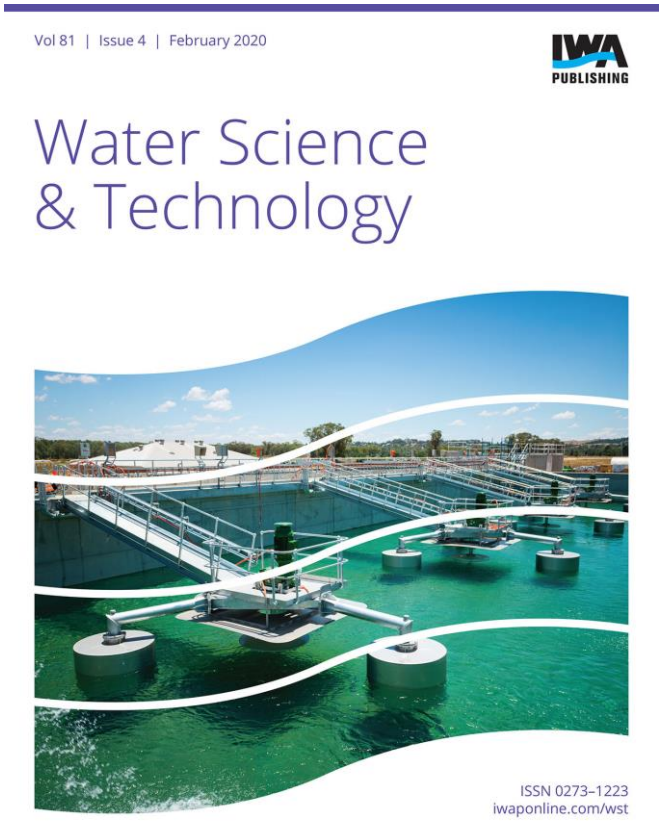

Application of cell entrapping beads for Quorum Quenching technique in submerged membrane bioreactor

S. Ahmed, S. Chung, N. Sohail, I. A. Qazi and A. Justin

ABSTRACT

Biofouling is unwanted accumulation of microbial population on the membrane surface which limits the use of membrane bioreactor (MBR) in the market. Disruption of the biofilm formation by Quorum Quenching (QQ) by using cell entrapping beads (CEBs) is an approach with great potential to control membrane biofouling as the beads used provide not only mitigating effect on biofilm formation, by interfering Quorum Sensing, but also physical forces to detach the biofilm from the membrane surface. This research aimed to develop QQ-CEB with locally available chemicals in Pakistan and its application to evaluate the QQ effect together with physical and chemical cleaning. Various CEBs were made of different mixtures of sodium alginate and polyvinyl alcohol (PVA) and their quality was tested considering physical and biological aspects. *Rhodococcus* sp. BH4 and *Pseudomonas putida* were entrapped in the CEBs and then introduced in MBR as one of biofouling control methods along with standard backwash and chemical backwash. The CEBs made of specific concentration of PVA were proven to be more durable and helpful in mitigating biofouling as compared to that of sodium alginate. An MBR operated with PVA-alginate QQ CEBs together with chemical backwash showed the best performance without deterioration of effluent quality.

Key words | biofouling control, cell entrapping beads (CEBs), membrane bioreactor, polyvinyl alcohol, Quorum Quenching

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INTRODUCTION

Membrane bioreactor (MBR) technology is an advanced innovative approach for wastewater treatment due to its high effluent quality and smaller footprint (Iorhemen *et al.* 2016). However, the extensive use of MBR is hindered because of microbial attachment on the membrane surface (Jiang *et al.* 2013) and difficulty in removing the attachment (i.e. biofilms) and increasing antibiotic resistance (Paluch *et al.* 2020). Quorum Sensing (QS) mechanism as an environmental sensing system plays an important role towards the development of biofilm on membrane that causes fouling of the membrane (Perveen 2018). So a promising approach is to target the root cause which is QS mechanism (Paluch *et al.* 2020). A number of studies and experiments have been designed and conducted to battle against biofouling but all these strategies have limits to battle against biofouling as all these strategies increase the unit treatment cost (Mutlu *et al.* 2019). Membrane biofouling resulting from QS mechanism is a major bottle neck which limits the efficiency of MBR (Lee *et al.* 2018). The most important factor is to introduce a mechanism which combats microorganisms' communication by interrupting signalization mechanism (Mutlu *et al.* 2019). Quorum Quenching is one of the mechanisms and an emerging novel strategy which appears to be successful in recent years for biofouling control in wastewater (Lee *et al.* 2018). Quorum Quenching based approaches have been reported recently to mitigate biofouling effectively by reducing the level of QS (Weerasekara *et al.* 2016) and by targeting inhibition of generation of N-Acyl Homoserine Lactones (AHLs), AHLs molecule itself or AHLs receptors (Nahm *et al.* 2017). Different Quorum Quenching (QQ) media (vessel, bead, cylinder, hollow cylinder, sheet, etc.) have been applied and studied extensively by Korean researchers (Oh & Lee 2018). According to researches, the surface area of QQ media is a dominant parameter in enhancing QQ activity (Bouayed *et al.* 2016). Lee and fellow researchers (Lee *et al.* 2016a) concluded in their study that hollow cylinders as QQ media showed greater efficiency in delaying

