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Changes of microbial community structures and functional genes during biodegradation of phenolic compounds under high salt condition

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Abstract

The changes of microbial community structures and functional genes during the biodegradation of single phenol and phenol plus *p*-cresol under high salt condition were explored. It was found that the phenol-fed system (PFS) exhibited stronger degrading abilities and more stable biomass than that of the phenol plus *p*-cresol-fed system (PCFS). The microbial community structures were revealed by a modern DNA fingerprint technique, ribosomal intergenic spacer analysis (RISA). The results indicated that the microbial community of PFS changed obviously when gradually increased phenol concentration, while PCFS showed a little change. 16S rRNA sequence analysis of the major bands showed that *Alcanivorax* sp. genus was predominant species during phenolic compounds degradation. Furthermore, amplified functional DNA restriction analysis (AFDRA) on phenol hydroxylase genes showed that the fingerprints were substantially different in the two systems, and the fingerprints were not the same during the different operational periods.

Key words: phenolic compounds; high salt; phenol hydroxylase; ribosomal intergenic spacer analysis; amplified functional DNA restriction analysis

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Introduction

Phenolic compounds are the most common representatives of toxic organic pollutants, which have resulted in cumulative hazardous effects in environments. They are encountered in the waste effluents of several processes and operations such as petroleum refineries, petrochemical plants and phenolic resin industries (Schie and Young, 2000). In addition to being potential carcinogens, phenolics are toxic (reducing enzyme activity) or lethal to fish at relatively low level (5–25 mg/L) (Brown *et al*., 1967). Thus, these effluents require proper treatment prior to being discharged to the water bodies. Among various techniques available for the removal of phenolic compounds, biodegradation is an environment friendly and cost effective technology. Biological treatment of phenolic compounds has been an increasingly important process in pollution control (Evans, 1947; Ahmed, 1995; Annadurai *et al*., 2002; Arutchelvan *et al*., 2003; Jiang *et al*., 2007). In contaminated groundwater and effluents from industrials, organic matter mixtures and high concentration of salts are prevalent. For example, the pharmaceutical industrial wastewater contains 1000 mg/L phenolic compounds and 1176 mg Na^+/L, 1572 mg Cl^-/L and 1612 mg K^+/L, etc. The occurrence of pollutants in the mixture is an important issue, because microbial degradation of one component could be inhibited by other compounds in the mixture and high concentration of salts would affect the growth of microorganism (Meyer *et al*., 1984). Despite of many reports on microbes for the mixed phenolic compounds remediation (Bielefeldt and Stensel, 1999; Reardon and Mosteller, 2000; Alvarez and Vogel, 1991), little is known about the diversity of organisms in the process, or how the balance between pollutant loading and treatment efficiency is maintained with the respect to microbial community dynamics.

Several studies on the microbial community structures of wastewater treatment have recently been conducted using culture-independent molecular techniques (Torsvik and Ovreas, 2002). Culture-independent methods such as ribosomal intergenic spacer analysis (RISA), and denaturing gradient gel electrophoresis (DGGE), have been used to describe bacterial community structures in soil and water (Futamata *et al*., 2003; Jiang *et al*., 2004; Enwall *et al*., 2005). Furthermore, PCR-based techniques have been used to detect functional/catabolic genes in environmental isolates or environmental DNA, and diversity is usually assessed by the sequencing of genes from isolates or PCR clone libraries (Buchan *et al*., 2001; Duarte *et al*., 2001). These culture-independent molecular studies can significantly improve the understanding of microbial function and degradation processes in the environment, which would help to design new bioremediation strategies

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(Widada *et al*., 2002).

As phenol and cresol are the most important components in phenolic wastewater, the biodegradation of the mixtures have been investigated (Kar *et al*., 1997; Perron and Welander, 2004). But very few studies focused on the microbial community structure and functional genes diversity under high salt condition. This study aimed to determine the changes of microbial community structures of activated sludge as well as the diversity of functional genes in different phenolic treatment systems, and to link the changes with the process performance under high salt condition. To our knowledge, it is the first report of utilizing the molecular methods of RISA and amplified functional DNA restriction analysis (AFDRA) to analyze the degradation of phenolic compounds under high salt condition.

