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Potential cause of aerobic granular sludge breakdown at high organic loading rates

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Abstract Aerobic sludge granules are compact, strong microbial aggregates that have excellent settling ability and capability to efficiently treat high-strength and toxic wastewaters. Aerobic granules disintegrate under high organic loading rates (OLR). This study cultivated aerobic granules using acetate as the sole carbon and energy source in three identical sequencing batch reactors operated under OLR of 9–21.3 kg chemical oxygen demand (COD) m^{-3} day^{-1}. The cultivated granules removed 94–96% of fed COD at OLR up to 9–19.5 kg COD m^{-3} day^{-1}, and disintegrated at OLR of 21.3 kg COD m^{-3} day^{-1}. Most tested isolates did not grow in the medium at >3,000 mg COD l^{-1}; additionally, these strains lost capability for auto-aggregation and protein or polysaccharide productivity. This critical COD regime correlates strongly with the OLR range in which granules started disintegrating. Reduced protein quantity secreted by isolates was associated with the noted poor granule integrity under high OLR. This work identified a potential cause of biological nature for aerobic granules breakdown.

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Introduction

The aerobic sludge granulation process is superior to the conventional activated sludge processes in terms of its great granule settleability (Morgenroth et al. 1997; Tay et al. 2001; Yang et al. 2003; Liu and Tay 2004; Adav et al. 2007a), high biomass retention (Adav et al. 2008a), and ability to withstand high organic loading rates (OLR) (Moy et al. 2002; Tay et al. 2002) and to resist inhibitory and toxic compounds (Adav et al. 2007a, b). State-of-the-art granulation processes have been commented (Liu and Tay 2004; Maximova and Dahl 2006; de Kreuk et al. 2007; Adav et al. 2008a).

The OLR is essential to determining granule stability (Arrojo et al. 2004; McSwain et al. 2004; de Kreuk et al. 2005). Tay et al. (2004a) was unable to cultivate aerobic granules at <4 kg chemical oxygen demand (COD) m^{-3} day^{-1} using glucose and peptone as substrates. Conversely, granules subjected to high OLR would disintegrate (Moy et al. 2002; Liu and Liu 2006; Zheng et al. 2006; Thanh et al. 2008). Moy et al. (2002) noted that acetate-fed granules did not withstand an OLR >9 kg COD m^{-3} day^{-1}. Zheng et al. (2006), who cultivated sucrose-fed aerobic granules at an OLR of 6 kg COD m^{-3} day^{-1}, observed that the granules converted from bacteria-dominated to filamentous granules prior to disintegration. Chen et al. (2008) demonstrated that aeration air velocity and OLR affect aerobic granulation. Kim et al. (2008), who cultivated aerobic granules at different OLR, identified the optimal COD loading for granulation processes.

Liu and Liu (2006) demonstrated that overgrowth of filamentous microorganisms could result in reactor failure when the amount of dissolved oxygen is inadequate, which commonly occurs in reactors with high OLR. Zheng et al. (2006) proposed that the mass transfer limitation of large granules cultivated under high OLR lead to anaerobe activity inside granules; hence, granules disintegrate. Adav et al. (2009) experimentally confirmed the existence of proteolytic bacteria belonging to genera *Pseudomonas*, *Raoultella*, *Acinetobacter*, *Pandoraea*, *Klebsiella*, *Bacillus*, and uncultured bacterium inside aerobic granules. These bacteria were demonstrated as responsible for granule core deterioration, resulting in granule disintegration. To the authors' best knowledge, no study has identified the biological causes of granule failure under high OLR.

This work identified the biological causes of granule breakdown under high OLR. Aerobic granules were cultivated in three geometrically identical sequencing batch reactors (SBRs). The bacterial strains were isolated and identified. The auto-aggregation indices and the capability of isolates to secrete extracellular polymeric substances (EPS) were determined under high OLR to demonstrate the biological breakdown of granules.

Materials and methods

Experimental setup and seeding

Activated sludge acquired from a local municipal wastewater treatment plant in Taipei, Taiwan, was used as the seed inoculum. This study used three geometrically identical column-type SBRs (R1–3), each 120 cm high and 6 cm in diameter, with 2-l working volume. Fine air bubbles for aeration and mixing were supplied through a dispenser at the reactor bottom at superficial velocity of 3.4 cm s^{-1}. All reactors were operated in 4-h cycles, each cycle consisting of 5-min settling, 10-min effluent withdrawal, 10-min filling, and 215 min of reaction. The volumetric exchange ratio, defined as the fraction of supernatant that was withdrawn, was 50%.

