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Fate of aerobic bacterial granules with fungal contamination under different organic loading conditions

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Running Head: Special aerobic sludge granules

Abstract

Aerobic sludge granulation is an attractive new technology for biological wastewater treatment. However, the instability of aerobic granules caused by fungal growth is still one of the main problems encountered in granular bioreactors. In this study, laboratory experiments were conducted to investigate the fate and transformation of aerobic granules under different organic loading conditions. Bacterial granules (2-3 mm) in a poor condition with fungi-like black filamentous growth were seeded into two 1-L batch reactors. After more than 100 d of cultivation, the small seed granules in the two reactors had grown into two different types of large granules (> 20 mm) with different and unique morphological features. In reactor R1 with a high organic loading rate of $2.0 \text{ g COD L}^{-1} \text{ d}^{-1}$, the black filaments mostly disappeared from the granules, and the dominance of rod-shaped bacteria was recovered. In contrast, at a low loading of $0.5 \text{ g COD L}^{-1} \text{ d}^{-1}$ in reactor R2, the filaments eventually became dominant in the black fungal granules. The bacteria in R1 granules had a unique web-like structure with large pores of a few hundred μm in size, which would allow for effective substrate and oxygen transport into the interior of the granules. DNA-based molecular analysis indicated the evolution of the bacterial population in R1 and that of the eukaryal community in R2. The experimental results suggest that a high loading rate can be an effective means of helping to control fungal bloom, recover bacterial domination and restore the stability of aerobic granules that suffer from fungal contamination.

Keywords: Aerobic granulation; bacterial granules; biological wastewater treatment; FISH-CLSM; fungal granules; PCR-DGGE.

24 **1. Introduction**

25 The granulation of aerobic sludge in a sequencing batch reactor (SBR) is an attractive
26 new technology for biological wastewater treatment. It is characterised by an excellent sludge
27 settling rate, great biomass enrichment, a low sludge yield, nitrogen removal and fast start-up
28 compared to anaerobic granulation (Morgenroth et al., 1997; Arrojo et al., 2004; Qin and Liu,
29 2006). However, one of the main problems encountered in the operation of a granular sludge
30 SBR is the instability of the aerobic granules. Filamentous growth, or fungal bloom, has often
31 been observed on such granules (Tay et al., 2001; Schwarzenbeck et al., 2005). Once
32 filamentous growth dominates the reactor, the granules begin to deteriorate in quality and
33 settleability, which leads to subsequent biomass washout and the eventual failure of the
34 granular system. Hence, the instability and deterioration of aerobic granules is a major
35 concern in the application of aerobic granulation technology in wastewater treatment.

36 Filamentous bacterial and fungal species have been implicated in the structural
37 development of granules (Beun et al., 1999; Weber et al., 2007; Yang et al., 2008). Liu and
38 Tay (2004) reported that the structure and species diversity of granules may be related to the
39 type of carbon source. In their study, glucose-fed aerobic granules exhibited a loose
40 morphology and were dominated by filamentous bacteria, whereas acetate-fed aerobic
41 granules had a compact structure and were dominated by rod-shaped bacteria with little
42 filamentous growth. However, other studies have shown that a filamentous structure is not
43 necessary for the formation of glucose-fed aerobic granules and that these granules still have
44 a compact structure dominated by rod-shaped bacteria (Li et al., 2008a; Yang et al., 2008).
45 However, a low-pH glucose-based growth medium may lead to the formation of filamentous
46 fungal granules (Yang et al., 2008). Thus, the factors that encourage filamentous growth
47 during aerobic granulation remain unclear, and the types and properties of filamentous
48 microorganisms that affect the structural features and microbial communities of aerobic

49 granules need to be investigated. Effective measures for controlling the filamentous species
50 in granules and recovering granules that suffer from filamentous growth remain to be
51 developed.

52 Filamentous microorganisms, including fungi, are commonly slow-growing species
53 compared to non-filamentous bacteria. It has been reported that the settling problems of
54 activated sludge that result from excessive filamentous growth always appear in wastewater
55 treatment plants that have a low organic loading rate (Knoop and Kunst, 1998). Filamentous
56 growth has also been observed in a granular sludge SBR under conditions of a high biomass
57 concentration or a low substrate loading rate (Liu et al., 2005). Filamentous species have an
58 advantage over non-filamentous species in granules, as they are able to take up more
59 nutrients from media with a low level of nutrients (Liu and Liu, 2006). As noted by
60 Eckenfelder (2000), the growth of filamentous species is favoured in substrates at a low
61 concentration. Hence, the organic loading rate can be an important factor in the control of
62 filamentous bloom and in the recovery of granules having the problem of filamentous growth.

63 In the experimental study reported herein, aerobic sludge granules cultivated in a SBR
64 were placed as seed granules into two batch reactors with two different organic loading rates.
65 These seed bacterial granules had already begun to deteriorate in quality with fungi-like black
66 filamentous growth on their surface. The changes in the morphology, structure and microbial
67 community of the granules under different growth conditions were characterised via scanning
68 electron microscopy (SEM), confocal laser scanning microscopy (CLSM), fluorescence in-
69 situ hybridisation (FISH) and CLSM observation, microbial DNA extractions followed by
70 polymerase chain reactions (PCR), denaturing gradient gel electrophoresis (DGGE) and clone
71 library analysis. The aim of the study was to investigate the fate of aerobic granules with
72 filamentous contamination under different substrate loading conditions and to evaluate the
73 operating measures for the recovery and improvement of the stability of such granules.

74

75 **2. Materials and methods**

76 *2.1. Experimental set-up and operation*

77 Two 1 L glass beakers (H 11 cm × D 11 cm) were used as batch reactors for the granule
78 growth experiment. Small granules of 2-3 mm in diameter collected from a laboratory SBR
79 were placed as seed granules into the two batch reactors, R1 and R2. These seed granules,
80 which are used to treat glucose-based synthetic wastewater, were typical yellow-coloured
81 bacterial granules. However, the granules had begun to deteriorate in quality with apparent
82 black fungal growth on their surface (Figs. 1a and 1b). The initial suspended solids (SS)
83 concentration of the seed granules in the two batch reactors was 1 g L⁻¹. The reactors were
84 fed once a day, after effluent withdrawal, with a substrate solution that consisted of glucose
85 and other nutrients (Tay et al., 2002; Li et al., 2008a). The operating conditions for the two
86 reactors were the same except for the feeding substrate concentration. Two different organic
87 concentrations in terms of chemical oxygen demand (COD) - 2000 and 500 mg L⁻¹ - were
88 used for R1 and R2, resulting in COD loading rates of 2.0 g L⁻¹ d⁻¹ (R1) and 0.5 g L⁻¹ d⁻¹ (R2),
89 respectively. NaHCO₃ was dosed into the feed solution to maintain the pH of the reactors in
90 the neutral range between 7.0 and 7.5. Aeration was supplied through an air diffuser at the
91 bottom of each reactor, and the dissolved oxygen (DO) concentration in the sludge
92 suspension was about 5 mg L⁻¹. The reactors were operated at room temperature, and the
93 water temperature was 20-22 °C.

