Formation and structure of granulated microbial aggregates used in aerobic wastewater treatment

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Abstract Granular microbial aggregates are used in aerobic treatment of wastewater. The granules have diverse microbial community and complex spatial structure. The structural elements are radial sub-aggregates, concentric layers, channels, pores, polysaccharide plugs, and an anaerobic core of lysed cells. Aerobic bacteria, consisting of 69–84% of microbial biomass, were concentrated in a layer to the depth of 550 μm from the surface of the granule. Facultative anaerobic bacteria, consisting of 9–13% of microbial biomass, dominated in a layer at a depth from 550 μm to 850 μm from the surface of the granule. Obligate anaerobic bacteria, consisting of 2% of microbial biomass, dominated in a layer on the depth from 850 μm to 1,000 μm from the surface of the granule. A core of dead and lysed cells was at a depth greater than 1,000 μm from the surface of the granule. The depth of the anaerobic layer correlated with the appearance of polysaccharide plugs in the pores. Enrichment cultures of microorganisms with high cell surface hydrophobicity or self-aggregation ability can be used to facilitate the formation of microbial granules.
Keywords Lipophilic tracer; microbial granule; oligonucleotide probe; structure; wastewater treatment

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Introduction

There are known granular microbial aggregates of medical importance (mycetome of pathogenic fungi or actinomycetes in human body), biological importance (bacterial mycetome of insects), and the granules, which are used in food biotechnology (kefir "grains"). Aerobically grown microbial granules are used also for wastewater treatment. The granules with diameters from 1 mm to 10 mm (Tay *et al.*, 2001; Toh *et al.*, 2002) have diverse structure (Tay *et al.*, 2002a, b; Tay *et al.*, 2003). These microbial aggregates have a short settling time and have been actively studied from 1997 (Morgenroth *et al.*, 1997) as the bioagents in the biological treatment of wastewater (Beun *et al.*, 2002; Peng *et al.*, 1999; Etterer and Wilderer, 2001). A first review on the microbial granulation in wastewater treatment was published in 2004 (Liu and Tay, 2004).

One problem in the practical application of microbial granules in aerobic wastewater treatment is the low depth of oxygen penetration into the granules. Due to the dense aggregation of cells the rate of the mass transfer between the medium and the granular matrix for the nutrients and metabolites may not be sufficient to ensure normal cell metabolism at some distance from the edge of the granule. The typical depth of the aerobic zone in a thick microbial biofilm aerated by air is between 50 to 200 μm (Villaverde and Fernandez-Polanco, 1999; Gieseke *et al.*, 2001). It was demonstrated earlier that obligate anaerobic bacteria can grow in the interior of aerobically grown granules (Tay *et al.*, 2002a, b; Tay *et al.*, 2003). Diffusion of nutrients and metabolites in the granule is complicated by the presence of radial sub-aggregates, spherical sub-granules, concentric layers, pores, channels and plugs in the channels.

Another problem in the practical application of microbial granules in aerobic wastewater treatment is the long duration of granule formation lasting usually from 10 to 20 days. Selected microbial cultures ("starter cultures", "seeds"), which enhance the

formation and stability of the granules, can be used to solve this problem. An aim of the research was the study of the optimal structure and formation of microbial granules used in the aerobic treatment of wastewater.

Materials and methods

The microbial granules were produced in a column sequencing batch reactor with a medium containing glucose (for all experiments) or ethanol/acetate (for the experiments on biomass distribution in the granules), as the main source of carbon, as described earlier (Tay *et al.*, 2002a, b). Settling time was 2 min. The diameter of the studied granules varied from 450 μm to 3,000 μm. Selection of cells with high cell surface hydrophobicity was made as described earlier (Stabnikova *et al.*, 1991) using four cycles including the following steps: (a) disintegration of microbial aggregates in the beater (Biospec, USA) for 5 min and filtration through a 35 mm cell strainer cap (Becton Dickinson, USA) to remove the particles larger than 35 mm; (b) collection of cells from air-suspension interphase by the ring of microbiological loop and transfer of these cells in liquid medium; (c) cultivation of cells in liquid medium for 48 h and removal of microbial aggregates after 5 min of sedimentation. Selection of cells with high aggregation ability was made using four cycles including following steps: (a) disintegration of microbial aggregates in the beater for 5 min and filtration through a 35 mm cell strainer cap (Becton Dickinson, USA) to remove the particles larger than 35 mm; (b) collection of cells from the tube bottom after 5 min of sedimentation and transfer of these cells in liquid medium; (c) cultivation of cells in liquid medium for 48 h and removal of microbial aggregates after 5 min of sedimentation. Due to this selection procedure the aggregation index of cells was increased from 5–20% to 60–80%.

