RESEARCH ARTICLE

The diversity of fungi in aerobic sewage granules assessed by 18S rRNA gene and ITS sequence analyses

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Abstract

Aerobic sewage granules are dense, spherical biofilms, regarded as a useful and promising tool in wastewater treatment processes. Recent studies revealed that fungi can be implemented in biofilm formation. This study attempts to uncover the fungal diversity in aerobic granules by sequence analysis of the 18S and 5.8S rRNA genes and the internal transcribed spacer regions. For this purpose, appropriate PCR and sequencing primer sets were selected and an improved DNA isolation protocol was used. The sequences of 41 isolates were assigned to the taxonomic groups *Pleosporaceae*, *Xylariales*, *Theleobolaceae*, *Claviceps*, *Aureobasidium*, *Candida boleticola*, and *Tremellomycetes* within the fungi. It turned out that the fungal community composition in granules depended on the wastewater type and the phase of granule development.

Introduction

Aerobic sewage granules are spherical, compact aggregates of microorganisms, mainly bacteria, but also protozoa and fungi, and extracellular polymeric substances (Morgenroth *et al.*, 1997; Beun *et al.*, 1999; Weber *et al.*, 2007). Because of their compact and dense structure, granular biofilms allow fast settling of the sludge, while the biomass is almost completely retained in the system and comprises a high and stable metabolism. Recent studies showed that an implementation of fungi in suspended granular biofilms usually occurs in sequencing batch reactors (SBRs) (Beun *et al.*, 1999; Etterer & Wilderer, 2001; Wang *et al.*, 2004; Weber *et al.*, 2007). Thereby, fungal hyphae act like skeletal elements in the structural formation process of the granules, providing a substratum for bacterial growth (Fig. 1).

Besides the use of particular fungi for industrial wastewater treatment, the overall fungal diversity in activated sludge remained mainly unidentified because these organisms were generally unwanted in conventional activated sludge processes. The filamentous hyphae were known to cause bulking problems and clog tubes and filters in the plants (Subramanian, 1983; Mudrack & Kunst, 1988). However, recent studies reported on the useful implementation of fungi in domestic wastewater treatment plants (Fakhru'l-Razi *et al.*, 2002; Guest & Smith, 2002; Molla *et al.*, 2002). Fungi contributed to sludge dewaterability and toxic resistance, COD removal, nitrification, and denitrification (Guest & Smith, 2002; Alam *et al.*, 2003; Mannan *et al.*, 2005). Some of them show even higher denitrification rates than bacteria (Guest & Smith, 2002). These observations endorse the need to investigate the fungal diversity in wastewater more closely and to integrate these organisms systematically in the treatment process to tap their possible bioconversion potential without bulking problems. For the latter task, novel treatment techniques as the above-mentioned aerobic granular sludge in SBR systems can be considered as appropriate (Weber *et al.*, 2007).

Because there is limited knowledge of the identity of fungi in granular wastewater biofilms, the aim of the present study was to examine the fungal diversity in two SBRs, operated with malthouse and artificial wastewater. Experience from numerous studies on fungal diversity in natural habitats has shown that the identification of fungi is often very complex

