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Bioaugmentation of bromoamine acid degradation with Sphingomonas *xenophaga* QYY and DNA fingerprint analysis of augmented systems

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Abstract

One high-effective bromoamine acid (1-amino-4-bromoanthraquinone-2-sulfonic acid, BAA) degrading strain was isolated previously with the ability to use BAA as sole source of carbon and nitrogen. It was identified as *Sphingomonas xenophaga* QYY by 16S rDNA sequence analysis and physio-biochemical tests. In this study, bioaugmentation of BAA degradation with suspended and immobilized cells of strain QYY was investigated. The optimal degradation conditions were as follows: temperature 30 °C, pH 6.0–7.0, 150 rev min^{-1} and the immobilized cells maintained degradation activity to BAA after 60 days storage at 4 °C. The structure of BAA was evidently changed according to the analysis of total organic carbon removal of BAA (about 50%) and the UV–VIS spectra changes during the biodegradation. Bioaugmented systems exhibited stronger abilities degrading BAA than the non-bioaugmented control ones. And microbial community dynamics of augmented systems was revealed by amplified ribosomal DNA restriction analysis (ARDRA), a modern DNA fingerprint technique. The results indicated that the microbial community dynamics was substantially changed throughout the augmentation process. This study suggests that it is feasible and potentially useful to enhance BAA degradation using bioaugmentation with the immobilized cells of BAA-degrading bacterium.

Abbreviations: ARDRA – amplified ribosomal DNA restriction analysis; AS – activated sludge; BAA – bromoamine acid; CGMCC – China General Microorganism Culture Center; IC – immobilized cells; SBRs – sequencing batch reactors; SC – suspended cells; TOC – total organic carbon; UV – ultraviolet; VIS – visible.

Introduction

Synthetic dyes are extensively used in textile dyeing, paper, printing, color photography, pharmaceutics, cosmetics and other industries (Banat et al. 1996; Kabadasil et al. 1999; Wrobel et al. 2001). During production, 10–15% of synthetic dyes produced are discharged into the environment (Pearce et al. 2003). Since some of the dyes are harmful and xenobiotic, colored wastewater poses an important environmental problem (Panswad & Luangdilok 2000; Forgacs et al. 2004). However, much of the work undertaken in bio-treatment of dye-containing wastewater concerned azo dyes (Chang et al. 2000; Zee van der et al. 2001). There is little information on biodegradation of anthraquinone dyes (Walker & Weatherley 2000). Bromoamine acid (1-amino-4-bromoanthraquinone-2-sulfonic acid), BAA, a major intermediate, is widely used in synthesis of anthraquinone dyes.

It is water-soluble and represents red in waterbody, which causes serious environmental pollution (Pearce et al. 2003; Forgacs et al. 2004). Nevertheless, there have been few studies on BAA biodegradation up to now. Efficient and reliable color removal is critically important for dyes wastewater treatment. Although the conventional activated sludge systems are generally considered effective to treat colored wastewater, they often fail to achieve high efficiency due to toxicity or recalcitrance of these compounds (Makinen et al. 1993). Bioaugmentation is expected as the most straightforward strategy to remedy such systems (Van Limbergen et al. 1998).

Bioaugmentation of activated sludge systems with specialized bacterial strains could be a powerful tool to improve several aspects of wastewater treatment (Rittman & Whiteman 1994; Van Limbergen et al. 1998). It has been reported to enhance degradation and removal of specific pollutants such as phenol, 3-chloroaniline, phosphate, and aromatic hydrocarbons (McClure et al. 1991; Boon et al. 2000; Yu & Mohn 2001b). The bacteria used in bioaugmentation have to meet at least three criteria (i.e. active, persistent and compatible) as described previously (Yu & Mohn 2001b). However, there are few microorganisms suitable for bioaugmentation. Nowadays, the genus *Sphingomonas* has received a lot of attention and became biotechnologically interesting microorganism for its degradation capabilities of various xenobiotic compounds, especially polycyclic aromatic hydrocarbons (David et al. 1996; Busse et al. 1999). In our laboratory, a new bacterial strain identified as *S. xenophaga* QYY demonstrated higher degrading ability to BAA, and the initial study showed that it was candidate for bioaugmentation of BAA in laboratory sequencing batch reactors.