To study distribution of microorganisms in the granules by fluorescence *in situ* hybridization (FISH) the granules with average diameter 2.2 mm were selected manually. Application of oligonucleotide labelled probes, FISH procedures, detection of exopolysaccharide (EPS) matrix, distribution of living organisms, detection of the channels and pores with diameters greater than 0.1 μm, and regimes of Fluoview300 confocal laser scanning microscope (CLSM) (Olympus, Japan) were described (Sghir *et al.*, 2000; Tay *et al.*, 2002a, b). The life cycle of the granules was studied using labeling of cells by fluorescent lipophilic tracer $DiIC_{18}(3)$-DS (Molecular Probes, Inc., Eugene, OR, USA) at final concentration 1 $\mu g\,L^{-1}$. The quantity of fluorescent lipophilic trace was measured using the Luminescence Spectrometer LS-50B (Perkin-Elmer, Boston, MA 02118, USA). The size of the granules was measured by a laser particle size analysis system (Malvern Mastersizer Series 2600), or an image analysis system (Quantimet 500, Leica Cambridge Instruments). Cell surface hydrophobicity was determined by the hydrocarbon adherence method (Rosenberg *et al.*, 1980) after disintegration of the granules in the beater for 5 min and filtration through a 35 mm cell strainer cap (Becton Dickinson, USA) to remove the particles larger than 35 mm. The mechanical strength of the compared granules was measured by the increase of optical density (OD) of suspension in 250 ml flasks with the samples during mechanical stirring by magnetic bar (5 cm) at 300 rpm. Aggregation index (AI) was determined in our experiments as relative decrease of suspension OD at 660 nm with initial OD 1.2–1.4 after 2 min of centrifugation at 650 × *g*. AI was expressed as percents of initial OD and reflected the percentage of aggregated cells precipitated in aggregates.

Results and discussion

Structure of the granules

The matrix of aerobically grown microbial granules is not homogenous. There were different microaggregates inside the granules. They were arranged randomly, in radial direction, or formed concentric layers. In some cases the granule was an aggregate of

smaller sub-granules. The concentric layers in the granules were revealed using CLSM after staining by specific fluorochromes or using FISH with specific oligonucleotide probes. The description of these layers is given in Table 1.

Considering a microbial granule as a sphere with 2.4 mm diameter, the volumes of different zones can be calculated and compared with microbial diversity of the granules (Table 2). Statistically reliable correlation was determined for the calculated percentage of total volume of the granule occupied by aerobic, facultative anaerobic and anaerobic bacteria and experimentally determined percentage of aerobic, facultative anaerobic and anaerobic bacteria isolated from the granules.

Low concentration of substrates and high concentration of metabolites in microbial aggregates may be overcome by the formation of channels and pores that interconnect the surface and the interior. Such channels and pores have been previously observed in aerobic biofilms (Massol-Deya *et al.*, 1995). The aerobic granules also have channels and pores (Table 1).

The thickness of the porous biomass layer was proportional to the granule diameter (Table 1). The formation of the anaerobic layer (Table 1) and cell death in the central core of the granule (Figures 1 and 2) were probably promoted by the plugging of the channels and pores with polysaccharides (Table 1).

CLSM study demonstrated that there was slow formation of new granules after labeling of cells with lipophilic tracer. Changes of fluorescence of the granules can be interpreted as detachment of labeled biomass and attachment of unlabeled microbial biomass during the cultivation of the granules. The life cycle of the granules was evaluated by decrease of the content of fluorescent lipophilic tracer in biomass of granules and flocs. Concentrations of granular biomass ($6.5 \pm 0.2\,g\,L^{-1}$) and suspended biomass of flocs ($0.15 \pm 0.02\,g\,L^{-1}$) were stable. It shows permanent formation and destruction of