Medium

The synthetic wastewater consisted of acetate as the sole carbon and energy source. The composition of wastewater for initial sludge acclimation is as follows: acetate, 0.4 g l^{-1}; $(NH_4)_2SO_4$, 1.0 g l^{-1}; NaCl, 0.2 g l^{-1}; $MgSO_4 \cdot 7H_2O$, 0.2 g l^{-1}; $FeCl_3$, 0.02 g l^{-1}; $CaCl_2 \cdot 2H_2O$, 0.01 g l^{-1}; buffer (K_2HPO_4, 1.65 g l^{-1}; KH_2PO_4, 1.35 g l^{-1}); and micronutrients, 1.0 ml l^{-1} (Adav et al. 2007b). The feed OLR were altered by proportionally altering concentrations of acetate and all ingredients, except for those of the buffer and $FeCl_3$. Following acclimation, the sludge was fed into R1–3 at an OLR of 9.0 kg COD m^{-3} day^{-1} (COD of 2,990 mg l^{-1}) for 1 week. The OLR for R2 and R3 were step increased to 12.6 and 16.7 kg COD m^{-3} day^{-1} (COD of 4,185 and 5,550 mg l^{-1}), respectively. After 60 days of operation, the OLR for R3 was increased to 19.5 kg COD m^{-3} day^{-1} (COD of 6,470 mg l^{-1}), and operated until it reached COD removal performance of 94–95%; then the OLR was further increased to 21.3 kg COD m^{-3} day^{-1} (COD of 7,070 mg l^{-1}) for a period of only 2 weeks, as reactor operation failed due to excessive foaming and disintegration of granules.

Strain isolation and identification

Granules were cultivated in R1–3 for a 60-day period of feeding at an OLR of 9.0 kg COD m^{-3} day^{-1}. Granules were collected and were aseptically broken in sterilized tubes containing sterile reactor-fed medium. Supernatant was serially diluted (10^1–10^9-fold dilutions), and 1 ml of each 10^5–10^9 dilution was spread onto an agar plate containing reactor-fed medium. The plates were placed in an incubator at 37 °C, and 52 morphologically identified colonies were isolated via several cycles of replating on reactor-fed agar medium.

The DNA from the isolated strain was extracted, and polymerase chain reaction (PCR)-amplified using primers 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1,492r (5′-GGTTACCTTGTTACGACTT-3′). Amplification was conducted in an Eppendorf mastercycler (Eppendorf AG, Hamburg, Germany) via denaturation at 94 °C for 3 min, 25 cycles at 94 °C for 20 s, 54 °C for 30 s, 72 °C for 45 s, and final extension at 72 °C for 7 min. The PCR-amplified 16S rRNA was sequenced using the ABI Prism model 3730 (version 3.2) DNA sequencer. The 16S rDNA sequences of isolates were compared with 16S rDNA sequences obtained via BLAST searches of the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov). Multiple sequence alignments were performed using Clustal W version 1.8, and phylogenetic trees were generated using the neighbor-joining method. The gene sequences of the isolates deposited in GenBank with accession numbers listed in Table 1.

The isolates noted were cultivated individually in medium ("Medium" section) at 400 mg l^{-1} acetate (equivalent to 232 mg COD l^{-1}) for 18 h. One representative isolate from one operational taxonomic unit was tested under COD of up to of 3,966 mg l^{-1}.

The auto-aggregation index of individual tested isolates was determined (Adav et al. 2008b). The OD ($OD_{(initial)}$) of cultures grown for 18 h was measured at 600 nm. The test solutions were left to stand at 25 °C for 30 min. In total, 1 ml supernatant was carefully drawn and placed in a

Table 1 Classification and sequence similarities to closest relatives of the isolates