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biofouling because of its larger surface area. QQ-MBR related researches have been conducted in Pakistan as well (Hasnain *et al.* 2017; Waheed *et al.* 2017). However, PVA based QQ beads that are known to be affordable and more stable for entrapping living cells (Van Pham & Bach 2014) never have been used in Pakistan so far. Therefore this research aimed to make good quality Quorum Quenching cell entrapping beads (QQ-CEBs) with locally available PVA to utilize these durable QQ-media for biofouling control and to examine their effect on the performance of MBR.

METHODOLOGY

PHASE 1 – development and evaluation of cell entrapping beads

Preparation of sodium alginate beads

Sodium alginate beads were prepared according to Kim's method (Kim *et al.* 2013) with a little modification. Sodium alginate solution (2w/v%) was added dropwise to $CaCl_2$ solution (4w/v%) through nozzle by peristaltic pump to obtain spherical beads of average diameter 2.78 mm. Beads were then stored in deionized water at 4 °C. Beads of this group were labelled as Exp0.

Preparation of polyvinyl alcohol (PVA)-alginate beads

PVA-alginate bead which is highly durable in water and nontoxic to microorganisms has been developed as cell immobilizing material for biological treatment of wastewater by Takei *et al.* (2011) and further tested by Van Pham & Bach (2014). This method was utilized for entrapping Quorum Quenching bacteria by Nahm *et al.* (2017) and others. However, the PVA (Wako brand polymerization degree 2000) that was used for the aforementioned researches is not available in Pakistan, and the same method to make PVA-alginate bead with a different brand of PVA did not work. Therefore, different conditions with three varying brand PVAs were tested to make the best PVA-alginate bead that would entrap Quorum Quenching bacteria in it. The different condition to prepare PVA-alginate bead and their evaluation methods were summarized in Table 1.

After optimizing the bead making technique, the PVA-alginate beads were characterized by structure restoration test, survival test of bacteria after immobilization and scanning electron microscopy (NOVA Nano SEM 450) to confirm the suitability of the bead for entrapping QQ bacteria.

Immobilization of QQ bacteria

Bacterial strains used in this study i.e. *Rhodococcus* sp. BH4 (Accession no. CP014941, Gram positive) and *Pseudomonas putida* (Accession no. KR058848, Gram negative) were received from Microbiology Lab., Institute of Environmental Science and Engineering, National University of Science and Technology, Islamabad, Pakistan. Two bacterial strains were grown in separate LB broth, centrifuged at 4,000 rpm for 30 min and re-suspended with autoclaved water to get the bacterial suspension. This suspension was added to bead-making solution (sodium alginate or PVA-alginate mixture) before cross linking and mixed thoroughly. Following this, the bacterial mixture solution was cross linked by dropwise addition as described above. Beads were washed and stored in deionized water at 4 °C. Dry cell concentration was 28.75 mg for 70 mL of bacterial mixture solution. These beads were called QQ-CEBs.

PHASE 2 – operation of membrane bioreactor with QQ-CEBs

Two parallel, submerged MBRs were operated with and without the QQ-CEBs in combination with standard backwash (SBW, simple back pulse 1 min after every 10 min filtration), chemical backwash (CBW, chemically enhanced backwash, in-line, 1 min, twice a day with 500 ppm NaClO) at 22LMH flux, 8 g/L mixed liquor suspended solids (MLSS), 5.2 h HRT, in 8.1 L working volume of bioreactors, with 0.1 μm pore hollow fibre PVDF membrane (PHILOS Korea) to treat synthetic wastewater which simulates domestic wastewater. The composition of wastewater used in this research was taken from reference (Weerasekara *et al.* 2014) and is given in Table 2. All the valves and pumps were operated by programable logic controller according to operational setting. Schematic diagram of the MBR plant is shown in Figure 1.

