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Aerobic granules with inhibitory strains and role of extracellular polymeric substances

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ABSTRACT

Microorganisms compete with other species by secreting antimicrobial compounds. The compact structure of aerobic granules was generally assumed to provide spatial isolation, resulting in the co-occurrence of diverse strains that have similar or dissimilar functions. No studies have investigated whether stable, mature aerobic granules can be formed with two mutually inhibitory strains. The strain *Acinetobacter* sp. I8 competes with *Bacillus sphaericus* I5 in a well-mixed environment, but can form stable and mature granules at 400 mg L^{-1} phenol by repeatedly replenishing fresh medium in a sequencing batch reactor. The supernatants collected from the I8 medium in its exponential-growth phase or from the I5 + I8 medium cultivated for 12 or 24 h significantly inhibited I5 growth. Addition of tightly bound extracellular polymeric substances (TBEPS) or loosely bound extracellular polymeric substances (LBEPS) extracted from I5 + I8 granules effectively suppressed the inhibitory effects of I8 on I5. The TBEPS or LBEPS physically separate strain I5 from I8 in the granule, and effectively adsorb the inhibitory substance(s) in the suspension.

1. Introduction

The application of aerobic granular sludge is considered as a promising biotechnology in wastewater treatment [1–3]. The first patent for the use of aerobic granules was granted to Heijnen and van Loosdrecht [4]. Aerobic granules have dense and strong structures, good settleability, high biomass retention, and high tolerance to medium toxicity [5,6]. Although granulation process has been characterized [7–10], little is known about the microbial interactions and adaptive mechanism for microbial survival within aerobic granules. The two populations associate tightly under some conditions or location indicating the beneficial association. In contrast, the clusters of population remain at a distance from each other in the community within aerobic granules when the productions of antagonist or competition exist. During such conditions, microbial cells get entrapped within the matrix of extracellular polymeric substances (EPS) secreted by themselves, a universal survival strategy adopted by microbes. EPS are metabolic products that are major components of activated sludge flocs, biofilms and microbial granules [11,12] and their layer forms a protective shield for aerobic granule cells against harsh external environments [13].

Aerobic granules are composed of numerous microbial strains [14]. Jiang et al. [15] isolated 10 strains from phenol-degrading granules that were either good phenol reducers or good flocculators. Jiang et al. [16] demonstrated that the two functionally dissimilar isolates, *Propioniferax*-like PG-02 (fast-growing strain in phenol) and *Comamonas* sp. PG-08 (strong coagulator) cannot co-exist at 250 mg L^{-1} phenol in a completely mixed environment, but can co-exist in a spatially heterogeneous environment. Jiang et al. [17] determined that two functionally similar strains, *Pandoraea* sp. PG-01 and *Propioniferax*-like PG-02, are fast-growing strains in phenol; however, they cannot co-exist in a well-mixed medium due to mutual competition. Zhou et al. [18] and Treves et al. [19] indicated that competitively inferior strains can co-exist when physical isolation is provided. Jiang et al. [17] demonstrated that two functionally similar strains, *Pandoraea* sp. PG-01 and *Rhodococcus erythropolis* PG-03 obtained from their phenol-fed aerobic granules, cannot co-exist in a well-mixed medium due to mutual competition, but can co-exist in the spatially heterogeneous structure of aerobic granules. However, these studies did not investigate whether the strains studied are mutually inhibitory.

Adav and Lee [20] isolated nine strains (I1–I9) from their aerobic phenol-degrading granules; strains *Bacillus thuringiensis* I2, *Acinetobacter calcoaceticus* I6 and *Acinetobacter* sp. I8 have high auto-aggregation capabilities and can form single-strain granules

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[21]. These authors also determined that the strains *Acinetobacter* sp. I8 and *Bacillus sphaericus* I5 were mutually inhibitory. Zhou et al. [18] and Treves et al. [19] claimed that EPS physically isolate strains from mutual competition and/or reduce local mass transfer of toxins to cells.

No studies have investigated whether stable, mature granules can be formed using inhibitory strains. This study tested two strains, one inhibits the growth of another, in homogeneous (well mixed) and heterogeneous (still) media; the two strains were cultivated to form stable and mature aerobic granules. This work provides experimental evidence that the EPS extracted from the I5 + I8 granules effectively eliminated the secreted inhibitory substance(s). The EPS provided physical isolation for strain I5 from I8, and eliminated the secreted inhibitory substance(s).