Isolate	Classification Family, class, phylum	Base pairs used for identity	GenBank accession no.	Close relative	Ref strain GenBank accession no.	Similarity (%)
AOLR01	*Flavobacteriaceae*, *Flavobacteria*, *Bacteroidetes*	1067	GQ916505	*Chryseobacterium* sp. MN13.3d	AM159535	99
AOLR03	*Moraxellaceae*, *Gammaproteobacteria*, *Proteobacteria*	1119	GQ916506	*Acinetobacter* sp. TDWCW6	AM421905	98
AOLR04	*Neisseriaceae*, *Betaproteobacteria*, *Proteobacteria*	1069	GQ916507	*Aquitalea* sp. PGP-1	AB277847	99
AOLR07	*Enterobacteriaceae*, *Gammaproteobacteria*, *Proteobacteria*	1044	GQ916508	*Enterobacter aerogenes*	AF395913	96
AOLR09	*Neisseriaceae*, *Betaproteobacteria*, *Proteobacteria*	1051	GQ916509	*Aquitalea magnusonii* strain NBRAJG85	EU661705	99
AOLR10	*Rhodocyclaceae*, *Betaproteobacteria*, *Proteobacteria*	1021	GQ916510	*Zoogloea resiniphila*	AJ011506	98
AOLR12	*Moraxellaceae*, *Gammaproteobacteria*, *Proteobacteria*	1026	GQ916511	*Acinetobacter* sp. LMG TH120	AJ633638	99
AOLR13	*Pseudomonadaceae*, *Gammaproteobacteria*, *Proteobacteria*	1051	GQ916512	*Pseudomonas otitidis*	EF687744	96
AOLR14	*Comamonadaceae*, *Betaproteobacteria*, *Proteobacteria*		GQ916513	*Comamonas aquatica* strain F-1	FJ493173	96
AOLR15	*Neisseriaceae*, *Betaproteobacteria*, *Proteobacteria*	1035	GQ916514	*Aquitalea magnusonii* strain TRO-001DR8	DQ018117	99
AOLR16	*Rhodocyclaceae*, *Betaproteobacteria*, *Proteobacteria*	1031	GQ916515	*Zoogloea oryzae*	AB201044	95
AOLR19	*Pseudomonadaceae*, *Gammaproteobacteria*, *Proteobacteria*	1035	GQ916516	*Pseudomonas nitroreducens*	AM088473	99
AOLR22	*Rhodocyclaceae*, *Betaproteobacteria*, *Proteobacteria*	1045	GQ916517	*Zoogloea resiniphila*	AJ011506	99
AOLR24	Bacteria, environmental samples, –	1028	GQ916518	Iron-reducing bacterium enrichment culture clone HN104	FJ269070	99
AOLR28	*Neisseriaceae*, *Betaproteobacteria*, *Proteobacteria*	1058	GQ916519	*Aquitalea* sp. PGP-1g	AB277847	98
AOLR29	*Burkholderiales*, *Betaproteobacteria*, *Proteobacteria*	1060	GQ916520	*Ideonella* sp. B513	AB049107	98
AOLR30	*Neisseriaceae*, *Betaproteobacteria*, *Proteobacteria*	1046	GQ916521	*Aquitalea magnusonii* isolate 12	EU430988	99
A0LR31	*Flavobacteriaceae*, *Flavobacteria*, *Bacteroidetes*	1065	GQ916522	*Chryseobacterium gleum*	AM232812	98
AOLR32	*Moraxellaceae*, *Gammaproteobacteria*, *Proteobacteria*	1052	GQ916523	*Acinetobacter baumannii* strain Ab8	AY847284	96
AOLR36	*Moraxellaceae*, *Gammaproteobacteria*, *Proteobacteria*	1050	GQ916524	*Acinetobacter brisoui* strain 5YN5-8	DQ832256	99
AOLR37	*Flexibacteraceae*, *Sphingobacteria*,	1020	GQ916525	*Flexibacteraceae* bacterium P2	FJ177421	99

Table 1 (continued)

Isolate	Classification Family, class, phylum	Base pairs used for identity	GenBank accession no.	Close relative	Ref strain GenBank accession no.	Similarity (%)
	Bacteroidetes					
AOLR39	*Moraxellaceae*, *Gammaproteobacteria*, *Proteobacteria*	1050	GQ916526	*Acinetobacter brisoui* strain 5YN5-8	DQ832256	97
AOLR41	*Moraxellaceae*, *Gammaproteobacteria*, *Proteobacteria*	1064	GQ916527	*Acinetobacter* sp. TDWCW6	AM421905	97
AOLR42	*Moraxellaceae*, *Gammaproteobacteria*, *Proteobacteria*	1079	GQ916528	*Acinetobacter brisoui* strain 5YN5-8	DQ832256	96
AOLR44	*Moraxellaceae*, *Gammaproteobacteria*, *Proteobacteria*	1089	GQ916529	*Acinetobacter* sp. AQ5NOL 1	EU828623	96
AOLR48	*Comamonadaceae*, *Betaproteobacteria*, *Proteobacteria*	1055	GQ916530	*Comamonas terrigena*	EU841530	95
AOLR50	*Moraxellaceae*, *Gammaproteobacteria*, *Proteobacteria*	1040	GQ916531	*Acinetobacter* sp. LMG TH120	AJ633638	98
AOLR51	*Moraxellaceae*, *Gammaproteobacteria*, *Proteobacteria*	1059	GQ916532	*Acinetobacter* sp. TDWCW6	AM421905	98
AOLR52	*Moraxellaceae*, *Gammaproteobacteria*, *Proteobacteria*	1082	GQ916533	*Acinetobacter baumannii* strain LUH	FJ867361	97
AOLR56	*Comamonadaceae*, *Betaproteobacteria*, *Proteobacteria*	1089	GQ916534	*Rubrivivax*-like sp. PC5.4B	X89910	97
AOLR57	*Flavobacteriaceae*, *Flavobacteria*, *Bacteroidetes*	1101	GQ916535	*Chryseobacterium* sp. Atl-24	EF028127	98
AOLR58	*Xanthomonadaceae*, *Gammaproteobacteria*, *Proteobacteria*	1093	GQ916505	*Stenotrophomonas acidaminiphila* strain NK 2. Ha-5	EU352763	98

microcuvette. The optical density of the suspension was measured at 600 nm. The auto-aggregation index for isolates was calculated by comparing the initial OD ($OD_{(initial)}$) and OD value after 30 min ($OD_{(30)}$) as follows: $\text{auto}-\text{aggregation index}\ (\%) = \frac{OD_{(initial)} - OD_{(30)}}{OD_{(initial)}} \times 100$.

