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Single-culture aerobic granules with *Acinetobacter calcoaceticus*

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Abstract Aerobic granules are cultivated by a single bacterial strain, *Acinetobacter calcoaceticus*, in a sequencing batch reactor (SBR). This strain presents as a good phenol reducer and an efficient auto coagulator in the presence of phenol, mediated by heat-sensitive adhesins proteins. Stable 2.3-mm granules were formed in the SBR following a 7-week cultivation. These granules exhibit excellent settling attributes and degrade phenol efficiently at concentrations of 250–2,000 mg l^{-1}. The corresponding phenol degradation rate reached 993.6 mg phenol g^{-1} volatile suspended solids (VSS) day^{-1} at 250 mg l^{-1} phenol and 519.3 mg phenol g^{-1} VSS day^{-1} at 2,000 mg l^{-1} phenol concentration. Meanwhile, free *A. calcoaceticus* cells were fully inhibited at phenol >1,500 mg l^{-1}. Denaturing gradient gel electrophoresis fingerprint profile demonstrated no genetic modification in the strain during aerobic granulation. The present single-strain granules showed long-term structural stability and performed high phenol degrading capacity and high phenol tolerance. The confocal laser scanning microscopic test revealed that live *A. calcoaceticus* cells principally distributed at 200–250 μm beneath the outer surface, with an extracellular polymeric substance layer covering them to defend phenol toxicity. Autoaggregation assay tests demonstrated the possibly significant role of secreted proteins on the formation of single-culture *A. calcoaceticus* granules.

Keywords Aerobic granule · Single culture · Autoaggregation · *Acinetobactor calcoaceticus*

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Introduction

Aerobic sludge granulation is a novel wastewater treatment technology that can quickly decontaminate high-strength wastewater (Beun et al. 1999; Peng et al. 1999). Aerobic granules have a dense and strong microbial structure, good settling ability, high biomass retention, and tolerance of highly toxic substrates (Tay et al. 2001; Su and Yu 2005; Adav et al. 2007a, b, c).

Jiang et al. (2004) isolated ten strains from phenol-degrading granules using culture-based and culture-independent techniques. These strains were either good phenol reducers or good flocculators, but not both. Jiang et al. (2006a) examined the impact of coaggregation of two isolates, *Propioniferax*-like PG-02 (fast-growing strain in phenol) and *Comamonas* sp. PG-08 (strong coagulation capability), on granule formation and on yielded phenol degradation rate. The coculture could degrade phenol faster than each strain separately when these two strains were incubated together. Furthermore, Jiang et al. (2006b) found that the strains PG-02 and the PG-08 could not coexist at 250 mg l^{-1} phenol in a complete mixed environment, but they could coexist in a spatially heterogeneous environment at low phenol concentrations. Jiang et al. (2007) cocultured two functionally similar strains, PG-01 and *Rhodococcus erythropolis* PG-03, both with high phenol-degrading rates but low aggregation capability, and observed that these could only coexist in the spatially heterogeneous frameworks of aerobic granules.

A single-culture aerobic phenol-degrading granule may be produced from a strain that is both a good phenol reducer and a good autoaggregator. The formation of aerobic granules with a single culture has not previously been explored to our knowledge. Adav and Lee (manuscript submitted for publication) isolated from their phenol-

degrading granules a strain of *Acinetobacter calcoaceticus*, which is both a good phenol reducer and a good autoaggregator. This investigation cultivated single-cultured aerobic granules with strain *A. calcoaceticus*, which could effectively degrade phenol at high phenol concentrations.

