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Efficacy of Quorum Quenching Bacteria Bacillus cereus 1306 Encapsulated Sodium Alginate - Magnetic Iron Nanocomposites in controlling Quorum Sensing Mediated Biofilm Formation

Jaskiran Kaur and K.N.Yogalakshmi*
Centre for Environmental Sciences and Technology, School of Environment and Earth Sciences, Central University of Punjab, Bathinda 151001, India
*Email: yogalakshmi25@gmail.com
Telephone number- (M)-91-8427706161, (O)- +91-1642864132
Fax number- +91-1642864111

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Abstract

Biofilm formation on membranes is a major issue of wastewater treatment processes. The signalling molecules produced by the quorum sensing (QS) bacteria is the main player behind the membrane biofilm formation. Degardation of signalling molecules via quorum quenching (QQ) bacteria is considered an effective alternative in controlling membrane biofouling for wastewater treatment processes. In the present study, QQ bacteria *Bacillus cereus* 1306 was immobilized in sodium alginate with magnetic iron nanoparticles and checked for its efficiency to control membrane biofouling. The scanning electron microscopy (SEM) analysis of immobilized magnetic iron nanocomposites (IMN) beads revealed the successful entrapment of *Bacillus cereus* 1306 within the beads. In membrane filtration experiments, the *B. cereus* 1306 IMN beads showed membrane flux decline of 64% as compared to control membrane (without *B. cereus* 1306 IMN beads) that showed decline in membrane flux by 73% in 30 days. Hence, a QQ bacterium IMN beads is an effective approach in controlling membrane biofouling.

Introduction

Membrane biofouling obstructs the prevalent application of various membrane separation processes (Baker and Dudley, 1998). The term membrane biofouling refers to the blockage of membrane pores due to formation of microbial biofilm. Quorum sensing,

a communication circuit utilized by micro-organisms play a noteworthy role in the development of biofilm. The microbes secrete various signaling molecules termed autoinducers which regulate the biofilm formation. The biofilm formation decreases the filtration performance with time. Henceforth, membrane biofouling is considered as one of the major issue which needs to be addressed on priority. Various methods including physical and chemical cleaning of membranes, sludge disintegration, membrane modification and biological methods were studied to control microbial biofilm formation (Bouhabila *et al.*, 2001; Woo *et al.*, 2013; Huang *et al.*, 2008; Liu *et al.*, 2010; Choudhary and Schmidt- Dannert, 2010). Among them, biological control method to embark upon biofouling problem has been proven to be the efficient method.

Biofouling control by biological method via disruption of signalling molecules through quorum quenching (QQ) mechanism is done by various researchers in the past few years (Choudhary and Schmidt- Dannert, 2010; Oh *et al.*, 2012; Kim *et al.*, 2013; Siddiqui *et al.*, 2015). Cheong *et al.*, (2013) encapsulated QQ bacteria *Pseudomonas sp. 1A1* in microbial vessel and observed membrane biofouling inhibition on polyvinylidene fluoride hollow fiber membrane. Khan *et al.*, (2016) immobilized QQ bacteria *Bacillus methylotrophicus* sp. WY in sodium alginate beads and reported 3 to 4 times increased membrane flux when compared to vacant beads indicating membrane biofouling ability of the bacteria. In view of this, we immobilized QQ bacteria *Bacillus cereus* 1306 in sodium alginate with magnetic iron nanoparticles and studied it ability to control membrane biofouling in filtration experiments.

Materials and Methods

Bacterial strain and culture conditions

The QQ bacteria *Bacillus cereus* 1306 and biofilm forming bacteria *Pseudomonas aeruginosa 3541* purchased from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India was used in the present study. The cultures were grown in Luria Bertani (LB) broth overnight at 30 ˚C and at 220 rpm.

Preparation of magnetic nanoparticles

The magnetic nanoparticles were prepared by co-precipitation of ferric and ferrous chloride (25M concentration each) salts in 2:1 ratio with 3M NaOH (pH 10). The mixture was kept on hot plate at 80˚C for 35 minutes under continuous shaking. The magnetite particles were separated with 20 megaoersted strength magnet and washed using distilled water and ethanol. The precipitates were kept in oven at 100 ˚C for drying (Kouassi *et al.*, 2005).

