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Enhancing the stability of fungal-bacterial aerobic granular sludge using nitrogen-fixing consortia

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Abstract
The stability of aerobic granular sludge can depend on nitrogen availability and changes in cultivation conditions. In this study, model fungal-bacterial aerobic granules were obtained using *Penicillium brasilianum*, *Engyodontium album* and *Fusarium oxysporum* together with a nitrogen-fixing bacterial community comprising representatives of the genera *Agrobacterium*, *Bacillus* and *Microbacterium*. The model systems were cultivated under nitrate-containing and nitrate-free conditions and subjected to hydrogen peroxide stress and prolonged substrate limitation. Aggregate morphology, nitrogen compound transformation, COD and microbial composition were evaluated during cultivation. The presence of the bacterial community was associated with the formation and preservation of denser aggregates during prolonged cultivation and under nitrogen-limited conditions. Stable granules contained both fungal and bacterial components, with fungal CFU accounting for 54–61% and bacterial CFU for 39–46% of the culturable microbial fraction. In nitrate-free systems, up to 0.7 g/L ammonium was transformed within 3–4 days without marked nitrate accumulation, whereas ammonium transformation in nitrate-containing systems was slower and accompanied by nitrate accumulation. COD decreased to almost zero within approximately 4 days in the more stable granular systems. Quantitative PCR confirmed the retention of *Agrobacterium*, *Bacillus* and *Microbacterium* in the model granules. The results show that fungal-bacterial granular structures can be maintained under nitrate-limited conditions. The observed nitrogen transformation may be associated with the combined activity of fungal and bacterial components, which makes such model systems promising for further investigation of nitrogen transformation processes in fungal-bacterial granules.

Keywords Aerobic granular sludge · AGS · Fungal-bacterial granules · Nitrogen-fixing community

1 Introduction

Aerobic granular sludge is a form of activated sludge characterized by the formation of compact microbial aggregates. Compared with conventional activated sludge, granular sludge has a number of advantages, including high biomass concentration, good settling properties and the possibility of simultaneous removal of organic matter and nitrogen compounds [1–4].

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The formation and stability of aerobic granules depend on cultivation conditions and microbial composition. Granulation is usually carried out in sequencing batch or fill-and-draw reactors, where repeated cycles of feeding, settling and removal of part of the culture liquid create conditions for selection of dense aggregates [1, 2, 5]. Extracellular polymeric substances are also involved in adhesion of microbial cells and formation and stabilization of the granule structure [6–8].

The structure of aerobic granules is heterogeneous. Differences in oxygen and substrate concentrations can occur between the outer and inner parts of the granule, which allows microorganisms with different metabolic properties to coexist within one aggregate [3, 4, 9]. At the same time, changes in cultivation conditions can affect the structure and stability of the formed granules [5, 10, 11].

Filamentous microorganisms, including fungi, can also be involved in the formation of granular structures. Their presence may have both positive and negative effects on granule formation. Excessive development of filamentous microorganisms can lead to loosening of the granule structure, whereas fungal hyphae can provide a surface for bacterial attachment and development of mixed microbial aggregates [12, 13]. Fungi have also been detected in aerobic sewage granules using molecular methods, including analysis of 18 S rRNA genes and ITS regions [14].

Fungal-based granular structures may have specific properties that distinguish them from conventional bacterial aerobic granules. The filamentous growth of fungi provides an extended surface for microbial attachment and can contribute to the formation of larger and more porous aggregates. Fungal components have also been associated with nitrogen transformation, including nitrate and nitrite reduction. Several fungal species are capable of dissimilatory nitrate reduction with formation of nitric oxide (NO), nitrous oxide (N_2O) and, in some cases, dinitrogen (N_2) [25–27]. In particular, *Fusarium oxysporum* has been shown to perform respiratory nitrate and nitrite reduction, with N_2O reported as a major product under the studied conditions [26, 27]. These processes indicate that fungi may contribute to nitrogen transformation through pathways that are different from those usually considered for bacterial nitrification and denitrification.