94

95 *2.2. Analytical methods*

96 The COD and SS concentrations were measured according to Standard Methods (APHA,
97 1998). The DO concentration was determined with a DO probe (5010 BOD Probe, YSI) and

98 a DO meter (5000 DO meter, YSI), and the pH was measured with a pH meter (420A, Orion).
99 The morphology of the aerobic granules was observed under a stereomicroscope (S8 APO,
100 Leica, Wetzlar, Germany) equipped with a digital camera (EC3, Leica). A digital camera
101 (Kodak V530, Kodak, Rochester, NY, USA) was also used to take photographs of the large
102 granules for characterisation. The projected images of the granules were analysed for their
103 sizes and surface roughness using a computer-based image analysis system (AnalySIS 3.1,
104 Olympus Soft Imaging Solutions, Germany). The roughness of a granule was determined
105 from the ratio between the actual boundary of the granule image and the perimeter of a circle
106 that covers the same area of the granule. In addition, the microstructure of the mature
107 granules was examined under SEM (Cambridge S440, Oxford Instruments, Cambridge, UK)
108 following the sample pre-treatment detailed by [Diao et al. \(2004\)](#) and [Chu and Li \(2005\)](#). The
109 total organic carbon (TOC) concentration was measured using a TOC analyser (IL550,
110 HACH-Lachat, Milwaukee, WI, USA).

111

112 *2.3. FISH and CLSM examinations*

113 The 3-D structure of the mature granules, particularly the distributions of the microbial
114 cells and extracellular polymeric substances (EPS) within the granules, was examined via
115 CLSM (LSM 5 Pascal, Zeiss, Jena, Germany) following the procedures described in previous
116 studies ([Zhang and Fang, 2004](#); [Yang et al., 2008](#)). In brief, for the fluorescent staining of the
117 cells and EPS, two probes were applied together: SYTO9 (25 μM , Molecular Probe) to target
118 all of the microbes and ConA-TRITC lectin (250 mg L^{-1} , Sigma) to target the polysaccharides.
119 When excited by a laser at proper wavelengths, SYTO9 and ConA-TRITC probes emit green
120 and red light, respectively. For sample preparation, a granule was embedded in Tissue-Tek
121 OCT Compound (Miles, Elkhart, IN, USA) and frozen overnight at $-20\text{ }^{\circ}\text{C}$. This frozen
122 granule was sectioned into thicknesses of 50 μm using a rotary cryo-microtome (CM 1510-

123 Cryostat, Leica, Germany), and the section specimens were then stained and examined under
124 CLSM.

125 In addition, the bacterial and fungal cells in the mature granules were distinguished via
126 FISH with specific probes for an estimation of their relative abundance. A sample with a few
127 granules was homogenised with a beadbeater (Mini-beadbeater, Biospec, Bartlesville, OK,
128 USA) without beads, and the microbial cells were suspended with a vortex mixer. The cell
129 suspension was fixed using paraformaldehyde (4%) and then placed onto a microscopic slide.
130 FISH staining was conducted at 20% formamide using fluorescein isothiocyanate (FITC) -
131 labelled probe Eub338 (5'-GCTGCCTCCCGTAGGAGT-3') for the bacteria (green) and
132 Cy3-labelled probe Euk516 (5'-ACCAGACTTGCCCTCC-3') for the fungi (red). The FISH-
133 CLSM images of the cells after staining were processed with an image analysis system
134 (LSM5 Pascal, V2.8 SP1, Zeiss, Jena, Germany). The relative abundance of the bacteria and
135 fungi in the granules was estimated based on the total projected areas of the bacterial and
136 fungal cells, respectively.

137

138 *2.4. DNA extraction, PCR-DGGE and microbial species identification*

139 The genomic DNA of the biomass was extracted from the granules following the protocol
140 described by [Zhuang et al. \(2005\)](#) using a beadbeater (Mini-beadbeater, Biospec, Bartlesville,
141 OK, USA) and a micro-centrifuge (MiniSpin plus, Eppendorf, Hamburg, Germany). The
142 extracted DNA was then used as the template for PCR amplification. For the bacterial species,
143 the variable V3 region of the 16S rDNA was amplified using primers 341f-GC and 518r
144 ([Muyzer et al., 1993](#)) with a DNA Engine Peltier Thermal Cycler (PTC-200, MJ Research,
145 Waltham, MA, USA) following a touchdown thermal profile ([Watanabe et al., 1998](#)). For the
146 eukaryal species, primers Euk1A and Euk516r-GC were used in the PCR programme.
147 Amplification began with initial denaturation at 94 °C for 130 s, followed by 35 cycles of

148 denaturation at 94 °C for 30 s, annealing at 56 °C for 45 s and extension at 72 °C for 130 s. It
149 ended with a final elongation step at 72 °C for 7 min (Diez et al., 2001).

150 The PCR-amplified DNA products were separated via DGGE, and the DGGE images
151 were acquired using the ChemiDoc (Bio-Rad, Hercules, CA, USA) gel documentation system.
152 For bacterial DNA, the samples were run on 8% polyacrylamide gels in a linear gradient with
153 a 30-50% denaturing condition at 130 v for 6 h at 60 °C. For eukaryal DNA, the samples
154 were run in a gradient with a 20-40% denaturing condition at 100 v for 16 h at 60 °C. The
155 DGGE gels were scanned and the scanned images were analysed for the band patterns using
156 the Quantity One 1-D analysis software (Bio-Rad). The relative abundance of the possible
157 species in a sludge sample was determined from its DGGE gel image based on the peak value
158 of the band brightness.

159 To identify the phylogeny of the bacterial DGGE bands, a 16S rRNA gene clone library
160 was constructed. PCR was performed using universal bacterial primers 27f and 1495r,
161 according to the programme used by Liu et al. (2006). The PCR products, which were
162 approximately 1450 bps long, were purified using a DNA gel extraction kit (MEGA-spin,
163 iNtRON Biotechnology, Korea). The purified PCR products were then cloned into
164 *Escherichia coli* TOP10 using the pCRII-TOPO vector system (Invitrogen, Carlsbad, CA,
165 USA). A total of 98 recombinant clones were selected randomly for plasmid recovery and
166 analysis, and the extraction and sequencing of the plasmids were carried out by a commercial
167 laboratory (Tech Dragon, <http://www.techdragon.com.hk/index.htm>). All of the sequences
168 obtained were compared with the 16S rRNA gene sequences in the GenBank using a BLAST
169 search (National Center for Biotechnology Information, U.S. National Library of Medicine)
170 for identification of the closest bacterial species.

171 Each band on the DGGE profile was defined as an operational taxonomic unit (OTU).
172 Representative plasmids in the clone library were used to perform DGGE together with the

173 PCR products of the DNA from the granules. Based on the migration position, the sequence
174 of a plasmid and its closest species known in the clone library was assigned to an OTU
175 (particular band) in the DGGE profile of the granule sample. A small number of DGGE
176 bands, which had no matching plasmids in the clone library, was sliced out, purified, re-
177 amplified and sequenced. These sequences were then analysed with an ABI PRISM 3700
178 DNA Analyser (Applied Biosystems, Foster City, CA, USA) for species identification ([Li et](#)
179 [al., 2008a](#)).