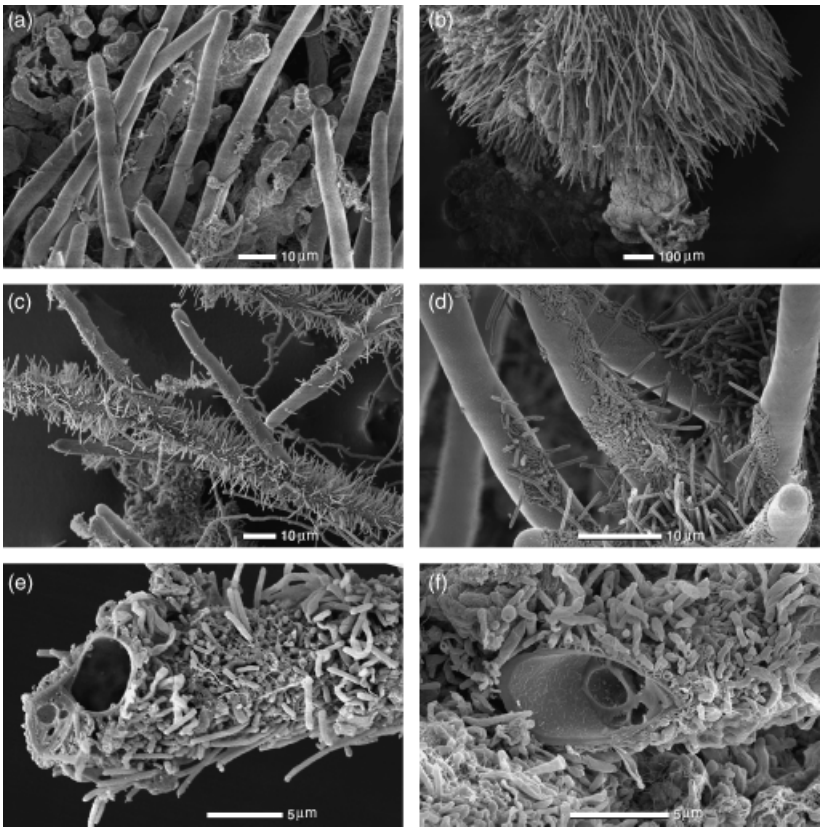

Fig. 1. Scanning electron micrographs of biofilm samples from SBR1 (malthouse wastewater; a, b) and SBR2 (artificial wastewater; c, d, e, f). Fungal filaments (hyphae) are implemented in the granule structure (a), with hyphae tips protruding from the granule surface (b). Hyphae are densely covered with rod-shaped bacterial cells both on the surface (c, d) and within the granule (e, f; visualized after cryo-fracture treatment).

due to their species richness and the high micromorphological similarities. Furthermore, these organisms exhibit multiple life-cycle types and often cannot be cultured using standard culturing techniques (Kowalchuk, 1999; Borneman & Hartin, 2000; van Elsas *et al.*, 2000). Therefore, this study applies 18S rRNA and 5.8S rRNA gene sequences and internal transcribed spacer (ITS) sequence analyses as recommended molecular methods for diversity studies on fungi within a broad taxonomic range (Kowalchuk, 1999). Because it became apparent that fungi play a meaningful role in wastewater treatment, appropriate molecular methods for the identification of these organisms will be of interest for a broad research community. Thus, this study includes improvements in methods such as DNA extraction for filamentous fungi from wastewater, evaluation and preparation of appropriate primer sets to assess the diversity of fungi in the samples and an evaluation of the phylogenetic markers used.

Materials and methods

Reactor setup and sampling

Biomass was enriched in two cylindric lab-scale SBRs. Seed sludge was obtained from the municipal wastewater treatment plant Garching, Germany. The SBRs were operated with different wastewater. SBR1 with a volume of 12 L was fed with malthouse wastewater prepared by mixing barley dust together with tap water as described elsewhere (Wilderer & McSwain, 2004). SBR2 with a volume of 9 L was operated with synthetic wastewater according to previous recommendations (Moy *et al.*, 2002). The mean pH values in the SBRs were between 7.5 and 8.0. All further physical and chemical reactor operation parameters were set as described previously (Weber *et al.*, 2007). Samples of 10 mL sludge from SBR1 were taken continuously two times per week from reactor start (early phase, floccular seed sludge) to 2 weeks after granulation (late phase, floccules have condensed to compact spherical granules with a dense core zone and a loosely structured fringe zone as described in Weber *et al.*, 2007). At the time of sampling, SBR2 was already operated since 18 months as a fully granulated 'steady state' reactor as described elsewhere (Weber *et al.*, 2007). Therefore, exclusively granular sludge in samples of 10 mL volume from SBR2 was taken twice per month over a period of 4 months. Fifty samples from SBR1 and 20 samples from SBR2 received from all granulation phases were subjected to microscopic investigations and DNA isolation. The presence of fungal spores and hyphae was monitored microscopically using a stereo microscope

(Stemi SV11, Carl Zeiss AG, Oberkochen, Germany; magnification up to $\times 105$). Single granules, which contained many fungi, were selected for further examination of their 18S rRNA gene sequence. Scanning electron microscopy was performed as described previously (Weber *et al.*, 2007).