Recent studies have also shown that filamentous fungal pellets can develop in aerobic granular sludge and may contribute to nitrogen removal. In a long-term sequencing batch reactor study, fungal pellets containing *Bradymyces*and *Knufia* were associated with denitrification activity, with stronger activity observed toward nitrite than nitrate [28]. However, fungal nitrogen transformation in aerobic granular sludge remains insufficiently studied, and the contribution of individual fungal species to nitrogen removal under different cultivation conditions requires further investigation.

An additional problem in biological wastewater treatment is the lack of available nitrogen. Some industrial wastewaters contain sufficient amounts of organic carbon but have a low concentration of nitrogen compounds required for microbial growth. Under such conditions, biomass formation and biological treatment can be limited by nitrogen deficiency [15]. One possible approach is the use of nitrogen-fixing microorganisms capable of providing biologically available nitrogen under nitrogen-limited conditions [16, 17].

The use of nitrogen-fixing microorganisms in granular systems is of particular interest because the bacterial component can be associated with the structural microbial matrix of the granule. Fungal-bacterial interactions may be especially important under nitrogen-deficient conditions. Recent studies have shown that nitrogen-fixing bacteria can be associated with fungal components in aerobic granular systems and participate in their functioning under nitrogen limitation [18].

Previously, a nitrogen-fixing bacterial community associated with aerobic granular sludge was isolated and characterized. The community included representatives of the genera *Agrobacterium*, *Bacillus* and *Microbacterium* [19, 20]. Dense aerobic granular sludge obtained in a previous study [21] was used as the source of fungal cultures. The isolated fungal cultures were subsequently characterized using molecular methods. Three cultures were selected from the isolated fungal cultures and used to construct model fungal-bacterial systems.

In the present study, model systems were prepared using the fungal cultures *Penicillium brasilianum*, *Engyodontium album* and *Fusarium oxysporum* together with the nitrogen-fixing bacterial community. The systems

were cultivated under nitrate-containing and nitrate-free conditions. The effects of nitrogen availability, hydrogen peroxide stress and starvation on aggregate formation, nitrogen compound transformation and microbial composition were studied.

The aim of this work was to study the formation and functioning of model fungal-bacterial aerobic granules based on filamentous fungal cultures and a nitrogen-fixing bacterial community under nitrate-containing and nitrate-free conditions.

2 Materials and methods

2.1 Microbial cultures

The fungal cultures used in this study were isolated from dense, stable aerobic granules obtained in a previous study on the formation and stability of aerobic granular sludge under stress conditions [21]. From the fungal cultures isolated from these granules, three cultures were selected to construct the model fungal-bacterial systems.

Three filamentous fungi were used as structure-forming cultures: *Penicillium brasilianum* SK28, *Engyodontium album* SK29, and *Fusarium oxysporum* SK30. The fungal cultures were used individually or in combination for the formation of model aggregates.

The bacterial component was represented by a nitrogen-fixing community comprising *Agrobacterium tumefaciens* SK25, *Bacillus amyloliquefaciens* SK27, and *Microbacterium oxydans* SK26. The bacterial community had been isolated and characterized previously [19, 20]. The nitrogen-fixing community was maintained on modified Ashby medium. Before use in the experiments, inoculum was prepared from freshly cultivated biomass.

The corresponding bacterial sequences were deposited in GenBank under the following accession numbers: *(A) tumefaciens* SK25, PZ896037; *M. oxydans* SK26, PZ896038; and *(B) amyloliquefaciens* SK27, PZ896039. The fungal sequences were deposited under the following accession numbers: *P. brasilianum* SK28, PZ896270; *E. album* SK29, PZ896271; and *F. oxysporum* SK30, PZ896272.

2.2 Model sludge cultivation

Twelve model aerobic sludge systems were prepared to evaluate the effect of fungal composition, the presence of the nitrogen-fixing bacterial community, and the availability of external nitrate nitrogen. The composition of the model systems is presented in Table 1.

