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ABSTRACT

The present work aims to evaluate a strategy to solve *Galactomyces geotrichum* bulking based on the incorporation of a biofilm carrier in activated sludge systems. For this study, four sequencing batch reactors (SBR) were operated without (SBR1) and with a carrier for biofilm growth (5% [SBR2], 10% [SBR3], and 20% [SBR4] of the reactor volume). As expected, *G. geotrichum* bulking was observed in the SBR operating just with suspended biomass, as ascertained by direct microscopic inspection. The incorporation of an optimized amount of biofilm carrier (10 and 20%) suppressed the overgrowth of the filamentous fungus probably due to the combined effect of a decreased biomass loading rate and an increased shear stress. Polymerase chain reaction–denaturing gradient gel electrophoresis (PCR–DGGE) analysis of the bacterial community suggested that the incorporation of a biofilm carrier induced increasing differences in the bacterial community structure as the concentration of carrier increased in the SBR. However, the observed differences did not seem to affect the activated sludge system since bacterial groups usually present in these systems prevailed.

Keywords: Bacterial community composition; Carrier concentration; Filamentous bulking control; *Galactomyces geotrichum*; Sequencing batch reactors (SBR)

1. Introduction

Activated sludge systems are among the most common technology used for the biological treatment of wastewaters. The conventional system consists of two stages: (i) the removal of organic compounds and nutrients from the wastewater by the activated sludge and (ii) the activated sludge separation from the effluent through gravity sedimentation. The latter often becomes the critical stage of the system due to the frequent problems that severely affect the settling and compaction of the sludge. The most common cause of settling problems is the filamentous bulking—a term used to describe the overgrowth of filamentous microorganisms (bacteria or/and fungi). Filamentous bulking promotes the formation of open aggregates with low settling velocity as well as low compaction which cause serious difficulties in the retention of sludge within the reactor, thus impairing the overall performance and efficiency of the treatment system.

Several approaches have been used to reduce the overgrowth of filamentous microorganisms. For instance, the compartmentalization of the aeration tank (i.e. plug-flow reactors), or its conversion to a

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batch system [such as sequencing batch reactors (SBR)] has been used to increase sludge settleability and compaction, being the use of a selector reactor the most widespread engineering tool to control filamentous bulking [1,2]. Although these technologies have been successful and have reduced filamentous bulking in many activated sludge systems, there are some reports that point out their failure [3–5].

An alternative to the existing technologies for filamentous bulking control might be the incorporation of a carrier for biofilm growth into suspended biomass reactors. Interestingly, no problems with excessive growth of filamentous microorganisms have been reported in the cases where the activated sludge systems were combined with biofilm growth. Wanner et al. [6] found experimental evidence that the presence of a biofilm in the activated sludge systems significantly improves the settling properties of the flocs, especially in systems with plastic foam carriers. According to the authors, the effect of decreasing the biomass loading rate and the predominance of the fixed form of biomass contributed to suppress the overgrowth of filamentous microorganisms in the mixed liquor.

Filamentous bulking studies have been focused mainly on bacteria. Fungi have not been considered as the important members of the community being indicated as the dominant or secondary bulking filament in approximately 1 and 2% of wastewater treatment plants; however, it is generally agreed that they are probably more common than reported because researchers are not looking for fungi and they have atypical forms in wastewaters [7]. *Galactomyces geotrichum*, whose anamorph is the *Geotrichum candidum*, is a fungus that can be found in the activated sludge systems [8]. Filamentous bulking due to *G. geotrichum* has been reported [9]. For instance, Matos et al. [10] reported *G. geotrichum* bulking in a SBR fed with an easily biodegradable substrate (acetate) and operated at a high organic loading rate ($4.3\,g\,COD\,L^{-1}\,day^{-1}$), while Meyers [11] observed it in an industrial wastewater treatment system.

The present work aims to evaluate *G. geotrichum* bulking in systems combining suspended biomass with biofilm growth. For this study, SBR fed with an easily biodegradable substrate (acetate) operating with or without (control unit) biofilm growth were used. The biofilm grew on small polyethylene carriers that were moving freely along with liquid in the reactor. Microscopic and molecular techniques were used to follow the changes in the microbial community developed in each reactor. The use of different carrier concentrations in the SBR together with the characterization of the bacterial community involved in the experimented systems allowed the assessment of the conditions that promote the control of *G. geotrichum* bulking without negatively affecting activated sludge systems.