180 To identify the eukaryal species in the granules, the DGGE bands were cut off from the
181 gels, purified and re-amplified using the same PCR procedures as those used for eukaryal
182 DNA. The PCR products were sequenced with BigDye Terminator Reactions (ABI PRISM
183 BigDye Terminators V3.1 Kit, Applied Biosystems, Foster City, CA, USA) and the ABI
184 PRISM 3700 DNA Analyser. The sequences were analysed by comparing them with the 18S
185 rRNA gene sequences in the GenBank by a BLAST search to identify the fungi and other
186 eukaryal species.

187

188 *2.5. Accession Numbers*

189 The DNA sequences obtained in this study were deposited in GenBank and were assigned
190 accession numbers FJ588654-FJ588680.

191

192 **3. Results**

193 *3.1. Formation of different types of aerobic granules in the two reactors*

194 The seed aerobic granules collected from a granular sludge SBR already showed signs of
195 deterioration. Most of these yellow bacterial granules were partially covered with a fungi-like
196 black filamentous growth ([Figs. 1a and 1b](#)). Small granules were cultivated under a high

197 organic loading ($2.0 \text{ g COD L}^{-1} \text{ d}^{-1}$) condition in reactor R1 and under a low organic loading
198 ($0.5 \text{ g COD L}^{-1} \text{ d}^{-1}$) condition in reactor R2. The resulting initial food-to-microorganism (F/M)
199 ratios were $2.0 \text{ g COD g}^{-1} \text{ SS d}^{-1}$ in R1 and $0.5 \text{ g COD g}^{-1} \text{ SS d}^{-1}$ in R2. After 120 d, the
200 granules in the two reactors had both become much larger, but had grown into two different
201 types of microbial entities with entirely different and unique morphological features (Figs. 1c
202 and 1d). More importantly, the large granules in R1 were recovered as bacterial granules with
203 a smooth and yellow surface, whilst those in R2 appeared to be puffy balls of black fungal
204 filaments. The sludge SS concentration increased gradually from 1 to 2.5 g L^{-1} for both
205 reactors during the growth process. Accordingly, the F/M ratio decreased to around 0.8 g
206 $\text{COD g}^{-1} \text{ SS d}^{-1}$ in R1 and about $0.2 \text{ g COD g}^{-1} \text{ SS d}^{-1}$ in R2. Both reactors performed well in
207 wastewater treatment with an effluent COD of less than 100 mg L^{-1} from R1 and less than 60
208 mg L^{-1} from R2, corresponding to organic removal efficiencies of 95% (R1) and 88% (R2).

209 The large granules in R1 ranged from 12 to 21 mm in diameter, and those in R2 from 20
210 to 35 mm. The R1 granules had a smooth surface with a roughness value of 1.03 ± 0.05 , which
211 was much lower than the roughness value of 1.84 ± 0.08 amongst the R2 granules. Despite
212 their large size, neither type of granule was of a shell structure with an empty interior. The R2
213 granules were rather uniform in structural appearance from the inside out (Fig. 1d). In
214 comparison, the R1 granules had an apparently layered structure from the centre to the
215 surface. No dark or black zones were found at the centre of the yellow R1 granules, thus
216 signifying a non-anaerobic condition inside the large granules (Fig. 1c). This is rather
217 remarkable, as an anaerobic condition is expected for the centre of a microbial granule as
218 large as 20 mm due to the common DO transfer limitation. For aerobic granules, it has been
219 reported that DO could penetrate only partially through $500 \mu\text{m}$ from the granule surface into
220 the granules under a substrate-sufficient condition (Li et al., 2008b).

221 SEM microscopic examination revealed different microbial communities for the two
222 types of large granules. The yellow granules in R1 were dominated by rod-shaped bacteria
223 (Figs. 2a and 2b), and most of the bacterial cells were clustered together, with only a few
224 filamentous microbial species found inside the granules. In contrast, the large black granules
225 in R2 were primarily formed by fungi-like long filamentous cells (Figs. 2c and 2d).

226

227 *3.2. Physical and physiological structures of the aerobic granules*

228 The CLSM examination of the spatial distributions of the cells and EPS in the granules
229 indicated different structural configurations for the two types of granules cultivated in R1 and
230 R2. Based on the CLSM cryosections after fluorescent staining, the R1 granules were not
231 empty inside, but had abundant bacterial cells towards their centres. In fact, there was a rather
232 uniform distribution of bacterial cells and EPS throughout these granules (Figs. 3a-c). In
233 contrast, filamentous cells were found to grow throughout the R2 granules, and EPS were
234 present (Figs. 3d-f).

235 Closer CLSM examination revealed a web-like structure within the bacterial granules
236 from R1 (Figs. 4a and 4b). Apparently, the bacteria were clustered together by EPS to build
237 the members of the granules' web network. It has been suggested that gel-forming
238 polysaccharides play an important role in the construction of a stable structure for aerobic
239 granules (Yang et al., 2005; Wang et al., 2006). Large pores formed between the members of
240 the granules' web structure, and many of these large pores were a few hundred μm in size,
241 which would allow effective material transfers, including substrates and DO, into the interior
242 of the granules. As a result, no anaerobic condition was observed at the centre of the large
243 granules in which abundant bacterial growth was evidenced. In comparison, the fungal
244 granules from R2 had a much looser and more porous configuration. The filamentous cells

245 and the bundles of the filaments tangled with one another to form a fluffy and loose granule
246 structure (Figs. 4c and 4d).

247

248 *3.3. Microbial communities for the two types of granules*

249 For additional analysis, the granules were homogenised into cell mixtures in suspensions.
250 The FISH-CLSM images of the cell mixtures indicated clearly different microbial
251 communities for the two types of granules. Those from R1 were mainly composed of rod-
252 shaped bacteria (Fig. 5a), although a few eukaryotes were also found (Fig. 5b). The granules
253 from R2, in contrast, were dominated by filamentous eukaryal species (Fig. 5c), and some of
254 the hyphae were attached to a few bacterial cells (Fig. 5d). According to area-based image
255 analysis of the FISH images, bacteria accounted for approximately 84% of the microbial
256 population in the R1 granules, whilst eukaryotes accounted for the remaining 17%. In
257 comparison, the black R2 granules were approximately 84% eukaryotes and only 16%
258 bacteria. This analysis confirmed that the high organic loading rate enhanced the growth of
259 bacteria to form large bacterial granules in R1. Meanwhile, the low COD loading condition in
260 R2 was unfavourable to such growth, but more favourable to fungi and other eukaryal species,
261 thus leading to the formation of large filamentous granules.