Table 1 The concentric layers in aerobically grown microbial granules

Specific layer	Average depth of layer from surface of granule and average thickness
Obligate aerobic ammonia-oxidizing bacteria *Nitrosomonas spp.*	70 μm (depth); 30 μm (thickness)
Facultative anaerobic enterobacteria	Concentration of enterobacterial rRNAs linearly increased from 0 to maximum at the depth 450 μm and then is stable at depth from 450 to 850 μm. Thickness was 1,200 μm
Obligate anaerobic bacteria *Bacteroides spp.*	850 μm (depth); 150 μm (thickness)
Channels and pores by penetration of 0.1 μm microspheres	Volume of the channels and pores linearly increased to the depth from 0 to 250 μm depending on size of the granule, then reached a minimum at depth from 250 to 500 μm. Analysis of the experimental data for the granules of different diameters showed that depth and thickness (H_l) of the layer of porous biomass linearly correlated with granule diameter (D_g) by equation: $H_l = 0.15\,mm + 0.2D_g$
Layer of active biomass with high concentration of ribosomes detected by staining with SYTO™	This layer coincides with the layer of porous biomass. Analysis of the experimental data for the granules of different diameters showed that depth and thickness (H_2) of the layer of active biomass linearly correlated with granule diameter (D_g) by equation: $H_2 = 0.15\,mm + 0.2D_g$
Polysaccharides detected by labeled wheat-germ agglutinin	Low content to the depth 500 μm, reached maximum at 650 μm, stable low content at depth from 800 to 1,200 μm. Thickness was 300 μm
Core of dying cells in the centre of granule	Depth was 1,000 μm. Diameter of this core depended on granule diameter

Table 2 Average geometrical and biological parameters of 2.4 mm spherical granule

Concentric layer in microbial granule	Geometrical parameters	Volume, mm^3	% of total volume of the granule	% of related bacterial clones isolated from the granules
Aerobic microorganisms in porous layer	At a depth of 0.55 mm from the granule surface	6.08	84	69 ± 7
Facultative anaerobic microorganisms between layer of porous biomass and layer of obligate anaerobic bacteria	Between 0.55 mm and 0.85 mm from the granule surface	0.97	13	9 ± 7
Layer of obligate anaerobic bacteria	Between 0.85 mm and 1.0 mm from the granule surface	0.15	2	2 ± 1

the granules. Content of lipophilic tracer in the flocs decreased and, by linear extrapolation, 100% of the labeled granules or their labeled surface material will be degraded after 12 days of cultivation.

Enhanced formation of the granules

Microbially enriched culture with high cell surface hydrophobicity was produced and tested as inoculum for faster formation of the granules. Cell hydrophobicity, measured by hydrocarbon adherence test, was increased from 33% to 80% after four steps of selection of cells from the air–water interphase. The granules with mean diameter 1 mm were formed from cells with high cell surface hydrophobicity for 2 days. The granules with mean diameter 1 mm were formed from the flocs of activated sludge for 8 days (Figures 3 and 4).

Formation of the granules from the flocs of activated sludge was accompanied by increase of cell hydrophobicity from 33% to 68%. Hydrophobicity of the granules, which were produced from cells with high hydrophobicity, remained at the same level of 80%.

The granules produced in bioreactor, where cells with high hydrophobicity were used as inoculum, were not destroyed under mechanical stirring (Figure 5). However, the granules, produced from the flocs of activated sludge, released cells to medium during mechanical stirring (Figure 5). Therefore, selected inoculum with high cell hydrophobicity can

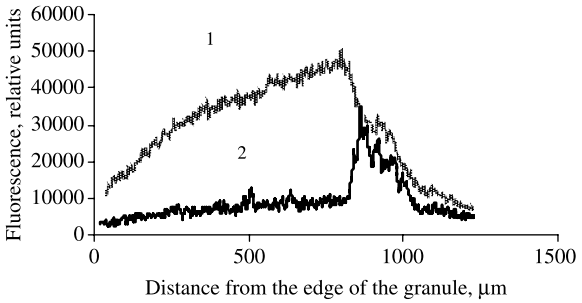

Figure 1 Distribution of active microbial biomass (1) and biomass of obligate anaerobic bacteria (2) in aerobically grown microbial granule

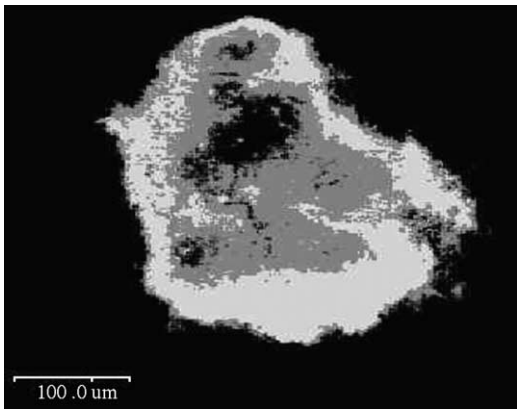

Figure 2 Microbial granule with core of lysed cells (black central part), active biomass (grey color) covered with a "skin", which is a layer of dead cells (brighter layer)