In order to find out the effect of chemical backwash, the effect of bead material and the effect of Quorum Quenching Cell Entrapping Bead (QQ-CEB), MBR plant was operated under a total of eight different conditions (Table 3). Performance of each operation was evaluated on the basis of length of operational duration until an MBR gets fouled by monitoring transmembrane pressure (TMP) profile. Removal efficiencies of biochemical oxygen demand (BOD), chemical oxygen demand (COD) and ammonia were determined by comparing

Table 1 | Tests on sodium alginate and various PVA-alginate beads

Experiment no.	Bead material	Polymerization	Mixing temp.	Composition (%)		Cross linking (h)		Evaluation method
				SA[a]	PVA[b]	1st[c]	2nd[d]	
Exp0	SA	–	60 °C	2	–	2	–	0–2 points were given to each experiment by five physical tests. Then total marks of the points obtained from the tests were compared.
Exp1-a	PVA-SA	1500 (PVA #1)	60 °C	1.50	10	2	8	
Exp1-b	PVA-SA	1500 (PVA #1)	60 °C	1	10	2	8	
Exp2-a	PVA-SA	1637 (PVA #2)	60 °C	1.50	8	2	8	
Exp2-b	PVA-SA	1637 (PVA #2)	60 °C	1.50	10	2	8	
Exp3-a	PVA-SA	2270 (PVA #3)	60 °C	1	5	2	8	Physical appearance
Exp3-b	PVA-SA	2270 (PVA #3)	60 °C	1	8	2	8	Agglomeration
Exp3-c	PVA-SA	2270 (PVA #3)	60 °C	1	10	2	8	Bead formation
Exp4-a	PVA-SA	2270 (PVA #3)	105 °C	1	10	0.5	2	Physical strength by centrifugation
Exp4-b	PVA-SA	2270 (PVA #3)	105 °C	1	10	0.5	8	Swelling in water
Exp4-c	PVA-SA	2270 (PVA #3)	105 °C	1	10	2	2	
Exp4-d	PVA-SA	2270 (PVA #3)	105 °C	1	10	2	8	
Exp5-a	PVA-SA	2270 (PVA #3)	105 °C	1	8	0.5	2	
Exp5-b	PVA-SA	2270 (PVA #3)	105 °C	1	9	0.5	2	
Exp5-c	PVA-SA	2270 (PVA #3)	105 °C	1	10	0.5	2	

[a]SA = Sodium Alginate.
[b]PVA = Polyvinyl Alcohol.
[c]1st cross linking solution = 4w/v% $CaCl_2$ for Exp0, 4w/v% $CaCl_2$ + 7w/v% H_3BO_3 for Exp1–Exp5.
[d]2nd cross linking solution = Na_2SO_4 0.5M for Exp1–Exp5.

influent and effluent permeate. Analyses were conducted according to *Standard Methods for the Examination of Water and Wastewater*, 22nd edition (APHA 2012).

Table 2 | Composition of synthetic wastewater as influent of MBR

Components	Value (mg/L)
Glucose	120
Peptone	90
Yeast extract	12
$(NH_4)_2SO_4$	96
$(KH_2)PO_4$	17
$NaHCO_3$	300
$CaCl_2{\cdot}2H_2O$	2.40
$MgSO_4{\cdot}7H_2O$	24
$MnSO_4{\cdot}5H_2O$	2.16
$FeCl_3{\cdot}6H_2O$	0.12
pH	7–8
BOD	130–190
COD	200–250
Ammonia-N	25–33

RESULTS AND DISCUSSION

Evaluation of cell entrapping beads

Result of physical strength test and aggregated points of five tests, i.e. physical appearance, agglomeration, bead formation, physical strength by centrifugation and swelling in water are shown in graphical ways in Figures 2 and 3, respectively. Sodium alginate beads of Exp0 were good enough for most tests but their physical strength is slightly lower than PVA-alginate beads of Exp3-b, c, Exp4 and Exp5 as they all broke at 4,500 rpm while the PVA-alginate beads did not break.