2. Materials and methods

2.1. Experimental setup

Four SBR with a working volume of 1.5 L were operated with a constant cycle time of 4 h. The sequence of the operating phases was as follows: 5 min fill, 225 min aerated, 5 min settle, and 5 min draw. At the end of each cycle, 0.75 L of effluent was pumped out of the reactors. One reactor was operated just with suspended biomass (SBR1—control reactor) while the others combined suspended biomass with biofilm growth. The biofilm was formed on a polyethylene carrier developed by the University of Minho, consisting of a hollow, star-shaped carrier with an external diameter of 17 mm, a height of 10 mm, and a specific surface area of $407\,m^2\,m^{-3}$ [12]. The density of the carrier is about $510\,g\,L^{-1}$, so it can be easily suspended by aeration. The carrier concentration was 5% (SBR2), 10% (SBR3), and 20% (SBR4) of the reactor working volume and the resulting hydraulic retention time (HRT) was 8 , 7.6 , 7.2 , and 6.4 h for SBR1, SBR2, SBR3, and SBR4, respectively. During the aerated phase, airflow of $2\,L\,min^{-1}$ was applied through membrane diffusers, making the reactor contents, including the carriers, to circulate. The dissolved oxygen (DO) in the SBR was above $2\,mg\,L^{-1}$. The reactors were operated with synthetic wastewater (not sterilized) containing acetate as the only carbon source and other necessary elements. The composition of the synthetic wastewater was $4{,}270\,mg\,L^{-1}$ $NaCH_3COO{\cdot}3H_2O$, $776\,mg\,L^{-1}$ NH_4Cl, $89\,mg\,L^{-1}$ KH_2PO_4, $45\,mg\,L^{-1}$ $MgCl_2{\cdot}6H2O$, $40\,mg\,L^{-1}$ $FeCl_3{\cdot}6H_2O$, $55\,mg\,L^{-1}$ $CaCl_2{\cdot}2H_2O$, and $1\,mg\,L^{-1}$ of the following trace elements $MnCl_2{\cdot}4H_2O$, $(Ni)_2SO_4{\cdot}6H_2O$, $CoCl_2{\cdot}6H_2O$, $Cu(NO_3)_2{\cdot}\,3H_2O$, and $ZnSO_4{\cdot}7H_2O$ [13]. The measured volumetric organic loading rate was 4.3 , 4.5, 4.8, and $5.4\,g\,COD\,L^{-1}\,day^{-1}$ for SBR1, SBR2, SBR3, and SBR4, respectively. The slight differences in the organic loading rates are due to the different HRT resulting from the different liquid volume of the reactors. The mean pH values in the SBR were between 7.5 and 8.5. The experimental conditions of the SBR1 (control unit) were known to promote the growth of *G. geotrichum* [10].

The reactors were inoculated with activated sludge coming from the Serzedelo I Wastewater Treatment Plant (Guimarães, Portugal) at an initial total suspended solids concentration of $1\,g\,L^{-1}$.

Regular cleaning of feed storage vessels, feed lines, and reactors was performed to avoid the proliferation of microorganisms on tubes and walls.

2.2. Analytical methods

Acetate, suspended biomass and biofilm concentration, standard sludge volume index (SVI), pH, and DO were determined weekly during the experiments (120 days).

Acetate concentration was analyzed using a HPLC system (Knauer, Berlin, Germany) with UV detection at 210 nm and an organic acid column (PL Hi-Plex H 8 μm, 100 mm × 7.7 mm, Polymer Laboratories) at 65° C. The mobile phase consisted of an aqueous H_2SO_4 solution (2 mmol L^{-1}) at a flow rate of 0.7 mL min^{-1}. Samples for acetate analysis were previously filtered through 0.45 μm mixed cellulose esters membrane filters (Advantec, Dublin, CA, USA). SVI of suspended biomass was determined using Standard Methods [14]. Considering the volume of the reactors, the SVI was determined in unstirred 25 mL cylinders. Suspended biomass concentration was estimated as total suspended solids [14] using 47 mm fiber glass membrane filters (Whatman, Maidstone, UK), and the biofilm concentration was measured as total solids according to Lazarova et al. [15]. Dissolved oxygen (DO) was measured with a DO meter (YSI 5000, YSI, Yellow Spring, OH, USA) and pH was monitored using a pH meter (model 420A, Thermo Scientific, Whaltham, MA, USA).

2.3. Microscopic observations

Microscopic observations of the microbial communities of the suspended biomass as well as those of the biofilm were carried out at least once a week in all systems in a phase contrast microscope (Leitz, Laborlux S). Fifty representative fields were analyzed per sample. Floc shape was evaluated qualitatively regarding size, openness, and porosity. The simplified counting technique developed by Jenkins et al. [2] was applied to quantify the occurrence of filaments. Additionally, the presence of fungi was analyzed with Calcofluor White M2R (American Cyanamid, Eugene, OR, USA) stain. Calcofluor was added to samples to a final concentration of 10 μM, and the binding of calcofluor to sample cell walls was practically immediate. After calcofluor staining, the filaments were observed under an epifluorescence microscope (Olympus BX51) using an excitation wavelength of 365–370 nm and an emission longpass filter by 421 nm.

2.4. Microbial analysis

The presence of *G. geotrichum* in the seed sludge and in the suspended biomass was assessed using conventional plating, isolation, and microscopic techniques [10] and comparing the results with the descriptions available in the literature [16].