262 The evolution in the microbial community during the formation of the special granules
263 was indicated by the DGGE profiles. The DGGE band pattern of the bacteria in the large
264 granules from R1 was largely different from that of the seed granules (Fig. 6), and at the high
265 COD loading rate, this change in the DGGE profile was rather dynamic. Only four of the
266 dominant bacterial bands (Bands 16, 18, 20 and 22) for the seed granules remained for the
267 mature R1 granules after 120 d of cultivation.

268 To identify the bacterial DGGE bands of the R1 granules, they were compared with 24
269 OTUs selected from 98 clones in the library. The bands that did not match any of the OTUs

270 in the library were excised and sequenced for identification. Of the 31 bands that appeared in
271 the DGGE profiles (Fig. 6), 22 dominant bands were identified (Table SM1 in Supplementary
272 Material), accounting for > 85% of the total bacterial community of the R1 granules. The
273 majority of the bacteria grouped with members of *Proteobacteria*, with three in the
274 *Alphaproteobacteria*, nine in the *Betaproteobacteria*, and one in the *Gammaproteobacteria*.
275 The next three groups clustered with *Flavobacteria*, followed by two with *Firmicutes*, two
276 with *Sphingobacteria*, one with *Actinobacteria* and one with *Planctomycetes*.

277 Bacteria from the classes *Alphaproteobacteria* and *Betaproteobacteria* have commonly
278 been found in conventional activated sludge (Bond et al., 1995; Snaidr et al., 1997; Vigeant et
279 al., 2002). Bacteria from *Flavobacteria* have also been reported to be dominant in aerobic
280 granules (Li et al., 2008a). After 120 d, Band 15, which corresponds to a close relative of
281 *Acidovorax*, became dominant in R1, accounting for 25% of the total bacterial community
282 judging from the DGGE band intensity. Bands 20 and 21, which were present as major bands
283 during the entire process, were identified as *Riemerella anatipestifer* and *Pedobacter* sp.,
284 respectively. *Lactococcus* (Band 2), *Streptococcus* (Band 5) and *Flavobacterium* sp. (Band 9),
285 which were less significant in the seed granules, became dominant (accounting for 4, 2 and
286 5%, respectively) in the mature R1 granules after 120 d.

287 The eukaryal DGGE band pattern for the black granules formed in R2 also indicated
288 evolution in the eukaryal community during the growth process (Fig. 7). At a low COD
289 loading rate of 0.5 g L⁻¹ d⁻¹, however, the change of the eukaryal population structure was
290 less dramatic. Most of the major bands could be found in both the seed granules and the large
291 granules after 120 d of cultivation, although the positions of some of them had shifted during
292 the process (Fig. 7).

293 Band 1 was identified as a *Cercozoa* species (Table SM2), a motile protist with a filose
294 pseudopodal morphology. The protozoan phylum *Cercozoa* has been found to be a major

295 component of marine, freshwater and, especially, soil ecosystems. They are grazers that feed
296 on bacterial cells and detritus (Cavalier-Smith and Chao, 2003). Bands 2, 6 and 17 were
297 identified as *Geotrichum fragrans*, *Dipodascus ingens* and *Cochlonema euryblastum*,
298 respectively, all of which belong to *Fungi* and occurred in the seed granules. The microscopic
299 examinations indicated that fungal species were clearly the most important eukaryotes in the
300 large black granules in R2. *C. euryblastum*, however, disappeared from R2 after 120 d, and *D.*
301 *ingens* was always dominant. Band 11, *Epistylis urceolata*, also became more abundant in R2.
302 The genus *Epistylis* is a non-motile stalked ciliate similar to *Vorticella* that is common in
303 activated sludge, and *Cercozoa*, *G. fragrans* and *E. urceolata* have been found dominating in
304 aerobic filamentous granules (Williams and de los Reyes, 2006).

305

306 **4. Discussion**

307 It is generally believed that fungi are able to grow with an extremely low level of
308 nutrients and that it is wasteful to supply rich substrates (Deacon, 2006). Hence, fungal cells
309 may have an advantage over bacteria in substrate uptake under a low loading condition (such
310 as that in R2). A higher organic loading rate (such as that in R1), in contrast, allows bacterial
311 cells to outgrow fungal filaments in granules. When exposed to a high organic loading
312 condition at $2.0 \text{ g L}^{-1} \text{ d}^{-1}$, the granules in R1 had an initial F/M ratio of $2.0 \text{ g COD g}^{-1} \text{ SS d}^{-1}$.
313 This F/M ratio decreased gradually to around $0.8 \text{ g COD g}^{-1} \text{ SS d}^{-1}$ with granular biomass
314 growth. In R2, which was subject to a lower organic loading condition of $0.5 \text{ g L}^{-1} \text{ d}^{-1}$, the
315 granules had an initial F/M ratio of $0.5 \text{ g COD g}^{-1} \text{ SS d}^{-1}$, dropping gradually to about 0.2 g
316 $\text{COD g}^{-1} \text{ SS d}^{-1}$ with biomass growth. Thus, the granules in R1 always had a higher F/M ratio
317 that was around four times that of those in R2. As a result, large non-filamentous bacterial
318 granules stabilised in R1, in which the black filaments gradually disappeared (Fig. 1c). In R2,

319 there was a low F/M ratio, and the fungal filaments outgrew the bacteria to form large
320 filamentous granules (Fig. 1d).

321 The DGGE profiles for bacteria in R2 and eukaryal species in R1 during the experimental
322 process were also analysed (Fig. SM-1 and Fig. SM-2 in Supplementary Material). The two
323 reactors, R1 and R2, did not differ greatly in terms of the microbial diversity. However, the
324 main difference between the two reactors was the dominance of the microbial communities
325 by different types of species, although many other species were also present in the granules.
326 The granules in R1 were dominated by bacterial cells, whilst those in R2 were dominated by
327 fungal filaments (Fig 1, Fig. 5). This comparison between two batch reactors with the same
328 seed granules suggests that a higher organic loading condition helps non-filamentous bacteria
329 to out-compete filaments, whilst filamentous fungi become dominant under a low substrate
330 loading condition. It is apparent that bacterial granules that have deteriorated with fungal
331 growth can be recovered by increasing the organic loading rate. Some of these fungal
332 filaments may have had a few rod-shaped bacteria attached. Liu and Tay (2004) reported that
333 glucose-fed aerobic granules exhibited a loose morphology and were dominated by
334 filamentous bacteria. However, our previous experiments showed that, with proper pH
335 control to a level close to 8.0, a filamentous structure is unnecessary for the formation of
336 glucose-fed granules dominated by rod-shaped bacteria (Li et al., 2008a; Yang et al., 2008).
337 The present study suggests that, in addition to pH, the organic loading rate can also affect the
338 growth of dominant microbial species in aerobic sludge granules.