DNA extraction

Fungi possess a diverse and very stable chemical and physical cell wall. For this reason, no universal DNA extraction method for fungi exists (Selitrennikoff, 2001; Chen *et al.*, 2002; Yeo & Wong, 2002; Karakousis *et al.*, 2005). DNA extraction was carried out applying three different methods in order to compare the received DNA yield and to identify the most adequate isolation protocol for fungi from granular sludge samples, using: (1) The Dneasy® Tissue Kit (Qiagen GmbH, Hilden, Germany), following the guidelines of the manufacturer for 'purification of genomic DNA from yeast', which includes enzymatic lysis of the fungal cell wall with Lyticase. (2) A bead beater to break cell walls mechanically following the protocol 'Simultaneous RNA/DNA extraction from soil samples and pure cultures' (Lüders *et al.*, 2004). (3) A modified protocol, which combines enzymatic and mechanical cell wall lysis, implementing a bead-beating step in the kit protocol after enzymatic treatment of the cells with Lyticase. The success of genomic DNA purification was proved by documentation with 1% agarose gel electrophoresis. Seventy samples were subjected to DNA isolation.

PCR and cloning

Fungal DNA was specifically amplified from the crude DNA extract with fungi-specific primers targeting the 18S rRNA gene and the conterminal ITS region, including ITS1, ITS2, and the 5.8S rRNA gene. The organization of the fungal rRNA operon is shown elsewhere (White *et al.*, 1990). The target sites of the primers were chosen to receive preferably the complete 18S rRNA gene sequence and the ITS region. Primers and target sequences were selected and evaluated using the ARB software (Ludwig *et al.*, 2004; http://www.arb-home.de) by comparing $> 41\,000$ rRNA gene sequences of prokaryotes and eukaryotes. Different primer pairs have been selected for the best possible phylogenetic coverage of the fungal diversity in the samples. The forward primers EF4 (Smit *et al.*, 1999) and NS1f (White *et al.*, 1990), both targeting the 18S rRNA gene, and the reverse primers ITS4r (White *et al.*, 1990) and LR1r (Vilgalys & Hester, 1990), both targeting the 28S rRNA gene, were selected for PCR amplification. Additionally, the forward primer EF60f (5′-TGTCTAAGTATAAGCAATT-3′, the homologous primer position for reference organism *Saccharomyces cerevisiae*, EMBL accession number V01335, for the target molecule 18S rRNA gene is 60 bp starting from the 5′-end) was designed and used for this study. EF60f was constructed for this study as a new fungi-specific forward primer. The primers FF390r (Vainio & Hantula, 2000), 18S-1101-CIL-S-f (Fried, 2002), and the primers M13f and M13r (specific for the TOPO TA cloning vector, TOPO TA Cloning Kit, Invitrogen, Carlsbad, CA) were selected for sequencing purposes. Considering the ARB probe match tool, the primers EF60f and EF4f were fungi-specific while NS1f may also detect some protozoan species. The amplification of the 18S rRNA gene, together with the conterminal ITS region, was carried out with NS1f/ITS4r, NS1f/LR1r, EF4f/ITS4r, and EF60f/ITS4r to select for the best primer combinations. PCR was carried out with genomic DNA samples using the TaKaRa ExTaq™ polymerase system (TaKaRa Shuzo Co., Otsu, Japan) according to Chou *et al.* (1992). The PCR reaction of every sample was performed with 5 μL ExTaq™ buffer ($10\times$), 4 μL dNTP-Mix (2.5 mM per nucleotide), 0.5 μL ExTaq™ polymerase enzyme, 0.5 μL primers, each 50 pmol μL^{-1}, 100 ng of DNA, and water added to a total volume of 50 μL. PCR included an initial denaturation step (94 °C, 10 min), followed by 30 cycles (94 °C, 1-min denaturation; '*x*' °C, 1-min primer annealing; 72 °C, 3-min elongation), and a final elongation step (72 °C, 10 min). The optimal annealing temperature ('*x*') for every primer pair was determined by performing gradient PCRs in steps of 0.5 and ± 4 °C around the primer-specific melting temperature (T_m). The optimal annealing temperatures determined for the primer pairs are 53 °C for NS1f/ITS4r, 60 °C for EF4f/ITS4r, and 55 °C for EF60f/ITS4r. Subsequent to PCR, a clone library of PCR fragments with 145 clones was generated for sequencing purposes, using the TOPO TA cloning kit. From 145 PCR fragments, 119 were successfully cloned.