Analytical methods

The dry weight of granules and volatile suspended solids and the sludge volume index (SVI) were measured using standard methods (APHA 1998). The size of sludge granules was determined by a particle sizer (Mastersizer Series 2600; Malvern Instruments, Worcestershire, UK). Washed granules were prepared for scanning electron microscopy (SEM) by fixing with 2.5% glutaraldehyde for 2 h, and dehydrated via successive passages through 30%, 50%, 75%, 85%, 90%, 95%, and 100% ethanol before being subjected to critical drying.

Cultures were centrifuged at 14 °C at 6,000×*g* for 10 min. The supernatant containing soluble EPS was separated from the pellet. To extract the bound EPS, the pellet phase was resuspended in 5 ml water and incubated for 1 h in a shaking water bath at 50 °C. Following centrifugation at 6,000×*g* for 10 min, the obtained supernatant contained bound EPS. The carbohydrate content in EPS was measured using the anthrone method (Gaudy 1962) with glucose as the standard. The protein content in EPS was measured using the modified Lowry method (Frolund et al. 1995) with bovine serum albumin as the standard.

Results

Granulation process

At the end of week 2, tiny aggregates with clear, spherical shapes were developed in R1–3 operated at OLR of 9, 12.6, and 16.7 kg COD m^{-3} day^{-1}, respectively. Figure 1 shows the biomass, SVI and R3 performance under high OLR.

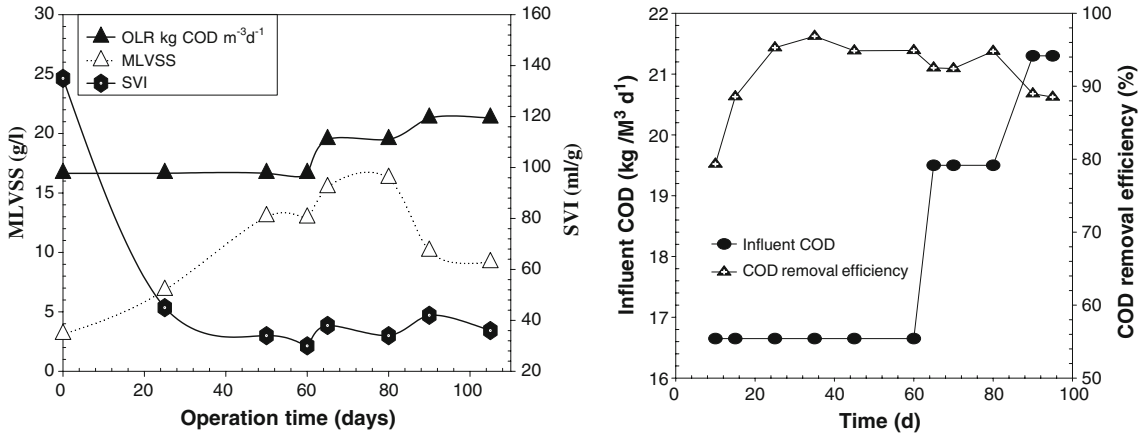

Fig. 1 Time profile of biomass and SVI and reactor performance with organic loading rate

The SVI was 30 ml g^{-1} on day 60, increased to 38 ml g^{-1} due to increase in OLR, and stabilized at 34 ml g^{-1} with reactor stabilization (Fig. 1). The SVI values fluctuated immediately after further increase in OLR and improved with column operation time. The size of granules increased to 3–4 mm following cultivation for 60 days (data not shown). On day 60, the granules in all reactors were compact and removed influent COD at a rate of 94–96%. At an OLR of 19.5 kg COD m^{-3} day^{-1}, filamentous outgrowth existed in R3. Further increasing the OLR to 21.3 kg COD m^{-3} day^{-1} caused the granules to start disintegrating (Fig. 2) and decreased the COD removal rate.

Through SEM, bacteria were identified as the predominant microbial component in the granular sludge (images not shown). The bacteria have varied morphologically as rods, cocci, and filaments, and were embedded in a thick EPS matrix as single cells or dense clusters. Cocci frequently occurred in spherical clusters in large cell groups. Colonization of fungal filaments and ciliate stalks was observed; ciliated outgrowth was also observed. The composition of the bacterial community and distribution of distinct morphotypes differed with OLR. At a high OLR, bacterial cells were embedded in thick EPS, while at low OLR, filamentous bacteria were observed.