339 Despite the large size of the bacterial granules from R1, no anaerobic condition was
340 evidenced towards the granule centres, as no dark or black zones were found at the centre.
341 Under anaerobic conditions, sulphate-reducing bacteria would reduce sulphate to hydrogen
342 sulphide, which precipitates trace metals as metal sulphides with black colour (Peiffer, 1994;
343 Kaksonen et al., 2003). The large granules however had a special web-like structure in which

344 the bacteria were glued together by EPS to build the members of the web network. Large
345 pores of a few hundred μm in size were formed between the members to allow effective
346 substrate and DO transport into the interior of the granules for aerobic bacterial growth. Such
347 a unique web structure has not previously been reported for microbial granules in biological
348 wastewater treatment. For the black granules from R2, the filamentous fungi and other
349 eukaryal species formed a much looser structure, and material transport limitation was less
350 expected.

351 DNA-based molecular analysis indicated a rather dynamic evolution in the bacterial
352 population within the R1 granules under a high organic loading condition. At the same time,
353 the fungal bloom that occurred in the seed granules was effectively suppressed. At a low
354 loading rate, the change of the eukaryal community structure in the R2 granules was less
355 dynamic. Nonetheless, the fungal species outgrew the bacterial species in R2, thus
356 transforming the yellow bacterial granules into black fungal granules. These experimental
357 results suggest that the organic loading rate may be an important factor in the fate of aerobic
358 bacterial granules that have deteriorated with fungal growth. A high organic loading
359 condition helps to minimise the growth of filamentous species, restore bacterial domination
360 and hence re-stabilise aerobic granules that suffer from deterioration caused by fungal bloom.

361

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367

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456

457 **Figure captions**

- 458
- 459 **Fig. 1.** Photographs of the aerobic granular sludge in the two batch reactors: (a) and (b) seed
460 granules from a SBR and (c) large bacterial granules from R1 and (d) large fungal
461 granules from R2, respectively, after 120 d of cultivation.
- 462 **Fig. 2.** SEM images of the microstructure of (a) and (b) mature granules from R1 and (c) and
463 (d) mature granules from R2.
- 464 **Fig. 3.** CLSM images of the cryosections of the granules cultivated in (a-c) R1 and (d-f) R2.
465 Depth: (a) 0.2 mm, (b) 3 mm, (c) 7 mm, (d) 3 mm; (e) 7 mm and (f) 12 mm. The cells
466 were stained with SYTO9 (green) and the EPS polysaccharides with ConA-TRITC
467 (red).
- 468 **Fig. 4.** CLSM images of the cryosections towards the centre of the granules from (a) and (b)
469 R1 and from (c) and (d) R2. The cells were stained with SYTO9 (green) and the EPS
470 polysaccharides with ConA-TRITC (red).
- 471 **Fig. 5.** FISH-CLSM images of the microbial cells in the granules from (a) and (b) R1 and
472 from (c) and (d) R2. The bacteria were labelled by probe Eub338-FITC (green), and
473 the fungi were shown to be eukaryal by probe Euk516-Cy3 (red).
- 474 **Fig. 6.** DGGE profiles and the abundance of the major bacterial species in the R1 granules
475 during the growth process: (a) DGGE image, (b) DGGE schematic and (c) the relative
476 abundance of the dominant bacterial species, as obtained through analysis of the
477 DGGE banding profiles (SG - seed granules; d - days of batch cultivation).
- 478 **Fig. 7.** DGGE profiles and the abundance of the major eukaryal species in the R2 granules
479 during the growth process: (a) DGGE image, (b) DGGE schematic and (c) the relative
480 abundance of the dominant eukaryal species, as obtained through analysis of the
481 DGGE banding profiles (SG - seed granules; d - days of the batch cultivation).