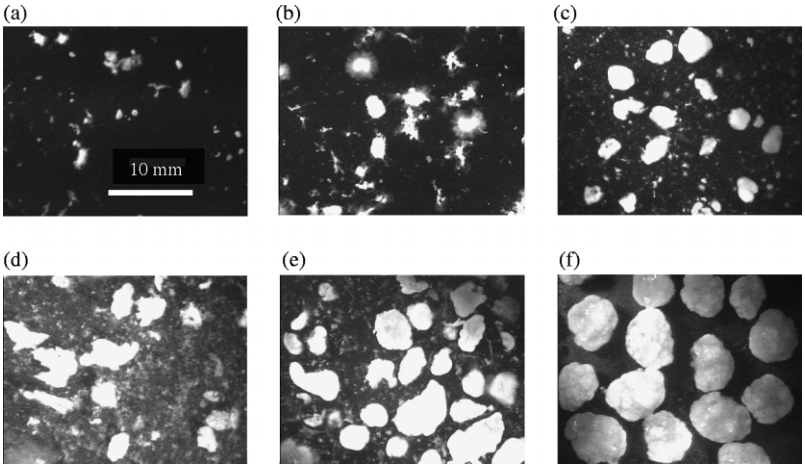

Figure 3 Formation of granules after 5 days (A, D), 10 days (B, E) and 20 days (C, F) in the reactors inoculated with flocs of activated sludge (A–C) or selected cells of high hydrophobicity (D–F)

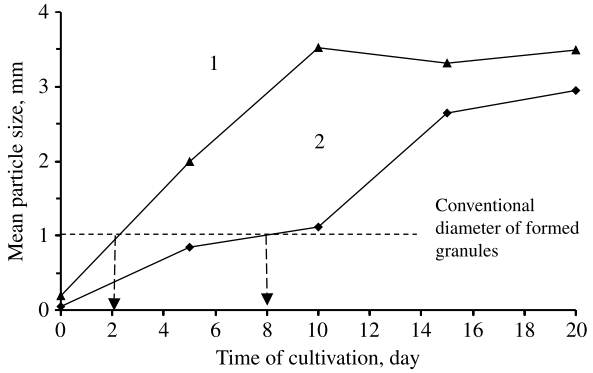

Figure 4 Formation of the granules from selected cells with high cell surface hydrophobicity (1) and from the flocs of activated sludge (2)

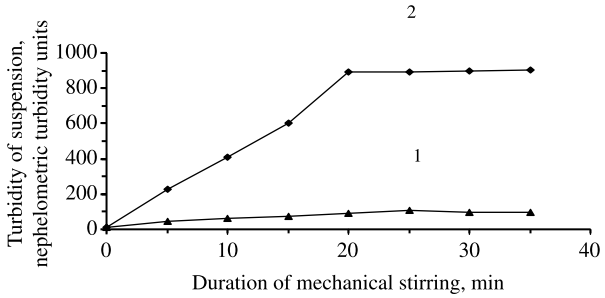

Figure 5 Changes of suspension turbidity during mechanical stirring of the granules produced in the reactor where cells of high hydrophobicity were used as inoculum (1), and where the flocs of activated sludge were inoculated (2)

be used in the case when the granules will be used in the bioreactors with intensive stirring.

To facilitate formation of the fast-settling granules 20 pure cultures with high self-aggregation ability were isolated. High self-aggregation ability was specific for capsular Gram-negative gliding bacteria and some not slimy sporogenic Gram-positive strains. Average AI index of gliding bacteria was 6.6 times bigger than average AI of non-gliding Gram-negative bacteria. Some pure cultures of gliding bacteria and *Bacillus sp*. were able to form granular aggregates in a few days instead of the several weeks needed for the production of microbial granules from the flocs of activated sludge. These data demonstrated that application of specific inoculum ("starter culture", "seeds") may be a useful approach in the practical application of microbial granules for the wastewater treatment.

Conclusions

Microbial granules used in aerobic wastewater treatment have a diverse microbial community and complex spatial structure. Typically, it is a sequence of the concentric layers of obligate aerobic microorganisms, facultative anaerobic, obligate anaerobic bacteria, and a core of dead and lysed cells in microbial granules with diameter larger than 1.6 mm. Cells with high hydrophobicity or self-aggregation ability were isolated from the granules. Inoculation of such cells can significantly diminish the duration of granule formation and improve the properties of produced granules.

Acknowledgements

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