Beads of Exp1 group were not stable and got dissolved in the 2nd cross linking solution (sodium sulphate). These were very weak as all of them broke at 1,000 rpm. Beads of Exp2 group were stable in the second cross linking solution but were not stable in distilled water. These were also weak as they all broke at 2,000–2,500 rpm. Beads of Exp3 group were stable both in the second cross linking solution and distilled water. Exp3-a was soft and all broke at 1,500 rpm. Exp3-b and Exp3-c were hard enough as none broke even at

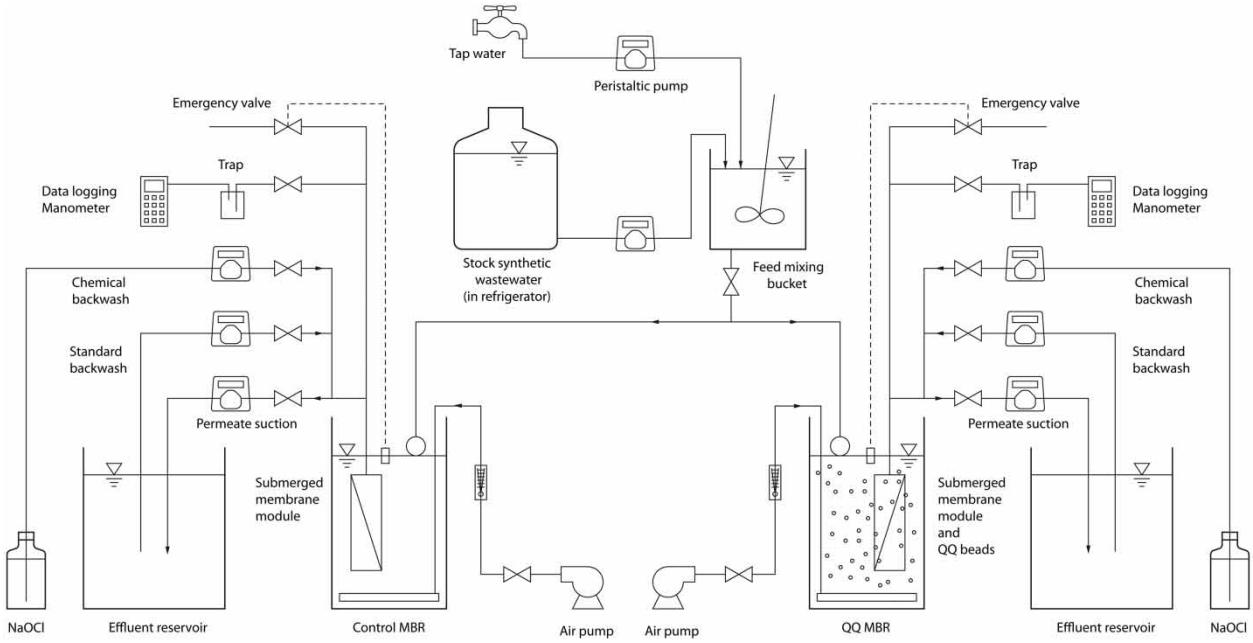

Figure 1 | Schematic diagram of MBR plant.

Table 3 | Operational conditions of MBRs

Operation name	Bead type	Bead filling ratio	Backwash mode
Control	No Beads	0	SBW
Operation 1	Vacant Sodium Alginate Beads	1%	SBW
Operation 2	Vacant Sodium Alginate Beads	1%	SBW, 2CBW
Operation 3	Vacant PVA-alginate Beads	1%	SBW, 2CBW
Operation 4	Sodium Alginate Beads with *Rhodococcus* sp. BH4	1%	SBW, 2CBW
Operation 5	Sodium Alginate Beads with *Pseudomonas putida*	1%	SBW, 2CBW
Operation 6	Sodium Alginate Beads with *Rhodococcus* sp. BH4 + *Pseudomonas putida*	0.5% + 0.5%	SBW, 2CBW
Operation 7	PVA-alginate Beads with *Rhodococcus* sp. BH4 + *Pseudomonas putida*	0.5% + 0.5%	SBW, 2CBW

13,000 rpm. However, these swelled in distilled water and the bead shape of Exp3-c was irregular and the beads agglomerated during cross linking as the bead making solution was too sticky. Beads of Exp4 groups were stable in both the second cross linking solution and distilled water, and hard enough and did not swell. But they made tails on one side of the bead as the bead making solution was too sticky. Beads of Exp5 groups were stable in both the second cross linking solution and distilled water, and hard enough, and did not swell. They did not break even at 13,000 rpm. Beads of Exp5-a, Exp5-b had a good spherical shape but Exp5-c had an oval shape or tails. During the structure restoration test, PVA-alginate beads of Exp5-a and b recovered their original shape after drying and dipping in distilled water. Sodium alginate beads did not recover their original shape once they got dried. So, it was considered that the use of PVA of polymerization degree 2,270, PVA 8–9%, sodium alginate 1%, mixing temperature 105 °C, first cross linking 30 min, second cross linking 2 h is the best condition to make QQ-CEBs. Although sodium alginate beads of Exp0 were not as good as PVA-alginate beads of Exp5-a,b (the best method found in this research), it was also selected to compare its performance with PVA-alginate beads. Finally, sodium alginate beads of Exp0 and PVA-alginate beads of Exp5-b (PVA 9%) were used to make QQ-CEB for MBR operation.

