Ecological role of *Acinetobacter calcoaceticus* GSN3 in natural biofilm formation and its advantages in bioremediation

Sahar Irankhaha,b, Ahya Abdi Ali,a, Megharaj Mallavarapu b,c, Mohammad Reza Soudia, Suresh Subashchandrabose b,c, Sara Gharavid and Bita Ayatie

aDepartment of Microbiology, Faculty of Biological Sciences, Alzahra University, Tehran, Iran; bGlobal Centre for Environmental Remediation (GCER), Faculty of Science and Information Technology, University of Newcastle, Callaghan, NSW, Australia; cCRC CARE, Newcastle University LPO, Callaghan, NSW, Australia; dDepartment of Biotechnology, Faculty of Biological Sciences, Alzahra University, Tehran, Iran; eEnvironmental Engineering Division, Civil and Environmental Engineering Faculty, Tarbiat Modares University, Tehran, Iran

**ABSTRACT**

This study assessed the role of a new *Acinetobacter calcoaceticus* strain, GSN3, with biofilm-forming and phenol-degrading abilities. Three biofilm reactors were spiked with activated sludge (R1), green fluorescent plasmid (GFP) tagged GSN3 (R2), and their combination (R3). More than 99% phenol removal was achieved during four weeks in R3 while this efficiency was reached after two and four further operational weeks in R2 and R1, respectively. Confocal scanning electron microscopy revealed that GSN3-gfp strains appeared mostly in the deeper layers of the biofilm in R3. After four weeks, almost $7.07 \times 10^7$ more attached sludge cells were counted per carrier in R3 in comparison to R1. Additionally, the higher numbers of GSN3-gfp in R2 were unable to increase the efficiency as much as measured in R3. The presence of GSN3-gfp in R3 conveyed advantages, including enhancement of cell immobilization, population diversity, metabolic cooperation and ultimately treatment efficiency.

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**Introduction**

There are a variety of technologies involving physical (Hemmati et al. 2015; Lin et al. 2007), chemical (Aneggi et al. 2017; Loloi et al. 2016; Liu et al. 2018) and biological processes developed for remediation of contaminated environments (Moussavi et al. 2014; Rafiei et al. 2014). Amongst these, biological processes are preferable due to their satisfactory efficiency, and environmentally sound and cost-effective properties, and in particular those which benefit from biofilm treatment (Pookpoosa et al. 2015; Ferro Orozco et al. 2016; Zielinska et al. 2014). In recent years, the use of specific contaminant-degrading microorganisms in wastewater treatment systems has increased and has provided an effective method to enhance the degradation of toxic organic contaminants (Zhang et al. 2012; Duque et al. 2011). For instance, some strong phenol-degrading microorganisms, such as *Pseudomonas* sp. and *Rhodococcus* sp., have been successfully used to treat phenol-containing wastewaters (González et al. 2001, Prieto et al. 2002). However, some undesirable events such as protozoan grazing or flow washing of favorable microorganisms can lead to their elimination from the system, and as a consequence, the high degradation rate of pollutants does not continue for a long time. Since the presence and performance of bioaugmented microorganisms is a major prerequisite for high treatment efficiency, their immobilization has been suggested as an effective strategy. Different methods of immobilization have been proposed to increase the persistence of desired cells in treatment systems, including immobilization in alginate beads (Guiot et al. 2000) or polyvinyl alcohol (PVA) (El-Naas et al. 2009). However, these strategies may be cost prohibitive and/or complex to apply, and most importantly, they have a defined capacity, longevity and stability which all limit their application. Biofilm, as a common form of bacterial life, offers a natural, efficient and feasible way of immobilization which enables immobilized cells to proliferate and tolerate adverse environments. Biofilm immobilization of degrading-bacteria has been suggested as a key method for achieving efficient degradation in a bioaugmentation system (Li et al. 2013).
Biological treatment by biofilm remediation has advantages including the high active biomass in a compact unit, tolerance to hostile environmental stresses, low sludge production and elimination of clogging problems (Leiknes and Odegaard 2007; Bassin et al. 2016; Moussavi et al. 2009). The most critical drawback of such biofilm systems is the weakness of the native flora, especially the desired ones, in terms of biofilm formation which eventually results in their elimination from the system and causes a substantial decline in removal efficiency (Eldyasti et al. 2013; Boon et al. 2000; McLaughlin et al. 2006; Dueholm et al. 2015; Li et al. 2008, 2013). This issue becomes even more severe when sudden loading shocks of contaminants are imposed on the system; these can lead to detachment of the biofilm from the surface, making the process ineffective (Moussavi et al. 2009; Li et al. 2013). Immobilization of weak biofilm forming cells using strains with strong biofilm forming properties has been proposed as a solution (Li et al. 2008, 2013). Recently, the microflora of a variety of natural or wastewater biofilms was studied, and some strong biofilm forming strains with a bridging function were introduced (Ramalingam et al. 2013; Metzger et al. 2009; Kwak et al. 2013; Cheng et al. 2014; Simões et al. 2008). These bacteria contribute to the development of biofilms through specific surface molecules which play a significant role in the production of multispecies biofilms in aquatic environments such as drinking water systems. It has been proven that bridging bacteria influence biofilm formation and population diversity during the development of complex multispecies biofilms by aiding the incorporation of other cells into the biofilm (Rickard et al. 2003; Simões et al. 2007, 2008). However, those investigations mainly attempted to screen the strains with coaggregation ability and/or explore the coaggregation mechanism used by them (Stevens et al. 2015; Ren et al. 2015; Cheng et al. 2014; Kwak et al. 2013; Min and Rickard 2009). Nevertheless, the ecological role and function of these bacteria in the development of biofilms in wastewater treatment systems remain unclear. Additionally, information on potential applications of these bacteria, especially those with further contaminant degrading abilities, is not available. Hence, in this study, an attempt has been made to survey the role of a strong biofilm producing and phenol degrading strain Acinetobacter calcoaceticus GSN3 in biofilm formation and immobilization of other sludge cells in a moving bed biofilm reactor (MBBR). In addition, the authors were interested in determining whether the co-inoculation of this bacterium with activated sludge cells could enhance phenol removal. Moreover, a sudden phenol loading shock was imposed to evaluate the presence and activity of this bacterium on the overall resistance.