1 Materials and methods

1.1 Medium and culture condition

The medium used in this study was basal salts medium, which contained (g/L): 50 NaCl, 2.0 KH_2PO_4, 1.3 Na_2HPO_4, 2.0 $(NH_4)_2SO_4$, 0.01 $FeCl_3$, phenol or phenol plus *p*-cresol was 200 to 1500 mg/L, pH 6.8–7.2. Both cultures were incubated at 30°C with shaking at 150 r/min.

1.2 Experimental systems

Samples of fresh activated sludge (AS) were obtained from Dalian Chunliu River Wastewater Treatment Plant, China, which were used as the indigenous populations in the sequencing batch reactors (SBRs). The SBRs were simulated with 250 mL flasks containing 100 mL synthetic wastewater medium with different substrates. The initial concentration of AS was 2000 mg/L (mixed liquor suspended solid, MLSS). It was cultured under two different conditions: (1) basal salts medium with phenol as sole carbon source; (2) basal salts medium with phenol and *p*-cresol mixture as carbon sources. In order to definite the effect of *p*-cresol on phenol degradation, the phenol concentration was gradually increased and *p*-cresol concentration was kept constant about 100 mg/L in phenol plus *p*-cresol-fed system (PCFS). In all experiments, a new cycle of the treatment system was initiated whenever the removal efficiency of phenolic compounds was higher than 90%. Once the substrate was degraded, the supernatant was discharged and replaced with the same fresh medium containing an equivalent amount or high concentration of phenolic compounds.

1.3 Genomic DNA extraction from activated sludge

Genomic DNA was extracted from 5 mL of sludge samples by the method described previously (Mufiel *et al*., 1999). The quantity and quality of the extracted DNA were checked by measuring the UV absorption spectrum of DNA solution (Sambrook *et al*., 1989), and DNA was finally dissolved in TE buffer at a concentration 100 μg/mL.

1.4 Ribosomal intergenic spacer analysis

The universal primers used in the RISA were S926f (5′-CTY AAA KGA ATT GAC GG-3′) and L189r (5′- TAC TGA GAT GYT TMA RTT C-3′). The PCR amplification of RIS was conducted in a volume of 50 μL containing 5 μL of 10× buffer, 0.5 pmol of each primer per μL, 20 μmol/L of each dNTP, 0.5 μL LA *taq* (Takara, Dalian Co., Ltd., China), and 1.0 μL DNA template. The DNA templates were first subjected to an initial denaturation step at 94°C for 2 min. The subsequent cycles consisted of denaturation step at 94°C for 1 min, annealing step at 50°C for 1 min, and extension step at 72°C for 1.5 min, a final extension at 72°C for 5 min was included after 30 cycles of PCR amplification. The resultant PCR products of RIS were resolved on 5% polyacrylamide gels, which were stained with ethidium bromide and photographed. The sequences of major bands were determined as described previously by Watanabe *et al*. (1998) and compared with the relative sequences in the GenBank database using the BLAST program. The obtained sequences were deposited in the database with accession numbers EU621884 and EU621887–EU621890.

1.5 Amplified functional DNA restriction analysis

Primers CTB686F (5′- AGG CAT CAA GAT CAC CGA CTG-3′) and CTB686R (5′- CGC CAG AAC CAT TTA TCG ATC-3′) were used for PCR amplification of the phenol hydroxylase gene. The PCR amplification of functional DNA was conducted in the same media as for RIS. PCR amplification was performed as follows: initial step at 94°C for 1 min, followed by 35 cycles of denaturation at 98°C for 10 s, annealing at 55°C for 30 s, and extension at 72°C for 60 s, and final extension step at 72°C for 5 min. PCR products were purified with Microspin S-400 HR columns (Amersham Biosciences, Sweden) and then digested with restriction enzyme Alu I for 4 h. The restriction fragments patterns were resolved on 5% polyacrylamide gels, at 120 V (80 mA), until the bromophenol blue dye in the loading buffer reach the front edge (approximately 5 h). For staining, the gel was placed in ethidium bromide solution (10 mg/L) for 1 h. The gel was visualized, and images were acquired and stored on a gel documentation system (Molecular Imager FX, Bio-Rad Laboratories, Inc., USA).

