# Functional analysis of microbial community in phenol-degrading aerobic granules cultivated in SBR

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**Abstract** Phenol-degrading aerobic granules were cultivated in a sequencing batch reactor with an influent phenol concentration of 500 mg $l^{-1}$. Eight strains were isolated from aerobic granules to characterize the functional redundancy of the microbial community in the granules. The specific oxygen utilization kinetics show the eight strains possessed different phenol-degrading activities, with half-saturation constants ($K_s$) ranging from 0.4 to 70.5 mg phenol $l^{-1}$. Two isolates belonging to dominant populations expressed differing functions. The first strain was linked to the function of phenol degradation as this strain has the highest phenol-degrading ability among all isolates, while the second strain was linked to the maintenance of the granule structure because of its strong self-flocculation activity. This study could be used to exploit the granule-based system for treating high-strength wastewaters.

**Keywords** Aerobic granules; function; microbial community; phenol degradation

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## Introduction

Phenol wastes are present in effluents from industries such as petroleum and petrochemical, coal gasification, pharmaceutical, and dye manufacturing. Phenol-containing wastewater is difficult to treat biologically because of substrate toxicity and inhibition. However, these substrate inhibition difficulties can be overcome by strategies such as cell immobilization (Loh *et al*., 2000). Aerobic granulation represents a relatively new form of cell immobilization that has attracted recent research attention (Beun *et al*., 1999; Peng *et al*., 1999; Tay *et al*., 2001). The aggregation of microbial cells into compact granules in sequencing batch reactors (SBRs) can serve as an effective protection against phenol toxicity. Phenol-degrading aerobic granules have been shown to treat wastewater at high phenol loads that normally lead to upsets in conventional activated sludge systems (Jiang *et al*., 2002, 2003).

An in-depth knowledge of the microbial community can enhance the design and performance of these phenol-degrading aerobic granules. During the last decade, the application of molecular tools in wastewater microbiology has revolutionized our view on the microbial ecology of these systems. Cultivation-based methods such as isolation, however, cannot be totally supplanted. Isolates are certainly needed for a better understanding of their physiology and function. Gaining an understanding of the functions of microbial communities is important as population diversity alone does not drive ecosystem stability (Briones and Raskin, 2003). This can be aided by the isolation and characterization of functionally important microbial populations.

In this study, eight pure cultures were isolated from phenol-degrading aerobic granules cultivated in an SBR. The phenol-degrading and aggregation abilities of these strains were characterized. Then the complementary functional roles of two bacterial strains belonging to dominant populations were discussed with respect to granule function. This work is expected to be useful in understanding the microbial community of aerobic granules and developing management strategies for aerobic granule systems.

## Materials and methods

### Reactor operation and sampling phenol-degrading aerobic granules

A column-type SBR with a working volume of 2.0 l was operated under an organic loading rate of 1.5 kg phenol $m^{-3}$ $day^{-1}$ with an influent phenol concentration of 500 mg $l^{-1}$ (Jiang *et al.*, 2002). Aerobic granules first appeared on day 9 of reactor operation. After about two months of operation, the reactor reached a steady state, as evidenced by stable biomass concentrations and almost complete phenol removal. The matured granules with diameters from 0.5 to 1.0 mm were separated manually and used for isolation.

### Isolation procedure

The culture medium used for isolation and growth on phenol was prepared using MP medium (Watanabe *et al.*, 1998) and phosphate buffer, supplemented with trace elements and vitamins (Cote and Gherna, 1994). The medium was sterilized by autoclaving for 20 min at 121°C. Phenol solution was sterilized by sterile filtration (0.2 μm) and added to the medium after autoclaving.

Portions of matured granules were added to 15 ml of medium as described above, and aseptically mixed in a sterilized beaker in order to detach granules. All operations were performed in a biohazard flow cabinet. The supernatant was then diluted with medium, and 150 μl of each dilution was spread onto an MP medium-based agar plate and supplemented with 500 mg $l^{-1}$ phenol. Plates were inverted and incubated in a 25°C incubator, and monitored over 4 weeks. Visible colonies were observed after 1 week of incubation. Pure cultures of phenol-degrading bacteria were isolated by several cycles of replating onto agar plates. Purity was confirmed by microscopic examination. Gram-staining was performed as previously described (Tay *et al.*, 1998).