**Material and methods**

**Bacterial strains and growth conditions**

A bacterial strain designated Acinetobacter calcoaceticus GSN3 was used in this study. This strain was previously isolated from petroleum-contaminated soil in Tehran Refinery, Tehran, Iran and demonstrated an ability to degrade efficiently high phenol concentrations (Nafian et al. 2016) and form strong biofilms in a wastewater context (data not shown here). The activated sludge was obtained from Nelson Bay wastewater treatment plant (Newcastle, Australia; 80,000 population equivalent). After sampling, the sludge was allowed to settle for 1 h, following which the supernatant was discarded and replaced with an equal volume of sterile synthetic wastewater (SWW). The total bacterial count of the sludge was 6.79 × 10^11 bacteria ml^-1, estimated using the LIVE/DEAD® Bacterial Viability Kit (BacLight™, Thermo Fisher Scientific, Waltham, MA, USA) according to Boulos et al. (1999).

**Chemicals and media**

All chemicals used in this study were of analytical grade and supplied by Merck company (Frenchs Forest, NSW, Australia). Synthetic wastewater (SWW) containing (mg l^-1) Na_2HPO_4 (16.7), CaCl_2.2H_2O (23.5), MnSO_4.7H_2O (9.45), CoCl_2 (0.8), ZnCl_2 (10.0), CuSO_4.5H_2O (392.0), FeCl_3.6H_2O (3.3), NaHCO_3 (1,000.0), MgSO_4.7H_2O (3.1) as trace elements and urea and a mixture of K_2HPO_4 and KH_2PO_4 as the main source of nitrogen and phosphorus, respectively was used in this study. Phenol and glucose (or their combinations) were applied as the carbon source in the acclimation phase while phenol was used only in the remainder of the test and filter-sterilized (0.22 μm pore size) before mixing with the steam sterilized (15 min at 121°C) SWW base solution. The ratio of chemical oxygen demand (COD):N:P was retained at 100:5:1 in the feeding tank where 1 mg l^-1 of phenol is equal to a COD of 2.15 mg l^-1 (Hosseini and Borghei 2005). Luria–Bertani broth (LLB) and plate count agar (PCA) were also used for microbial growth and counts, respectively.
Construction of GFP-expressing bacterial strain