1.6 Analytical methods

Concentration of phenolic compounds in all cultures were periodically measured with a high-performance liquid chromatograph (HPLC, System Gold, Beckman instruments, Fullerton, USA) equipped with a Hypersil ODS column (4.6 × 250 mm, Agilent Technologies, USA). The mobile phase consisted of methanol and water (60:40, *V/V*) at a flow rate of 1.0 mL/min. The UV absorbance spectrum was obtained at 254 nm.

MLSS was measured periodically according to standard method (Andrew *et al*., 1995). Cluster analysis of RISA and AFDRA was generated using UVI-Band-Map softwar (Version 99).

2 Results and discussion

2.1 Performance of treatment systems for phenolic compounds biodegradation

The changes of concentration and removal rate of phenolic compounds in the two systems are shown in Fig. 1. In phenol-fed system (PFS), the removal rates of phenol increased gradually during the first and second shock loadings (Fig. 1a). When the concentration of phenol increased to 1000 mg/L, the degradation efficiency of PFS was decreased and maintained about 0.05 mg/(mg MLSS·d) during the rest of the operational period. Compared with PFS, PCFS demonstrated a lower efficiency to cope with a higher phenolics shock loading (Fig. 1b). When the concentration of mixed substrates was increased to 1000 mg/L, total phenolics removal rates maintained at a lower level about 0.035 mg/(mg MLSS·d). It was clear that PCFS had lower phenolics removal rates range 0.0225–0.0509 mg/(mg MLSS·d), with an average rate of 0.039 mg/(mg MLSS·d). In contrast, the phenol removal rate in PFS was significant higher, ranging from 0.0304 to 0.0621 mg/(mg MLSS·d) with an average removal rate of 0.051 mg/(mg MLSS·d).

Furthermore, total biomass concentrations remained relatively stable in PFS (MLSS, 2107 ± 247 mg/L) (Fig. 2a). But fluctuation was observed in PCFS range 1757–2341 mg/L, with an average of 2083 mg/L (Fig. 2b). It was concluded that both phenol and *p*-cresol could be consumed simultaneously, but the presence of a small amount (100 mg/L) of *p*-cresol retarded phenol biodegradation particularly at high substrate concentration. It was reported that the effects of other compounds in a mixture of homologous carbon and energy substrates on biodegradation is generally negative (Yu and Loh, 2002; Klecka and Maier, 1988). The pattern of mixed substrates utilization observed in this work was similar to the results of Reardon and Mosteller (2000). It was indicated that the reasons for decreasing removal rate include competitive inhibition and toxicity of phenolic mixtures.

2.2 Ribosomal intergenic spacer analysis profiles of microbial community

RISA analysis of bacterial communities in the two systems showed different community fingerprint patterns (Fig. 3). It was noted that the original microbial community structure was changed by adding phenolic compounds as the sole carbon source, and there were differences in community structures between the two systems. In PFS, the intensity of predominant band (approximately 1.5 kb) was decreased and other three bands (1.8–2.0 kb) disappeared due to abrupt increase of the substrates concentration. However, these bands reappeared after 55 d of operation and four new bands occurred, and the system maintained stable removal rates. Cluster analyses supported that samples from different systems or different time points could be distinguished from each other, but PFS-35 and PFS-55 were clustered together with 90% similarity (Fig. 3b). Such changes may be attributed to the adaptation of the