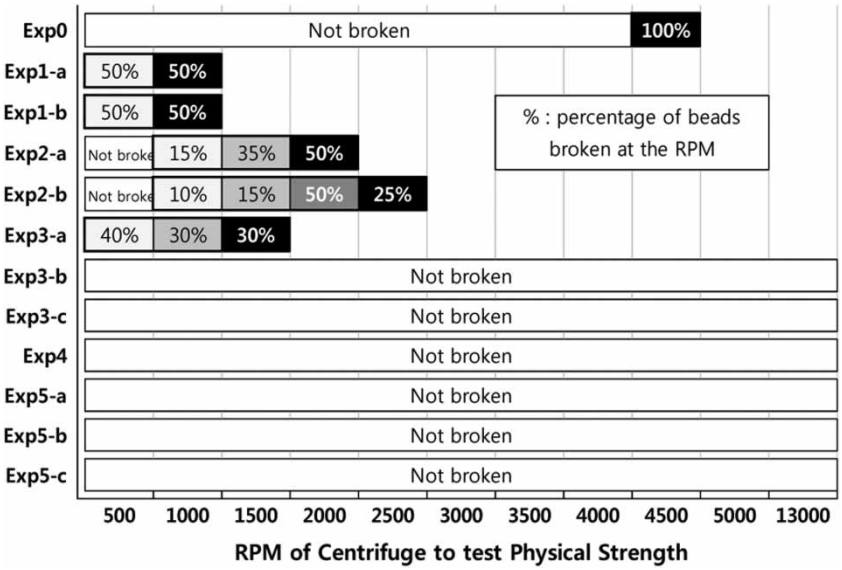

Figure 2 | Physical strength of various beads.

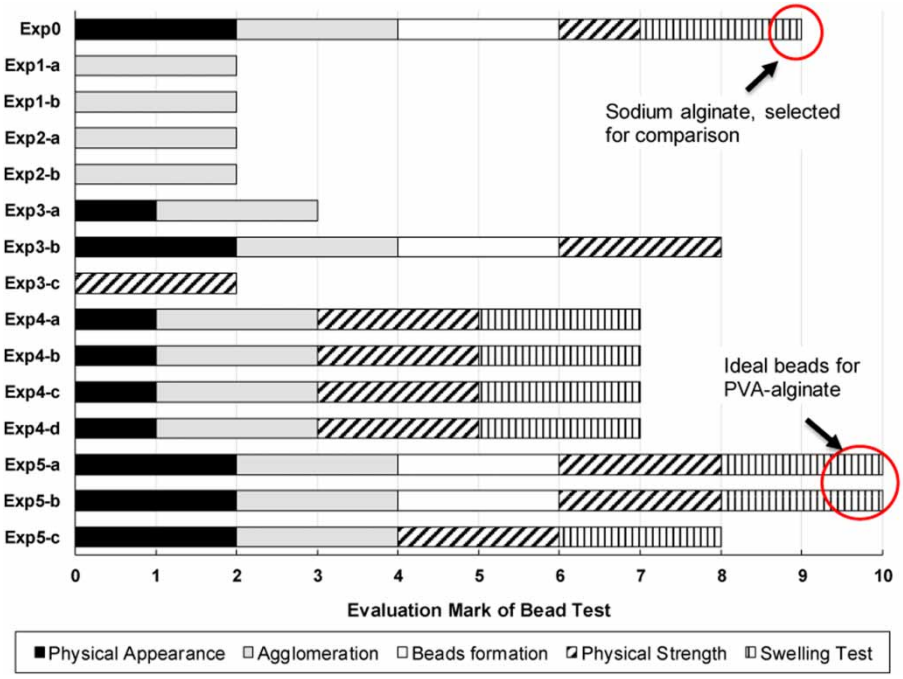

Figure 3 | Evaluation of various beads.

Scanning electron microscopy (SEM) image

After successful immobilization of QQ bacteria in the PVA-alginate beads by the selected best method (Exp5-b), SEM was used to observe and monitor the presence of bacteria from internal and external side of beads (Figure 4) as Li and his colleagues did (Li *et al.* 2014). It can be observed that bacteria were well immobilized in the bead.