In order to monitor the survival and competitiveness of GSN3 exposed to a natural wastewater flora, it was marked with a green fluorescent plasmid (GFP). GSN3 strain was transformed with the pGFPuv plasmid vector (Clontech, Palo Alto, CA, USA) according to the calcium chloride transformation method described by Sambrook et al. (1989). This plasmid confers resistance to 100 mg l⁻¹ ampicillin as a selective marker. Afterwards, the expression, brightness, and uniformity of GFP-labeled cells were checked with an epifluorescent microscope (IX-71, Olympus, Tokyo, Japan). The stability and expression of the GFP plasmid were evaluated over 20 days. For this purpose, transformed GSN3 strains were inoculated in a flask containing 50 ml of sterile synthetic wastewater containing 900 mg l⁻¹ of phenol. Every other day, a sample was taken from the previous culture and inoculated into a similar fresh culture (OD₆₀₀=1); however, before any inoculation, the viable cell count of bacteria was determined as follows: 10 ml of sample were centrifuged for 1 min at 12,000 g, washed three times with sterile PBS and re-suspended in it. A serial dilution of suspension was provided and then plated onto LB agar supplemented with and without 100 µg ml⁻¹ of ampicillin for counting GSN3-gfp and total bacteria, respectively.

Reactor design

Four cylindrical MBBR reactors made from Plexiglas with characteristics including internal diameter, height and wall thickness of 10, 70 and 0.4 cm, respectively, were used in this work (refer to Figure 1 for more details). The total volume of each reactor was 5.5 l, and the working volume was 5 l and they were half filled with free-swimming biofilm carrier elements. The carriers (Pakan Ghatreh Co., Tehran, Iran) were composed of high-density polyethylene (HDPE), shaped as small corrugated cylinders with two crosses inside, giving an effective specific surface area of 389.6 m² m⁻³ and a density of 0.96 g cm⁻³, slightly lower than the density of water. The aeration system supplied fine mixing bubbles to meet the oxygen demand and keep the carriers moving throughout the reactor as well. Sterile air was supplied from the bottom through a 0.22 µm pore size filter.

MBBR reactors

In order to survey the survival, persistence, and activity of the bioaugmented strain facing the natural flora of the activated sludge, different experiments were designed. Three parallel reactors were set up and each was seeded with different inocula so as to make a final suspended solid concentration of 24 × 10⁸ cell ml⁻¹. The reactors were inoculated with activated sludge (R1), the overnight culture of GFP-tagged GSN3 strain (GSN3-gfp) (R2) and a 3:1 ratio of activated sludge plus GSN3-gfp (R3), respectively. The latter resulted from a test which surveyed different inoculation times (0 to 24 h) and proportions (1:1 to 1:10) of GSN3-gfp and activated sludge cells in a flask containing phenol synthetic wastewater and carriers.

**Figure 1.** MBBR schematic diagram. Five-liter effective volume, with ~50% carrier fill ratio.
(the results are not shown here). The fourth reactor was marked as a non-inoculated control (RC) and filled with only the sterile synthetic wastewater to monitor the amount of phenol lost through either leaking or absorption to the rubber stoppers. However, as the results indicated, these effects were all negligible.

The reactors were operated in batch mode with the mixture of phenol and glucose for 15 days to allow the seeded cells to adapt to the increasing amount of phenol and achieve constant wastewater that was rich in cell populations. During this phase, a combination of phenol and glucose with a COD of 1,500 mg l\(^{-1}\) was used as feed for the reactors so that a 10% phenol increase was followed by the same decrease in the amount of glucose. The higher amount of phenol was not added until the removal efficiency of the last one exceeded 90%. Subsequently, all reactors were switched to continuous mode and fed with just SWW containing 900 mg l\(^{-1}\) of phenol as the sole carbon source. All reactors were operated under the same controlled conditions including room temperature (23 ± 3 °C), 4.0 ± 1 mg O\(_2\) l\(^{-1}\) of dissolved oxygen (DO) concentration and a neutral pH 7 ± 0.5. A hydraulic retention time (HRT) of 24 h was used to partially simulate the situation in the real wastewater treatment plant. Sterile carriers were added to the reactors once the batch mode was switched to the continuous mode. This permitted microorganisms to grow and increase before confronting the shear force stress. All reactors were conducted in duplicate. No significant differences were observed between the two duplicate reactors; nonetheless, all the given data were presented as the averages for all.