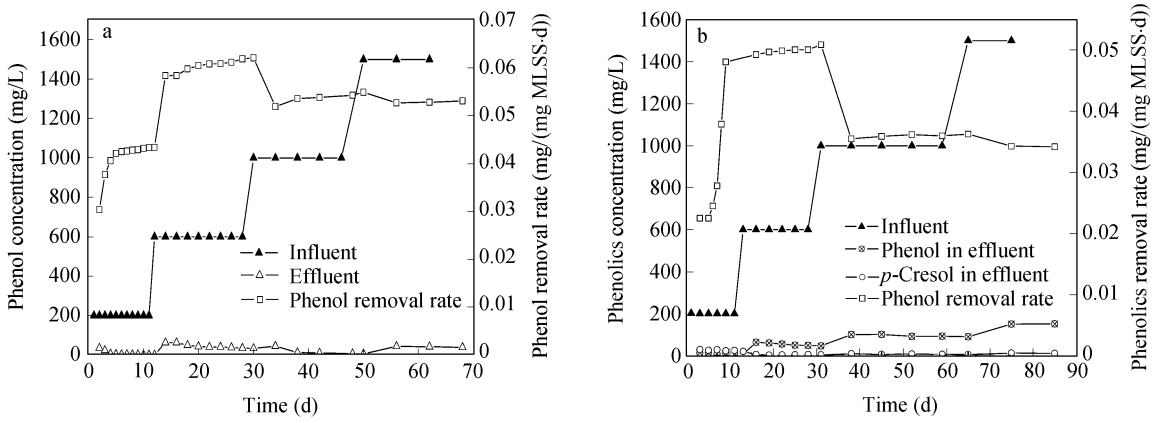

Fig. 1 Phenolic compounds degradation in phenol-fed system (PFS) (a) and phenol plus *p*-cresol-fed system (PCFS) (b).

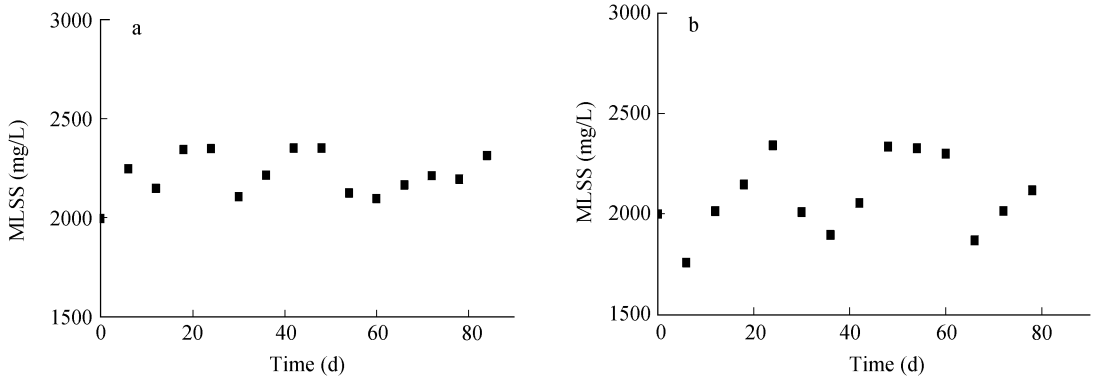

Fig. 2 Variations in biomass concentration from PFS (a) and PCFS (b).