Systems 1–4 contained fungal cultures without the nitrogen-fixing community and were cultivated in the presence of KNO_3. Systems 5–8 contained fungal cultures together with the nitrogen-fixing community and were

Table 1 Composition of the model systems

System	*F. oxysporum* SK30	*E. album* SK29	*P. brasilianum* SK28	Nitrogen-fixing community	KNO_3
1	+	+	+	−	+
2	+	−	−	−	+
3	−	+	−	−	+
4	−	−	+	−	+
5	+	+	+	+	+
6	+	−	−	+	+
7	−	+	−	+	+
8	−	−	+	+	+
9	+	+	+	+	−
10	+	−	−	+	−
11	−	+	−	+	−
12	−	−	+	+	−

cultivated in the presence of KNO_3. Systems 9–12 contained fungal cultures together with the nitrogen-fixing community and were cultivated without addition of KNO_3.

Cultivation was carried out in 250-mL Erlenmeyer flasks containing 100 mL of culture medium. The process was performed in a periodic fill-and-draw mode. After cultivation, the biomass was allowed to settle for 5–10 min, after which 60–70% of the culture liquid was removed and replaced with fresh medium. Subculturing was performed every 4–7 days depending on the experimental stage and condition of the biomass.

Modified Gause medium was used for cultivation. The medium contained 2.5 g/L starch, 0.5 g/L K_2HPO_4, 0.5 g/L $MgSO_4 \cdot 7\ H_2O$, and 10^{-4} g/L $FeSO_4 \cdot H_2O$. For nitrate-containing systems, KNO_3 was added at a concentration of 0.5 g/L, whereas nitrate-free medium did not contain KNO_3.

The COD load in the model systems was approximately 400 ± 50 mg/L. The model systems were prepared under aseptic conditions using the defined fungal cultures and the previously characterized nitrogen-fixing bacterial community.

2.3 Stress exposure and starvation

Hydrogen peroxide was used as an oxidative stress factor. The stress-adaptation procedure was performed according to the conditions established in a previous study [21]. H_2O_2 was initially added at a concentration of 0.3 g/L. Following addition of hydrogen peroxide, the cultures were incubated for 2 h on a shaker, after which the culture medium was replaced with fresh medium. During subsequent adaptation passages, the H_2O_2 concentration was increased to 0.6 g/L.

The formed aggregates were subsequently cultivated under conditions of prolonged substrate limitation. The starvation stage was continued for 4 weeks, with sampling of the culture liquid and aggregates at the defined experimental points.

2.4 pH regulation and morphological analysis

The pH of the culture medium was monitored during cultivation. When necessary, the pH was adjusted manually using hydrochloric acid (HCl) or sodium hydroxide (NaOH). During the experimental procedure, the pH was adjusted to 6.5 and subsequently to 7.0.

The morphology of the aggregates was monitored by visual observation and light microscopy. The shape, size, compactness, and general structural characteristics of the aggregates were recorded during cultivation.

Light microscopic observations were performed using a Nikon Eclipse TE2000-E microscope (Japan).

Selected aggregates were examined by scanning electron microscopy (SEM). SEM was used to characterize the structure of the aggregates, including the distribution of fungal hyphae, bacterial cells and internal inclusions.

2.5 Chemical analysis

The concentrations of ammonium, nitrite, and nitrate in the culture liquid were determined by photometric methods. Ammonium nitrogen was additionally determined using Nessler's reagent.

Total nitrogen in biomass was determined by the Kjeldahl method. Crude protein content was calculated using a conversion factor of 6.25.

Chemical oxygen demand (COD) was determined by the dichromate method and expressed as mg/L.

Samples of the culture liquid were collected at the defined stages of cultivation, including active cultivation, stress adaptation, and starvation.

2.6 Microbiological and molecular-genetic analysis

The microbial composition of selected model aggregates was analyzed by cultivation methods and molecular-genetic approaches.

For microbiological analysis, samples of the formed aggregates were inoculated onto solid nutrient media. The resulting colonies were counted and expressed as colony-forming units (CFU). The fungal and bacterial fractions were determined for selected stable model aggregates.