Figures

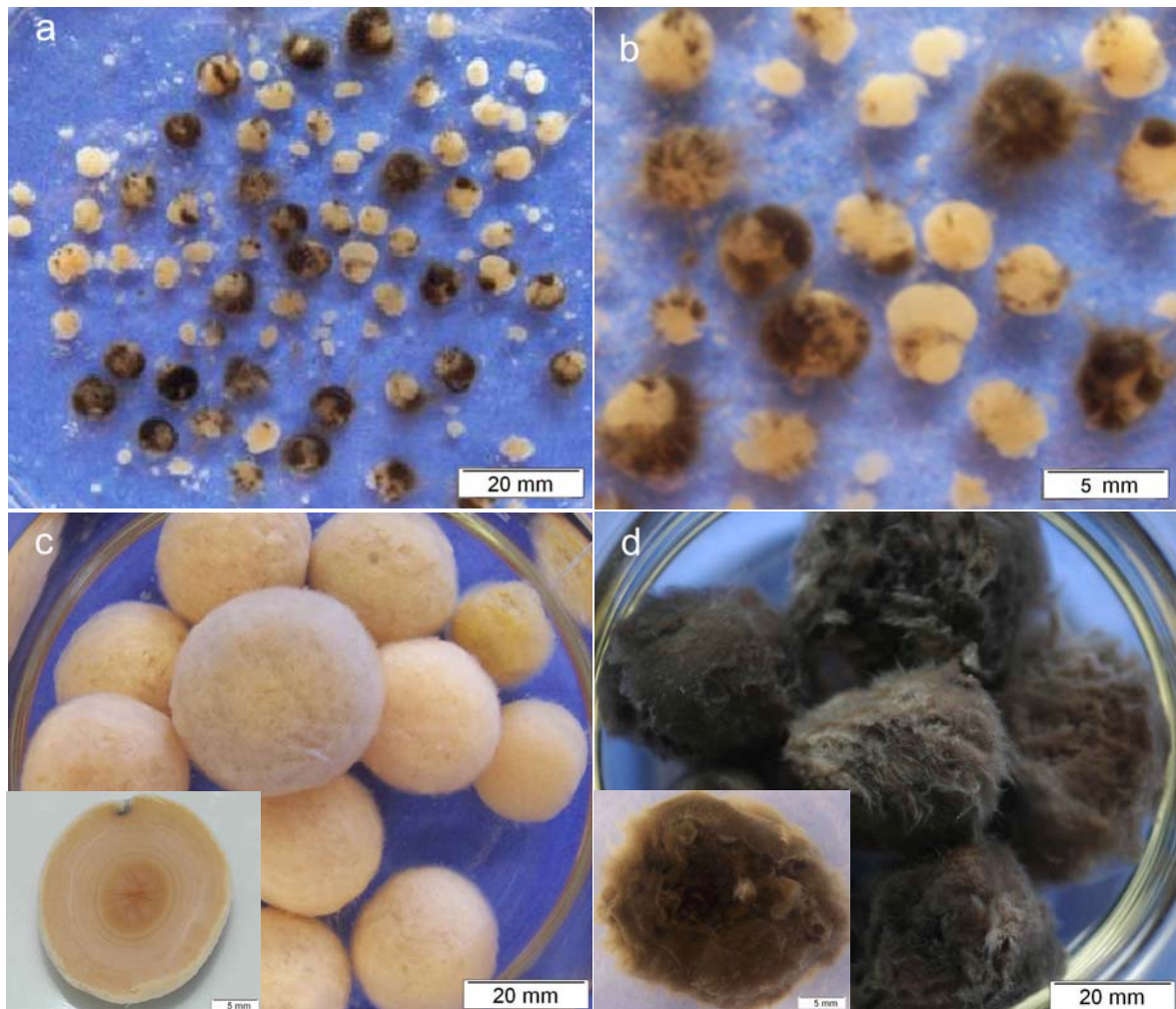


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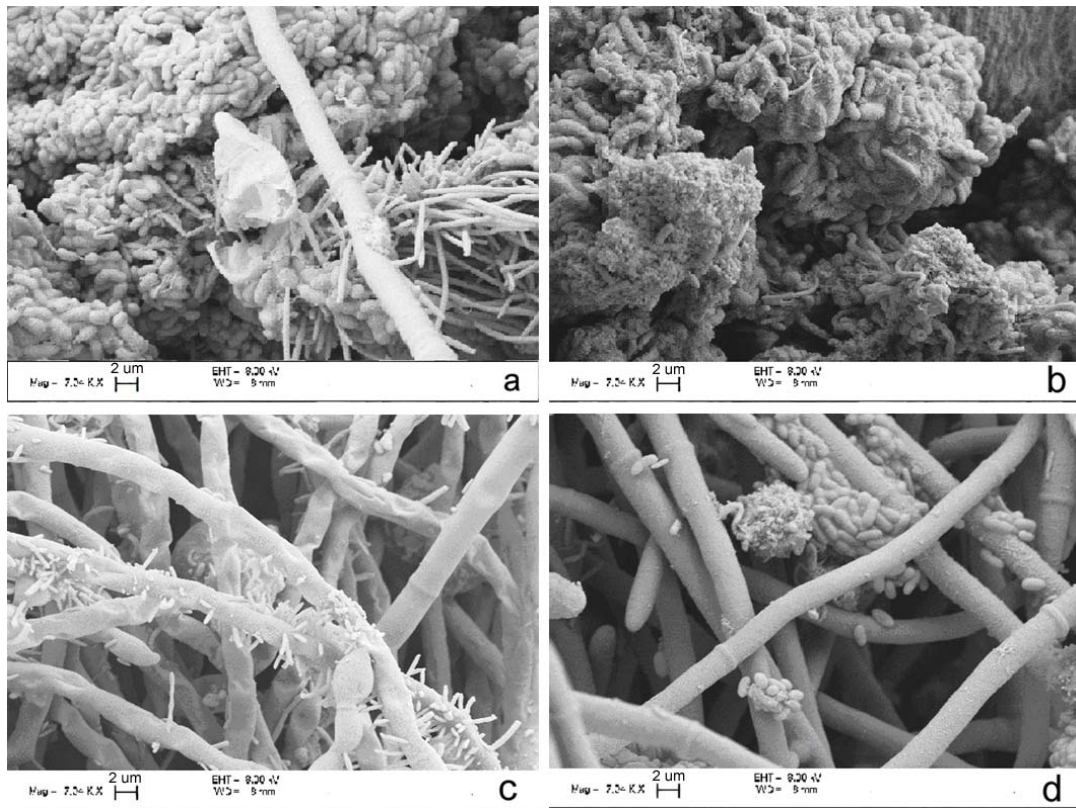


Fig. 2. SEM images of the microstructure of (a) and (b) mature granules from R1 and (c) and (d) mature granules from R2.

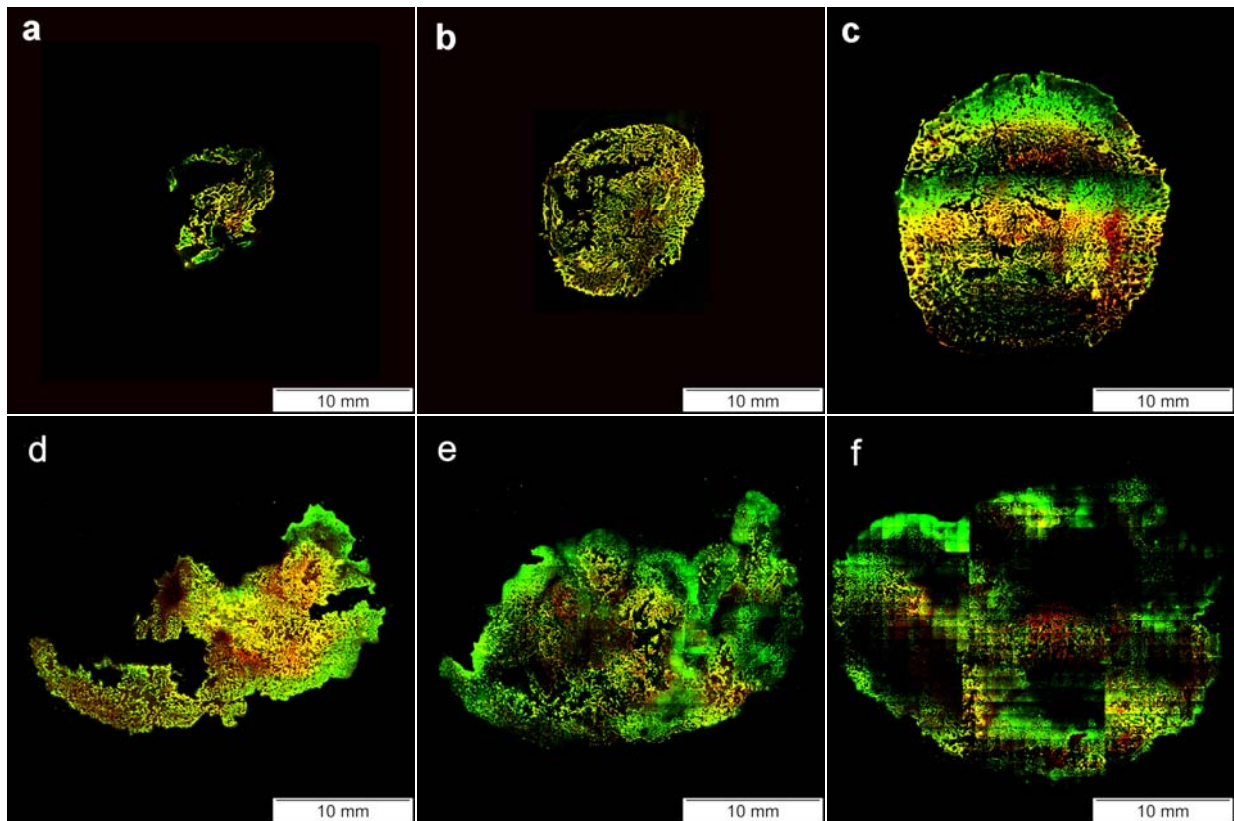


Fig. 3. CLSM images of the cryosections of the granules cultivated in (a-c) R1 and (d-f) R2. Depth: (a) 0.2 mm, (b) 3 mm, (c) 7 mm, (d) 3 mm; (e) 7 mm and (f) 12 mm. The cells were stained with SYTO9 (green) and the EPS polysaccharides with ConA-TRITC (red).