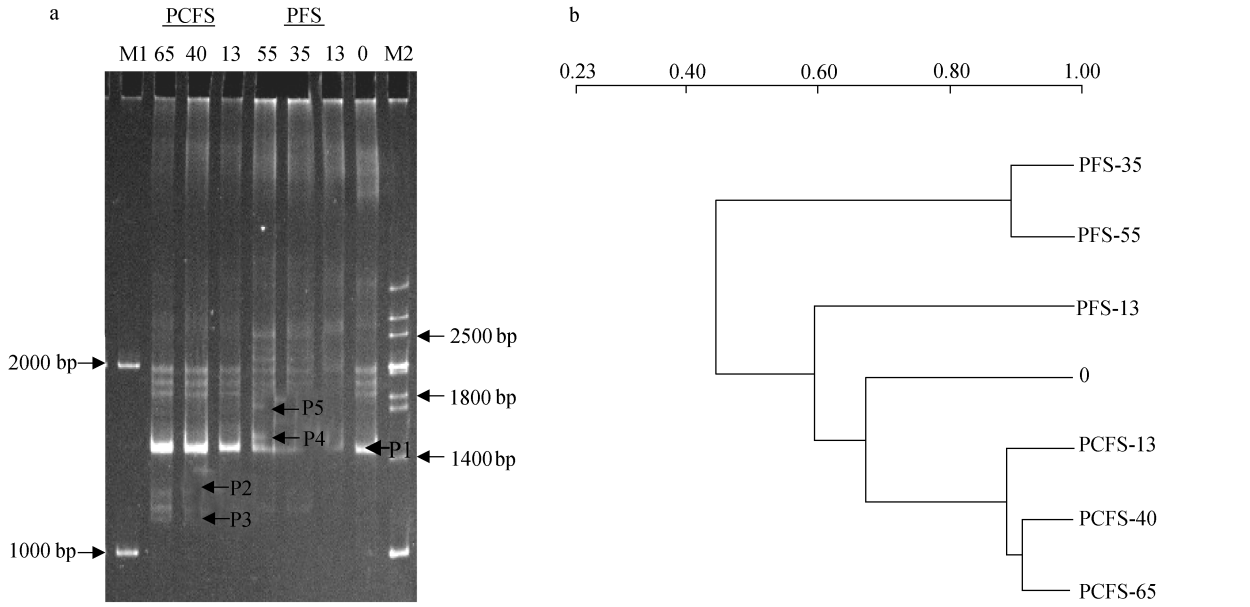

Fig. 3 Ribosomal intergenic spacer analysis (RISA) fingerprints (a) and clusters analysis (b). M1: DL2000 DNA marker; M2: 200 bp DNA ladder marker. The numbers 0, 13, 35, and 55, etc., indicate the sampling time (d).

communities to the shock loading of phenol during the two months operation. Some studies have concluded that there was no, or only transient, improvement of process performance and it always correlated with the decrease or disappearance of certain populations (Bouchez *et al*., 2000).

However, there were minor changes of the community structure in PCFS (Fig. 3a). The intensity of main bands from PCFS gradually increased after long term operation, and two new bands of approximately 1.2–1.3 kb appeared in the sample taken at day 40. These bands still existed at the end of the operation (day 65). Such changes demonstrated that the indigenous species capable of utilizing the mixed substrates gradually increase in proportion and become the dominant populations. Cluster analyses showed that PCFS-40 and PCFS-65 were clustered together with 90% similarity (Fig. 3b), indicating that the community structures were relatively stable during this period. These results of RISA not only verified that different substrates led to the changes of microbial community during long term operation, but also demonstrated that single substrate would increased the diversity of microbial community under high salt condition.

Although there were differences within the community structures of the two systems, prominent RISA bands derived from the two systems were excised and used for nucleotide sequence analysis (numbered RISA bands in Fig. 3). The affiliations of the 16S rRNA sequences were determined by comparison against the GenBank database, as shown in Table 1. P1 was always present during the process of operation and most closely related (99% similarity) to *Alcanivorax* sp. Therefore, the microorganism corresponding to P1 was assumed to be mainly responsible for phenolic compounds treatment. It was reported that *Alcanivorax* sp. is globally distributed genus of marine petroleum degrading bacteria, which is extreme halophiles (Cappello *et al*., 2007; Liu and Shao, 2005). Among the other bands with relatively weak intensities, P2 and P3 were most closely related (98% similarity) to *Arthrobacter* sp. W1 and *Achromobacter xylosoxidans* (99% similarity), respectively. The sequences obtained from P4 and P5 bands of RISA were related (98% similarity) to *Mesorhizobium* sp. and *Alcanivorax indicus* (96% similarity). As the two bands occurred in PCFS, the microorganism corresponding to P2 and P3 might participate in the degradation of mixed phenolic compounds.