For molecular identification, DNA was isolated from pure microbial cultures. Bacterial isolates were identified by amplification and sequencing of fragments of the 16 S rRNA gene.

For fungal cultures, short fragments of the 18 S rRNA gene were initially used for preliminary identification. Since these fragments did not provide sufficient taxonomic resolution, the fungal isolates were subsequently analyzed using the internal transcribed spacer (ITS) region. The final taxonomic assignments reported in the present study are based on the updated ITS analysis.

The bacterial components of the nitrogen-fixing community were quantified by real-time PCR using TaqMan technology. The analysis targeted the 16 S rRNA genes of *Agrobacterium*, *Microbacterium*, and *Bacillus*. Genus-specific primer–probe systems were used for quantitative detection. The primer–probe systems were designed based on 16 S rRNA gene sequences available in GenBank and have been described previously [22]. The qPCR procedure and amplification conditions were performed as described previously [22].

3 Results and discussion

3.1 Formation of model granules

Granule formation depended on the fungal composition of the system, the presence of nitrate in the medium and the addition of the nitrogen-fixing community. Systems without the bacterial community did not preserve stable granules during prolonged cultivation. System 1, which contained all three fungal cultures without the bacterial community, mainly formed loose irregular aggregates.

Systems with *Engyodontium album* showed weak granulation. In systems 3, 7 and 11, large loose conglomerates appeared at the initial stages. After pH regulation, the aggregates partly changed their structure: the main loose biomass was disrupted, and smaller dense aggregates appeared. These structures, however, remained less uniform than the aggregates formed in systems with *Penicillium brasilianum*.

Systems based on *Fusarium oxysporum* showed more active aggregation. In system 2, large rounded aggregates up to 10 mm in diameter were formed. In system 10, smaller granules of about 1–6 mm were obtained. These granules had a denser central region but were heterogeneous in size and morphology.

The most uniform granules were obtained in systems containing *P. brasilianum*. Systems 4, 8 and 12 formed rounded granules of about 2–3 mm. In the presence of the bacterial community, these structures were preserved better during further cultivation.

After prolonged cultivation, starvation and hydrogen peroxide adaptation, the most stable granules were observed in systems 8, 9 and 12. These systems retained dense, smooth granules at the final observation stage. The granules were white, gray or gray-brown depending on the system and cultivation stage. The appearance of a representative granule from system 8 is shown in Fig. 1.

These results are consistent with previous observations that filamentous microorganisms can affect the structure of aerobic granules [12–14]. In the present model systems, the formation and preservation of compact aggregates depended on the composition of both fungal and bacterial components. Fungal cultures alone formed aggregates, but stable compact structures were observed mainly in systems containing the bacterial community.



Fig. 1 Granules from model system 8: biomass in the cultivation flask, isolated granule and granule cross-section. The granule diameter was about 5 mm

3.2 Effect of pH on aggregate morphology

pH changes affected aggregate morphology. At the early cultivation stages, most systems had slightly alkaline pH values. A stronger decrease was observed in nitrate-free systems containing the nitrogen-fixing community. The pH decreased to about 7.6 in system 11 and to about 6.4 in system 12.

During cultivation, pH was adjusted first to 6.5 and subsequently to 7.0. This regulation was accompanied by changes in aggregate morphology. The effect was particularly evident in systems 3, 7 and 11, where the initial large loose conglomerates were partly disrupted and smaller, denser aggregates appeared.

Thus, pH regulation affected aggregate morphology but was not the only factor determining stable granule formation. This is consistent with the general view that aerobic granulation depends on a combination of operating conditions, microbial composition and extracellular matrix formation [6–8, 10].

3.3 Granule microstructure

Microscopy showed that fungal hyphae formed the main framework of the granules. The hyphal network provided attachment sites for bacterial cells and shaped the spatial structure of the aggregate. Bacteria were also detected in matrix-like material in the inner regions of the granules.

Scanning electron microscopy confirmed a close association between the fungal and bacterial components. In compact granules, bacterial cells and clusters were embedded in matrix-like material, while fungal hyphae provided structural connectivity.