2. Materials and methods

2.1. Strains and medium

This study used bacterial strains *Acinetobacter* sp. I8 and *B. sphaericus* I5, which were isolated previously from phenol-degrading granules [20]. Strain I8 is a Gram-negative bacterium that has a short rod shape, and strain I5 is a Gram-positive bacterium with a rod-shaped morphology. These two strains have high phenol-degrading capability. The composition of the MP medium used in this work was (in mg L^{-1}): 1000, $(NH_4)_2SO_4$; 200, $MgCl_2$; 100, NaCl; 20, $FeCl_3$; 10, $CaCl_2$; 1350 KH_2PO_4 and 1650 K_2HPO_4 (pH 6.8). The micronutrients were (mg L^{-1}): 50, H_3BO_3; 50, $ZnCl_2$; 30, $CuCl_2$; 50, $MnSO_4{\cdot}H_2O$; 50, $(NH_4)Mo_7O_{24}{\cdot}4H_2O$; 50, $AlCl_3$; 50, $CoCl_2{\cdot}6H_2O$ and 50, $NiCl_2$.

2.2. Granules cultivation and EPS extraction

Aerobic granules were cultivated in column-type sequential batch reactors (SBR) 6 cm in diameter and 120 cm in height. These reactors seeded with 2L of I5 + I8 and fed with sterilized MP medium at pH 6.8 ± 0.2 with 250 mg L^{-1} phenol as the sole carbon source. Fine air bubbles at a flow rate of 3 L min^{-1} were supplied at the reactor bottom, and the air outlet was immersed in sterilized water. The column was operated at cycle time of 6 h (5 min settling, 5 min filling, 5 min effluent withdrawal, and 5 h and 45 min of aeration). The volumetric exchange ratio 50% was applied by discharging the effluent above 50 cm from the reactor bottom followed by replenishing the reactor with the same volume of fresh sterilized medium in each cycle. The reactor was operated for 50 days.

The EPS were extracted from cultured I5 + I8 aerobic granules as described in [22]. In brief, the samples were washed with water, and loosely bound EPS (LBPES) were obtained by centrifugation at 5000 × *g* for 10 min. The residues were resuspended to their original volume using saline solution (0.05% NaCl), and were extracted again using low-frequency ultrasound at 20 W for 5 min in an ice bath. Following ultrasonication, suspensions were collected by centrifugation and filtered through a 0.2 μm filter (Advanced Microdevices, Ambala Cantt, India). The EPS in the collected filtrate were considered the tightly bound EPS (TBEPS) of the sample. Via quantification of the 2-keto-3-deoxyoctonate (KDO) in the extract, the quantities of DNA in all extracted EPS samples were <0.2 mg g^{-1} volatile suspended solids (VSS), indicating negligible contamination of the collected EPS by intracellular matter. The carbohydrate content in EPS was measured by the Anthrone method [23] with glucose as the standard. The protein and humic content in EPS was measured by the modified Lowry method [24] using bovine serum albumin and humic acid (Fluka, USA) as the respective standards.

The granules were collected, washed with phosphate buffered saline (PBS, pH 7.2) and fixed with 4% paraformaldehyde for 3 h at 4 °C. The fixed granules were washed with PBS buffer and stained for β-polysaccharides by adding calcofluor white (fluorescent brightener 28, Sigma, USA) solution (300 mg L^{-1}, 100 μL) for 30 min. The stained granule was washed twice with PBS to remove excess stain and hybridized for FISH as described by [25] with hybridization buffer containing 5 ng μL^{-1} of each of the specific probes—*Acinetobacter* sp. (ATC CTC TCC CAT ACT CTA) and *B. sphaericus* (ATG AGA AAT TTG GAT TTT ATT)—labeled with FAM (green) and Cy3 (red). The granule was then embedded for cryosectioning in embedded medium (Shandon Cryomatrix, Pittsburgh, PA, USA). Embedded samples were frozen at −20 °C, after which 40-μm sections were cut on a cryomicrotome and mounted onto a gelatin-coated (0.1% gelatin and 0.01% chromium potassium sulfate) microscopic slide and analyzed by confocal laser scanning microscopy (CLSM) (Leica TCS SP5, Confocal Spectral Microscope Imaging System, GmbH, Germany).

