011) in GC-MS (SHIMADZU GC-MS-Model QP, 2010 Ultra). For the analysis of C6-HSL, capillary column RTxi-1ms (30 m × 1 mm X 0.1 μm) was used. Conditions of mass spectrometer were: electron ionization source at 70 eV, MS ion source temperature at 200 °C and solvent cut time was 3.5 min. The mass spectrometer was run in full scan mode (*m/z* 30–500) and in Selected Ion Monitoring (SIM) mode at 143 *m/z*. The spectra obtained were compared for their retention times with the synthetic C6-HSL standard and their degradation percentages were calculated using equation (1).

$$\text{Amount of C6-HSL degraded (\%)} = \frac{Ai-Af}{Ai} \text{ X } 100 \tag{1}$$

where Ai = initial concentration of C6-HSL and Af = C6-HSL concentration after degradation.

2.6. Biofilm growth inhibition studies on glass slides

The biofilm inhibition by QQ bacteria was assessed as per the

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