Table 1 Sequence length and closest phylogenetic affiliation of RISA bands

Band	Sequence length (bases)	Close relative	Similarity (%)	Accession No.
P1	500	*Alcanivorax* sp.	99	EU621884
P2	491	*Arthrobacter* sp. W1	98	EU621887
P3	492	*Achromobacter xylosoxidans*	99	EU621888
P4	494	*Mesorhizobium* sp.	98	EU621889
P5	500	*Alcanivorax indicus*	96	EU621890

2.3 Variations of functional genes during phenol and phenol plus *p*-cresol degradation

Phenol hydroxylase genes is well known to be of crucial for aromatic degradation, therefore, it was used as target gene to adapt the amplified ribosomal DNA restriction analysis. To determine the changes in community structure at the functional level, phenol hydroxylase genes were amplified by PCR, digested with restriction enzymes Alu I, and separated by electrophoresis (Fig. 4a). The fingerprints from day 0 to 35 of PFS were obviously changed, the intensity of two bands (440 and 500 bp) decreased and three new bands appeared in the system. However, the fingerprints from day 35 to 55 showed little change, which were corresponding to the changes of microbial communities (Fig. 3a). For PCFS, the fingerprints of PCFS-13 were different from others (Fig. 4b). The intensity of the main

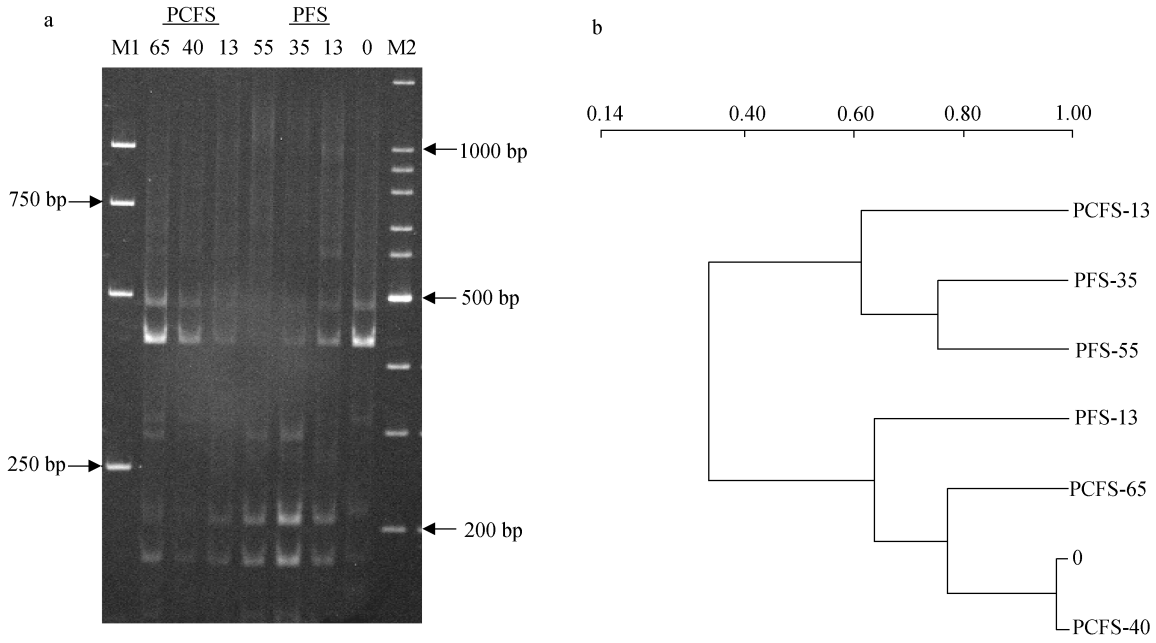

Fig. 4 Community restriction patterns of PCR-amplified phenol hydroxylase genes digested with Alu I (a) and clusters analysis (b). M1: DL2000 DNA marker; M2: 100 bp DNA ladder marker. The numbers 0, 13, 35, and 55, etc., indicate the sampling time (d).

bands (440–500 bp) was decreased and two new bands at 180 and 200 bp appeared in the system. The intensity of initial main bands were gradually increased and became the dominant bands at the end of the operation (day 65), which also coincided with the changes of microbial community structure in PCFS.