Materials and methods

Strain and autoaggregation test

The strain *A. calcoaceticus* isolated in our previous study (Adav and Lee, manuscript submitted for publication), deposited under accession number EU250016 in GenBank, was applied to cultivate granules. Autoaggregation activity of the studied *A. calcoaceticus* strain was estimated by incubating the strain in the medium with the following composition (per liter): 1.0 g of $(NH_4)_2SO_4$, 0.2 g of $MgCl_2 \cdot 6H_2O$, 0.02 g of $FeCl_3 \cdot 6H_2O$, 0.01 g of $CaCl_2$, 0.4 g of peptone, and phosphate buffer (1.35 g of KH_2PO_4 and 1.65 g of K_2HPO_4) with different levels of phenol or glucose as sole carbon source. An equal volume of (1 ml) strain suspension was mixed with fresh, sterilized medium at 30 °C. The initial optical density ($OD_{(initial)}$) of strains was measured (600 nm) and the test solutions were allowed to stand at room temperature (25±2 °C) for 30 min. The supernatant with a volume of 1 ml was carefully pipetted into a microcuvette and the optical density was measured at 600 nm. The autoaggregation indices of the test strains were calculated by comparing initial OD ($OD_{(initial)}$) and OD value after 30 min ($OD_{(30)}$), as follows:

$$\text{Autoaggregation index}(\%) = \frac{OD_{(initial)} - OD_{(30)}}{OD_{(initial)}} \times 100$$

A high autoaggregation index denotes a strong tendency that the cells would agglomerate into an aggregate.

Granule cultivation

The aerobic granules were cultivated in a column-type sequential batch reactor (SBR, 6×80 cm). The sterilized reactor was seeded with 2 l of the free *A. calcoaceticus* cells and was fed with synthetic wastewater at pH 7.0±0.2 with 500 mg l^{-1} phenol as the sole carbon source. The composition of the synthetic wastewater was 1,000 mg l^{-1} $(NH_4)_2SO_4$, 200 mg l^{-1} $MgCl_2$, 100 mg l^{-1} NaCl, 20 mg l^{-1} $FeCl_3$, 10 mg l^{-1} $CaCl_2$, KH_2PO_4 1,350 mg l^{-1}, K_2HPO_4 1,650 mg l^{-1}, peptone 400 mg l^{-1}, and micronutrients as listed in Adav et al. (2007c).

Fine air bubbles for aeration were supplied through a filter device (0.2 μm, Advanced Microdevices, Ambala, India) via the reactor bottom, and the air outlet was immersed in sterilized water. The column was operated at a volumetric exchange ratio of 50% by discharging the effluent at 40 cm above the reactor bottom, followed by replenishing the reactor with the same volume of fresh, sterilized medium in every cycle. The granules were monitored for contamination by plating on agar synthetic wastewater medium (composition as above). The granules were aseptically broken in a sterilized tube, diluted to 10^6–10^7 folds with sterilized Milli-Q water, and 1 ml of the dilutions was plated on agar synthetic wastewater medium. The plates were incubated at 37 °C for 48 h and contamination was monitored by colony morphology and microscopic test. In addition, DNA isolation and polymerase chain reaction (PCR) amplification technique was used for contamination monitoring.

SBR degradation test

The formed granules in "Granule cultivation" were added into SBR fed with wastewater with 250–2,000 mg l^{-1} phenol as sole carbon source. The phenol degradation tests were undertaken for cultivated granules in the batch reactors. The phenol concentrations were periodically measured in triplicate.

DNA extraction and identification

Genomic DNA of samples was extracted by freezing and thawing as described previously (Adav et al. 2007a). PCR amplification of the 16S rRNA gene was undertaken using extracted DNA with forward primer P1 and reverse primer P2 as described earlier (Adav et al. 2007c). The GC-rich sequence of 40 nucleotides (GC clamp) was attached at the 5′ end of primer P1. Amplification was conducted with a Mastercycler (Eppendorf AG, Hamburg, Germany) using a 50-μl (total volume) mixture containing 1.25 U of *Taq* polymerase (Promega, Madison, WI, USA), 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 200 μM deoxynucleoside triphosphate, 25 pmol of each primer, and 50 ng DNA extracted from either mature granules or isolated strains. The resulting PCR-amplified products were confirmed using electrophoresis through 1.2% agarose gel in 1× TAE buffer (40 mM Tris base, 20 mM sodium acetate, 1 mM Na2–EDTA, pH 8.0) stained with ethidium bromide.