Confirmation of bacterial survival in MBR

Cell entrapping beads were taken out from MBR and streaked on LB plate at regular intervals after starting MBR operation to confirm the bacterial survival inside the bead (Figure 5). This survival test confirmed the presence of bacteria entrapped inside the bead throughout the operation.

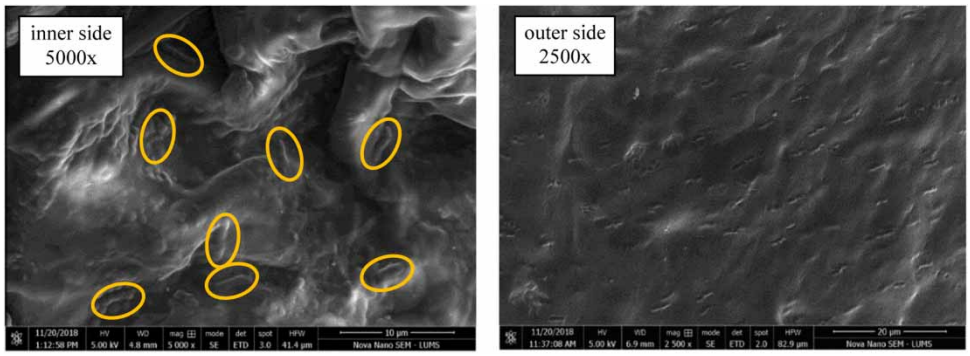

Figure 4 | SEM Image of PVA bead entrapping QQ bacteria.

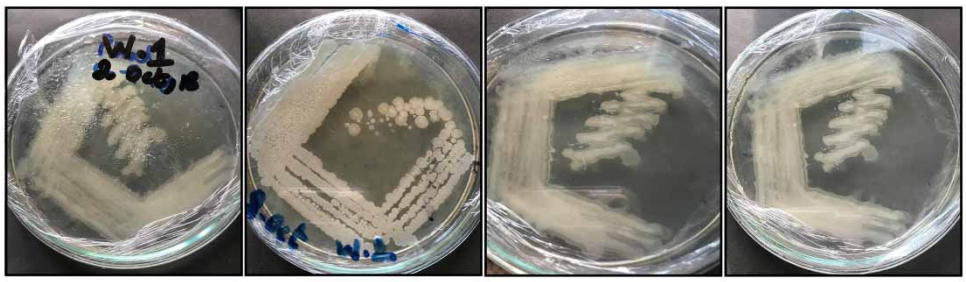

Figure 5 | Confirmation of bacterial survival in MBR.

Comparison of removal efficiencies of different operations of MBR

Average removal efficiencies of COD, BOD and ammonia for all the operations were fairly good to be 91.2–95.9%, 96.6–98.9% and 95.3–99.3%, respectively, with small differences between the operations. In order to check whether there is statistically meaningful difference in average removal, one-way analysis of variance (ANOVA) and Kruskal-Wallis tests were applied. As shown in Table 4, *p*-values of both tests for COD, BOD and ammonia are all greater than the significance level 0.05 which means the small differences in removal efficiencies between each operation are insignificant. This result confirms that different operational conditions that were applied to control biofouling of MBR (by interfering bacterial communication through Quorum Quenching and by chemical backwash) did not give adverse effect on organic and ammonia removal ability of bacteria in the bioreactor.

Comparison of performance of different operations of MBR

Transmembrane pressure profiles

TMP profiles increased trends and operational durations until membrane become fouled of each operation are shown in Figure 6. Control operation worked for 13.5 days. When vacant sodium alginate beads were introduced in MBR (Operation 1), it worked for 18.3 days (4.8 days longer than control operation) because of the physical cleaning effect of the moving beads. When the chemical backwash was added (Operation 2), it worked for 24.6 days (11.2 days longer) because of synergic effect of physical and chemical cleaning. Hasnain *et al.* (2017) also reported that QQ-MBR with backwashing have greater capability to eliminate biofouling and increasing filtration time comparative to QQ-MBR without backwashing. Backwash minimized the production of EPS concentration and delayed the TMP rise and thus it is considered as a standard operating strategy to be incorporated to delay biofouling in waste water as Wang and his colleagues suggested (Wang *et al.* 2014). When vacant PVA-alginate beads instead of sodium alginate beads were introduced in MBR, it worked for 44.3 days (29.8 days longer). This noticeable improvement was because of durability of PVA-alginate beads. PVA-alginate beads were recovered 100% from the sludge of MBR after completing operation while sodium alginate beads were recovered only 40–70%. These were broken in the mixed liquor during the operation since these are not strong enough to maintain their original strength in the harsh environment. Lee *et al.* (2016b) reported similar phenomenon in full-scale MBR.