### DNA extraction and DGGE

Genomic DNA of isolates and granules was extracted by the bead beating method as described previously (Tay *et al.*, 2002). PCR primers P2 and P3 (containing 40 bp of GC clamp) (Muyzer *et al.*, 1993) were used to amplify the variable V3 region of bacterial 16S rDNA (corresponding to positions 341–534 in the *Escherichia coli* sequence). The PCR conditions and thermal programs for DGGE have been previously described (Watanabe *et al.*, 1998). 16S rDNA sequencing of isolates and bands in the gel were obtained using the ABI model 310A DNA sequencer (Applied Biosystems, Perkin-Elmer) and the ABI PRISM BigDye Terminator Cycle Sequencing ready-reaction kit (Applied Biosystems, Perkin-Elmer) (Tay *et al.*, 2002).

### Analytical methods

The extraction of extracellular polymers (ECPs), and the measurement of phenol concentrations, polysaccharides, proteins, suspended solids (SS) and sludge volume index (SVI) have been described previously (Jiang *et al.*, 2003). To check flocculation activities of isolates grown on liquid phenol or YEPG medium, isolates were incubated for one day in a reciprocating shaker at 60 rpm and then settled for one minute. Flocculation activity was judged based on whether flocculated biomass can be observed. Determination of biomass concentrations on a dry weight (DW) basis for pure cultures has been described elsewhere (Onysko *et al.*, 2000).

To determine the oxygen utilization rates of bacteria, a DO meter with Clark-type polarographic oxygen electrodes (YSI 5300A, YSI Incorporated, USA) was used to measure the oxygen concentrations. Specific oxygen utilization rates were calculated from oxygen concentration curves obtained under different phenol concentrations. A kinetic analysis of the data was performed based on Haldane's formula for an inhibitory substrate,

$q = q_{max}\, s/[K_s + s + (s^2/K_i)]$, where q and $q_{max}$ are the specific and the theoretical maximum specific oxygen utilization rates (mg DO g $DW^{-1}$ $min^{-1}$), respectively, and s, $K_s$ and $K_i$ are the substrate concentration, half-saturation constant and inhibition constant (mg phenol $l^{-1}$), respectively (Jiang *et al*., 2002).

## Results

### Reactor performance and cultivation of phenol-degrading aerobic granules

The sequencing batch reactor operated under an organic loading rate of 1.5 kg phenol $m^{-3}$ $d^{-1}$ with an influent phenol concentration of 500 mg $l^{-1}$(Jiang *et al*., 2002). After about 20 days of operation, the phenol concentration in the effluent declined to below 0.2 mg $l^{-1}$. Aerobic granules first appeared on day 9 of reactor operation and quickly grew to displace the seed flocs as the dominant form of biomass in the reactor. These compact granules consisted of bacterial rods and cocci embedded in an extracellular polymeric matrix (Figure 1a, b). At steady state, biomass concentrations and SVI in the reactor averaged 6.5 g SS $l^{-1}$ and 40 ml g $SS^{-1}$, respectively.

### Physiological analyses of isolates from aerobic granules

Eight bacterial strains were isolated from phenol-degrading aerobic granules by direct isolation technique and partially characterized (Table 1). Three out of eight strains were gram-positive. DGGE results indicate that PG-01 and PG-08 were members of dominant genera in the microbial community in the aerobic granules, as each of these strains was represented in DGGE bands retrieved directly from granules.

Considering that aerobic granulation represents a form of self-immobilization of microbial cells, the flocculation abilities of these isolates were determined. Table 1 shows that most of the isolates are non-flocculating. PG-08 possessed the strongest flocculation ability amongst all the isolates. Auto-aggregation took place when PG-08 was cultivated in phenol medium or YEPG medium, with formation of compact cell aggregates with a mean size of 0.45 mm (Figure 2a, b). Polysaccharide contents in the ECP of auto-aggregates were high, with 152.3 mg g $DW^{-1}$ in phenol medium and 137.6 mg g $DW^{-1}$ in YEPG medium.

In order to compare their phenol-degrading abilities, oxygen utilization kinetics for the eight isolates were measured as oxygen utilization activities of bacteria corresponded with their phenol-degrading activities (Watanabe *et al*., 1996). Specific oxygen utilization rates of PG-01 and PG-09 are shown in Figure 3. Although PG-08 can grow fast in YEPG liquid medium, the ability of this strain to degrade phenol seems lowest as confirmed by the lowest $q_{max}$ (Table 1). Compared to PG-08, PG-01 possessed the highest phenol-degrading activity. The $q_{max}$ of PG-01was about 31 times that of PG-08. It is also seen that isolates expressed different $K_s$ values (Figure 4). The $K_s$ value for PG-09 was as low as 0.4 mg $l^{-1}$, while the $K_s$ value for PG-04 was as high as 70.5 mg $l^{-1}$.