**Analytical methods**

To assess the performance of the MBBR, samples were taken from the influent and effluent and analyzed at defined HRT intervals. The measured factors in both inlet and outlet samples were COD, phenol, pH, DO and temperature. The parameters including pH, DO and temperature were measured daily using specific electrodes (HACH, Düsseldorf, Germany) in order to monitor and establish the optimum conditions for bacterial growth in all reactors. To measure phenol and COD factors, samples were passed through a filter with 0.45 mm pore size before analysis. Phenol concentrations were measured spectrophotometrically, by the 4-aminoantipyrine colorimetric method (APHA, AWWA, WEF 2005), using a UV/vis spectrophotometer and COD was determined according to the 5220D standard method (APHA, AWWA, WEF 1998).

**Tracking the GSN3-gfp activity and survival**

The survival and function of GSN3-gfp were monitored by two methods: plating on LB medium with ampicillin and CLSM microscopy. Once a week, carrier samples were collected randomly from all reactors and cut into pieces very gently to prepare a flat part using sterile forceps and scalpel. Following analysis, sterile carriers were added to the reactors after being marked by a small incision to elude re-sampling. Throughout the test, <20% of the total carriers was taken as the sample.

**Confocal light scanning microscopy (CLSM)**

CLSM, a non-destructive visualizing method, was used weekly to observe the structure of established biofilms on carriers and estimate the biovolume and thickness. Samples from the control reactor were collected along with others. To observe the entire microbial community, carriers were stained with 100 ml of 15 mM red fluorescent nucleic acid stain SYTO 60 (Sigma) for 40 min in the dark to allow for deep penetration of the stain into the biofilm structure. Biofilms were then rinsed with phosphate buffered saline (PBS) for 30 min to remove excess stain in order to reduce the background fluorescence. CLSM was carried out using a Zeiss LSM 510 META confocal laser scanning microscope (Carl Zeiss, Jena, Germany). The 488-nm laser line and 515- to 545-nm band-pass emission filters were used to detect the introduced GFP strain. The 633-nm laser line and a 665-nm emission filter were used to detect SYTO 60 red signal. Biofilms attached to the substratum were scanned at 10 randomly selected regions per sample at various biofilm depths, analyzed and converted into 3-D reconstructions using IMARIS Software (Bitplane, Zürich, Switzerland) and counted by ImageJ image analysis software with a Volume Viewer plug-in (Abramoff et al. 2004).

**Viable cell counts for the biofilm**

Survival of GFP cells was monitored by viable plate counting of the total GFP cells and GFP cells attached to the carriers. In brief, 10 samples were taken from the reactors and gently rinsed in three changes of sterile PBS to remove suspended cells. Biofilm cells were then detached and released by pulsed low-intensity ultrasonication (5 min, 30 s on/off mode, SFX250
Sonifier, Branson, Danbury, CT, USA) and mixing (1 min). The serial dilution suspension was plated onto plate count agar for total bacterial CFU and onto LB medium with ampicillin for determining the CFU number of GFP-expressing cells. GFP strains were detectable by green fluorescence emission under a long-wavelength UV lamp.

**Organic loading shock**

Finally, organic loading shock was applied to investigate the rate of resistance in all reactors. For this purpose, a prompt enhancement of effluent phenol concentration from 900 to 1,500 mg phenol \( \text{L}^{-1} \) (1,935–3,225 mg COD \( \text{L}^{-1} \)) at 24 h was applied in all reactors. The shock load was kept constant for 4 h, and then the condition was returned to initial order. During and following the shock period, the amount of phenol and COD were measured every 1 h until the steady state condition was re-established.