2.3. Homogeneous and heterogeneous tests

Reagent bottles (500 ml) containing 200 ml sterilized MP medium and 400 mg L^{-1} phenol were utilized in tests. Equal quantities of strains I5 and I8 at their respective exponential-growth phases were inoculated and incubated at 35 °C and 150 rpm in a rotary shaker to produce a well-mixed (homogeneous) environment. Spatially heterogeneous tests were conducted following the procedures developed by Rainey and Travisano [26]. The experimental protocol was the same as in homogeneous environment tests, except that bottles were kept still and were mixed manually daily.

Suspension samples were collected from homogeneous environment tests and heterogeneous environment tests during their daily, manual mixing period. The bacteria in the collected samples were concentrated by centrifugation (8000 rpm at 4 °C), washed with 1× PBS and fixed with 4% paraformaldehyde for 30 min at 4 °C. The fixed samples were resuspended in 50% ethanol after washing with 1× PBS buffer. The resuspended cells were then hybridized using hybridization buffer (0.9 M NaCl, 20 mM Tris–HCl at pH 7.4, 0.01% sodium dodecyl sulfate and formamide) containing 5 ng μL^{-1} of the probes labeled with FAM and Cy3 probes, as stated in Section 2.2. The hybridized cells were imaged via CLSM and cell populations (%) were determined their respective fluorescent signals.

2.4. Inhibitory tests with extracted EPS

In total, 8 ml of strain I8 (OD = 0.8) was incubated in 500 ml reagent bottles containing 200 ml sterilized MP medium with 400 mg L^{-1} phenol at 35 °C in a well-mixed environment. The medium supernatant was collected separately during the exponential-growth phase (supernatant I8-E) and stationary phase (I8-S) of I8 cells following centrifugation and filtration. Moreover, the supernatants of mixed cultures collected (Section 2.3) with I5 + I8 under a well-mixed environment were collected separately at 12 h (I5 + I8-12 h) and 24 h (I5 + I8-24 h) of incubation.

In total, 8 ml of strain I5 was incubated at 35 °C in 500 ml reagent bottles containing 100 ml sterilized MP medium with 400 mg L^{-1} phenol, and 100 ml of one of the four collected supernatants (I8-E, I8-S, I5 + I8-12 h, and I5 + I8-24 h). Each set of bottles fed with a specific supernatant was further divided into three groups. The first group was fed with 150 ml of LBEPS from I5 + I8 granules (Section 2.2); the second group was fed with 150 ml of TBEPS from granules (Section 2.2); the third group without EPS was used as a control. All bottles were shaken at 150 rpm to generate a homogeneous environment.

2.5. Analytical methods

The dry weights of granules, VSS, SS, and the sludge volume index (SVI) in the suspension were measured according to Standard Methods [27]. The size of the granules was determined by a particle size analyzer (Mastersizer Series 2600; Malvern Instruments, Worcestershire, UK). Phenol concentrations in the reactor were determined by high-performance liquid chromatography (HPLC) equipped with a C18 column (Varian, Inc., CA, USA) at wavelength 276 nm. The mobile phase comprised of acetonitrile:water (300:700), 0.11 g heptane sulphonic acid, 0.29 g anhydrous sodium acetate, and 2.5 ml glacial acetic acid. The size exclusion chromatography system used for EPS analysis comprised a BETA 10 gradient pump (Ecom spol. s r. o., Prague, Czech Republic), a size exclusion TSK G3000SW$_{XL}$ column (TOSOH Bioscience, Stuttgart, Germany), on-line SAPPHIRE 600 UV–VIS variable wavelength detector (Ecom spol. s. r. o., Prague, Czech Republic) and a CHF 100SA fraction collector (Advantec MFS, Inc., Dublin, CA, USA).