Table 4 | Removal efficiency of COD, BOD and ammonia for all operations

	COD			BOD			Ammonia		
Operation name	**Avg (%)**	**Stdev**	***n***	**Avg (%)**	**Stdev**	***n***	**Avg (%)**	**Stdev**	***n***
Control	93.4	5.9	7	98.3	2.1	4	97.8	3.2	6
Operation 1	92.3	4.5	5	98.9	0.5	4	97.7	2.0	3
Operation 2	91.3	6.3	9	96.6	2.4	4	95.3	5.2	5
Operation 3	92.2	5.1	14	97.6	2.6	10	98.4	2.8	16
Operation 4	95.9	3.2	7	98.0	1.6	7	99.2	0.4	8
Operation 5	91.0	7.8	16	97.9	2.1	14	98.9	1.0	10
Operation 6	91.3	5.7	28	97.7	2.1	13	99.3	0.5	19
Operation 7	91.2	8.4	18	98.2	2.6	12	97.6	4.2	15
One-way ANOVA	P-value = 0.8977 > 0.05			P-value = 0.8743 > 0.05			P-value = 0.1169 > 0.05		
Kruskal-Wallis test	P-value = 0.741 > 0.05			P-value = 0.791 > 0.05			P-value = 0.401 > 0.05		

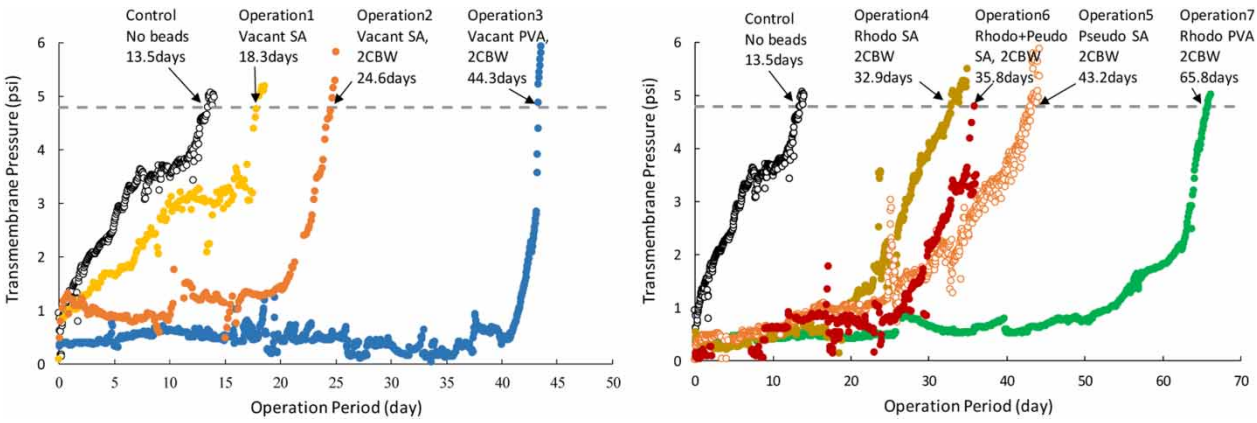

Figure 6 | TMP profiles with operational duration for each operations. (a) Operations 1, 2, 3 (vacant beads). (b) Operations 4, 5, 6, 7 (QQ-CEBs).

Because the beads made of alginate matrix were easily decomposed in MBR during the operation and it caused the TMP jump earlier than PVA-alginate beads while fouling the membranes.

When QQ CEBs were introduced in MBR, an MBR with *Rhodococcus* sp. BH4 alone in sodium alginate beads (Operation 4), *Pseudomonas putida* alone (Operation 5) and *Rhodococcus* sp. BH4, as well as *Pseudomonas putida* (Operation 6) worked for 32.9 days, 43.2 days and 35.7 days, respectively. *Pseudomonas*' QQ-CEB showed better performance on biofouling control, however the exact cause was not identified in this research. It is hypothesized that more bacteria might exist in the mixed liquor of MBR that produce signal molecules which are easily interfered by the Quorum Quenching effect of *Pseudomonas* than *Rhodococcus*. The combination of *Rhodococcus* and *Pseudomonas* (Operation 6) targeted to bacterial communication of both Gram positive and Gram negative did not show better performance than single *Pseudomonas*' QQ-CEB. When *Rhodococcus* and *Pseudomonas* QQ-CEBs in PVA-alginate beads were introduced in MBR (Operation 7), it worked for 65.8 days (52.3 days longer than control, 390% improvement). This noticeable improvement shows the superiority of PVA-alginate bead to ensure the activity of QQ bacteria entrapped in it, as well as excellent physical cleaning effect.