The denaturing gradient gel electrophoresis (DGGE) tests were conducted using the Bio-Rad (Hercules, CA, USA) universal mutation detection system with 10% (*w*/*v*) polyacrylamide gels. The range of denaturants [100% denaturant corresponds to 7 M urea and 40% (*v*/*v*) deionized formamide] was 35–65%. Electrophoresis was performed at 60 °C for 14 h at 120 V. Gels were stained with ethidium bromide and photographed using a UV transilluminator. The dominant bands were excised from

the gel and amplified and sequenced by an ABI model 3730 DNA sequencer.

Extracellular polymeric substance extraction

The extracellular polymeric substances (EPS) of samples were extracted using heat treatment (80 °C, 30 min) followed by high-speed centrifugation (10,000×*g*, 20 min; Adav and Lee 2007). The cell lysis during EPS extraction was estimated by analyzing the quantity of 2-keto-3-deoxyoctonate (KDO) in samples, using KDO (0.1–2 μg, Sigma, St. Louis, MO, USA) as a standard as described by Adav and Lee (2007), and we noted very negligible cell lysis. The carbohydrate content in EPS was measured by the anthrone method (Gaudy 1962) using glucose as the standard. The contents of protein and humic substances in EPS were measured by the modified Lowry method (Frolund et al. 1996) using bovine serum albumin and humic acid (Fluka, Milwaukee, WI, USA) as the respective standards.

EPS staining, flourescent in situ hybridization, and confocal laser scanning microscopy imaging

The collected granules were washed with Milli-Q water followed by phosphate saline buffer (PBS buffer, 130 mM NaCl, 10 mM sodium phosphate at pH 7.2). SYTO 63, which is a cell-wall-permeable nucleic acid stain, was applied to stain cells. The proteins and amino sugars were stained with fluoresceinisothiocyanate dye. The dead cells in the granules were then stained with SYTOX blue, a cell-wall-impermeable stain. The α-mannopyranosyl and α-glucopyranosyl sugar residues were detected using concanavalin tetramethylrhodamine conjugate (ConA). Lipids were stained by Nile red. Calcofluor white was applied to stain β-polysaccharides. All probes were purchased from Molecular Probes (Carlsbad, CA, USA).

The oligonucleotide probe designed to target the 16S rRNA gene of *A. calcoaceticus* was generated. The strain was washed with PBS and fixed for 3 h in 4% paraformaldehyde. Following fixation, paraformaldehyde-fixed strain was suspended in 50% ethanol in PBS buffer and incubated for 30 min at −20 °C, washed again in PBS, and hybridized using hybridization buffer (0.9 M NaCl, 20 mM Tris–HCl at pH 7.4, 0.01% SDS) containing 5 ng/ml of probe (5′-ATC CTC TCC CAT ACT CTA-3′ labeled at the 5′-end with the sulfoindocyanine dyes Cy3) for 30 min at 47 °C. This step was followed by a 20-min washing step at 37 °C in wash buffer prior to a final wash in 1× TE buffer (Tris 100M, EDTA 10 mM, pH 8.0).

Confocal laser scanning microscopy (CLSM; Leica TCS SP2 Confocal Spectral Microscope Imaging System, Gmbh, Wetzlar, Germany) was employed to visualize cell and EPS distributions in the granule. The granules were imaged using a ×10 objective and analyzed using Leica confocal software. Chen et al. (2007) describe staining procedures in detail.