Microbial diversity and phylogenetic analysis of isolates

Of the 52 bacterial isolates sequenced (accession numbers in GenBank listed in Table 1), 27 belonged to *Gammaproteobacteria* (*Enterobacteriaceae*, 1; *Xanthomonadaceae*, 1; *Pseudomonadaceae*, 2; *Moraxellaceae*, 22); 20 were *Proteobacteria* (*Rhodocyclaceae*, 4; *Comamonadaceae*, 2; *Neisseriaceae*, 10); and four were *Bacteroidetes* (*Flavobacteriaceae*, 3; *Flexibacteriaceae*, 1). Isolates AOLR42, AOLR39, AOLR32, AOLR44, AOLR52, AOLR51, and AOLR41 had 16S rRNA nucleotide sequences identical to the sequence from *Acinetobacter* sp. and grouped together with an 80–100% bootstrap confidence level (Fig. 3). Isolates AOLR57, AOLR31, and AOLR01 clustered with *Chryseobacterium gleum* with bootstrap values of 68–100%. The 16S rRNA sequences of isolates AOLR48 and

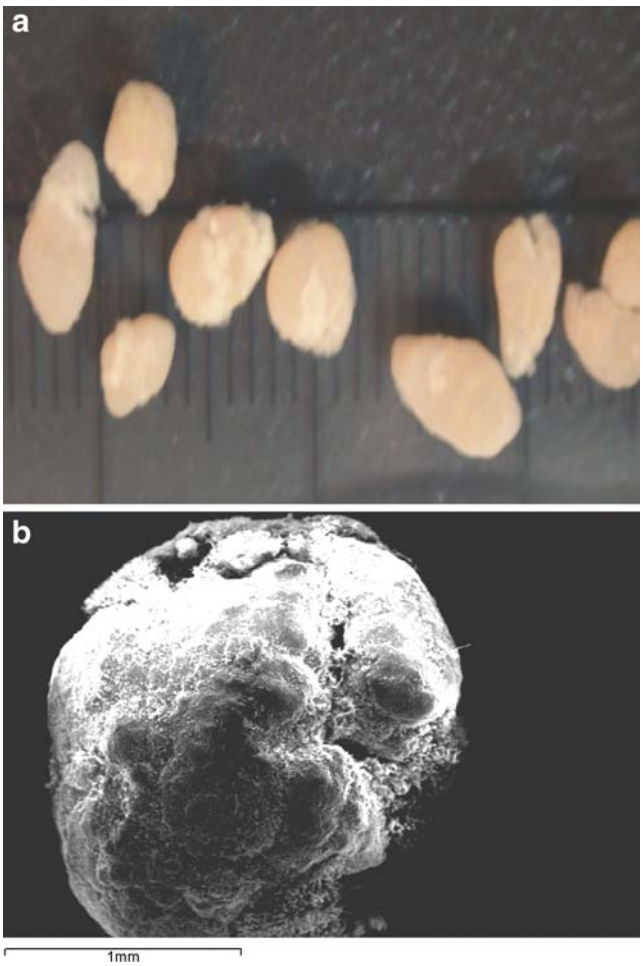

Fig. 2 Photograph and SEM image of the granules at OLR 21.3 kg COD m^{-3} day^{-1} for 10 days in reactor R3. Tiny scales in (**a**) indicate millimeters. *Bar* in (**b**) indicates 1 mm