Characterization of magnetic iron nanoparticles

Scanning electron microscopy (SEM; Merlin Compact 6073) was used for the characterization of surface morphology of magnetic nanoparticle. The size and magnetic property of synthesized nanoparticles was identified using Transmission electron

microscope (TEM; Model TECNAI G^2 2S-TWIN) and Vibrating Sample Magnetometer (VSM; Lakeshore Model 7410), respectively.

Immobilization of *B. cereus* 1306 in immobilized magnetic nanocomposite (IMN) beads

The *B. cereus* 1306 were inoculated in 50 mL of LB broth at 30 ˚C and 220 rpm. The overnight grown culture was centrifuged at 9400 rpm and at 4˚C for 10 minutes. Pellet was washed with distilled water. The immobilization procedure was done as per the method suggested by Ivanova *et al.*, (2011) with some modifications. The 20 mL of 2% (w/v) sterilized sodium alginate was mixed with 0.1 g of iron nanoparticles to which 1 g of *B. cereus* 1306 was added. The mixed suspension was then dripped into ice cold $CaCl_2$ solution (2% w/v) using silicone tubing (ID 3.5 mm/OD 7 mm) attached to the peristaltic pump (Masterflex, Cole Parmer). After keeping the beads for 2 hours at room temperature for hardening, the beads were kept in 2% (w/v) $CaCl_2$ solution for 12 hours and at 4 ˚C for cross linking. The beads were filtered and washed with distilled water. The average bead size as measured by screw gauge was in the range of 4-5 mm. The beads were then used for the flux studies.

Characterization of IMN beads

The IMN beads were characterized by SEM for evaluating the successful immobilization of *B. cereus* 1306 in the beads. For SEM analysis, the sample was prepared using method formulated by Chen *et al.*, (2012). The beads were fixed for two hours in glutaraldehyde (3%), washed with distilled water and then dehydrated in ethanol series from 30%, 50%, 70%, 90% up to 100%. The beads were kept in refrigerator for freeze drying overnight. Next day, the beads were coated with gold for SEM analysis.

Membrane biofilm inhibition studies

P. aeruginosa 3541 is a biofilm forming bacteria. The effect of *B. cereus* 1306 in inhibiting the biofilm formation of *P. aeruginosa* 3541 on the membrane surface was evaluated as per the method proposed by Lee *et al.*, (2014). Around 18 cellulose acetate membranes of pore size 0.45 µm was attached to glass slides and placed in 100 mL of three sterilized beakers (6 membranes in each beaker) labelled as A_1 to A_3. 49 mL of synthetic dairy wastewater and 1 mL of *P. aeruginosa* 3541 were filled in each beaker. The composition of synthetic dairy wastewater is given in Table 1. Beaker A_1 was operated as control without addition of beads. To the beaker A_2 and A_3, around 10 blank nanoparticle beads (without *B. cereus* 1306) and 10 beads of *B. cereus* 1306 were added, respectively. Second control A_2 was operated to confirm if the biofilm inhibition effect is either by QQ or by physicochemical adsorption by blank nanoparticle beads. Each beaker was incubated at 30 ˚C and at 150 rpm for 30 days. Every day, the substrate was replaced with fresh synthetic dairy wastewater in both beakers. One membrane sample was removed from each beaker after every 5^{th} day for flux studies.