Analytical methods

The dry weight of granules and cells and volatile suspended solids (VSS) in the suspension were measured according to standard methods (APHA 1998). Phenol concentrations in the reactor were determined with high-performance liquid chromatography equipped with a C18 column (Varian, Pacific Grove, CA, USA) and measured spectrographically at 276 nm. The mobile phase comprised acetonitrile and water (300:700), 0.11 g *n*-heptane sulphonic acid, 0.29 g anhydrous sodium acetate, and 2.5 ml glacial acetic acid. Granules were prepared for scanning electron microscopy (SEM) by washing with Milli-Q water followed by fixation in 2.5% glutaraldehyde for 2 h. The granules were suspended in osmium (VIII)-tetroxide, dehydrated via successive passages through 30, 50, 75, 85, 90, 95, and 100% ethanol, and subjected to critical drying. The granule structure and surface morphology were viewed using SEM (Jeol JSM-5310, Tokyo, Japan).

Results

Autoaggregation of cells

Figure 1a shows the flourescent in situ hybridization CLSM image of the pure strain. The strain appears in chain or in pair. By degrading phenol at an initial concentration of 500 mg l^{-1}, the strain *A. calcoaceticus* started to grow, as shown in the increase in nephelometric turbidity units in suspension (Fig. 1b). The cells began to aggregate at 9 h of incubation (Fig. 1c). Settleable aggregates were formed at about 27 h of phenol incubation (Fig. 1d). Hence, the present results correlate with those reported in Adav et al. (2007a) that the strain *A. calcoaceticus* is both a phenol reducer and an autoaggregator.

Figure 2 displays the CLSM images of the 27-h autoaggregate, presenting the distributions of proteins, lipids, polysaccharides, living cells, and dead cells. The proteins, living cells, α-polysaccharides, and β-polysaccharides were distributed uniformly over the aggregate interior. Besides the fractions associated closely with individual cells, a large quantity of substances was noted between cells. The fluorescence intensity of SYTO Blue was quite low, showing that most cells were alive in the aggregates.

Single-culture granules in SBR

In SBR operation, a single strain grew and aggregated into compact granules. The corresponding granule size rose