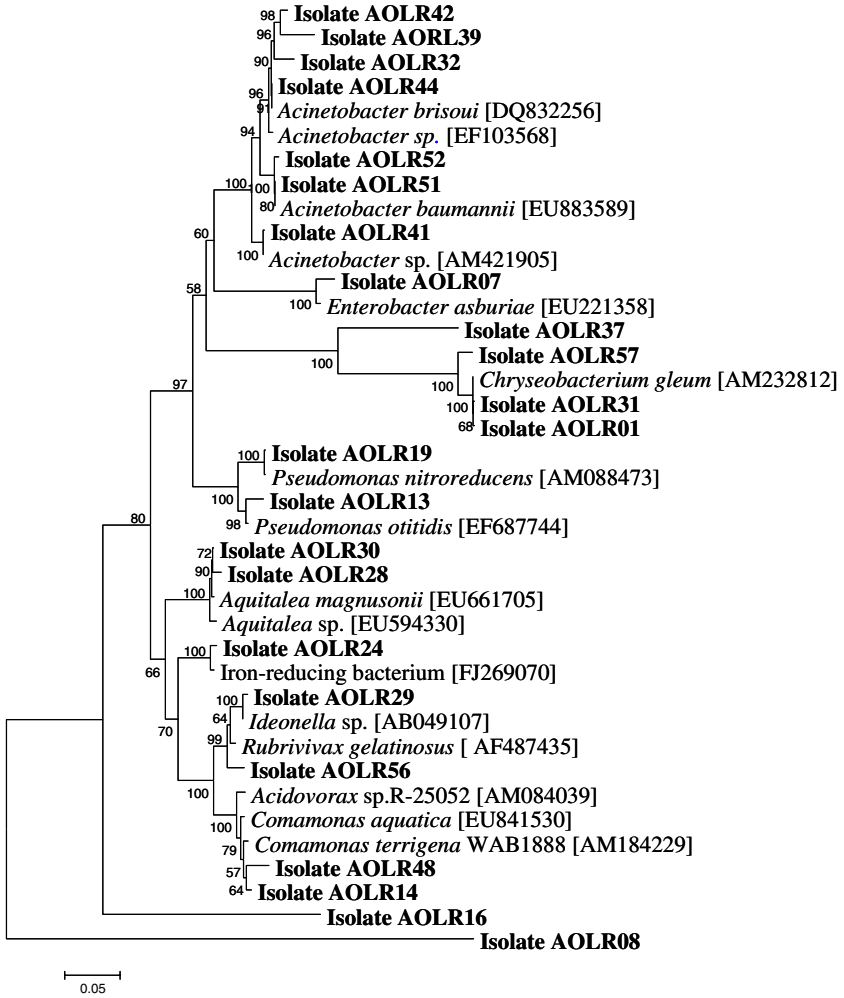

Fig. 3 Phylogenetic tree for the partial bacterial 16S rDNA sequences of the isolates. The phylogenetic tree was constructed based on the neighbor-joining method with bootstrapping. The bootstrap value that was greater than 50% was shown at each node

AOLR14 were similar to that of *Comamonas* sp. Isolates AOLR19 and AOLR13 clustered with members of the *Pseudomonadaceae* family with 100% bootstrap values. Isolate AOLR24 exhibited a 16S rRNA nucleotide sequence nearly identical to that from iron-reducing bacteria. Isolates AOLR30 and AOLR28 clustered with bootstrap values of 72–100% and contain a 16S rRNA nucleotide sequence identical to that of *Aquitalea* sp. The 16S rRNA nucleotide sequences of some isolates were identical to that of *Rubrivivax gelatinosus*.

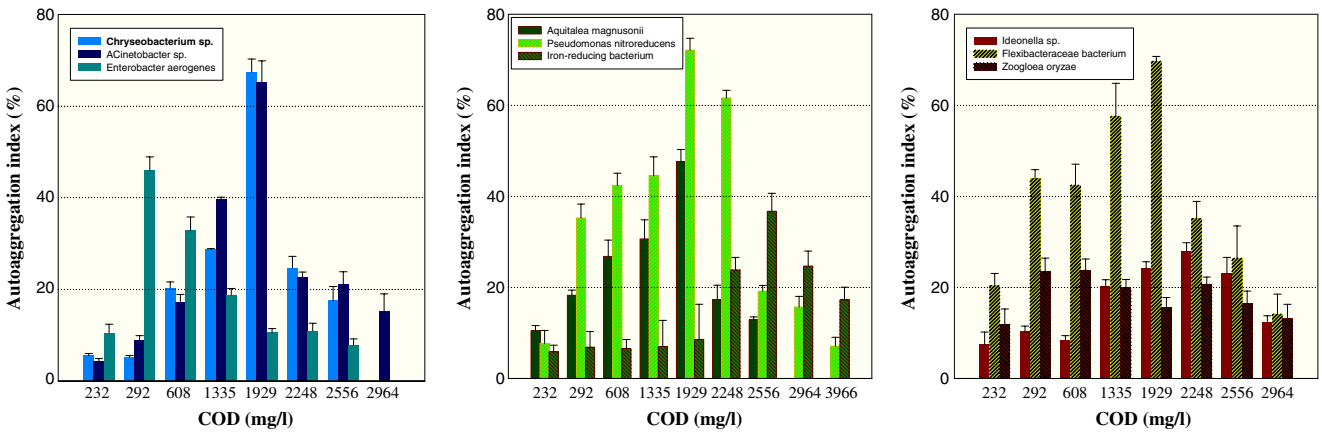

Fig. 4 Auto-aggregation index for individual isolated cultivated in 400 mg l^{-1} acetate for 18 h at 37 °C

Table 2 Quantities (mg l^{-1}) of proteins secreted by individual isolates cultivated in 400 mg l^{-1} acetate medium for 18 h at 37 °C

COD (mgl^{-1})	*Chryseobacterium* sp. MN13.3d	*Acinetobacter* sp.	*Enterobacter aerogenes*	*Aquitalea magnusonii*	*Pseudomonas nitroreducens*	Iron-reducing bacterium	*Ideonella* sp.	*Flexibacteraceae* bacterium	*Zoogloea oryzae*
232	8.66	5.70	2.67	18.80	6.85	8.44	10.39	13.06	18.66
292	13.01	11.47	22.13	21.25	14.62	13.68	24.03	18.26	19.32
608	3.50	14.06	25.75	24.40	19.93	12.91	24.89	14.43	19.78
1,335	10.59	14.03	34.96	27.71	20.98	58.92	27.78	14.82	27.95
1,929	36.89	36.82	49.80	42.10	66.01	52.25	63.03	56.63	104.13
2,248	61.32	53.13	34.62	44.01	33.37	43.74	36.85	33.74	80.24
2,556	41.74	37.29	50.81	64.11	45.38	43.52	45.26	40.68	50.71
2,964	29.63	32.62	12.44	52.49	22.71	47.14	43.89	17.26	14.38

Table 3 Quantities (mg l^{-1}) of polysaccharides secreted by individual isolates cultivated in 400 mg l^{-1} acetate medium for 18 h at 37 °C