Cluster analysis of the phenol hydroxylase fingerprints from both system confirmed that the two communities were different during the period of operation. PFS-35 and PFS-55 were clustered together with 75% similarity (Fig. 4b), whereas PCFS-40 and sample 0 were gathered in the same cluster with a relatively high similarity (95%). As mentioned above, PCFS showed lower removal rates at day 40. Accordingly, such changes would result in the function loss or decrease. Therefore, it was concluded that different substrates affected the expression of functional genes, which was consistent with the changes of microbial community structure and substrate degradation rate. AFDRA thus provided a rapid assessment of phenol hydroxylase genes diversity in phenolic compounds feeding systems. It was suggested that AFDRA can be used to monitor other functional gene families present in the environment DNA extracts.

3 Conclusions

In this study, changes of microbial community structure and functional genes in the phenolic compounds treatment system were investigated. The relationships among microbial communities, gene expression and performance were well documented. It was showed that the removal rate of PFS was higher than that of PCFS. The biomass concentration was relatively stable in PFS and significantly fluctuant in PCFS. The RISA patterns revealed shifts in microbial community structure with a complexity of banding pattern in PFS than that in PCFS. The presence of the dominant genus suggested that the bacteria affiliated with *Alcanivorax* sp. might have an important role in phenolic compounds degradation. AFDRA showed that PFS had obvious changes of phenol hydroxylase gene, while PCFS showed little change compared with the original activated sludge. It was further concluded that different substrates could play different roles in the system performance, microbial community structure and functional genes. To our knowledge, it is the first report providing a detailed analysis of microbial community structure and phenol hydroxylase gene associated with the changes of degradation rate in phenolic compounds feeding system under high salt condition.

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References

Ahmed A M, 1995. Phenol degradation by *Pseudomonas serugi-nosa*. *Journal of Environmental Science and Health*, 30(1): 99–103.

Alvarez P J J, Vogel T M, 1991. Substrate interactions of benzene, toluene and *p*-xylene during microbial degradation by pure cultures and mixed cultures aquifer slurries. *Applied and Environmental Microbiology*, 57(10): 2981–2985.

Andrew D E, Lenore S C, Arnold E G, 1995. Standard Methods for the Examination of Water and Wastewater. Washington, DC: American Public Health Association.

Annadurai G, Juang R S, Lee D J, 2002. Microbiological degradation of phenol using mixed liquors of *Pseudomonas putida* and activated sludge. *Waste Management*, 22(7): 703–710.

Arutchelvan V, Kanakasabai V, Nagarajan S, Muralikrishnan

V, 2003. Isolation and identification of novel high strength phenol degrading bacterial strains from phenol-formaldehyde resin manufacturing industrial wastewater. *Journal of Hazardous Materials*, 127(1-3): 238–243.

Bielefeldt A, Stensel H D, 1999. Modeling competitive inhibition effects during biodegradation of BTEX mixtures. *Water Research*, 33(3): 707–714.

Bouchez T, Patureau D, Dabert P, Juretschko S, Dore J, Delqenes P *et al*., 2000. Ecological study of a bioaugmentation failure. *Environmental Microbiology*, 2(2): 179–190.

Brown V M, Jordan D H M, Tiller B A, 1967. The effect of temperature on the acute toxicity of phenol to rainbow trout in hard water. *Water Research*, 1(8-9): 587–597.

Buchan A, Neidle E L, Moran M A, 2001. Diversity of the ring-cleaving dioxygenase gene *pcaH* in a salt marsh bacterial community. *Applied and Environmental Microbiology*, 67(12): 5801–5809.

Cappello S, Denaro R, Genovese M, Giuliano L, Yakimov M M, 2007. Predominant growth of *Alcanivorax* during experiments on "oil spill bioremediation" in mesocosms. *Microbiology Research*, 162(2): 185–190.