Table 1 Composition of synthetic dairy wastewater

Ingredient	Amount (g/L)
Powdered milk	2.5
NH_4Cl	1.4
$CaCl_2$	0.038
$MgSO_4.7H_2O$	0.05
KH_2PO_4	1
$NaHCO_3$	2

Membrane biofouling due to biofilm formation on the surface of membrane was determined by studying changes in membrane flux. The membrane flux was studied by filtering 100 mL distilled water through membrane at constant pressure (10 kPa). The membrane flux (flow of liquid per unit membrane area) is calculated by using formula given in Equation 1.

$$J = \frac{Q_p}{A_m} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

where J = Flux, $m^3/m^2/h$

Q_p = Filtrate flow rate through the membrane, m^3/h

A_m = Membrane surface area, m^2

Statistical analysis

The flux studies were carried in triplicates and data was investigated statistically. Results were given as mean±S.E. One way analysis of variance (ANOVA) was done for determining the significant difference in fouling potential of *B. cereus 1306* at different time intervals using SPSS Statistics 20. The differences were significant at $p<0.05$.

Results and Discussion

Characterization of magnetic iron nanoparticles

Figure 1 shows the SEM micrographs of magnetic nanoparticles. From the Figure, it was observed that the nanoparticles exhibited cubical shape. The cubical shape provide high surface-to-volume ratio. This permit strong interaction of bacteria with the nanoparticles (Zhao *et al.*, 2016). Further, the uniform distribution of particles was observed in SEM micrographs (Figure 1). The absence agglomeration indicated proper mixing of ferrous and ferric chloride. The nanoparticles showed an average size of 74 nm as analyzed by TEM. This confirms that the synthesized nanoparticles fall in the nanometer range (1-100 nm). From the VSM analysis, it was found that the saturation magnetization of magnetic nanoparticles was 39 emu/g, which is sufficient for the separation of nanoparticles from the mixture. The results are in complete agreement with Wu *et al.*, (2008) that reported saturation magnetization in the range of 30-80 emu/g.

Characterization of IMN beads

Figure 2 displays the visual examination of IMN beads. From the Figure, it is clear that the particles were light black in color showing the presence of nanoparticles. Figure 3 depicts the cross-sectional SEM images of vacant and *B. cereus* 1306 IMN beads.

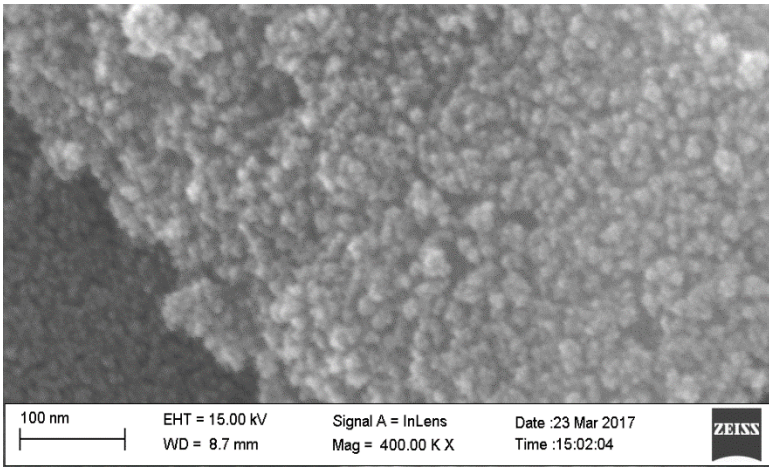

Figure 1 SEM analysis of synthesized magnetic nanoparticles

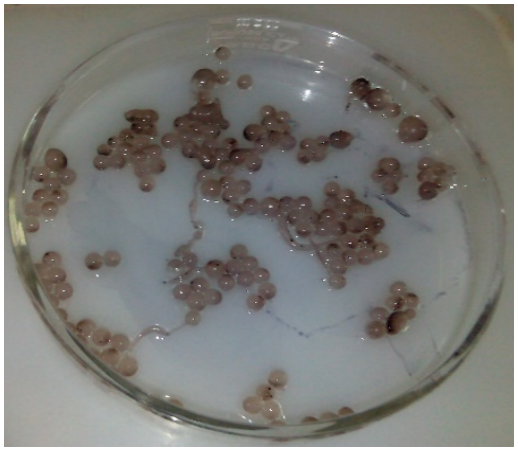

Figure 2 Visual examination Physical appearance of IMN beads

The entrapment of *B. cereus* 1306 within the beads can be clearly seen in Figure 3 (b). The alginate beads possessed micro-pores that allowed high biomass as well as high metabolic activity (Fraser and Bickerstaff, 1997). Past study by Maqbool *et al.*, (2015) also showed entrapment of *Rhodococcus* sp. within the alginate matrix.