## Discussion

Aerobic granule-based systems have the potential to treat wastewaters with high phenol loads because of the compact structure and good settling ability of aerobic granules (Jiang *et al*., 2002, 2003). In this study, the combination of molecular methods and conventional techniques was exploited to investigate the functional diversity of the microbial community in phenol-degrading aerobic granules. Eight isolates show different phenol degradation abilities in accordance with $K_s$ values fitted for specific oxygen utilization rates (Table 1 and Figure 4). The operating condition and substrate concentration strongly selected microorganisms based on $K_s$ values (Massol-Deya *et al*., 1997). It was reported that strains isolated from a continuous suspended reactor enriched by a low phenol concentration expressed phenol-oxygenating activities with low apparent $K_s$, while the strains

isolated from a batch system enriched by relatively high concentrations of phenol expressed phenol-oxygenating activities with relatively high apparent $K_s$ (Watanabe *et al.*, 1996). However, strains with either high or low apparent $K_s$ were isolated from aerobic granules cultivated in an SBR with relatively high initial phenol concentrations. Obviously, it was necessary to incorporate other factors to accurately explain these observations. Although the granule surface faced high phenol concentrations during the initial part of the SBR cycle, phenol concentrations would always be low in the granule interior because of diffusional resistance. Thus, both bacteria with high $K_s$ and low $K_s$ can grow in aerobic granules.

Recognizing the diversity and the links within each functional group of a system can lead to better ways to model diversity and function as well as helping to improve process stability (Hulot *et al.*, 2000). PG-01 may be numerically abundant in the aerobic granules as only colonies of PG-01 could be found in plates with highest dilution of inoculum prepared from aerobic granules. DGGE also suggested that PG-01 belonged to a dominant genus. Considering the high abundance of active cells and high specific oxygen utilization activity and phenol removal rate, PG-01 could be regarded as one of the functionally dominant strains and may have contributed significantly to phenol degradation in the granules. Although PG-08 was also identified as a strain of a dominant genus, the specific oxygen utilization activity was lowest among all isolates. However, PG-08 exhibited strong flocculation activity, and could form auto-aggregates with high extra-polysaccharide content. In fact, polysaccharides are known to contribute to the structure of aerobic granules (Tay *et al.*, 2001). So PG-08 might play an important role in maintaining the structure of phenol-degrading aerobic granules.

Phenol degradation and granule structure stabilization are two basic functions for the stability of granule systems exposed to high phenol concentrations. Although two strains