3. Results

3.1. Homogeneous and heterogeneous tests

The changes in populations of I5 and I8 cells over time in the homogeneous medium were monitored (Fig. 1a). In homogeneous medium, the number of I8 cells increased from 54.4 ± 3.5% initially to roughly 97 ± 1.1% after 24 h of incubation while I5 declined to 2.7% from its initial population of 45.5 ± 3.5% (Fig. 1a). In other words, strain I8 competes with strain I5 in the homogeneous medium. In the heterogeneous medium, conversely, the population of I5 decreased by 27–30% and stabilized after 24 h of incubation (Fig. 1b). The heterogeneous medium shielded strain I5 from strain I8. This experimental observation correlates with findings obtained by Jiang et al. [17], Zhou et al. [18], Treves et al. [19], indicating that competitive strains can co-exist when they are physically separated.

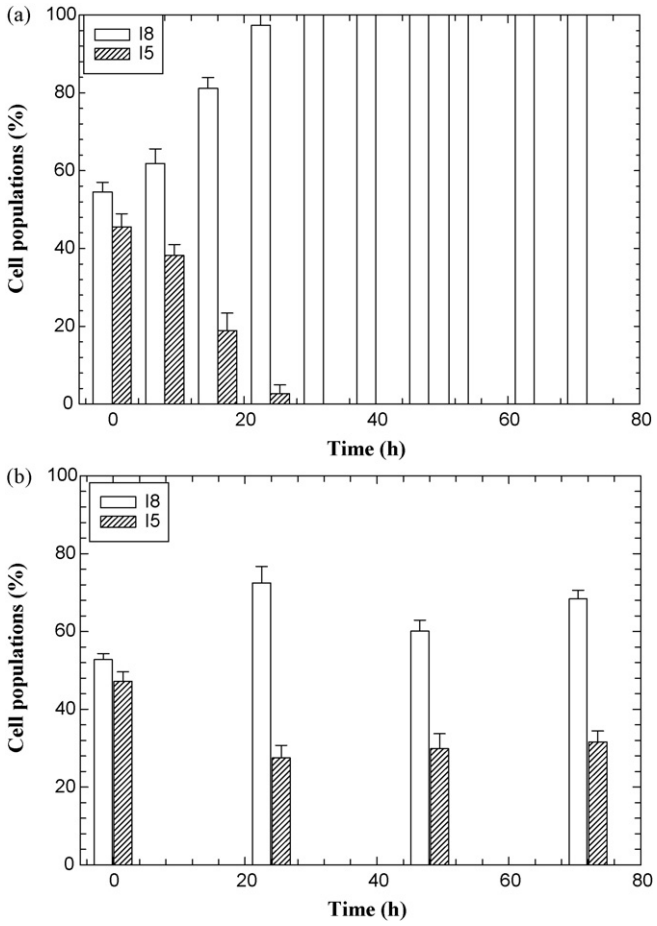

Fig. 1. Proportion of cell populations in different environment at 400 mg L^{-1} phenol concentration. Cells were hybridized with specific probes and the percentage of each strain was calculated. (a) Homogeneous medium and (b) heterogeneous medium.