Crystalline inclusions were found in the central regions of several compact granules. Their morphology suggested mineral deposits. These inclusions may have contributed to core densification; however, their composition was not determined in the present study and requires elemental analysis. Similar mineral-associated structures have been described in aerobic granular sludge [23]. Matrix material surrounding microbial cells may also contribute to aggregate cohesion and stability [24].

SEM images of selected granules are shown in Fig. 2.

3.4 Response to hydrogen peroxide stress

Hydrogen peroxide caused clear morphological changes in the model systems. During adaptation, the H_2O_2 concentration was sequentially increased from 0.3 to 0.6 g/L. After exposure, loose peripheral biomass gradually decreased, while compact aggregates were retained. The granules became denser, and the previously observed loose, “fluffy” structure decreased.

After adaptation, granules of approximately 2–5 mm were observed in several systems. Compact structures were preserved particularly well in systems containing the nitrogen-fixing community.

These observations indicate that hydrogen peroxide treatment was accompanied by restructuring of the aggregates and preservation of the more compact structures. However, the present data do not allow the effect of hydrogen peroxide to be considered independently from the other factors involved in granule development.

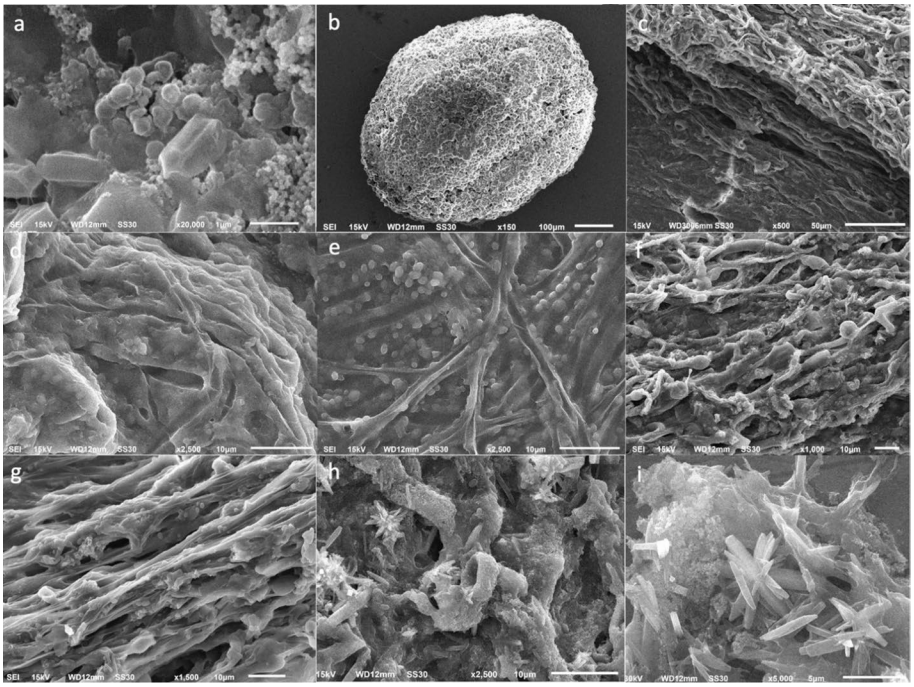


Fig. 2 Scanning electron microscopy images of fungal-based aerobic granules: **a** surface of a loose floc (MS7); **b** outer surface of the granule (MS11); **c** cross-section of the granule wall (MS12); **d** bacterial cluster embedded in extracellular polymeric substances in the granule center (MS12); **e** bacteria immobilized on fungal hyphae (MS12); **f** central section of the granule (MS12); **g** granule wall section (MS12); **h**, **i** crystalline inclusions in the central part of the granule (MS8)

3.5 Dynamics of nitrogen compounds

Nitrogen transformation differed between nitrate-containing and nitrate-free systems. In nitrate-containing variants, nitrate concentrations generally decreased during active cultivation. During starvation and deep starvation, nitrate accumulation was observed, which may have been associated with the release and transformation of nitrogen-containing compounds under carbon-limited conditions.