16S rRNA gene-targeting techniques, such as denaturing gradient gel electrophoresis (DGGE), cloning, and sequencing analysis were used as cultivation-independent tools for the analysis of bacterial diversity in the suspended biomass and biofilm.

2.4.1. DNA extraction and amplification

Approximately, 2 mL of well-homogenized suspended biomass was washed and resuspended in sterile phosphate-buffered saline solution (PBS) at the time of the sampling and stored at −20°C until further use. The biofilm was sampled by removing two carriers from the reactors and placing them into a falcon tube containing 40 mL of sterile PBS. The tube was vigorously vortexed for 1 min. Subsequently, the clean carriers were removed, and the biofilm suspension was stored at −20°C.

Total genomic DNA was extracted using a modified protocol of the PowerSoil® DNA Isolation Kit (MO BIO, Carlsbad, CA, USA) in which the cells were disrupted in a FastPrep® FP120 instrument (Qbiogen, Carlsbad, CA, USA). The rest of the DNA extraction steps were performed according to the original manufacturer's protocol.

Selected DNA target regions were amplified by polymerase chain reaction (PCR) using a *Taq* DNA Polymerase (Invitrogen, Carlsbad, CA, USA) in a 50 μL reaction mixture according to Rodrigues et al. [17]. For DGGE analysis, bacterial 16S rRNA gene fragments were amplified using the primers U968-GC-f (5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGAACGCGAAGAACCTTAC-3′) and L 1401-r (5′-CGGTGTGTAC- AAGACCC-3′), targeting the V6 to V8 region [18]. The thermocycling program used for amplification was previously described by Rodrigues et al. [17]. Bacterial 16S rRNA gene fragments were also amplified for cloning using the forward primer Bact27-f (5′-AGAGTTTGATCMTGGCTCAG-3′) and the universal primer Uni1492-r (5′-ACGCC TACCTTGTTACGACTT-3′) [19]. The program of amplification was similar to the one used above but with an annealing temperature of 52°C. The PCR products were examined by electrophoresis in a 1% (w/v) agarose gel.

2.4.2. DGGE analysis

DGGE analysis of the amplicons was carried out using the Dcode system (Bio-Rad, Hercules, CA, USA) in gels containing 8% (w/v) polyacrylamide (37.5:1 acrylamide/bis-acrylamide). A linear denaturant gradient of 35–60% was used for all analyses, where a denaturing strength solution of 100% was defined as 7 M urea (Sigma-Aldrich, St. Louis, MO, USA) and 40% formamide (Fluka Chemie, Buchs, Switzerland). Gels were run for 16 h at 85 V in a 0.5× TAE buffer (50× Tris acetate: 242 g Tris, 57.1 mL glacial acetic acid, 100 mL 0.5 M EDTA (pH 8.0) per liter) at 60°C. Subsequently, DGGE gels were stained with silver as previously described by Sanguinetti et al. [20], scanned in an Epson Perfection V750 PRO (Epson, USA), and the DGGE profiles were compared using the BIONUMERICS™ software package (version 5.0; AppliedMaths BVBA, Sint-Martens-Latem, Belgium). Similarity indices of the compared profiles were calculated from the densitometric curves of the scanned DGGE profiles using the Pearson product-moment correlation coefficient [21].

2.4.3. Cloning and sequencing of PCR-amplified products

PCR products obtained with the primers set Bact27f/1492r were purified with the QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany) and cloned into *E. coli* JM 109 (Invitrogen, Carlsbad, CA, USA) using the Promega pGEM-T Easy vector system I (Promega, Madison, WI, USA), according to the manufacturer's instructions. Ninety-six white-colored recombinant colonies were collected and screened by DGGE. PCR was carried out on the cell lysates using the primer pair described above for DGGE analysis. The DGGE mobility of amplicons was compared to the band pattern of the biomass samples. The clones whose amplicons corresponded to bands in the biomass samples community profile were selected for sequencing. Clones with sequences showing identical DGGE mobility were also selected for replicate sequencing. Plasmids of selected clones were purified using the QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany) and subjected to DNA sequence analysis. Sequencing reactions were performed at STAB Vida in a 3730xl DNA analyzer (Applied Biosystems, Foster City, CA, USA) using the BigDye Terminator v1.1 DNA sequencing kit and the SP6 (5′-ATTTAGGTGACACTATAG-3′) and T7 (5′-TAATACGACTCACTATAGG-3′) sequencing primers. The sequence information was imported into the BioEdit v7.0.5.3 software package [22] for assembly. Consensus sequences obtained were manually checked and corrected when necessary. They were also checked for potential chimera artefacts by the Mallard program v1.02 [23]. Similarity searches for the 16S rRNA gene sequences derived from the suspended biomass and biofilm clones were performed using the NCBI Blast search program within the GenBank database (http://www.ncbi.nlm.nih.gov/blast/). Sequence data have been deposited in the GenBank database under accession numbers JF826422 to JF826429.