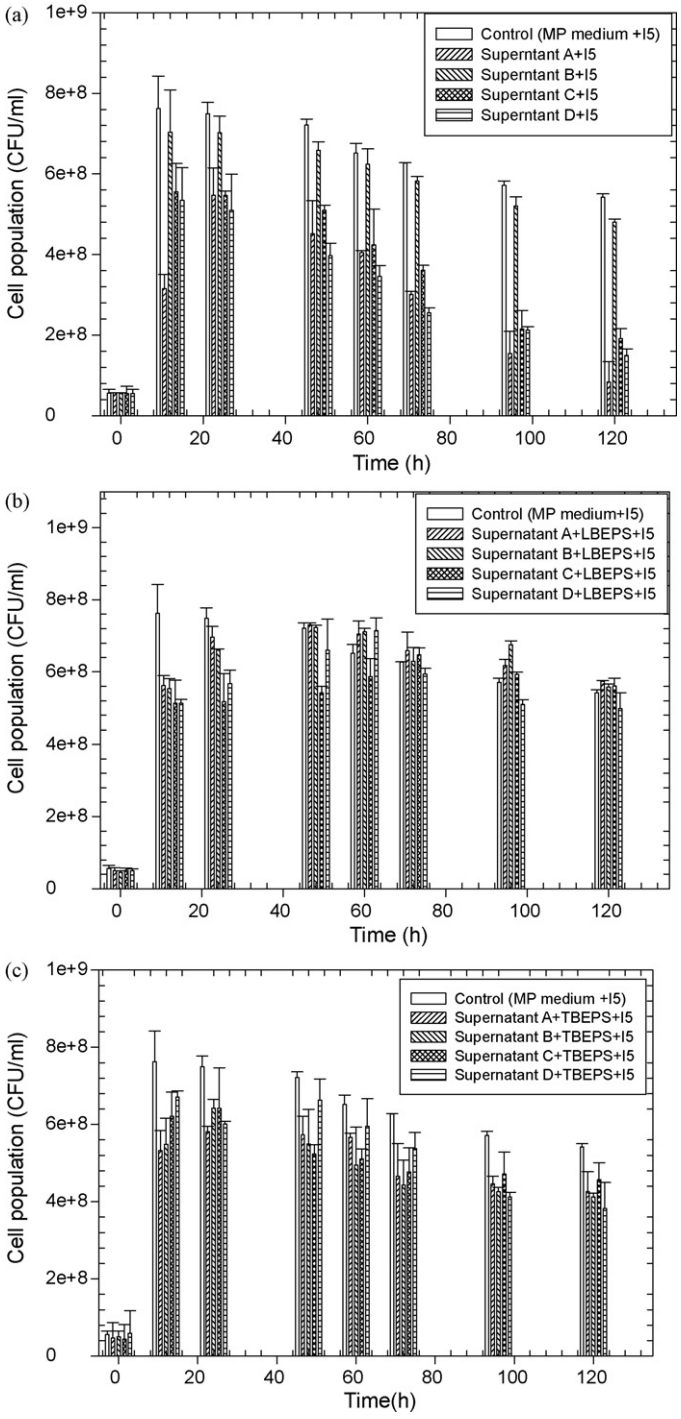

Fig. 2. Cell populations for strain I5 with MP medium and supernatants I8-E (supernatant A), I8-S (supernatant B), I5 + I8-12 h (supernatant C) and I5 + I8-24 h (supernatant D) (40:60, v/v). (a) With no added EPS; (b) with added TPEPS and (c) with added LBEPS.

3.2. Inhibition test

The I5 strain grew well in the MP medium, reaching $7.7 \pm 0.5 \times 10^8$ CFU ml^{-1} during cultivation for 16 h (control in Fig. 2a). With supernatant I8-E (supernatant collected at the exponential-growth phase of strain I8), I5 + I8-12 h (supernatant collected during the homogeneous test with I5 + I8 after 12 h of testing) or I5 + I8-24 h (supernatant collected during the homogeneous test with I5 + I8 after 24 h testing) added, the growth of I5 cells was inhibited significantly. For example, with I8-E added, the concentrations of I5 cells were $3.1 \pm 0.6 \times 10^8$ and $0.8 \pm 0.4 \times 10^8$ CFU ml^{-1} after 16 and 120 h cultivation, respectively, accounting for 40% and 15% of the control respectively.

Adding TBEPS or LBEPS collected from I5 + I8 granules (Section 2.2) markedly decreased the inhibitory effects of I8-E, I5 + I8-12 h or I5 + I8-24 h on strain I5 (Fig. 2b and c). For example, when TBEPS were added, the quantities of I5 cells in I8-E were $5.2 \pm 0.5 \times 10^8$ and $4.1 \pm 0.4 \times 10^8$ CFU ml^{-1} after 16 or 120 h cultivation, respectively, significantly higher than those without TBEPS added ($3.1 \pm 0.6 \times 10^8$ and $0.8 \pm 0.4 \times 10^8$ CFU ml^{-1}, respectively).

3.3. Cultivated granules and extract EPS

In granule cultivation tests, small aggregates of I5 + I8 appeared within 1 day in the SBR. The granules grew to 0.5–0.7 mm in size in 14 days, and the corresponding SVI values decreased from 210 to 112 ml g^{-1}. On day 30, the granules had grown to a mean size of 1.6 mm and SVI values declined to 52.8 ml g^{-1}. Microscopic observations demonstrate that the formed granules had a nearly spherical shape and smooth surface. The strains in granules were *Acinetobacter* sp. I8 and *B. sphaericus* I5 via analysis of their corresponding 16S rRNA sequences in extracted DNA.