COD (mgl^{-1})	*Chryseobacterium* sp. MN13.3d	*Acinetobacter* sp.	*Enterobacter aerogenes*	*Aquitalea magnusonii*	*Pseudomonas nitroreducens*	Iron-reducing bacterium	*Ideonella* sp.	*Flexibacteraceae* bacterium	*Zoogloea oryzae*
232	4.17	2.96	2.64	2.10	9.31	6.05	3.02	1.75	9.91
292	3.10	3.50	5.21	2.78	7.95	9.06	3.42	2.35	11.39
608	3.32	4.75	4.32	2.82	5.43	11.82	3.89	5.06	11.82
1,335	8.85	20.22	4.00	2.60	3.67	6.60	4.21	4.24	14.81
1,929	18.85	12.57	6.39	3.17	0.53	6.56	6.21	12.52	29.89
2,248	5.07	9.26	1.11	11.29	3.67	1.68	3.14	2.75	10.03
2,556	5.21	1.96	0.43	17.12	0.21	1.75	1.03	1.71	9.64
2,964	2.11	1.85	0.21	3.07	0.64	1.57	1.25	1.03	3.10

Auto-aggregation and EPS secretion by isolates

All tested isolates grew in the medium at up to 2,556 mg COD l^{-1}, whereas *Acinetobacter* sp., *Pseudomonas nitroreducens*, iron-reducing bacterium, *Ideonella* sp. *Flexibacteraceae* bacterium, and *Zoogloea oryzae* tolerated 2,964 mg COD l^{-1}. Only strain *P. nitroreducens* and iron-reducing bacterium tolerated a COD of 3,966 mg l^{-1}.

The tested isolates had a low auto-aggregation index at low COD; however, the auto-aggregation index increased as COD increased, achieving maximal aggregation at certain COD level, and then decreased as COD further increased (Fig. 4). The auto-aggregation index for both *Aquitalea magnusonii* and *Flexibacteraceae* bacterium peaked at a COD of 1,929 mg l^{-1}, whereas that for *Enterobacter aerogenes* peaked at a COD of 292 mgl^{-1}. Strains *Chryseobacterium* sp., *Acinetobacter* sp., *P. nitroreducens*, and *Flexibacteraceae* had higher auto-aggregation activity than other isolates. The auto-aggregation indices of these strains were 65–72% at a COD of 1,929 mg l^{-1}. At a COD of 3,966 mg l^{-1}, only strains *P. nitroreducens* and iron-reducing bacterium had limited auto-aggregation capability.

The quantities of proteins and polysaccharides secreted by *Flexibacteraceae* bacterium peaked at a COD of 1,929 g l^{-1}, whereas those by *A. magnusonii* peaked at a COD of 2,556 mg l^{-1} (Tables 2 and 3).

Discussion

Response of isolates under high OLR

The cultivated aerobic granules could keep their structural integrity and high COD degradation rates at an OLR of 19.5 kg m^{-3} day^{-1}, and started disintegrating at an OLR of 21.3 kg m^{-3} day^{-1}, corresponding to feed COD