2.5. Batch tests

In order to gain insights into the influence of the shear stress in the fungus morphology, batch tests with a pure culture of *G. geotrichum* were performed. *G. geotrichum* strain used in the batch tests was isolated from the activated sludge of SBR1. Erlenmeyer flasks (250 mL) containing 100 mL of the synthetic wastewater were covered with cotton and sterilized at 121°C for 20 min. After inoculation with *G. geotrichum*, the cultures were placed on a rotary shaker (100 rpm) or incubated under static conditions at 25°C for seven days. By then, microscopic observations of the cultures were carried out in a phase contrast microscope (Leitz, Laborlux S).

3. Results and discussion

3.1. SBR performance

Before reactors start up, the seed sludge was spread plated onto NGRBA medium [10] to verify the presence of fungal filaments, this being a crucial step to the subsequent experiments. The culture revealed a large abundance of filamentous fungal colonies, including *G. geotrichum* colonies. At the end of the experiments (day 120), the suspended biomass of the four reactors was also spread plated onto NGRBA medium. These cultures only revealed *G. geotrichum* colonies and a few counts of non-filamentous colonies that were considered to be yeasts (data not shown).

Four reactors (SBR1–SBR4) were operated with different amounts of carrier for biofilm growth. The microscopic observations showed that the suspended biomass in the four reactors started to differ very soon after the inoculation. The initial seed sludge gradually developed into large and compact flocs in SBR3 and SBR4 and flocs dominated by *G. geotrichum* filaments growing in profusion beyond the confines of the flocs into the bulk solution in SBR1 and SBR2 (with level 6 and 5 of filament abundance according to Jenkins et al. [2] for SBR1 and SBR2, respectively).

Additionally, the microscopic observations after calcofluor staining showed that the fungal filaments were present in SBR3 and SBR4; however, these filaments were integrated in the flocs structure (internal filaments) and were considerably shorter than in SBR1 and SBR2 (about 35 μm for SBR3 and SBR4 and about 245 μm for SBR1 and SBR2). Fig. 1 depicts the suspended biomass of the four reactors and Fig. 2 shows the suspended biomass of SBR1 and SBR4 after calcofluor staining, both at the end of the experiments.

The SVI values were in direct agreement with the microscopic observations. The SVI in SBR3 did not exceed 150 mL g^{-1} but in SBR1 and SBR2 ranged between 130 and 680 mL g^{-1} as a result of the excessive occurrence of fungal filaments. The SVI in SBR4 increased to 220 mL g^{-1} although no problems with excessive fungal filaments were observed. The SVI in SBR4 might have been negatively affected by the high carrier concentration. In fact, the high carrier concentration in SBR4 led to a high carrier-to-carrier collision frequency [24] which may have hindered the formation of larger and more compact flocs.

Due to the different settleability and compaction of the suspended biomass, the four reactors differed considerably in the suspended biomass content (Fig. 3a). The reactor presenting lower SVI values (SBR3) had higher suspended biomass concentration. A relatively high suspended biomass concentration between 2.8 and 6.7 g L^{-1} was maintained in SBR3. During the experiments, the suspended biomass concentration decreased to 0.6, 1.2, and 2.2 g L^{-1} in SBR1, SBR2, and SBR4, respectively.

Biofilm concentration increased with the increase in carrier concentration present in the reactors (Fig. 3b). These results are likely due to the larger surface area of carrier available for the attachment of microorganisms and mainly because the mentioned surface area is mostly internal which protected the biofilms from erosion and abrasion detachment mechanisms. When the carrier concentration was 5% (SBR2), the biofilm concentration was approximately 0.3 g L^{-1}. The biofilm concentration increased to 1.3 g L^{-1} in SBR3 (10%) and to 2.8 g/L in SBR4 (20%) during the operations of the reactors. Nevertheless, the suspended biomass was the prevalent form of