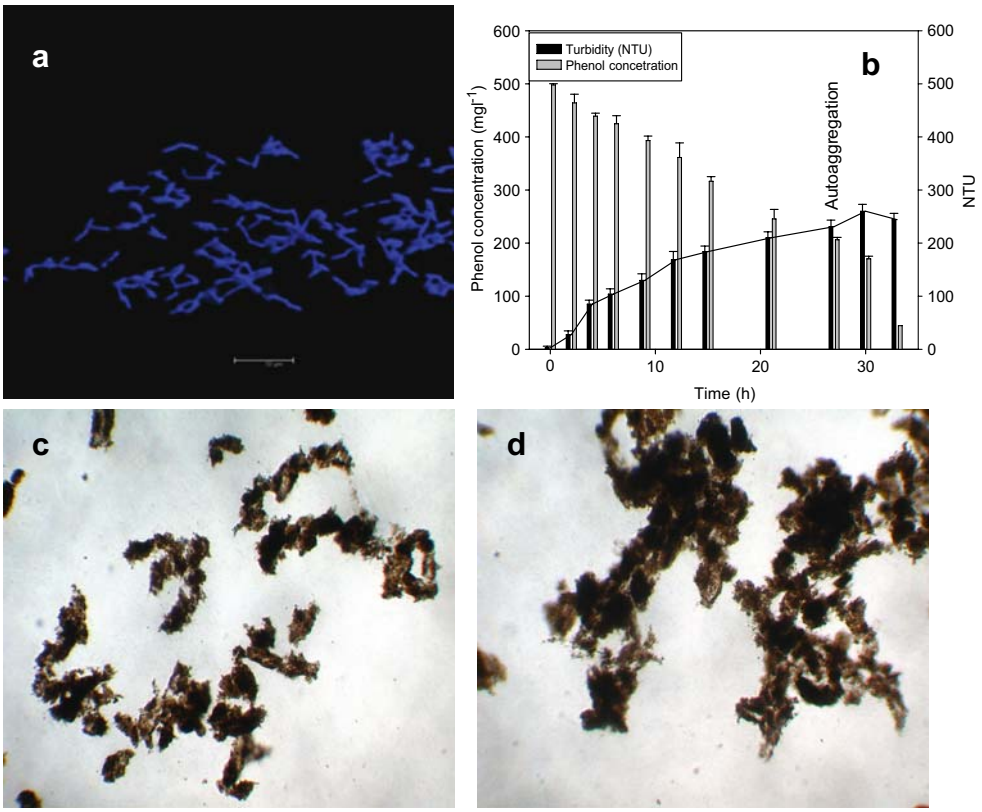

Fig. 1 Autoaggregation of *A. calcoaceticus AL 21* in 500 mg l^{-1} phenol. **a** Strain probed with specific molecular probe; **b** phenol biodegradation and turbidity; **c** aggregates after 9 h of incubation; **d** aggregates after 27 h of incubation

from 0.7 mm on week 1 to approximately 2.3 mm on week 7. Figure 3 shows the SEM image of the 49-day-old granule. Crowded cells with large intercluster channels were noted on the granule.

Table 2 lists the corresponding characteristics of the cultivated granules. The granules had a roundness of 84.5%, settling velocity of 25.6 m h^{-1}, and specific gravity of 1.002 g l^{-1}. The integrity coefficient of the granule was 86.4%.

The DGGE analysis was conducted on amplified 16S rDNA fragments and the DGGE profile of cultivated granules sampled at different incubation dates (data not shown for brevity). Over the 49-day testing time, gradient gel indicated only one band, revealing no genetic modifi-

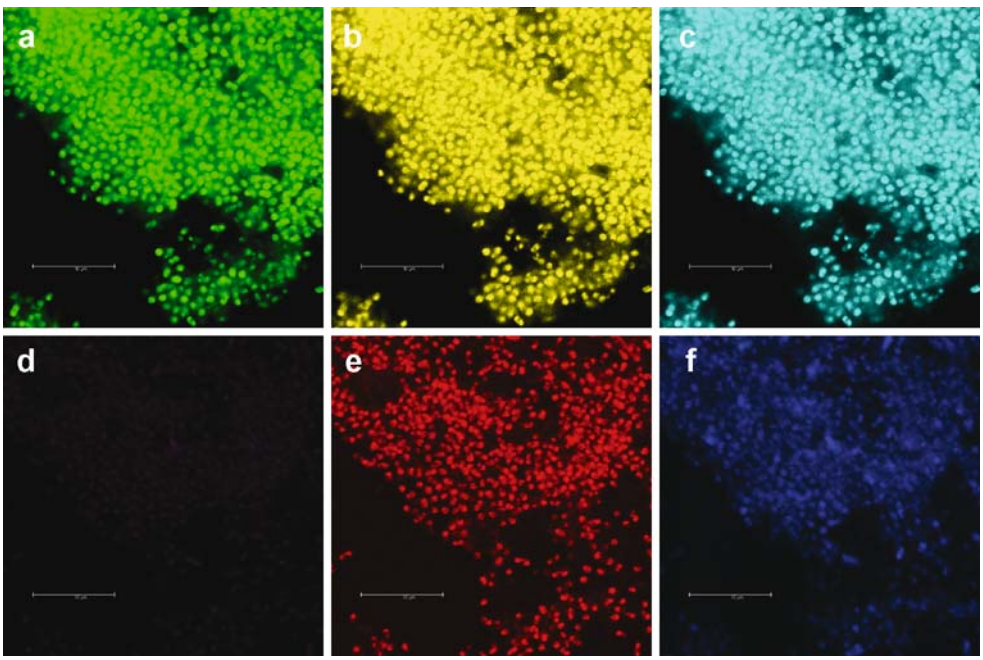

Fig. 2 CLSM images of the autoaggregate of *A. calcoaceticus*: **a** proteins (*green*), fluoresceinisothiocyanate; **b** lipids (*yellow*), Nile red; **c** α-polysaccharide (*light blue*), ConA rhodamine; **d** total cells (*red*), Syto63; **e** dead cells (*pink*), SytoX blue; **f** β-polysaccharide (*blue*), calcofluor white. *Bar*=10 μm