**HPLC**

High-performance liquid chromatography (HPLC) was utilized for monitoring phenol degradation during the organic loading shock. For phenol estimation by HPLC, 1.5 ml liquid from each reactor was taken as the sample in the steady state condition. Samples were transferred to tubes and centrifuged at 12,000 \( \times \) g at 4 °C for 10 min. After discarding the pellet, the supernatant was taken for analysis. The HPLC system (Model 2695, Waters, Rydalmere, NSW, Australia) was equipped with an UV/vis detector (Model 2487, Waters, Rydalmere, NSW, Australia) operating at 270 nm and the HPLC column used was a symmetry C-18 column (4.6 × 150 nm, 5 μm particle sizes). The mobile phase was composed of 55% (v/v) distilled water and 45% (v/v) acetonitrile and the flow rate was set at 1 ml min \(^{-1}\). The amount of degradation was estimated by comparing the peak area of the control with the desired samples (Kumar et al. 2013).

**Statistical analysis**

The values illustrated in all graphs are the means ± SD for duplicate samples. Statistically significant differences in values were tested by comparing at 95% confidence intervals and confirmed using the \( t \)-test and \( p \)-values (0.05 signifying statistical relevance).

**Result and discussion**

**Performance of GSN3 in the reactor**

To better understand the impact of the presence and activity of GSN3 strain augmentation in a continuous MBBR, sludge cells were inoculated or co-inoculated with these cells in three separate reactors and grown in 900 mg \( \text{L}^{-1} \) phenol containing SWW. Phenol degradation was relatively low in the first six days of operation in R3; however, a slight increase of \( \sim 200 \text{mg L}^{-1} \) in phenol concentration occurred as a result of its injection at 24 h (Figure 2A). Nevertheless, degradation started to speed up from day 13 and reached the amount of 70.3 ± 2.5 mg phenol \( \text{L}^{-1} \) (>90% degradation) on day 22. Degradation efficiency gradually increased in R2 and reached >90% over the 34 days. However, phenol was degraded rather slowly in R1 with very low initial efficiency. Nonetheless, it increased from day 21 and the amount of phenol was reduced to 79.6 ± 4.1 mg \( \text{L}^{-1} \) on the 47th day. The control reactor did not show any significant changes in phenol concentration during the trial.

The less pronounced enhancement of phenol degradation in R1 can be explained by the low ability of sludge cells to degrade phenol and the inhibitory effect of high phenol concentration on their activity which all resulted in limited biodegradation and probably the accumulation of intermediates (Ho et al. 2009). Although the removal efficiency in R2 was higher than those of two other reactors during the first week, it was not as efficient as that observed in R3 for the rest of the test. However, co-augmentation of the activated sludge with GSN3-gfp cells experimentally modified the degradation efficiency against phenol in R3. Moreover, the rate of COD removal was relatively close to phenol degradation in R3 while substantial differences were found among them in R2 and R1 (Figure 2B). This result confirmed the ability of the applied cells to co-metabolize phenol in R3.

Different studies have attempted to enhance the efficiency of degradation by mixed-culture inoculation (Jiang et al. 2006; Li et al. 2008, 2013). Cordova-Rosa et al. (2009) examined a bacterial consortium and Acinetobacter calcoaceticus, isolated from a coal wastewater treatment plant, in both continuous and batch systems and showed that the mixed culture was able to survive in higher phenol concentrations and promote higher degradation in comparison to their single cultures. In another study, it was demonstrated that when two strains of Acinetobacter sp. and Sphingomonas sp. mixed in the ratio of 1:1 were able
to remove 1,000 mg phenol l\(^{-1}\) within 60 h more efficiently (97.2%) than their single inoculation (78.3% and 68.1%, respectively) (Liu et al. 2009). From this, it could be proposed that the co-inoculation of GSN3-gfp strain and sludge consortium led to the development of a more diverse population of microorganisms, including beneficial cells of sludge in R3 and boosted the efficiency of phenol and COD degradation.

**Stability of the GFP plasmid in GSN3 strains**

GSN3 transformation with a high copy number GFP plasmid resulted in the production of bright fluorescent cells that were detectable by a UV lamp. During the incubation of GSN3-gfp strain in synthetic wastewater for 20 days, no significant differences were observed in CFU values of LB medium supplemented with and without antibiotic \((p > 0.05)\), indicating prolonged stability and expression of the plasmid in the desired cell population (Figure 3).