Sequence analysis

Sequencing of 119 clones was performed using the SequiTherm EXCELRII DNA Sequencing Kit-LC (Epicentre, Madison, WI) and using an LI-COR Global IR2 DNA sequencer (66 cm; LI-COR Biosciences, Bad Homburg, Germany). The primer combinations 18S-1101-Cil-S-f/M13r and M13f/FF390r were used to receive the complete sequence of the 18S rRNA gene and the ITS region. FF390r is a fungi-specific primer. 18S-1101-Cil-f is a forward primer that was already successfully used for sequencing the 18S rRNA gene of eukaryotes, for example ciliated protozoa (Fried, 2002). After verification of this primer in the database, it became apparent that it can also be used for sequencing of fungal 18S rRNA gene sequences. Incomplete sequences and, after a first phylogenetic comparison of all received clone sequences, those of protozoan origin were discarded. The sequences from 41 of 65 fungal clones, which were originally received with the PCR primer pairs EF4f/ITS4r and EF60f/ITS4r, were used for further phylogenetic

analysis. Raw sequence data were analyzed with the E-SEQ software (LI-COR Biosciences). All sequences were tested for chimeras with an ARB intrinsic software for the chimera check of prokaryotes and eukaryotes. No chimeras were found.

Phylogenetic analysis

Phylogenetic analysis was performed with 41 sequences using the alignment and tree calculation methods of the ARB software package. The new 18S rRNA gene sequences were added to an existing ARB-alignment for the 18S rRNA gene sequence with the ARB primary and secondary structure editor. For the ITS region sequences, an extra ITS database was created, together with public sequences obtained from the EMBL database (http://www.ebi.ac.uk/embl). A new alignment was created with the help of CLUSTAL W implemented in ARB. The ITS region and the 18S rRNA gene sequence were separately aligned. Likewise, phylogenetic trees of the 18S rRNA gene sequence and ITS region sequences of all 41 clones were calculated separately and compared afterwards. Phylogenetic consensus tree constructions were based on distance matrix, maximum parsimony, maximum likelihood, and tree puzzle methods following the recommendations for phylogenetic analysis as described previously (Ludwig *et al.*, 1998). A minimum similarity filter was chosen, which only retained positions conserved in at least 50% of the selected sequences.

Results and discussion

Phylogenetic analysis of the clones

Because very little is known about the diversity of fungi occurring in SBR systems, we attempted to characterize the fungi of aerobic sewage granules from two different wastewater treatment approaches. Both difficulties in culturing and in micromorphological differentiation required the application of molecular methods to assess the fungal diversity (Kowalchuk, 1999). The use of 18S rRNA gene analysis in the field of phylogeny is common and well established for many eukaryotic organisms (White *et al.*, 1990; Aleshin *et al.*, 1998; Smit *et al.*, 1999; Borneman & Hartin, 2000; Fried, 2002; Hughes & Piontkivska, 2003; Schaap *et al.*, 2006) and was applied for this study. Forty-one rRNA gene clones were received from malthouse and artificial wastewater samples. Their overall phylogenetic classification is shown in Fig. 2. Within both SBR wastewater approaches, representatives of the *Ascomycota* and *Basidiomycota* were identified. Sequences representing the *Basidiomycota* were assigned exclusively to the *Tremellomycetes*. Sequences representing the *Ascomycota* were assigned to the taxonomic groups *Pleosporaceae*, *Xylariales*, *Theleobolaceae*, *Claviceps* and relatives, *Aureobasidium* and relatives, and to the *Candida boleticola* cluster. Artificial wastewater samples contained representatives of *Tremellomycetes*, and *Aureobasidium* and relatives. The fungal diversity in malthouse samples was clearly higher. In these samples, all of the above-listed taxonomic groups of the *Ascomycota* and *Basidiomycota* were identified. Some studies reported that the fungal 18S rRNA gene sequence can only be used for analysis of species that are distantly related (White *et al.*, 1990). Here, the use of 18S rRNA gene sequence analysis allowed a phylogenetic differentiation down to the levels of families or genera. Some clones representing organisms assigned to the *Pleosporaceae* clustered closely with *Phoma herbarum* and *Pleospora rudis* and some others indicated a monophyletic group related to *Leptosphaeria* sp. Two clones from artificial wastewater and two clones from malthouse wastewater assigned to the *Aureobasidium* and relatives cluster showed sequence similarities to rRNA primary structures from *Aureobasidium pullulans* and *Discosphaerina phagi*. A member of the *Theleobolaceae* indicated by clone 5 clustered with *Theleobolus stercoreus* and a relative of *Monographella nivalis* within the *Xylariales* was represented by clone 15. A very clear relationship with all species of the genus *Candida* currently listed in the GenBank database was found for a