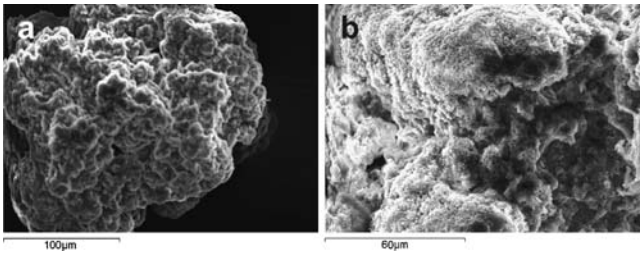

Fig. 3 SEM images of mature granules of *A. calcoaceticus* cultivated from SBR for 49 days. **a** *Bar*=100 μm. **b** *Bar*=60 μm

cation of the strain. The nucleotide sequences of all bands correspond to 100% similarity to 16s rRNA sequences of strain *A. calcoaceticus*.

Phenol degradation by single-cultured granules

The batch tests for phenol degradation using *A. calcoaceticus* granules cultivated in "Single-culture granules in SBR" were performed in mineral medium with a phenol concentration of 250–2,000 mg l^{-1} (Fig. 4a,b). The granules degraded phenol by following zero-order kinetics without lag at an initial phenol concentration of 250–2,000 mg l^{-1}. The maximum specific phenol degradation rate was 993.6 mg phenol g^{-1} VSS day^{-1} at 250 mg l^{-1} phenol concentration. At >1,500 mg l^{-1} phenol, the free *A. calcoaceticus* cells were fully inhibited (Fig. 4c). However, the *A. calcoaceticus* granules could degrade phenol at 519.3 mg phenol g^{-1} VSS day^{-1} under 2,000 mg l^{-1} phenol (Table 1). Thus, the granule "defends" the strain from phenol toxicity.

Discussion

Bioaugmented phenol degradation

Jiang et al. (2006a) claimed that the functionally dissimilar strains, a phenol reducer and a coagulator, could work together to form phenol-degrading granules. This work demonstrated that the single strain, *A. calcoaceticus*, which is both a phenol reducer and an autoaggregator, could form single-culture aerobic granules with high phenol degrading capability and with long-term stability. Because only one strain exists, the specific phenol degradation rate could be higher than those from multistrains.

Bioaugmentation was observed for the so-occurred granulation process. The CLSM images of the 40-μm-thick cryosection taken from the center of a mature granule (ca. 460 μm below the surface) revealed that live *A. calcoaceticus* cells were distributed 200–250 μm in depth from the surface, with a noncellular EPS layer composed of proteins, lipid, and α- and β-polysaccharides above them (image not shown for brevity). The studied *A. calcoaceticus* strain is a strict aerobe. To stay within a gel layer is not beneficial for easy oxygen uptake. One possible reason for this allocation is the presence of mass transfer shield to phenol toxicity, correlating with the higher phenol tolerance noted by granules compared with free *A. calcoaceticus* cells (Fig. 4).

The strain *Acinetobacter* sp. enhances biological phosphate removal (Kim et al. 1997; Lotter 1985; Buchan 1983) and degradation of toluene (Ishii et al. 2004), phenol (Adav et al. 2007a; Hao et al. 2002), and phenanthrene and pyrene (Gaoa et al. 2006). The present *A. calcoaceticus* strain assimilates a wide variety of carbon sources, as demonstrated by Biolog plate (date not shown). The single-culture granules of *A. calcoaceticus* are expected to perform well for industrial wastewater treatment containing a variety of organic pollutants.