**Survival and activity of GSN3 in MBBR**

**Plate counting method**

At the end of the first week, the concentration of GSN3-gfp strain was low in R3 \((4.7 \pm 0.3 \text{ Log }_{10} \text{ CFU/10 carriers})\); this was followed by an uptrend through

![Figure 2. Phenol (A) and COD (B) effluent removal efficiencies in three different seeded continuous MBBRs; R1 (▲) R2 (●) and R3 (■) spiked with 900 mg l\(^{-1}\) inlet phenol concentrations at a hydraulic retention time of 24 h.](image-url)
the second week, reaching the relative abundance of 68.6% of total cells (Figure 4). As observed on days 21 and 28, GFP cell numbers increased, but at a lower rate than that of total cells. Eventually, 40.7% (14.3 ± 0.9 Log$_{10}$ CFU/10 carriers) of the introduced GSN3-gfp cells were able to survive on day 28 of the experiment. At this time, the inoculum size stabilized at ~35.1 ± 1.5 Log$_{10}$ CFU/10 carriers. The number of cells in R2 increased gradually at a higher rate than their counterparts in R3 over the test. The estimated total cell numbers of day 42 was about 28.5 ± 1.9 Log$_{10}$ CFU/10 carriers. The activated sludge formed weaker biofilm at all time points, and the phenol degradation activity displayed low values except for the measurements started on day 23, indicating the toxicity of the phenol to the biofilm microbial population. A subsequent stagnation of activity in R1 was observed while the degradation activities in R2 and R3 were increasing. At the end of week three 6.8 ± 1.5 Log$_{10}$ CFU/10 carriers were counted in R1, indicating that indigenous cells of the activated sludge capable of degrading phenol emerged. However, phenol degradation activity was still rather low at the same time. By the end of the experiment, a total of 24.7 ± 1.2 Log$_{10}$ CFU/10 carrier cells were recorded in R1, which was 10.5 ± 1.7 Log$_{10}$ CFU/10 carriers lower than that estimated on day 28 in R3. The mixed strain biofilm in R3 performed steadily with a rather constant phenol degradation activity and increased biofilm formation.

Figure 4. Viable count of total cells formed biofilms in R1 (■), R2 (□) and R3 (▲) assessed by the plate counting method. GSN3-gfp cells in dual-species biofilms in R3 represented by the dotted column. Viable counts were determined until achievement of complete phenol degradation in each reactor. Error bars exhibit the SD and refer to the cell count on different carriers (n = 10) with two replicates.

Figure 3. Surveying the stability of GFP plasmid by counting CFU values of LB medium with and without antibiotic over 20 days cultivation of GSN3 strains in SWW containing phenol.
CLSM microscopy
CLSM microscopy was employed to explore the role of GSN3-gfp strain in biofilm formation and also to survey the presence, survival and spatiotemporal development of these tagged cells on carriers. Since biofilm formation on different areas of every single carrier was not uniform, 10 different random fields of view on each carrier were selected for biofilm thickness and biovolume determination. Observation of the 3-D biofilm structure showed that both GSN3-gfp and activated sludge cells were embedded in the biofilm structure (Figure 5). The thickness and biovolume of immobilized cells in all reactors were investigated and the results are shown in Figures 6 and 7. Co-inoculation in R3 resulted in development of GFP cell clusters which were partially covered by sludge cells to form the biofilm of about 22.60 ± 0.79 μm thick after seven days (Figures 5A and 6). Although only a slight increase was observed in the volume of sludge cells during the second week, it increased further augment over the following weeks. Nevertheless, the layer thickness and biovolume increased across the test. Analysis of CLSM fluorescence images (taken as five z-sections with a slices interval of 5 μm) in R3 at all time points revealed that a higher mass of GFP cells was positioned mostly within the deepest z-stack corresponding to the section of biofilm attached to the carriers (Figure 8). For instance, the mean volume of GFP per field of view on carriers (n = 10) in the first z-section (closest to the carrier surface) was 5,450 ± 141 compared to 3,237 ± 97, 1,750 ± 76, 990 ± 31 and 528 ± 25 μm³/μm² in the second to fifth z-sections, respectively, in 45.20 ± 1.5 μm thick biofilm. These results suggest that when the carriers were consistently exposed to phenol, biofilms continued to expand by an increase in the volume of sludge cells upon a conditioning layer of GSN3-gfp bacteria that had initially developed on the surface (Figure 5A, B and G). Conversely, the largest fraction of sludge cell mass was found in the outer layer of biofilm. Biofilm thickness as a function of the GFP/sludge ratio within 28 days is provided in Figure 9. Inoculation of R2 with GSN3 strains was followed by a growth of biomass to cover >70% of the carrier surface, yielding a thickness of 23.54 ± 2.6 μm after seven days (Figures 5E, F, G, H and 6). In comparison to the mixed species biofilm in R3, the single species biofilm in R2 contained higher amounts of GSN3 cell volume over time in all samples taken (Figure 7). The thickness of the biofilms in R2 was higher than that in R3 up to the second week, while it decreased after that (~39.14 μm compared to 45.20 μm thickness by 28 days). Statistical analysis shows a significant difference between biofilm thickness taken from R3 and R1. Sludge cells did not adhere to the carriers with high mass and thickness at the end of the first week (15.53 ± 1.12 μm) (Figures 5I, J, K, L and 6). No significant changes in biovolume and thickness were observed even after two weeks (18.88 ± 0.92 μm). However, it was observed that the thickness and volume of biofilm increased with further incubation time. The morphology and mass analysis showed the development of a thicker biofilm on carriers in R3 in comparison to R1 and R2.