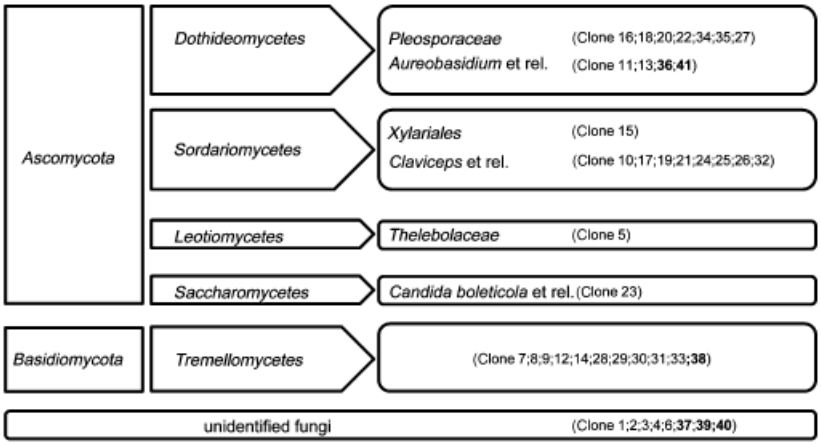

Fig. 2. Overview of the affiliation of clone sequences obtained from malthouse (marked in bold) and artificial wastewater samples to different fungal taxonomic groups according to 18S rRNA gene sequence analysis. Most clones are assigned to different groups of the *Ascomycota*. Clone sequences representing the *Basidiomycota* were assigned exclusively to the *Tremellomycetes*. Some clones derived from artificial wastewater could not be assigned to any defined group of fungi and are thus, listed as unidentified fungi.