Comparison of individual effects

Overall performance of MBR with QQ-CEBs in PVA-alginate beads (Operation 7) was better than the others. But the improvement was not only from the QQ effect but also the physical cleaning effect of the PVA-alginate beads and chemical cleaning. Therefore, the individual effect of

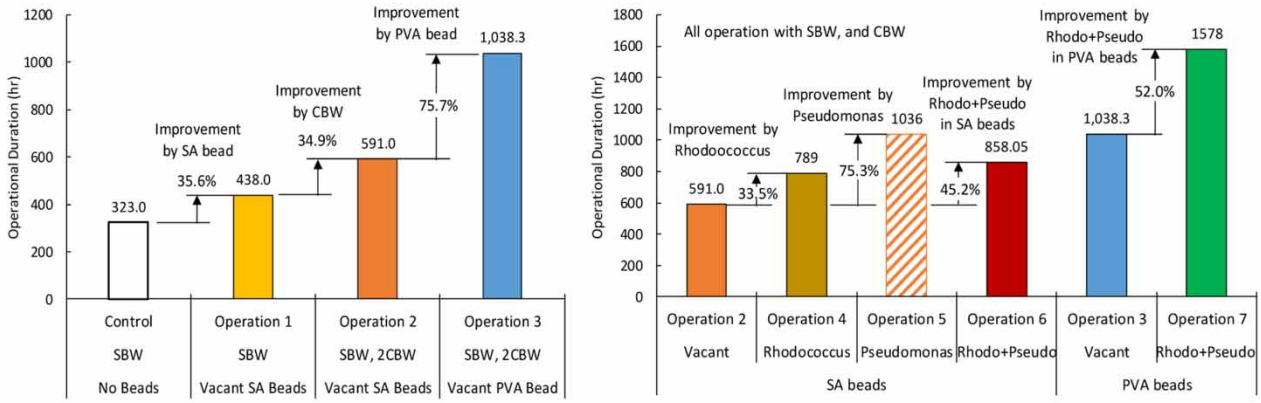

Figure 7 | Improvement of MBR performance by individual biofouling control methods. (a) Improvement by chemical backwash and bead making material. (b) Improvement by QQ effect in sodium alginate beads and in PVA beads.

biofouling control methods were examined by separate comparison as shown in Figure 7.

The introduction of sodium alginate beads improved operational duration by 36% over that without beads (Control vs Operation 1). Addition of chemical backwash improved it by 35% (Operations 1 vs 2). Replacing sodium alginate beads with PVA-alginate beads improved it by 76% (Operations 2 vs 3). The physical cleaning effect of PVA-alginate beads proved to be very effective (Figure 7(a)). Introduction of *Rhodococcus* sp. BH4 alone, *Pseudomonas putida* alone and *Rhodococcus* sp. BH4 together with *Pseudomonas putida* in sodium alginate beads improved operational duration by 34%, 75% and 45%, respectively (Operations 2 vs 4, 5, 6). The introduction of *Rhodococcus* sp. BH4 together with *Pseudomonas putida* in PVA-alginate beads improved it by 52% (Operations 3 vs 7) (Figure 7(b)).

CONCLUSION

A procedure to make QQ-CEBs was successfully developed with locally available chemicals in Pakistan. The superiority of PVA-alginate bead as QQ-CEB made of PVA 8–9% polyvinyl alcohol with polymerization degree 2,270, sodium alginate 1%, mixing temperature 105 °C, first cross linking 30 min, second cross linking 2 h was proved. It was tested by five physical tests, supported by SEM and survival test and further confirmed by performance improvement in MBR operations. PVA-alginate QQ-CEB showed excellent physical cleaning effect together with QQ effect. When they were combined with chemical backwash, MBR performance was improved by 390% compared to control operation. This improvement was achieved without deterioration of organic and ammonia removal efficiencies. Therefore it can be concluded that application of PVA-alginate QQ-CEB developed in this research has good potential to mitigate biofouling to improve performance of MBR.

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