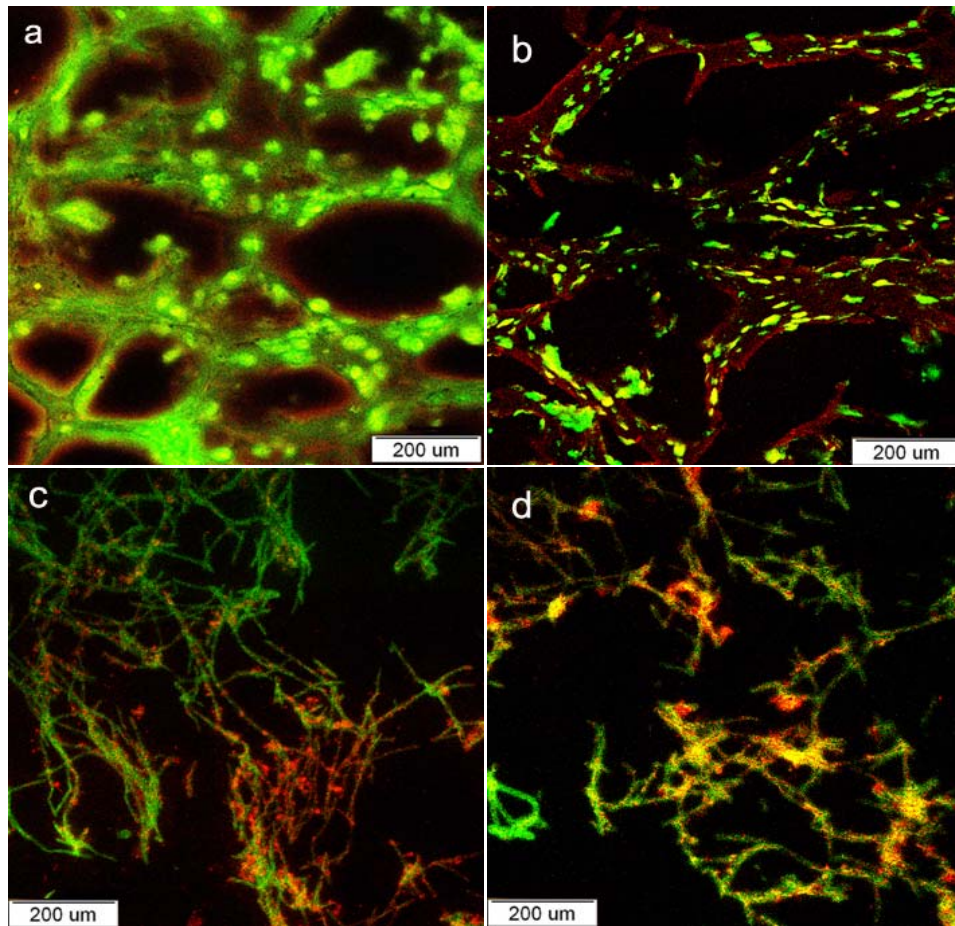


Fig. 4. CLSM images of the cryosections towards the centre of the granules from (a) and (b) R1 and from (c) and (d) R2. The cells were stained with SYTO9 (green) and the EPS polysaccharides with ConA-TRITC (red).