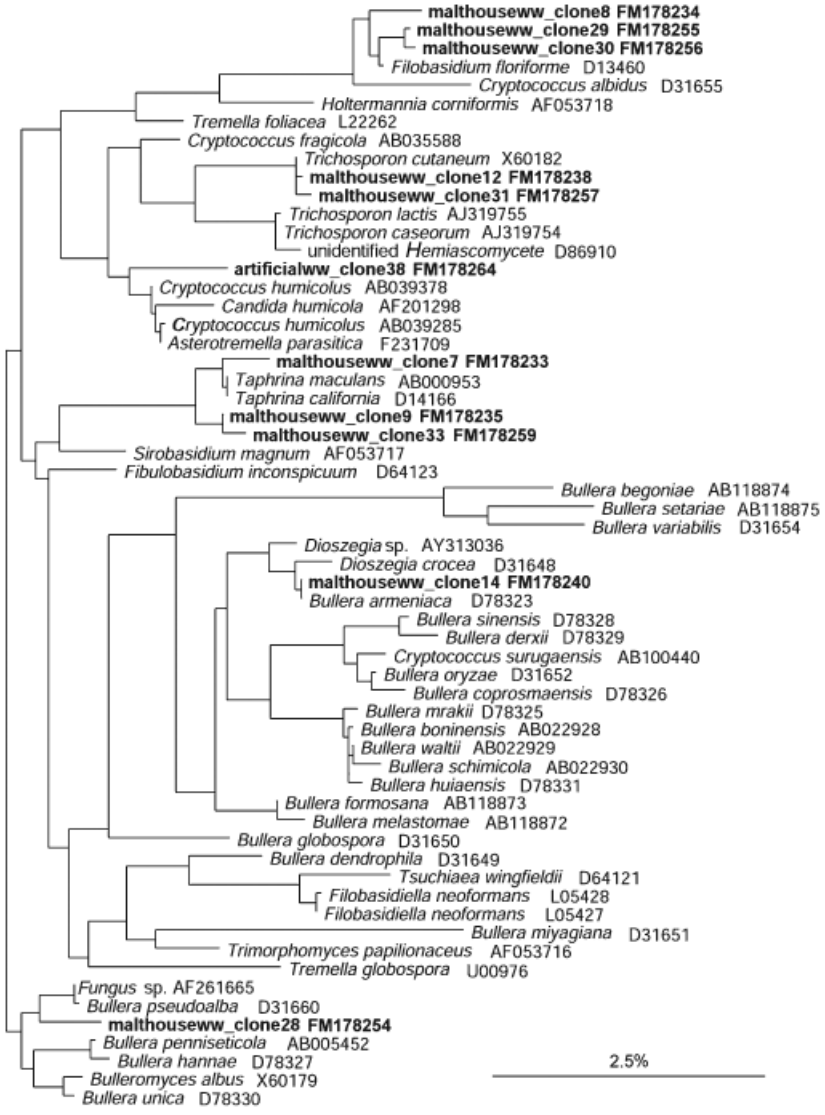

Fig. 3. Maximum likelihood-based tree reflecting the phylogeny of the *Tremellomycetes* based on 18S rRNA gene sequence comparisons. Bold indicates the sequences obtained during this study. Numbers behind taxonomic names represent EMBL sequence accession numbers. The scale bar indicates 2.5% estimated sequence divergence. Note: two representatives of the genus *Taphrina* (*Ascomycota*, *Taphrinomycotina*) group within the *Tremellomycetes*, which is not in accordance with its systematic assignments. Therefore, the GenBank sequence entries of *Taphrina maculans* AB000953 and of *Taphrina california* D14166 should be revised.

yeast taxon represented by clone 23. However, no stable intragroup branching pattern could be found. Therefore, the respective organism could not be clearly assigned to its next phylogenetic neighbors within the *C. boleticola* cluster. An example of a phylogenetic tree representing the 18S rRNA gene-based assignment of clones within the *Tremellomycetes* is shown in Fig. 3. Some of these clone sequences clustered with homologous sequences from *Filobasidium floriforme*, *Trichosporon cutaneum*, *Cryptococcus* sp., *Bullera* sp., and *Dioszegia* sp. Within this cluster, two representatives of the genus *Taphrina* (*Ascomycota* and *Taphrinomycotina*) can also be found. Systematically, *Taphrina* is assigned to the *Ascomycota*, *Taphrinomycotina* and does not fit into the *Tremellomycetes*. Thus, the GenBank sequence entries of *Taphrina maculans* AB000953 and of *Taphrina california* D14166 must be revised.

In both approaches, some fungal clone sequences, which could not be clearly assigned to those from any defined fungal taxon, were found. The overall sequence similarities of this unidentified group to their next phylogenetic neighbors *Rozella allomyctis*, *Physioderma dolicii*, and *Physioderma maculare* are 87.6%, 88.8%, and 87.2%, respectively.

In addition to 18S rRNA gene analysis, ITS markers were used for a more refined differentiation because the ITS regions by themselves were reported as highly variable and recommended to be adequate for analyses with high phylogenetic resolution (Chen *et al.*, 2001; Manter & Vivanco, 2007). In our study, the phylogenetic assignment of the 18S rRNA gene sequences to taxonomic groups was always consistent with the assignment of the associated ITS sequences in these taxa. However, the intragroup resolution power as well as branching pattern significance and stability

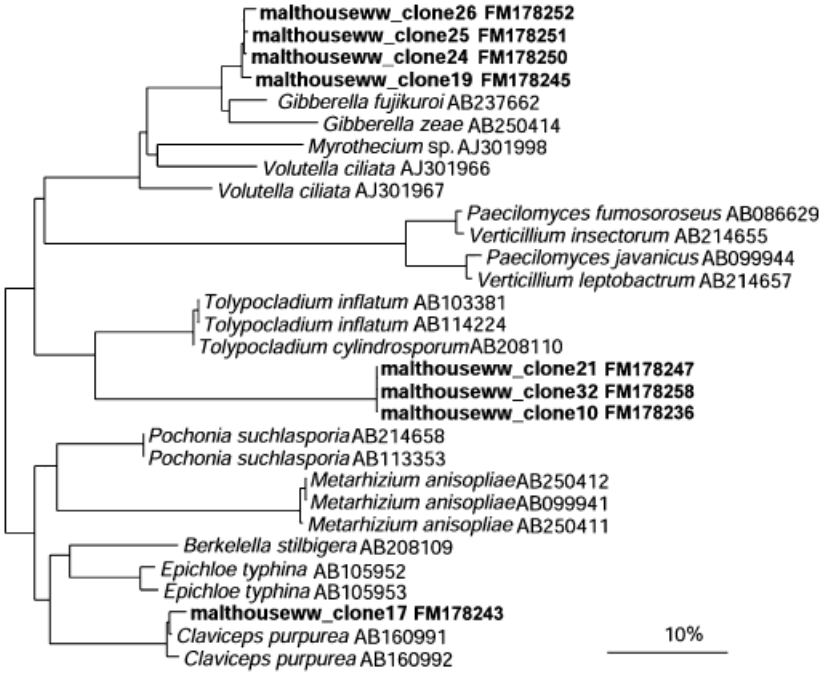

Fig. 4. Maximum likelihood-based phylogeny of the *Claviceps* et rel. cluster based on ITS sequences. Malthouse wastewater clones formed a monophyletic group with *Tolypocladium* sp. or with *Claviceps purpurea*. Some clones clustered with *Gibberella* spp. Bold indicates sequences obtained during this study. Numbers behind taxonomic names represent EMBL sequence accession numbers. The scale bar indicates 10% estimated sequence divergence.