During hydrogen peroxide adaptation, temporary ammonium accumulation was observed in several nitrate-containing systems. After adaptation, ammonium concentrations decreased to low or undetectable levels. During starvation, ammonium was not detected in the liquid phase.

Nitrate-free systems containing the nitrogen-fixing community showed a different pattern. In systems 9–12, up to 0.7 g/L NH_4^+ was transformed within 3–4 days without marked nitrate accumulation. In nitrate-containing systems 1–8, transformation of a similar ammonium concentration was accompanied by nitrate accumulation and required up to 8 days.

The observed nitrogen transformation may be related to the combined activity of the fungal and bacterial components of the model systems. Fungal nitrate and nitrite reduction has been described for several fungal species. In particular, *Fusarium oxysporum* is capable of dissimilatory nitrate reduction and denitrification under oxygen-limited conditions, with nitrous oxide formation reported as one of the products [25–27]. Fungal pellets have also been obtained in aerobic granular sludge, where their presence was associated with increased denitrification performance, particularly for nitrite [28]. Therefore, fungal nitrate and nitrite reduction may represent one of the possible pathways contributing to nitrogen transformation in the present model systems.

However, nitrate, nitrite and ammonium transformation alone do not allow the contribution of fungal denitrification to be distinguished from other microbial processes. In particular, gaseous nitrogen products were not determined in the present study. Therefore, the observed nitrogen dynamics should not be considered as direct evidence of fungal denitrification.

COD removal was also more pronounced in structurally stable granular systems. In these variants, COD decreased almost to zero within approximately 4 days. In less stable systems, residual COD ranged from 5 to 10 to 40–50 mg/L.

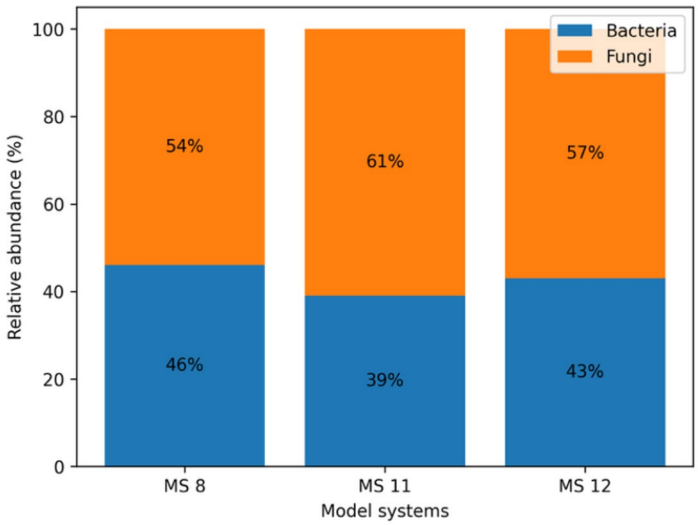


Fig. 3 Fungal and bacterial CFU fractions in selected model granules at the final cultivation stage: systems 8, 11 and 12

Thus, nitrate-free systems containing the bacterial community demonstrated faster ammonium transformation without marked nitrate accumulation. Since nitrogen fixation itself was not measured directly in the present study, these results should be regarded as indirect evidence based on nitrogen compound dynamics, biomass accumulation and preservation of the systems in the absence of an external nitrate source.

3.6 Retention of the nitrogen-fixing community

Quantitative PCR confirmed the presence of *Microbacterium*, *Agrobacterium* and *Bacillus* in model systems containing the nitrogen-fixing community. The bacterial community was retained in both nitrate-containing and nitrate-free systems.

The relative abundance of its individual components changed during cultivation. *Microbacterium* increased following nutrient supply, whereas *Agrobacterium* became more pronounced during starvation. In granular systems 8–12, the relative contribution of *Microbacterium* varied considerably between sampling points and reached 10–98% of the detected bacterial community under individual cultivation conditions.