The failure of sludge cells to form substantial biofilm can be ascribed to their loss of biofilm formation or phenol degradation ability, which led to their elimination from the system. As shown in Figures 6 and 7, sludge cells formed thicker biofilms in R3, while the same amount of phenol prevented them from significant biofilm formation in R1. The longevity data revealed that GSN3-gfp cells mainly appeared in the deeper layers of the biofilm and were gradually covered by a carpet of sludge cells after week two. It seems that at this point in the experiment, the emerged beneficial sludge cells were responsible for degradation rather than the GSN3 bacteria. This proposition stems from the observation that the greater volume of GSN3 cells in R2 was not successful in augmenting degradation as much as that measured in R3. It is clear that the performance of R3 was boosted in the presence of GSN3-gfp cells. Nevertheless, the mass of initially adhered GSN3 cells in R3 was not as much as that estimated in R2, especially during the first two weeks, suggesting that just a fraction of the inoculated GSN3-gfp were able to survive in competition with the natural sludge flora. However, only the presence of this amount of GSN3-gfp cells in R3 had a significant impact on sludge cell surface colonization, with a 9 × 10¹¹-fold increase in their volume in comparison to that estimated in R1 on the same day. This phenomenon could lead to the formation of diverse biofilm cell types in R3 which cooperated actively in degradation of phenol and its intermediates and subsequently boosted the removal efficiency of COD.

Simões et al. (2008) studied several bacteria isolated from drinking water systems and demonstrated that some strains such as Acinetobacter calcoaceticus have both autoaggregation and coaggregation abilities which promoted biofilm formation in aquatic environments. Moreover, particular aerobic bacteria are believed to be capable of playing a role as a bridging bacterium (Simões et al. 2008; Buswell et al. 1997).
Figure 5. CLSM 3-D micrograph of biofilms formed on carriers in R3 (A, B, C, D), R2 (E, F, G, H), and R1 (I, J, K, L) after 7, 14, 21, and 28 days, respectively. Green fluorescence represents GSN3-gfp cells, and red fluorescence represents activated sludge cells. Bars = 10 μm.
The present findings are in good agreement with studies which showed that *A. calcoaceticus* as a bridging bacterium can aggregate with other cells in water and wastewater systems, increasing the opportunity for biofilm formation (Simões et al. 2008; Anderson et al. 2008).

Based upon the present results, microorganisms with such a characteristic have the potential to be used as anchors for attachment of adherence-deficit strains in biofilm treatment systems without out-competing them. Degradation efficiency would be elevated if they were also equipped with a degradation capability. The high phenol degradation property of the GSN3 strain used in this study elucidates the reason for the enhanced degradation efficiency in R3.