are different for the two markers and a generally higher discriminatory power is expected for the ITS sequences. This could be shown for the *Claviceps* and relatives group (Fig. 4). The ITS tree of *Claviceps* and relatives contains one clone sequence with similarity to *Claviceps purpurea* and three clone sequences, clones 21, 32, and 10, representing organisms of a monophyletic group with *Tolypocladium* spp. The sister group status of *Tolypocladium* and the three clone sequences was significantly supported in the ITS tree, but not resolved in the 18S rRNA gene sequence phylogeny (data not shown). However, for all other ITS clone sequences, no refinement compared with 18S rRNA gene trees was possible because the number of publically available ITS reference sequences for the respective groups is still too small. Considering the current growth of available data and the enormously fast sequencing advances in molecular biology, the use of ITS sequences is also regarded as an adequate and important tool supporting and extending rRNA gene-based phylogenetic analysis. However, before it exerts its full use, there is an indispensable need to expand the dataset, especially for complex environmental samples that contain many phylogenetically quite diverse fungi. This study provides 41 new ITS region sequences and represents one more step toward the aim of an extended ITS database.

Fungi occurring in early- and late-phase granular biofilms

The use of granular sludge technology creates new possibilities for the application of fungi in wastewater treatment. Fungal hyphae cause no bulking problems when implemented in compact sewage granule biofilms. Concurrently, the manifold metabolic characteristics of fungi can be used for wastewater treatment purposes. Therefore, especially those fungi were of interest, that were present in the early phase with floccular seed sludge and also in the late phase after granular biofilm development. These fungi may contribute particularly to the biofilm structure of the sludge granules. The results of this study showed that most of the investigated fungi could be found before and after granulation.

In malthouse wastewater *Claviceps* and relatives, *Tremellomycetes* and *Pleosporaceae* were found in both phases. Some representatives of the order *Tremellomycetes*, which were the only fungi additionally identified in the granular samples from artificial wastewater, are saprotrophic organisms that find an adequate environment for growth in wastewater. Several other clones indicated organisms, assigned to the *Pleosporaceae*, which clustered, for example, with *P. herbarum*, which is a plant pathogen. They may be originally derived from the barley dust that was continuously supplied to the reactor.

Representatives of *Aureobasidium* and relatives could be found in malthouse wastewater samples before, but not after granulation. However, they were detected in granules from artificial wastewater when fungal filaments were isolated directly from single granules and identified by sequence analysis as representatives of the *Aureobasidium* and relatives taxonomic group. These clones showed high sequence similarities to *A. pullulans*, which occurs often in activated sludge (Subramanian, 1983). Here, they contributed to the

granular biofilm structure. The hyphae serve as a substratum for bacteria to grow and act like skeletal elements in the granule architecture (Weber *et al.*, 2007; Fig. 1). Hence, the interactions of fungi and bacteria represent another important aspect in biofilm development. Fungal–bacterial interactions were already investigated in the fields of medical science, food microbiology, or ecology of the soil and rhizosphere, and it was shown that bacteria can enhance or suppress the filamentation of fungi (Wargo & Hogan, 2006). Therefore, future research of complex wastewater biofilms should also focus on such bacterial–fungal relationships.

Methodological findings and improvements

This study provides some methodological aspects regarding DNA extraction and PCR primers.

We tested three different DNA extraction protocols to isolate fungal DNA from wastewater samples. The visualization of purified eukaryotic and prokaryotic genomic DNA on agarose gels showed that DNA extraction was feasible with all described methods. However, the yield of amplified 18S rRNA gene sequences and ITS fragments varied with the DNA isolation method. Fungal DNA fragments could successfully be amplified from 44% of the tested samples that were handled with the bead beater isolation protocol. After DNA purification with the DNA Tissue Kit or the modified protocol, a PCR fragment yield of 60% could be achieved, respectively. Therefore, the enzymatic and the combined enzymatic–mechanical approach can be used for the extraction of fungal DNA from wastewater samples. However, the authors recommend the use of the combined approach because enzymatic extraction alone may be sometimes poor for filamentous fungi. Thus, according to another study on the efficiency of DNA extraction methods for medically important fungi (Karakousis *et al.*, 2005), we succeeded with the use of a commercial enzymatic kit with an implemented mechanical step.