Duarte G F, Rosado A S, Seldin L, Araujo W, Elsas J D, 2001. Analysis of bacterial community structure in sulfurous-oil-containing soils and detection of species carrying dibenzothiophene desulfurization (*dsz*) genes. *Applied and Environmental Microbiology*, 67(3): 1052–1062.

Enwall K, Philippot L, Hallin S, 2005. Activity and composition of the denitrifying bacterial community respond differently to long-term fertilization. *Applied and Environmental Microbiology*, 71(12): 8335–8343.

Evans W C, 1947. Oxidation of phenol and benzoic acid by some soil bacteria. *Journal of Biological Chemistry*, 41(3): 373–382.

Futamata H, Harayama S, Hiraishi A, Watanabe K, 2003. Functional and structural analyses of trichloroethylene-degrading bacterial communities under different phenol-feeding conditions: laboratory experiments. *Applied Microbiology and Biotechnology*, 60(5): 594–600.

Jiang H L, Tay J H, Maszenan A M, Tay S T L, 2004. Bacterial diversity and function of aerobic granules engineered in a sequencing batch reactor for phenol degradation. *Applied and Environmental Microbiology*, 70(11): 6767–6775.

Jiang Y, Wen J, Bai J, Jia X Q, Hu Z D, 2007. Biodegradation of phenol at high initial concentration by *Alcaligenes faecalis*. *Journal of Hazardous Materials*, 147(1-2): 672–676.

Kar S, Swaminathan T, Baradarajan A, 1997. Biodegradation of phenol and cresol isomer mixtures by *Arthrobacter*. *World Journal of Microbiology and Biotechnology*, 13(6): 659–663.

Klecka G M, Maier W J, 1988. Kinetics of microbial growth on mixtures of pentachlorophenol and chlorinated aromatic compounds. *Biotechnology and Bioengineering*, 31(4): 328–335.

Liu C, Shao Z, 2005. *Alcanivorax dieselolei* sp. nov., a novel alkane-degrading bacterium isolated from sea water and deep-sea sediment. *International Journal of Systematic and Evolutionary Microbiology*, 55(3): 1181–1186.

Meyer J S, Marcus M D, Bergman H L, 1984. Inhibitory interactions of aromatic organics during microbial degradation. *Environmental Toxicology and Chemistry*, 3(4): 583–587.

Mufiel B, Wafa A, Vincent U, Thierry H, 1999. DNA extraction from activated sludges. *Current Microbiology*, 38(6): 315–319.

Perron N, Welander U, 2004. Degradation of phenol and cresols at low temperatures using a suspended-carrier biofim process. *Chemosphere*, 55(1): 45–50.

Reardon K F, Mosteller D C, 2000. Biodegradation kinetics of benzene, toluene, and phenol as single and mixed substrates for *Pseudomonas putida* F1. *Biotechnology and Bioengineering*, 69(4): 385–400.

Sambrook J, Fritsch E F, Maniatis T, 1989. Molecular Cloning: A Laboratory Manual. NY: Cold Spring Harbor Laboratory Press.

Schie P M, Young L Y, 2000. Biodegradation of phenol: mechanisms and applications. *Bioremediation Journal*, 4(1): 1–18.

Torsvik V, Ovreas L, 2002. Microbial diversity and function in soil: from genes to ecosystems. *Current Opinion in Microbiology*, 5(3): 240–245.

Watanabe K, Teramoto M, Futamata H, Harayama S, 1998. Molecular detection, isolation, and physiological characterization of functionally dominant phenol-degrading bacteria in activated sludge. *Applied and Environmental Microbiology*, 64(11): 4396–4402.

Widada J, Nojiri H, Omori T, 2002. Recent developments in molecular techniques for identification and monitoring of xenobiotic-degrading bacterial and their catabolic genes in bioremediation. *Applied Microbiology and Biotechnology*, 60(1-2): 45–59.

Yu Y G, Loh K C, 2002. Inhibition of *p*-cresol on aerobic biodegradation of carbazole and sodium salicylate by *Pseudomonas putida*. *Water Research*, 36(7): 1794–1802.