Chemical analysis reveals that the quantities of proteins, polysaccharides, and humic substances in the EPS extracted from I5 + I8 granules were 228.4 ± 21.8, 145.8 ± 18.2, and 109.2 ± 8.9 mg g^{-1} VSS, respectively. Retention times of SEC chromatograms of LBEPS were roughly 5 and 9.1 min, whereas that of TBEPS was at 10.9 min (single peak). The apparent molecular weights (AMWs) of LBEPS and TBEPS were estimated at >20,000 and 8000 Da, and about 2000 Da, respectively. The residue (pellet) after EPS extraction has a broad range of AMWs, ranging from <100 to >20,000 Da.

Fig. 3 shows the FISH–CLSM image of an I5 + I8 aerobic granule, cross-sectioned at 360 μm from the top surface. The β-polysaccharide was distributed in the entire granule structure forming a backbone. The β-polysaccharides (blue: calcofluor white) physically separated strain I8 (green) and strain I5 (red).

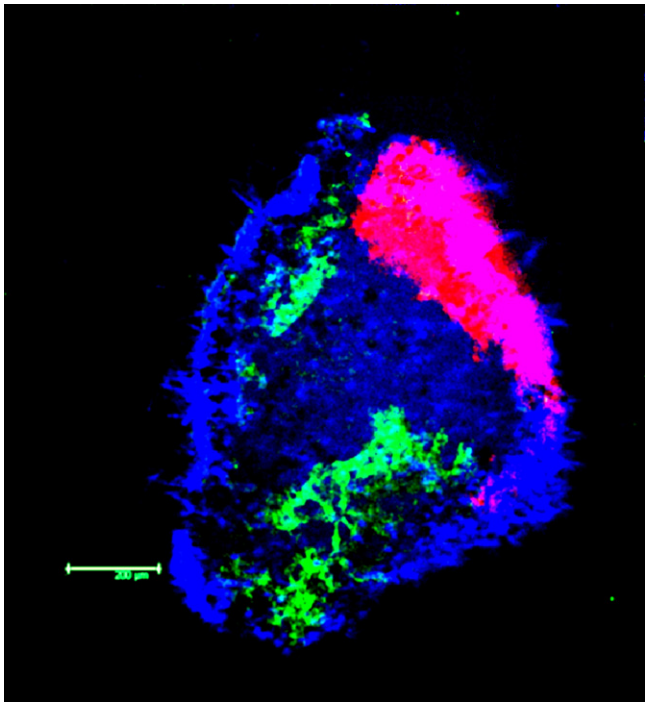

Fig. 3. FISH–CLSM image of aerobic granule cross-sectioned at 360 μm from top surface, stained for β-polysaccharides (blue: calcofluor white) and simultaneously hybridized with specific probes for strain I8 (green) and strain I5 (red). Bar represents 200 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

4.1. Inhibition of I8 on I5 in homogeneous and heterogeneous tests

The I5 and I8 cells cannot co-exist in a homogeneous medium, based on the inhibitory effects of I8 on I5 in plate tests [20]. The supernatant I8-E rather than I8-S inhibited I5 growth (Fig. 2a). Hence, the inhibitory substance(s) were primarily secreted/generated with phenol metabolism (intermediates of phenol metabolism) by I8 during its exponential-growth phase, rather than during its stationary phase.

The I5 + I8-12 h and I5 + I8-24 h supernatants inhibited I5 growth, demonstrating that I8 secreted and released inhibitory substance(s) into surrounding liquid when I5 is present. In the homogeneous medium, the inhibitory substance(s) efficiently reached I5 via the mixing current. When I5 and I8 were kept still in a bottle (heterogeneous tests), the I5 cells co-existed with I8 from 24 h and beyond (Fig. 1b) as the inhibitory substance(s) secreted by I8 that effectively inhibit I5 cells diffuse slowly or due to low concentration of inhibitory substance.

4.2. Granules with mutually inhibitory strains

When I5 + I8 are co-cultivated in an SBR to form granules, likely due to dilution via frequent replenishment of fresh MP medium during SBR operation, the inhibitory substance(s) secreted by I8 did not inhibit I5's growth.