Following the successful DNA extraction, adequate PCR primer pairs were tested. The primers used for PCR should fulfill three requirements: (1) they should cover the phylogenetic spectrum in the database as far as possible, (2) discriminate against other eukaryotes, and, due to their target sites, (3) allow amplification of the complete fungal 18S rRNA gene and the conterminal ITS region. Some previously published primers (NSA3, NSI1, 58A1F, 58A2F, 58A2r, 58A1F, 58A2F, and 58A2r; Kendall & Rygiewicz, 2005) could not be used because they amplify only parts of the 18S rRNA gene and the ITS regions. Additionally, due to the highly conserved nature of the 18S rRNA gene, no previously published primer pair could be used, nor could a new one be designed that would cover all fungal 18S rRNA gene sequences in the database and exclude other eukaryotic organisms. Thus, we followed the recommendation of Kendall & Rygiewicz (2005): it is necessary to use multiple primer sets for the identification of fungi in natural habitats to include the range of organisms under study.

The four primer combinations described in Materials and methods were tested for amplification of the 18S rRNA gene and the ITS regions. NS1f is known to detect a wide variety of fungi and other eukaryotes (White *et al.*, 1990). It was tested in our database with the probe match tool of ARB for the specificity for fungi. The primer matched without any mismatch most fungal sequences and additionally some protozoa, including species of the genera *Epistylis*, *Vorticella*, and *Carchesium* (phylum *Ciliophora*), which can often be found in wastewater. Nevertheless, we attempted to amplify fungal sequences with the primer combinations NS1f/ITS4r and NS1f/LR1r taking into account that some amplified DNA would be of protozoan origin because several protozoa were observed in our samples. However, the use of NS1f/LR1r did not lead to the amplification of DNA fragments and nearly all sequences received with NS1f/ITS4r turned out to be protozoan DNA even under modified PCR conditions with different annealing temperatures. Therefore, we decided that NS1f was not useful to detect fungi in our samples.

EF60f/ITS4r and EF4f/ITS4r were adequate for the specific amplification of fungal DNA. The EF60f primer was constructed during this study taking into consideration that the best possible coverage of fungi in our database was guaranteed and no protozoan rRNA gene sequence was amplified. Additionally, EF60f should allow the amplification of all fungi in the database that could not be covered with EF4f. In our database, EF60f and EF4f together covered 1372 different fungal sequences (*c.* 70%) from a total of 1991 without any mismatch. Performing PCR with EF60f and EF4f resulted in 18S rRNA gene sequence fragments of *c.* 1740 and 1600 bp, respectively. The length of the ITS region can vary between 50 and 1050 bp (Ranjard *et al.*, 2001) and was between 156 and 721 bp for our clones.

The results showed that sequences received with the primer pairs EF60f/ITS4r and EF4f/ITS4r from the same samples were assigned to different taxa, except for representatives of *Claviceps* and relatives, which could be amplified with both forward primers. The use of EF60f combinations did not lead to the amplification of DNA fragments from artificial wastewater samples, implying that specific target organisms for that primer could not be detected in these samples. All 18S rRNA gene sequences and ITS fragments from artificial wastewater were amplified only with the primer combination EF4f/ITS4r. A previously described preference of EF4f primer combinations to target the 18S rRNA gene of *Ascomycota* extracted from soil (Kowalchuk & Smit, 2004) could not be observed for the wastewater samples in our study. In contrast, all representatives of the *Basidiomycota* were detected with EF4f primer

combinations, and representatives of the *Ascomycota* were mainly identified with EF60f ones.

Conclusions

This study represents a combined approach to assess the diversity of fungi and their possible role in granular SBR biofilm samples by improving the required molecular techniques such as DNA extraction and the application of adequate primer sets for 18S rRNA gene and ITS amplification of fungi. The sequence data were used for phylogenetic analysis.

Regarding the common knowledge of the metabolic potential of fungi and the high fungal diversity presented in this study, the role, diversity, function, and application of fungi in activated sludge processes should be further examined. It was shown that some of the identified fungi might be involved in the structural buildup of granular sewage biofilms because they could be found before and after granulation and were implemented in the granules. The specific development of technical procedures to integrate fungal filaments in compact granular sludge will support the purposeful use of fungi in wastewater treatment.

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