In some studies, the bioaugmentation of activated sludge with a mixed culture of bacteria either with biofilm-forming or degrading capabilities has been proposed as an applicable solution to improve the contaminant removal efficiency in biofilm systems (Jiang et al. 2006; Li et al. 2008, 2013). For instance, a mixed culture of *Bacillus* strains promoted the acetonitrile degradation efficiency of a specific strain via their biofilm forming but not degrading abilities (Li et al. 2013). Nevertheless, for applying different microorganisms with diverse individual abilities it is
expensive and time-consuming to provide a common optimum growth condition for all, and help them to compete with indigenous flora, particularly in real wastewater containing complex compounds and harsh materials (Andersson et al. 2008; Dueholm et al. 2015). However, taking advantage of bacteria with dual abilities makes the process simple, affordable, and stable in comparison to artificial immobilization techniques that rely on embedding synthetic agents or using a mixture of microorganisms with diverse requirements.

**Response to organic shock loading**

The result of the loading shock resistance assay is depicted in Figure 10A, B and C. As the results indicate, the effluent phenol concentration increased from 3.4, 3.5 and 2.3 mg l$^{-1}$ to the maximum concentrations of 124.6, 100.4 and 98.6 mg l$^{-1}$ in R1, R2, and R3 respectively. The outlet phenol concentration gradually decreased to a near steady state condition around 9, 6 and 5 h after applying the shock and reached the level of 7.1, 5.4 and 4.9 mg l$^{-1}$ – where the removal efficiency was $>99\%$ – in R1, R2, and R3, respectively. According to the results, the highest resistance was observed in R3 while it was relatively low in R2. By contrast, once subjected to loading shock, R1 showed the lowest resistance with a significant reduction in degradation efficiency. The close results obtained in R2 and R3 suggest that the GSN3 strain had a significant effect in increasing the resistance to the phenol shock compared to sludge microorganisms. Indeed, this strain retained the integrity of the biofilm by attaching other microorganisms firmly to the surface of carriers in high loading shocks. The role of strong biofilm forming strains in resistance enhancement was demonstrated previously (Li et al. 2008, 2013; Matz and Kjelleberg 2005).

Once again, the COD removal rate was increased more efficiently in R3 than in the two others, suggesting an enhanced cooperation in phenol degradation and its efficient transformation to the end products.

**HPLC analysis**

The degradation of phenol was further confirmed by HPLC analysis. Samples were taken for HPLC analysis 3 h following the cessation of the shock load. The peak of the control sample was detected (retention time, 3.535 min) and compared to the control; the peaks appeared at 3.542 min in R3, 3.535 min in R2 and 3.537 min in R1. This observation correlates well with the result obtained by the spectrophotometric method. After 3 h post-shock, 50.6, 32.4 and 11.3 mg
Figure 10. Organic loading shock resistance; Phenol (▲) and COD (■) removal efficiencies during the sudden increase of inlet phenol concentration (●) at a hydraulic retention time of 24 h in (A) R1; (B) R2; and (C) R3.
$1^{-1}$ of the remaining phenol concentrations were estimated in R1, R2, and R3 samples, respectively in comparison to 100 mg $1^{-1}$ phenol in the control (see Figure 11 for further details).

Conclusions

In the present work, the activated sludge cells were inoculated with an Acinetobacter calcoaceticus strain, GSN3, possessing both strong biofilm producing and phenol degrading properties in an MBBR. The results revealed that the combination of activated sludge and GSN3 cells provided improved phenol and COD removal efficiencies in comparison to their individual cultures. The mixed species reactor was also very stable against phenol shock loading and performed well under various operational conditions. A. calcoaceticus GSN3 appeared in the deeper layers of the biofilm where more activated sludge cells covered them over time. Indeed, some strong biofilm producing microorganisms like A. calcoaceticus have the potential to aid other beneficial adhesion-deficit cells to immobilize within biofilms while allowing them to proliferate non-competitively. They can also enhance the removal efficiency if they are equipped with a degrading ability for the desired contaminant.

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Disclosure statement

The authors declare no conflict of interest.

ORCID

Megharaj Mallavarapu http://orcid.org/0000-0002-6230-518X

Bita Ayati http://orcid.org/0000-0001-7720-9863

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