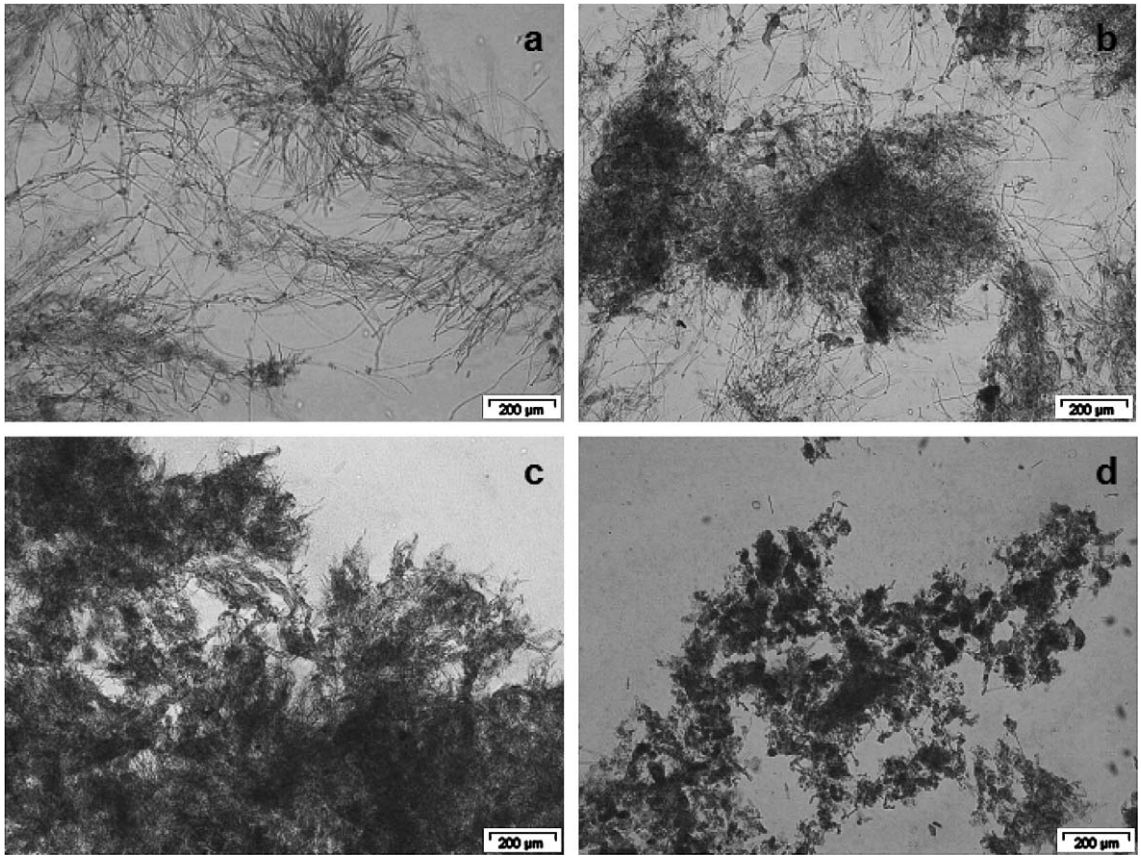


Fig. 1. Micrographs of the suspended biomass from SBR1 (a), SBR2 (b), SBR3 (c), and SBR4 (d) on day 120 taken with a Olympus Altra-20 Camera in a Leitz phase contrast microscope.

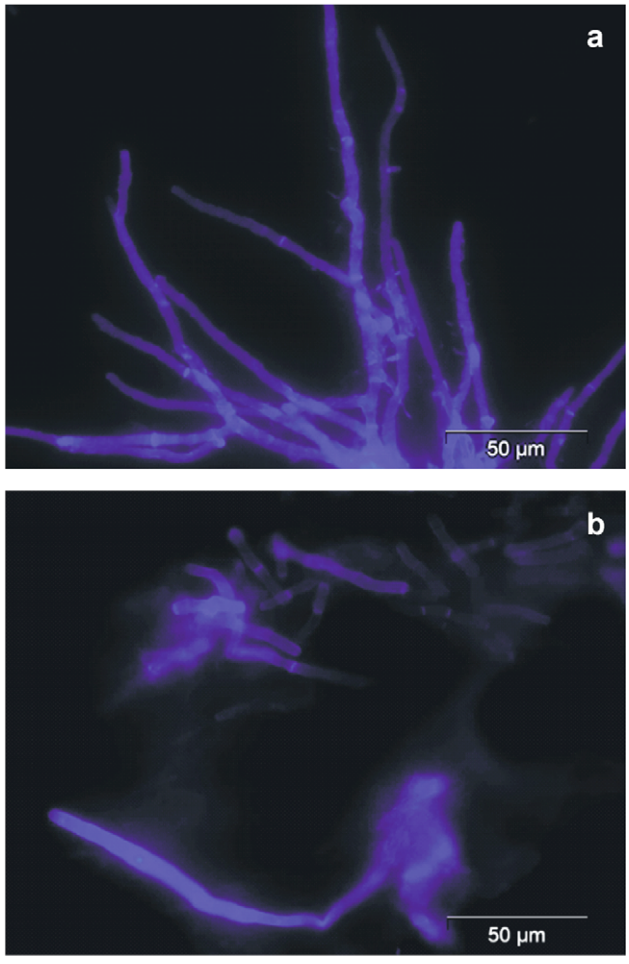


Fig. 2. Micrographs of the suspended biomass from SBR1 (a) and SBR4 (b) on day 120, after calcofluor staining, taken with an Olympus DP71 Camera in an epifluorescence Olympus BX51 microscope.

biomass during the entire period of experiments (about 90, 84, and 60% for SBR2, SBR3, and SBR4, respectively).

All reactors performed well in terms of carbon removal. The acetate was completely removed during a typical SBR cycle (Fig. 4). Acetate removal ran over the entire cycle in SBR1 and SBR2, while in SBR3 and SBR4 the acetate supplied was quickly consumed due to a higher overall quantity of biomass in the reactor (Fig. 3).