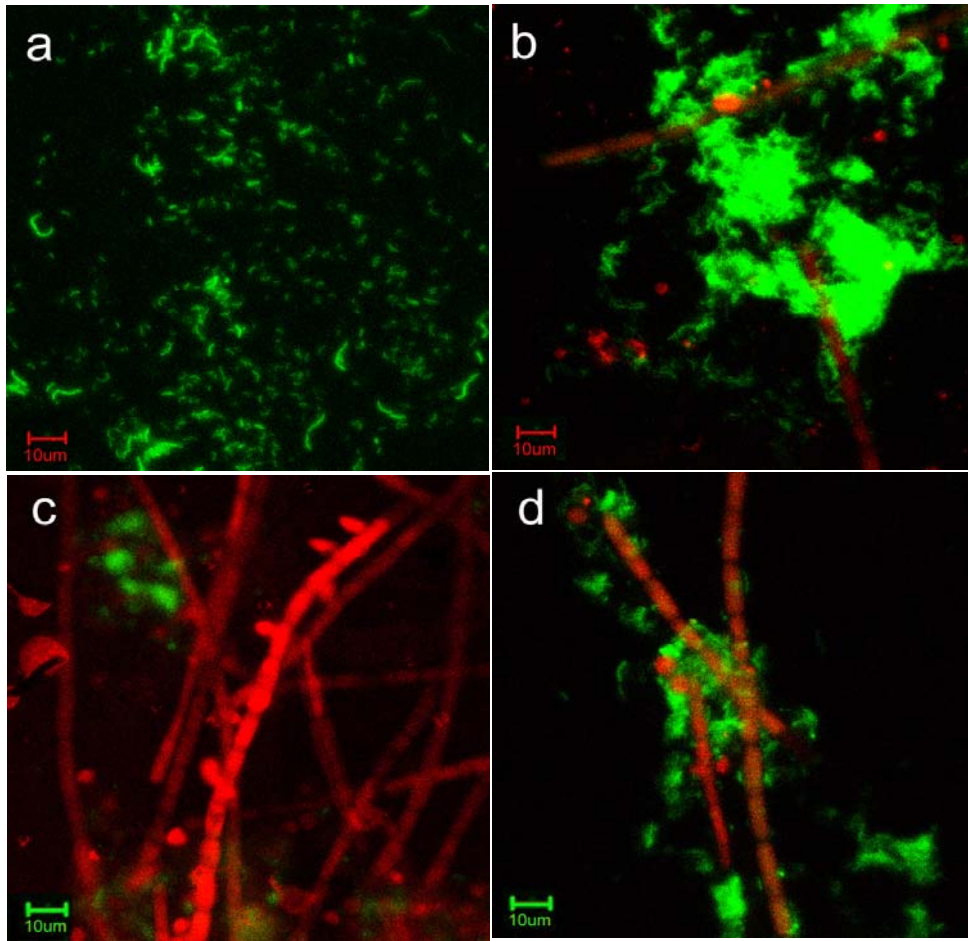


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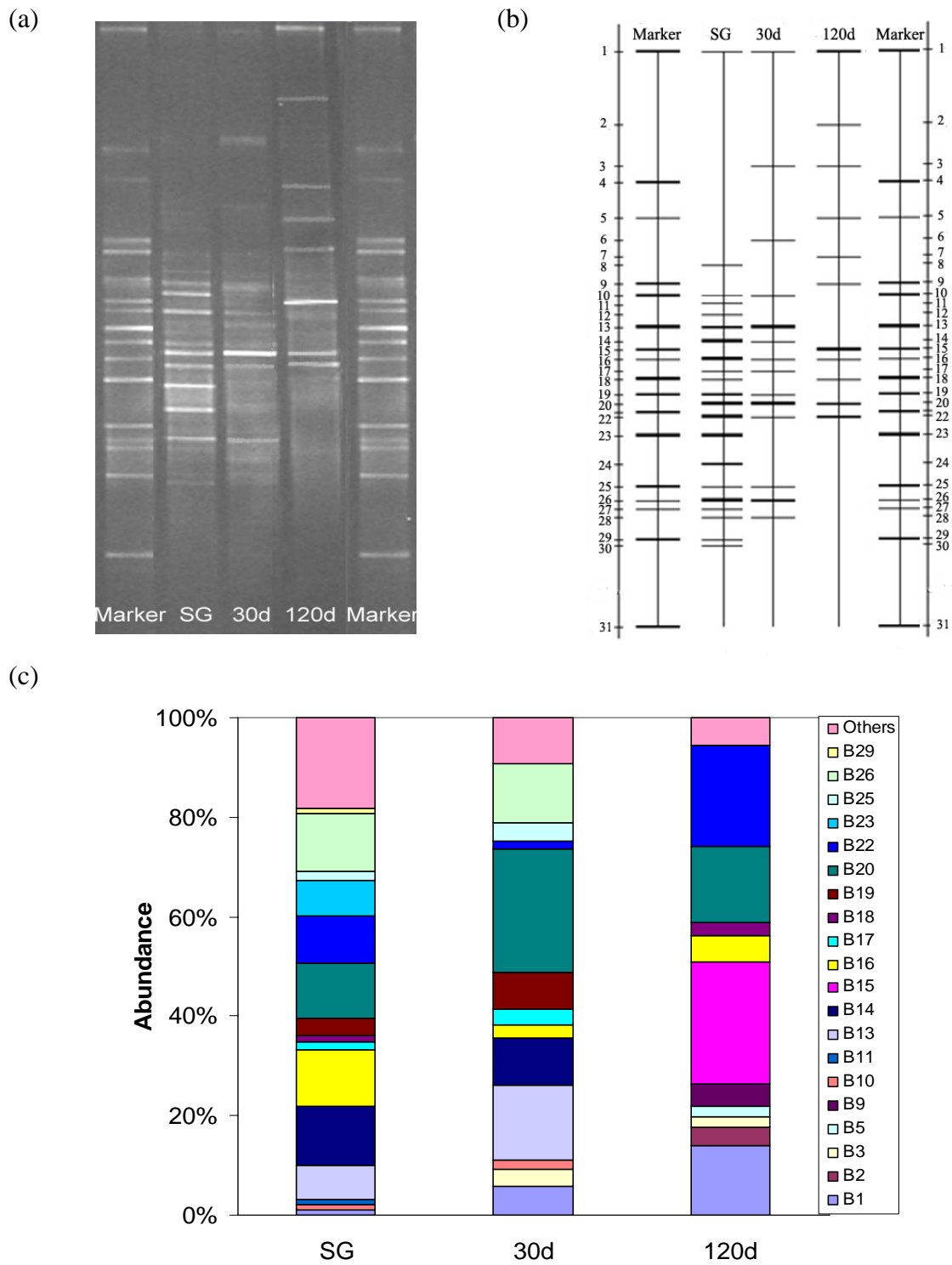


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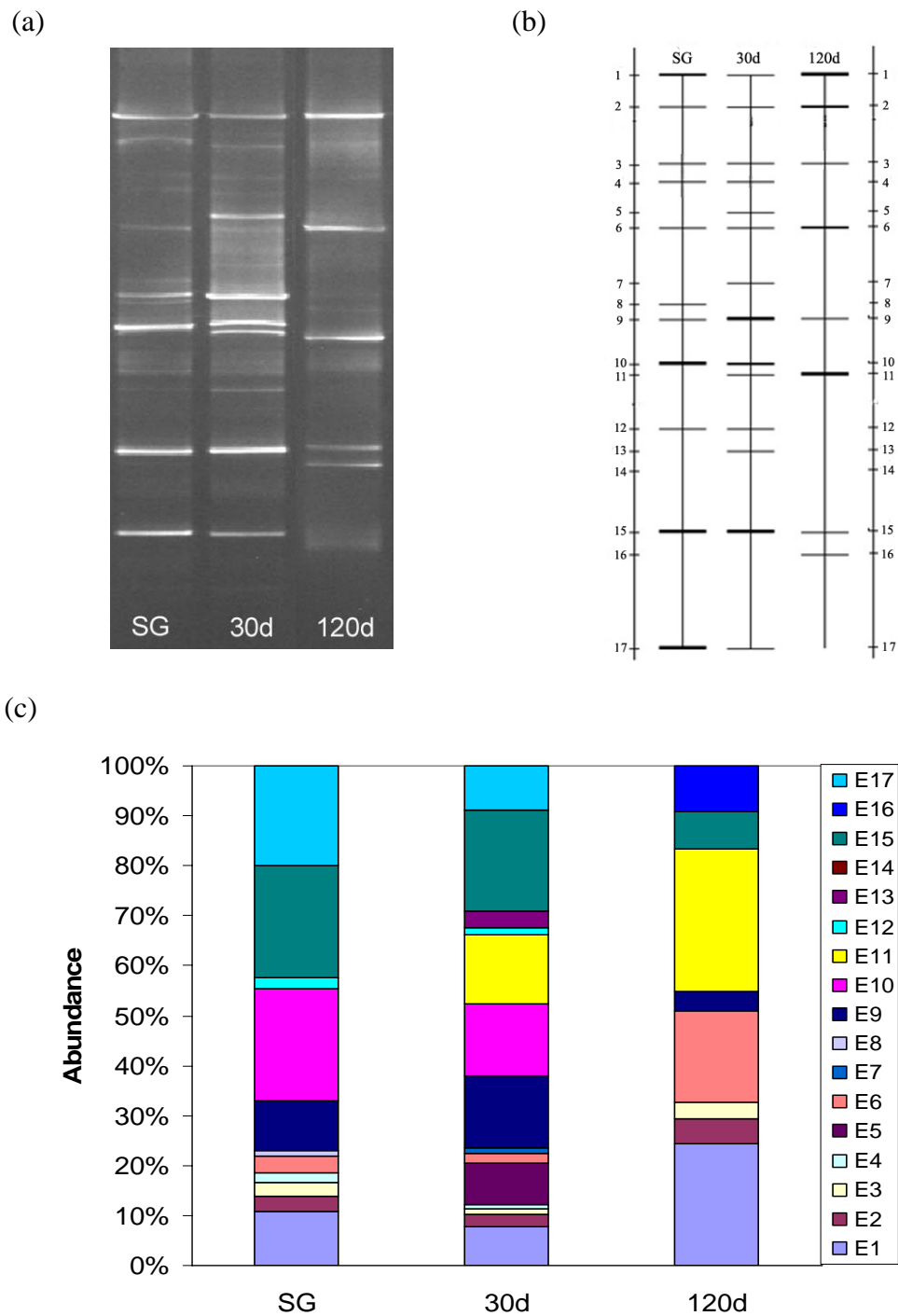


Fig. 7. DGGE profiles and the abundance of the major eukaryal species in the R2 granules during the growth process: (a) DGGE image, (b) DGGE schematic and (c) the relative abundance of the dominant eukaryal species, as obtained through analysis of the DGGE banding profiles (SG - seed granules; d - days of the batch cultivation).