The single-culture granules degrade phenol at a rate of 993.6 mg g^{-1} VSS day^{-1}, close to that noted of granules incubated by Adav et al. (2007b) with mixed cultures and significantly higher than those of activated sludge and of other aerobic granular sludge (Tay et al. 2005; Watanabe et al. 1996). The different process performances noted with the mixed-culture granules reveal the difficulty to precisely control the dominant strains in the granules, partly because of the presence of inhibiting strains. The single-culture granules can be biologically precise for engineered systems.

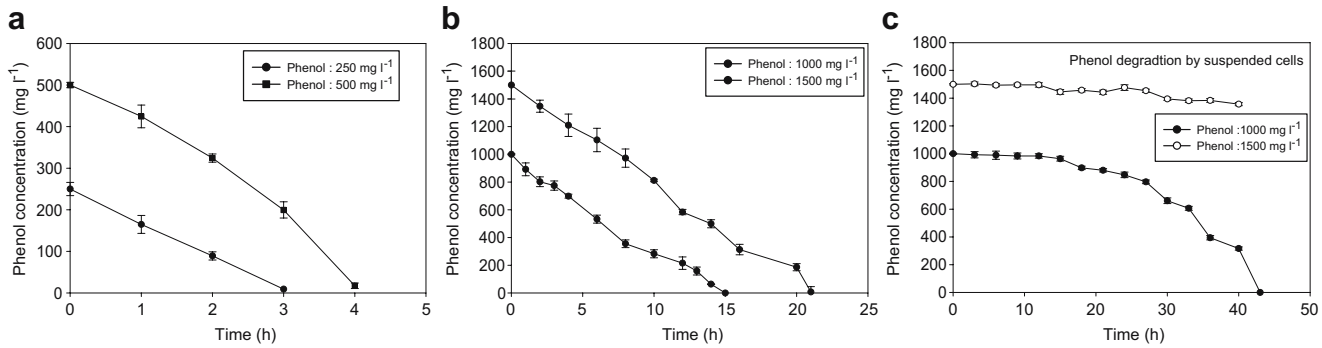

Fig. 4 Batch phenol degradation test with aerobic granules of *A. calcoaceticus* cultivated from SBR and suspended cells. **a** Granules at initial phenol concentrations of 250 and 500 mg l^{-1} phenol. **b** Granules at initial phenol concentrations of 1,000 and 1,500 mg l^{-1} phenol. **c** Free cells at initial phenol concentrations of 1,000 and 1,500 mg l^{-1} phenol

Table 1 Characteristics and phenol biodegradation rates of *A. calcoaceticus* granules

Characteristic	Granule	Phenol biodegradation rate (mg g^{-1} VSS day^{-1})
Granule characteristics		
Mean diameter (mm)	2.3±0.2	
Roundness (%)	84.5±7.89	
Integrity coefficient (%)	86.4±6.74	
Specific gravity (g l^{-1})	1.002±0.018	
Settling velocity (m h^{-1})	25.6±1.84	
Initial phenol concentration		
250 mg l^{-1}		993.6±38.1
500 mg l^{-1}		858.2±49.4
1,000 mg l^{-1}		745.2±57.6
1,500 mg l^{-1}		722.6±28.4
2,000 mg l^{-1}		519.3±45.6

Autoaggregation activity

The autoaggragation activity of the *A. calcoaceticus* cells corresponds closely to the noted capability to form single-culture granules. The autoaggregation indices of the *A. calcoaceticus* strain were measured in the batch tests (Table 2). In the presence of 540 mg l^{-1} phenol, the autoaggregation index reached a maximum value of 81.1%. At 720 mg l^{-1} phenol, conversely, the autoaggregation index reduced. The autoaggregation indices decreased with increasing glucose concentration and were generally lower than those with phenol as sole carbon source ($p<0.001$, ANOVA). The phenol thereby stimulates the autoaggregation of cells. The concentration of NaCl had no significant effects on the autoaggregation index, excluding the electrostatic repulsion force as determinant to aggregate free *A. calcoaceticus* cells. Independent tests revealed that the pretreatment with heat at 80 °C for 5 min and with protease for 30 min completely removed the autoaggregation potentials of the cells (data not shown). Hence, the origin of the *A. calcoaceticus* cells' autoaggregation is likely of chemical rather than physical nature.