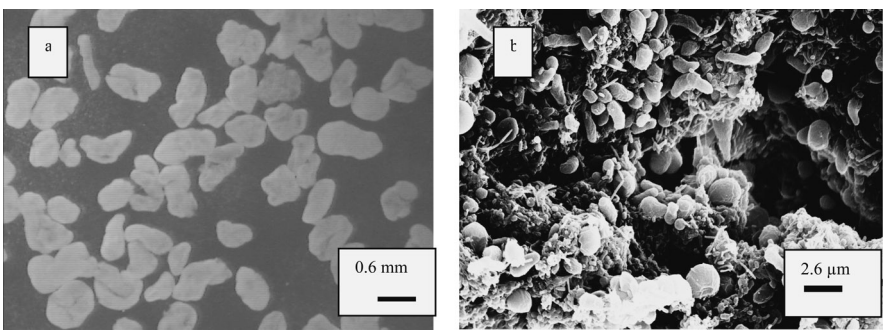


**Figure 1** Stereomicroscope image (a) and scanning electron micrograph (b) of aerobic granules

**Table 1** Characterization of isolates from phenol-degrading aerobic granules

| | Gram-stain[a] | Flocculation activity[b] | | | Kinetics constants | |
|---|---|---|---|---|---|---|
| | | Growth in phenol | Growth in YEPG medium | $q_{max}$ (mg DO mg $DW^{-1}$ $min^{-1}$) | $K_s$ (mg $l^{-1}$) | $K_i$ (mg $l^{-1}$) |
| PG-01 | – | – | – | 9.3 ± 0.7 | 36.2 ± 5.8 | 60.5 ± 5.2 |
| PG-02 | + | + | + | 1.5 ± 0.1 | 7.1 ± 0.4 | 50.6 ± 3.1 |
| PG-03 | + | – | – | 3.9 ± 0.3 | 12.8 ± 1.3 | 150.1 ± 12.2 |
| PG-04 | + | + | – | 3.0 ± 0.4 | 70.5 ± 6.2 | 165.2 ± 20.1 |
| PG-05 | – | – | – | 2.8 ± 0.7 | 45.6 ± 7.3 | 60.5 ± 4.9 |
| PG-08 | – | ++ | ++ | 0.3 ± 0.1 | 47.2 ± 7.8 | 20.2 ± 6.9 |
| PG-09 | – | – | – | 2.2 ± 0.1 | 0.4 ± 0.02 | 58.6 ± 2.2 |
| PG-10 | – | – | + | 2.8 ± 0.6 | 8.3 ± 0.2 | 70.3 ± 5.5 |

[a] Gram-negative (–), gram-positive (+);
[b] No flocculation activity (–), weak flocculation activity (+), very strong flocculation activity (++)

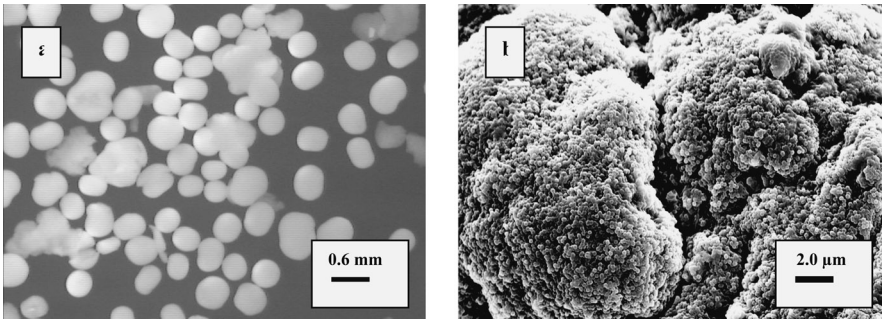


**Figure 2** Stereomicroscope image (a) and scanning electron micrograph (b) of PG-08 aggregates

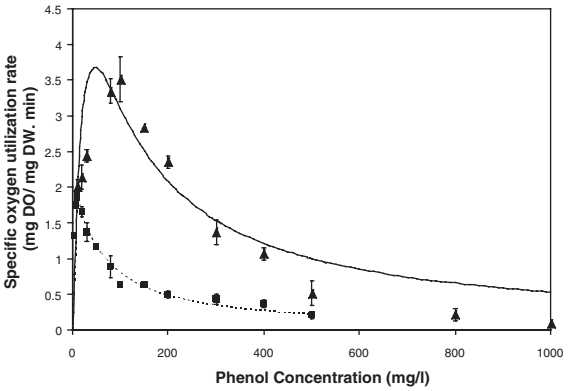


**Figure 3** Specific oxygen utilization rates of PG-01 (▲) and PG-09 (■)

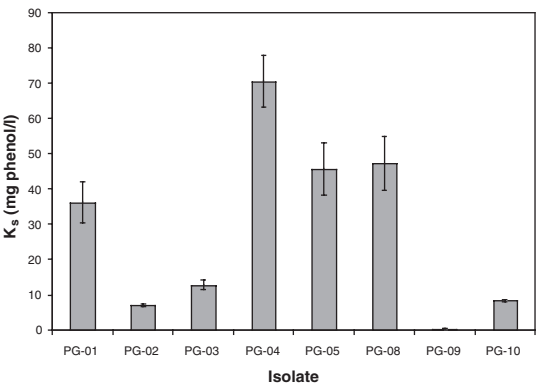


**Figure 4** Half-saturation constants ($K_s$) for isolates

were uncovered that could be associated with each of the above functions, it should be noted that significant diversity exists within different functional groups of bacteria (Wagner *et al.*, 2002). Actually, the arrangement of different microorganisms responsible for distinct functions within the granule matrix is not surprising as granules are similar to biofilms, which represent highly differentiated, well-organized, multicultural microbial communities (Watnick and Kolter, 2000).

In summary, eight isolates from phenol-degrading aerobic granules cultivated at a loading of 1.5 kg phenol $m^{-3}$ $d^{-1}$ were characterized. The eight strains showed different phenol-degrading activities. Two strains belonging to different dominant genera in the granules were linked with one of two basic functions, namely phenol degradation or maintenance of

granule structure. This study may have important implications for system management and for exploiting these novel microbiological systems to treat higher phenol substrate loadings.


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