The results obtained suggest that increasing the carrier concentration for biofilm growth in the reactors apparently suppressed the *G. geotrichum* bulking. During the entire period of experiments (120 days), the microscopic observations revealed that *G. geotrichum* filaments were quite common in SBR1 and SBR2, while in the other reactors (SBR3 and SBR4), their occurrence were negligible.

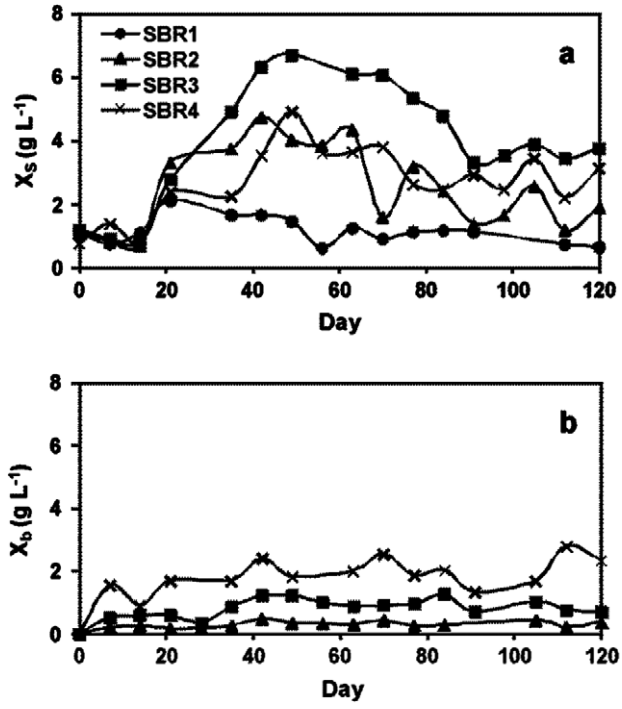


Fig. 3. Suspended biomass (a) and biofilm (b) concentration.

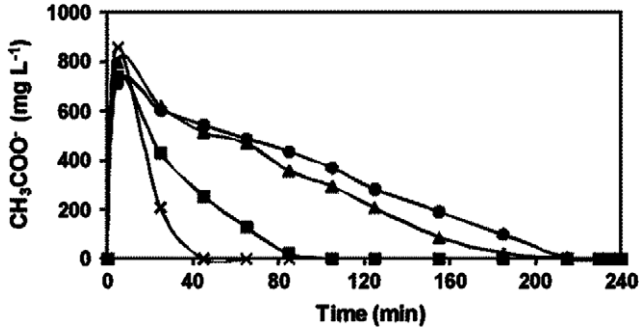


Fig. 4. Acetate concentration profiles during a representative SBR cycle on day 120 in SBR1 (●), SBR2 (▲), SBR3 (■) and SBR4 (×).

The results suggest that the suppression of the overgrowth of *G. geotrichum* filaments in SBR3 and SBR4 might be related to the increase in the total amount of biomass in the reactors and consequently, to the decrease in the biomass loading rate (food-to-microorganism (F/M) ratio). SBR3 and SBR4 had lower biomass loading rate (0.2–0.4 g COD g TS^{-1} day^{-1} and 0.8–1.4 g COD g TS^{-1} day^{-1}, respectively), and excessive occurrence of fungal filaments was not observed in these reactors. On the other hand, a higher biomass loading rate was