The EPS extraction procedures of aggregates in phenol medium yielded proteins of 105.4–126.0 mg g^{-1} VSS, carbohydrate of 14.2–14.6 mg g^{-1} VSS, and humic acid of 20.5–33.6 mg g^{-1} VSS. The protein content in glucose medium was significantly lower ($p<0.001$, ANOVA) than that in phenol medium. The protein content in the aggregates in 5 mM NaCl (no carbon source) was 84.8 mg g VSS^{-1} (Table 2).

Good linear relationship ($R^2>0.99$) was noted between the autoaggregation indices and the protein contents extracted from cell aggregates, incubated in phenol, glucose, or NaCl media (plot not shown). In our experience, cells with autoaggregation index <0.3 in batch culture tests had little chance to form mature granules in SBR operation owing to strong hydrodynamic shear. The redundant proteins secreted by the *A. calcoaceticus* cells with phenol may stick individual cells to form a protein–cells matrix, thereby producing mature granules in the subsequent SBR operation.

Conclusions

This work, for the first time, cultivated aerobic granules in SBR using the single bacterial strain *A. calcoaceticus*. In contrast with the bioaugmentation between two functionally alike or dislike strains proposed in the literature, the *A. calcoaceticus* strain examined herein is both a good phenol reducer and a good autocoagulator, revealing potential to form single cultured granules. Stable granules of size 2.3 mm were formed in 49 days of SBR operation. The granular biomass had excellent settling ability and could degrade phenol at 993.6 mg phenol g^{-1} VSS day^{-1} at 250 mg l^{-1} of phenol concentration and 519.3 mg phenol g^{-1} VSS day^{-1} at 2,000 mg l^{-1} of phenol concentration. Bioaugmentation by granulation was noted for the single cultured strain because the original suspended *A. calcoaceticus* cells would be inhibited at phenol concentration >1,500 mg l^{-1}.

The CLSM tests show that the *A. calcoaceticus* cells were mainly located 200–250 μm beneath the outer surface. The presence of a large quantity of EPS may defend the cells from phenol toxicity. The present single-strain granules were observed to have stable ecosystems with long-term structural stability and supreme phenol degrad-

Table 2 Effect of carbon sources and ionic strengths on autoaggregation activity and extracted EPS of *A. calcoaceticus* cells and granules

Conditions	Autoaggregation index (%)	EPS (mg g^{-1} VSS)		
		Proteins	Carbohydrates	Humic acids
Phenol (mg l^{-1})				
188.2	43.0±3.4	ND	ND	ND
376.4	73.4±9.5	105.4±21	14.6±7.6	20.5±3.8
564.6	81.1±3.4	126.0±16	14.2±6.1	33.6±4.1
752.8	41.7±16.9	ND	ND	ND
Glucose (mg l^{-1})				
9,008	25.8±12.9	6.6±2.2	19.0±4.2	13.0±2.2
18,016	24.9±14.8	6.3±2.0	16.4±5.0	13.2±2.8
36,032	8.7±4.8	ND	ND	ND
54,048	8.9±4.8	ND	ND	ND
NaCl (mg l^{-1})				
292.2	60.0±12.8	84.8±3.9	8.9±2.1	7.6±1.8
584.4	57.7±9.0	ND	ND	ND
876.6	57.8±12.4	ND	ND	ND

ND Not determined

ability. The secreted proteins by cells likely correspond to the noted granulation of the *A. calcoaceticus* cells. The long-term biostability of the single-culture granules needs to be confirmed in future research.

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