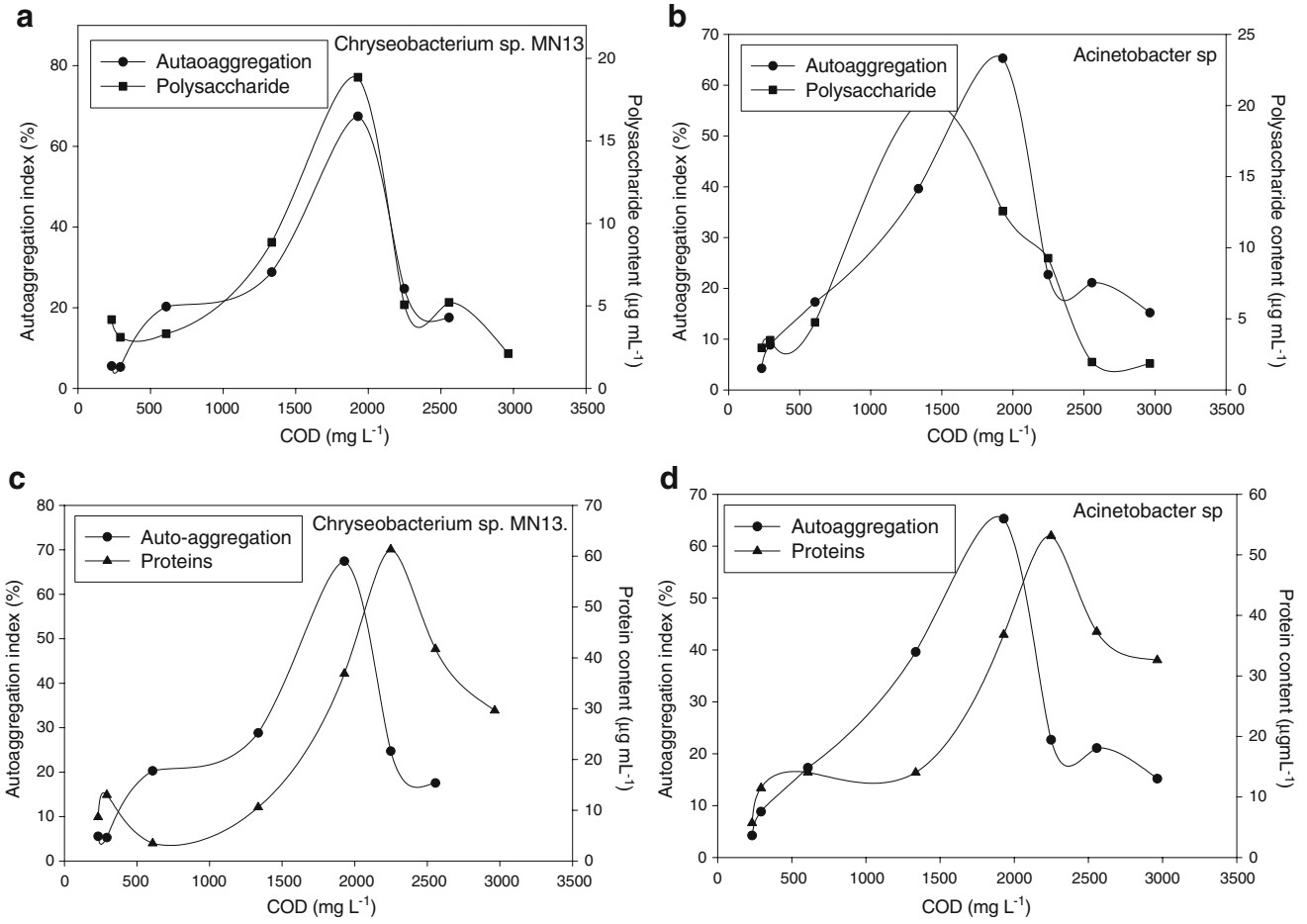

Fig. 5 Auto-aggregation index and protein or polysaccharide production quantities by individual isolates cultivated in 400 mg l^{-1} acetate medium for 18 h at 37 °C. **a** Polysaccharides by *Chryseobacterium* sp. MN13; **b** proteins by *Chryseobacterium* sp. MN13; **c** polysaccharides by *Acinetobacter* sp.; **d** proteins by *Acinetobacter* sp.

of 6,470 and 7,070 mgl^{-1}, respectively. As the volumetric exchange ratio in this study was 50%, the feed concentration was diluted by 50% when it was fed into the reactor. For example, at OLR of 19.5 and 21.3 kg $m^{-3}l^{-1}$, the effective COD concentration experienced by strains in granules would be roughly 3,240 and 3,530 mg l^{-1}, respectively. This COD regime corresponds to the critical COD values noted for granule disintegration (3,000–4,000 mg l^{-1}). In other words, this work demonstrates that failure of constituent cells in aerobic granules may be associated with the noted granule disintegration under high OLR.

The proposed breakdown mechanism under high OLR differs from those proposed in literature, including overgrowth of filamentous microorganisms (Liu and Liu 2006) and intracellular protein hydrolysis and degradation at the anaerobic granule core (Zheng et al. 2006; Adav et al. 2009). This study demonstrates that under a high OLR, most constituent members do not aggregate, which runs counter to the common assumption that cells typically agglomerate into clusters in harsh environments (Bossier and Vertraete 1996). The change in aggregation tendency led to granule disintegration in this study.

Secreted proteins and biological breakdown

Aerobic granules are enriched with proteins compared with conventional activated sludge flocs that are not enriched (Adav et al. 2007a). Extracellular enzymes can hydrolyze proteins and carbohydrates in sludge flocs (Frolund et al. 1995; Nielsen et al. 1996). Adav et al. (2007a) determined that large vacuoles exist in stored granules prior to breakdown. Adav et al. (2009) isolated and identified proteolytic bacteria in aerobic granules responding to protein hydrolysis inside stored granules. Li et al. (2008) combined immunohistochemical staining and a multicolor fluorescent scheme to confirm internal hydrolysis within the granule core; thus, both protein and polysaccharide content should correspond in principle to the loss of granule stability.

A strong correlation (Fig. 5c, d) exists between secreted protein quantity (Table 2) and auto-aggregation indices of individual isolates (Fig. 4). No such correlation was noted between polysaccharide quantity and auto-aggregation indices (data not shown). Additionally, the quantity of polysaccharides was much lower than that of proteins. Hence, the role of polysaccharides on granule stability should be minimal. The quantity of proteins secreted by isolates likely contributes to granule stability under high OLR. As Fig. 5c and d reveals, *Chryseobacterium* sp. and *Acinetobacter* sp. aggregate and then secrete protein to strengthen their aggregation. The decrease in protein productivity by isolates under high OLR indicates that the EPS structure of granules was weak, thereby leading to granule breakdown.

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