Effect of QQ bacteria on membrane flux

Determining membrane flux assessed the ability of QQ bacteria in preventing P. aeruginosa 3541 biofilm formation on the membrane surface. Flux was measured at constant pressure of 10KPa. Figure 4 illustrates the effect of QQ bacteria on flux studies. It was seen that the membrane flux of control 1 membrane (without any beads) showed abrupt flux decline by 57% in 10 days as compared to the 1st day membrane flux (26%).

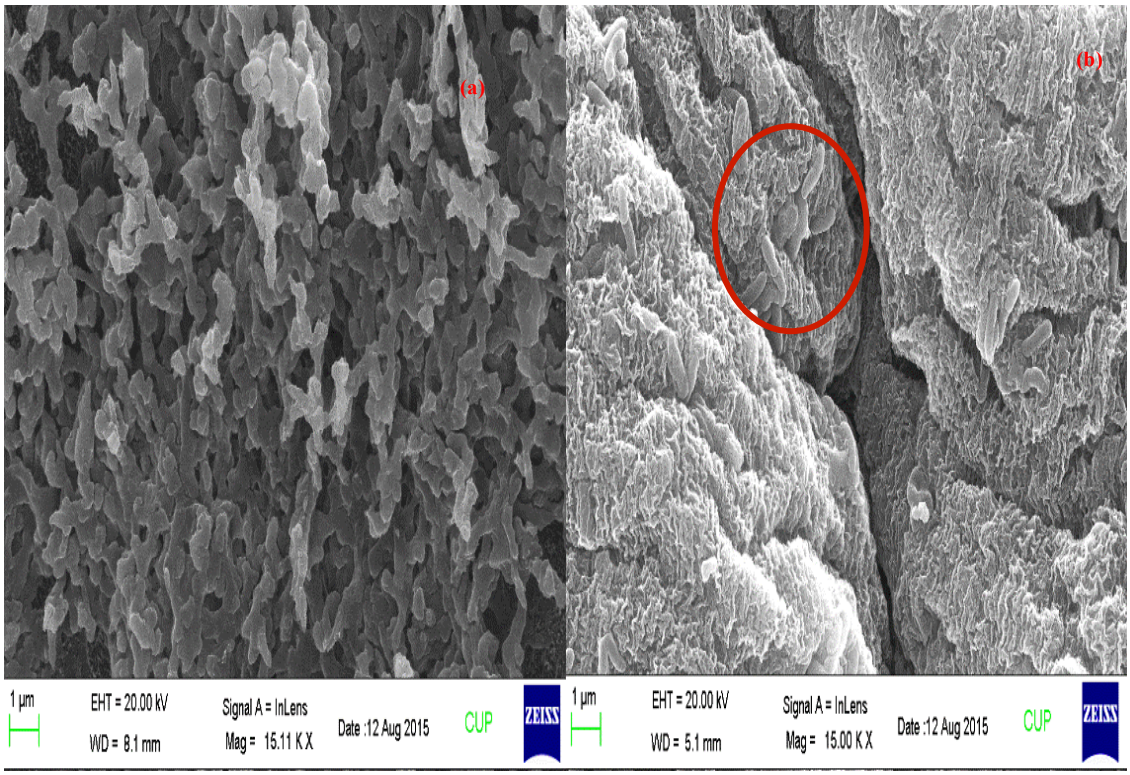

Figure 3 SEM images of (a) empty IMN beads (b) *Bacillus cereus* 1306 immobilized IMN beads.