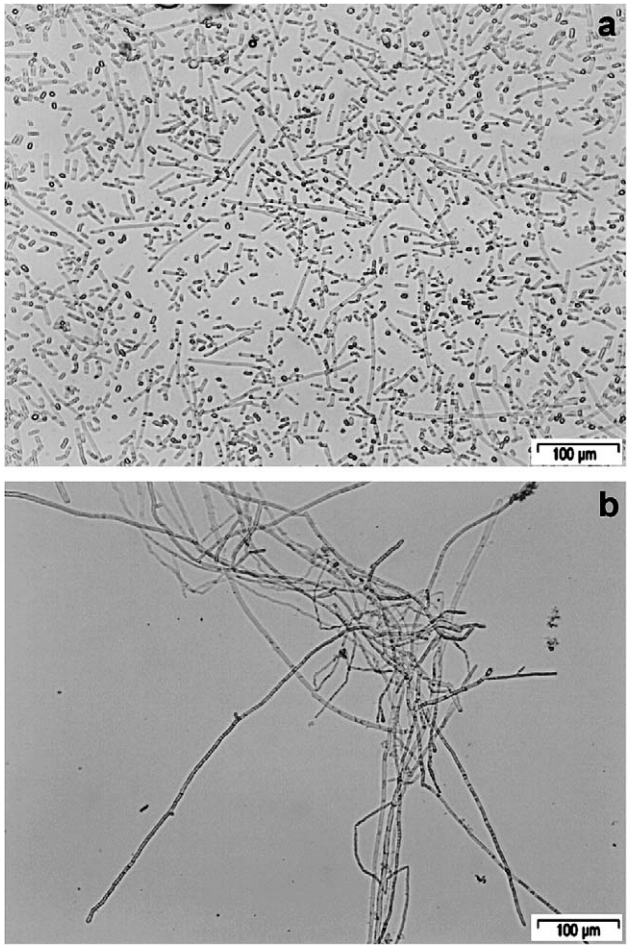


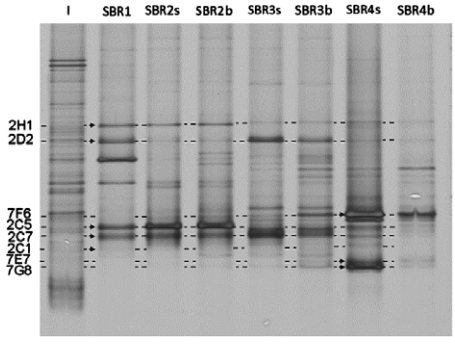
Fig. 5. Micrographs of *G. geotrichum* incubated with (a) and without agitation (b) taken with a Olympus Altra-20 Camera in a Leitz phase contrast microscope.

maintained in SBR1 and in SBR2 (2.0–6.9 g COD g TS^{-1} day^{-1} and 0.9–3.3 g COD g TS^{-1} day^{-1}, respectively) where a relatively high proliferation of fungal filaments was observed. Similar results have been reported in literature by Matos et al. [10].

It was also observed that the existing fungal filaments in SBR3 and SBR4 seemed to be considerably shorter than in SBR1 and SBR2. Additional batch tests with a pure culture of *G. geotrichum* demonstrated that a higher shear stress induced the growth of *G. geotrichum* with a different morphology (Fig. 5).

The tests showed that agitation enhanced the growth of *G. geotrichum* with high arthroconidia and few filaments, while the incubation without agitation enhanced filaments growth and ramification. These results suggest that *G. geotrichum* bulking in SBR3 and SBR4 was also suppressed due to the shear stress established by collisions between carriers. In SBR3 and SBR4, a carrier concentration of 10 and 20% was used, which led to a high carrier-to-carrier collision frequency and accordingly to a higher shear stress [24]. Consequently, *G. geotrichum* filaments might have become too short to cause filamentous bulking. In SBR2, it seemed that the carrier-to-carrier collisions established were not enough to control filamentous bulking as this reactor presented lower carrier concentration (5%).

The results obtained showed that the reactor operating without biofilm growth faced *G. geotrichum* bulking as previously assessed by Matos et al. [10]. It was observed that the incorporation of an optimized (10 and 20%) amount of carrier for biofilm growth seemed to overcome the fungal filamentous bulking, apparently due to the increase in the overall quantity of biomass and subsequent decrease in the biomass loading rate and the carrier-to-carrier collisions established. Both factors, biomass loading rate and shear stress, influenced the control of the fungal filamentous bulking; however, the experiments did not allow to conclude which one played the most meaningful role.



	SBR4b	SBR4s	I	SBR3b	SBR3s	SBR2b	SBR2s	SBR1
SBR4b	100							
SBR4s	62.4	100						
I	30.7	38.3	100					
SBR3b	55.2	47.4	33.2	100				
SBR3s	23.4	25.5	24.5	84.6	100			
SBR2b	39.4	36.1	30.5	60.5	59.6	100		
SBR2s	32.9	31.7	30.0	63.6	67.3	97.7	100	
SBR1	13.1	11.6	24.2	54.5	55.7	61.7	63.2	100

Fig. 6. DGGE patterns of bacterial 16S rRNA gene fragments amplified from suspended biomass (s) and biofilm (b), collected from the SBR (1, 2, 3, and 4, respectively) on day 120 and from the inoculum (I). Corresponding similarity (in%) matrix is also presented. Numbered DGGE bands were further identified by cloning and sequencing.

3.2. Molecular microbial analysis

In order to gain insights into the diversity and changes induced in the bacterial community by the increase in the reactor's carrier concentration, a molecular approach combining PCR–DGGE, cloning, and sequencing techniques was further used. Diversity and shifts in bacterial community was estimated based on the DGGE patterns of the partial 16S rRNA genes amplified from the inoculum (I) and from the suspended biomass (s) and the biofilm (b) collected from the SBR (1, 2, 3, and 4, respectively) at the end of the experiments (120 days). Fig. 6 depicts the obtained DGGE profiles as well as the correspondent similarity indices (SI).