In nitrate-free systems, fungal biomass reached 2–4 g/L, which was comparable with nitrate-containing variants. Thus, systems containing the bacterial community were able to maintain fungal biomass formation in the absence of externally supplied nitrate nitrogen.

Previous studies of the same nitrogen-fixing community demonstrated its ability to accumulate nitrogen under nitrogen-limited conditions and characterized the activity of its individual bacterial components [19, 20]. The present results show that these microorganisms were retained within the model fungal-bacterial systems during prolonged cultivation.

3.7 Fungal and bacterial fractions in stable granules

The microbial composition of selected stable granules was determined by plating on solid nutrient media and counting CFU. In all analyzed granules, fungal CFU predominated over bacterial CFU. The fungal fraction accounted for 54–61%, while the bacterial fraction accounted for 39–46%.

In system 8, fungal and bacterial fractions were 54% and 46%, respectively. In system 11, the fungal fraction reached 61%, while the bacterial fraction was 39%. In system 12, these values were 57% and 43%, respectively. The fungal and bacterial fractions in the selected granules are presented in Fig. 3.

Thus, the obtained granules were not purely fungal aggregates but represented mixed fungal-bacterial structures. The fungal component predominated in the culturable fraction, while bacterial cells constituted a substantial

part of the microbial population of the granules. Together with the microscopy and qPCR results, these data indicate that the bacterial community was retained within the formed granular structures.

4 Conclusions

Model fungal-bacterial aerobic granules were obtained using filamentous fungi and a nitrogen-fixing bacterial community under fill-and-draw cultivation.

Fungal cultures without the bacterial community formed loose or irregular aggregates and did not preserve stable granules during prolonged cultivation. The addition of the nitrogen-fixing community was associated with the formation of denser and more persistent fungal-bacterial aggregates.

The most stable dense and smooth granules were obtained in systems 8–12 after prolonged cultivation, starvation and adaptation to hydrogen peroxide. The fungal composition affected the morphology of the aggregates, with *Penicillium brasilianum* associated with the formation of relatively uniform rounded granules.

Nitrate-free systems containing the nitrogen-fixing community transformed up to 0.7 g/L NH_4^+ within 3–4 days without marked nitrate accumulation. In nitrate-containing systems, transformation of a similar ammonium concentration required up to 8 days and was accompanied by nitrate accumulation.

Microscopy showed that fungal hyphae formed the main structural framework of the granules, while bacterial cells were associated with fungal hyphae and matrix-like material. In selected stable granules, fungal CFU accounted for 54–61%, while bacterial CFU accounted for 39–46%.

The data demonstrate that the nitrogen-fixing bacterial community was retained within the fungal-based granules during cultivation under both nitrate-containing and nitrate-free conditions. The obtained results indicate the potential of fungal-bacterial systems for formation of stable granular biomass under nitrogen-limited conditions. However, since nitrogen fixation was not measured directly, the contribution of biological nitrogen fixation to the observed nitrogen transformation requires further investigation.

Author contributions L. S. performed the experimental work, analyzed the results, prepared the figures and wrote the first version of the manuscript.D. G. carried out PCR analysis and contributed to the interpretation of molecular genetic data.A. M. performed scanning electron microscopy and contributed to the interpretation of SEM images.N. K. contributed to the planning of the experimental work and discussion of the results.D. S. contributed to analytical measurements and processing of experimental data.S. K. supervised the work, contributed to the study design and revised the manuscript.All authors read and approved the final manuscript.

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Data availability The nucleotide sequences generated in this study are available in GenBank under the accession numbers listed in Sect. 2.1. The other data supporting the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethical approval and consent to participate Not applicable.

Consent for publication Not applicable.

Previous publication of preliminary data Preliminary data on the isolation, identification and activity of the nitrogen-fixing bacterial community used in this study were reported previously [19, 20]. The qPCR procedure and primer–probe systems used for quantitative analysis of the bacterial components were described previously [22]. The present work extends these studies by investigating the formation, morphology, nitrogen transformation and stability of model fungal-bacterial aerobic granules under nitrate-containing and nitrate-free conditions.

Competing interests The authors declare no competing interests.

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