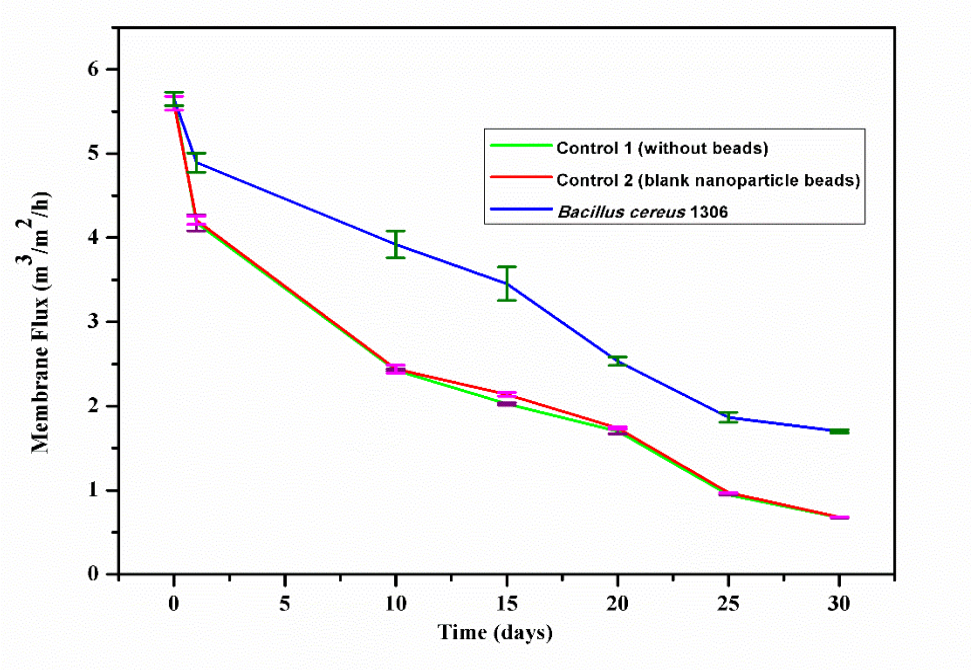

Figure 4 Flux studies with *Bacillus cereus* 1306 IMN beads in membrane filtration.

Further increase in incubation time to 15 and 20 days resulted in drop in flux values by 64% and 70%, respectively. Prolonged incubation of membranes for 30 days, resulted in 84% flux decline. The flux of control 2 membrane declined by 56.8% in 10 days which increased to 87.9% in 30 days incubation. This showed that the flux of control 2 membrane (blank nanoparticle beads) showed nearly same flux as that of control 1 membrane. Hence, the blank nanoparticle beads showed negligible biofilm inhibition. On the contrary, the *B. cereus* IMN beads incubated membrane exhibited gradual flux decline showing flux drop of 13% in 1 day incubation. Further increase in incubation time to 15, 20 and 30 days showed flux decline by 39%, 55% and 66%, respectively. When compared to control membrane, it was observed that the flux values of *B. cereus* IMN beads incubated membrane showed 47%, 39% and 21% increased flux.

The formation of biofilm by the microbes on the membrane surface block the membrane pores resulting in reduced water flow per unit area of the membrane. The reduced flux in control membranes as compared to *B. cereus* IMN beads incubated membrane clearly point out the effectiveness of *B. cereus* IMN beads in controlling membrane biofouling. According to the study by Lee *et al.*, (2014), the relative permeability of membrane was maintained for about 14 days incubation with nanoscale enzyme reactor of acylase (NER-AC) as compared to control (no addition of NER-AC) that showed flux drop in just 6 days incubation. In the present study, *B. cereus* IMN beads showed better ability in controlling membrane biofouling.

The present study concluded that the *B. cereus* 1306 IMN beads prevented the membrane biofilm formation for 30 days on cellulose acetate membrane. Hence, *B. cereus* 1306 have the ability to interfere with the QS in *P. aeruginosa* 3541.

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Author's Contribution: *Ms. Jaskiran Kaur (Research Scholar) performed the research experiments, interpreted the data and wrote the manuscript. Dr. Yogalakshmi K N (Supervisor and corresponding author) designed the work, supported in writing the article and corrected it.*

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