As revealed by Fig. 3, the EPS physically separated strain I5 and I8, as demonstrated by Zhou et al. [18] and Treves et al. [19]. Prolonged tests indicate that the I5 + I8 granules were stable over a subsequent 1-month test in MP + phenol medium in an SBR. The I5 + I8 granules were a stable eco-system for the co-existence of two inhibitory strains.

4.3. Role of EPS on inhibitory effects

Adding extracted TBEPS or LBEPS generally eliminate the inhibition effects of supernatants I8-E, I5 + I8-12 h or I5 + I8-24 h on I5 cells (Fig. 2b and c). Hence, the EPS shielded the I5 strain from inhibitory substance(s) secreted by I8 or generated during phenol metabolism.

A test with 200 ml sterilized MP medium and 400 mg L^{-1} phenol, 2 ml each for strains I5 and I8 at their respective exponential-growth phase, and 150 ml TBEPS extracted from I5 + I8 granules was conducted in reagent bottles at 35 °C and 150 rpm. Fig. 4 shows the FISH–CLSM images of strains I8 and I5 after 50 h incubation. The quantities of both I5 and I8 were considerable in the medium, implying that I8 only minimally inhibited I5. The I5 cells remained in a dispersed state (Fig. 4); hence, the TBEPS added in this test do not provide a physically isolated environment for I5 cells, as suggested by Zhou et al. [18] and Treves et al. [19], or provide

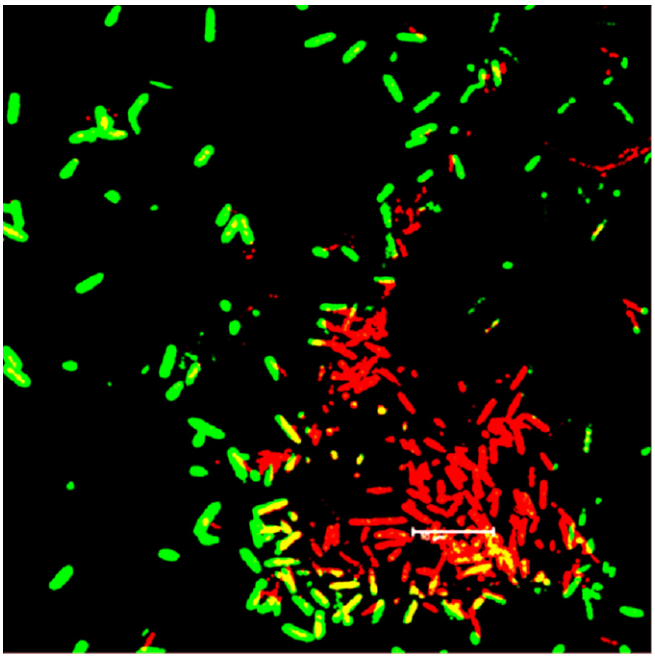

Fig. 4. FISH–CLSM image of strains I8 and I5 in homogeneous MP medium and extracted EPS from aerobic granules (40:60, v/v) after 50 h. In situ hybridization was performed simultaneously with specific probes labeled with Cy3 and FAM (red-strain I8 and green-strain I5). Bar represents 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

mass transfer resistance to reduce local concentrations of toxins in suspension, as argued by Chiu et al. [13]. Adsorption of inhibitory substance(s) on EPS molecules may be a mechanism for the noted shield effect by the EPS. However, although the extracted TBEPS or LBEPS have extremely different AWM distributions, both effectively "protect" I5 from I8, demonstrating that the hydrophobic interaction or "van der Walls interaction" between the functional groups on the EPS, and inhibitory substances did not likely correspond to the proposed adsorption mechanisms.

5. Conclusions

Microorganisms inhibit the outgrowth of other species by secreting antimicrobial compounds. This inhibition due to antimicrobial substance secreted by one species can be overcome by strategies such as cell immobilization to protect other microbial cells. In the compact structured aerobic sludge granules, EPS provide spatial isolation for the microbial strains having similar or dissimilar functions. In homogeneous environment, strain *Acinetobacter* sp. I8 inhibits *B. sphaericus* I5, while they co-exits in aerobic granules. Thus, role of EPS is proposed to effectively adsorb the inhibitory substance(s) and provide spatial isolation environment for the strain.

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