At the end of the experiments, the bacterial community patterns were significantly different from the initial pattern of the inoculum, as indicated by the comparison of the calculated similarity indices: bacterial similarity between the inoculum and the biomass (suspended and fixed) from the SBR ranged between 24.2 and 38.3%. This clear shift in the bacterial structure suggests that a decrease in the diversity resulting from the biomass adaptation to the laboratory conditions occurred. At the end of the experiments, the similarity index between the biomass from SBR1 and the suspended biomass from SBR2, SBR3 and SBR4 was 63.2, 55.7, and 11.6%, respectively, suggesting that higher differences in the bacterial community structure were obtained in the reactor with a higher carrier concentration. Furthermore, the increase in the concentration of carrier used in the reactor was found to induce increasing differences between the bacterial community present in the suspended biomass and in the biofilm (SBR2s/SBR2b—97.7%, SBR3s/SBR3b—84.8%, and SBR4s/SBR4b—62.4%).

To identify the prominent (more intense) bands in the bacterial community represented in the DGGE patterns, 16S rRNA genes of two representative suspended biomass samples (SBR1 and SBR4s) were amplified, cloned and sequenced. The DGGE mobility of amplicons obtained from a total of 192 clones was compared to DGGE profiles of both biomass samples to determine that to which fragments they corresponded. Table 1 summarizes the sequencing results and Fig. 6 depicts their corresponding position in each DGGE profile.

Sequencing and blast searching of the selected bacterial clones resulted mainly in matches with unknown and uncultured microorganisms assigned to the beta subclass of *Proteobacteria* group (clones 2C7, 2D2, 7E7, 7F6, and 7G8). In addition, close relatives to *Pseudomonas* sp. (clone 2C5) and *Rheinheimera* sp. (clone 2H1) belonging to the gamma subclass of *Prote-*

Table 1
Sequencing results of the selected bacterial clones

Clone	Accession number	Sequence length (bp)	Closest relatives (accession number, % sequence similarity)	Phylogenetic division[a]
2C1	JF826422	1,492	*Comamonas testosteroni* CNB-2 (CP001220, 99%)	Betaproteobacteria
2C5	JF826423	1,501	*Pseudomonas* sp. BBTR25 (DQ337603, 99%)	Gammaproteobacteria
2C7	JF826424	1,491	Uncultured beta proteobacterium clone OS1L-16 (AB076869, 99%), *Comamonas* sp. PG6–1 (AB277849, 99%)	Betaproteobacteria
2D2	JF826425	1,491	Bacterium rM5 (AB021340, 99%), *Simplicispira psychrophila* strain CA 1 (AB076845, 97%)	Betaproteobacteria
2H1	JF826426	1,484	*Rheinheimera sp.* JSM 083085 (FJ527418, 96%)	Gammaproteobacteria
7E7	JF826427	1,488	Uncultured bacterium clone UTFS-O04–62-07 (AB166776, 98%) *Alicycliphilus sp.* R-24611 (AM084014, 97%)	Betaproteobacteria
7F6	JF826428	1,496	Uncultured bacterium clone BE16FW031401IDW-SAK15 (DQ088753, 99%), *Thauera* sp. 27 (AY838760, 99%)	Betaproteobacteria
7G8	JF826429	1,492	Uncultured bacterium clone 65 (DQ413124, 98%), *Acidovorax ebreus* TPSY (CP001392, 97%)	Betaproteobacteria

[a]According to Wang et al. [25].

obacteria and a close relative to *Comamonas* sp. (clone 2C1) belonging to the beta subclass of the same group were also found. Bacteria from beta and gamma subclasses of *Proteobacteria* represent a collection of microorganisms that are commonly present in conventional activated sludge systems [26,27]. Bacteria of the genus *Rheinheimera* (clone 2H1) have been isolated from marine environments [28]. *Pseudomonas*-like organisms (clone 2C5) are known for their production of extracellular polymeric substances (EPS) and ability to bind cells together [29,30]. Other members related to *Thauera* genera (clone 7F6) and *Comamonadaceae* family (clone 2C1, 2C7, 2D2, 7E7, and 7G8) are generally present in biological organic oxidation and nitrifying–denitrifying activated sludge [31,32].

The results obtained from the molecular bacterial analysis showed clear shifts in the bacterial community induced by the increase in the reactor's carrier concentration. Phylogenetic analysis of the sequences corresponding to prominent ribotypes in the DGGE profiles suggests that the prevalent groups of bacteria present in the reactors are commonly related to bacteria known to thrive in conventional activated sludge systems. The incorporation of carrier for biofilm growth, which favored the control of the fungal filamentous bulking, caused clear shifts in the bacterial community; however, these shifts did not seem to affect the activated sludge system since bacterial groups usually present in these systems prevailed.

4. Conclusions

G. geotrichum bulking was successfully overcome through the incorporation of an optimized amount of carrier for biofilm growth. Two factors might have influenced the filamentous bulking control: decrease in the biomass loading rate as a result of the increase in the overall quantity of biomass and increase in the shear stress induced by carrier-to-carrier collisions.

Besides the observed differences in terms of the filamentous fungus, the incorporation of a carrier for biofilm growth into the SBR was also found to induce differences in the bacterial community structure: as the concentration of the carrier used in the SBR increased, increasing differences in the bacterial community were induced. The bacterial groups identified are commonly found in conventional activated sludge systems.

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