However, very little is known about the normal dynamics of microbial communities in augmented systems. Activated sludge, even though it is the key part of the process, is still too often considered as an impenetrable "black box" (Dabert et al. 2002). Ecological studies of microbial communities will advance our fundamental understanding of microbial ecology and provide useful information for improving the design and performance of treatment systems (Dabert et al. 2002; Wagner et al. 2002). DNA fingerprints provide a relatively effective way to monitor the dynamics of bacterial community (Amann et al. 1998). There are some DNA fingerprints methods (Yu & Mohn 2001a, 2002; Wagner et al. 2002; Smith et al. 2003), including ribosomal intergenic spacer analysis, terminal restriction fragment length polymorphisms analysis, denaturing and temperature gradient gel electrophoresis. Although each method is available to observe changes of microbial communities, there are still some pitfalls (Wintzingerode et al. 1997; Dabert et al. 2002). Amplified ribosomal DNA restriction analysis, ARDRA, is a currently developed method, which exhibits more efficient ability to reveal bacterial communities of various systems (Eric et al. 1997; Dabert et al. 2002).

The objective of this study was to evaluate the potential utility of BAA bioaugmentation with *S. xenophaga* QYY and monitor the microbial community dynamics of augmented systems. This is the first report of community dynamics revealed by ARDRA during bioaugmentation of BAA degradation.

Materials and methods

Chemical

BAA is a major synthetic intermediate of anthraquinone dyes. It was kindly presented by Dye Synthesis Laboratory of Dalian University of Technology. The maximum absorbance wavelength in visible fields of BAA is 485 nm and its molecular mass is 382.19. The exact chemical structure is shown in Figure 1.

Bacterial strain, media and activated sludge samples

Strain QYY was originally isolated from sludge samples in our laboratory and deposited in the

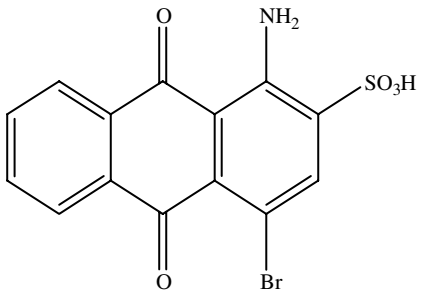

Figure 1. Chemical structure of BAA.

China General Microorganism Culture Center (CGMCC) with the accession number 1172. It was identified as *S. xenophaga* according to the physiological-biochemical characteristics and 16S rRNA gene sequencing. It was a Gram-negative, non-motile, rod-shaped, aerobic bacterium, wide (0.3–0.4) μm and long (1.0–1.4) μm. Colonies were mucoid, rounded and yellow. It was oxidase-positive, catalase-positive and denitrification-negative. Acid can be produced from glucose and esculin is hydrolyzed. The following compounds are hydrolyzed: glucose, DL-arginine, arabinose, galactose, maltose and leucine.

The media used in this study were BAA-Luria-Bertani medium, which contained (g l^{-1}): 10 Bactotryptone, 5 Bactoyeast extract, 10 NaCl, 0.1 BAA, pH 7.2, and the synthetic wastewater medium, which contained (g l^{-1}): 1.3 KH_2PO_4, 1.5 Na_2HPO_4, 0.1 $(NH_4)_2SO_4$, 0.01 $FeCl_3$, 0.02 tryptone, and BAA was 110 to 520 mg l^{-1}, pH 7.0.

Samples of fresh activated sludge (AS) were taken from Dalian Chunliu River Wastewater Treatment Plant, which were used as the indigenous populations in the sequencing batch reactors (SBRs).

Operation and construction of augmented SBRs

The SBRs were simulated with 250-ml flasks containing 50-ml synthetic wastewater medium with inoculants of different forms (see below) and were incubated at 30 °C on a shaker (Constant Temperature Rolling-beds, Model HYA, Wuhan, China). The SBRs were operated on a 12 h cycle, and each cycle consisted of 10 min fill, 11 h react, 40 min settle, 10 min decant. The mean cell residue time (sludge age) was controlled for about 10 days. Samples were taken once a day for both BAA degradation and DNA fingerprint analysis.

The augmented systems were (i) AS with suspended cells of strain QYY (ASSC), and (ii) AS plus immobilized cells (ASIC). The SBR without inocula was used as a control. The initial concentration of the AS was 3.08 g l^{-1}, and the inoculation amount was 9.07% (the dry weight of the supplemented culture to that of indigenous AS).

Before immobilization, strain QYY was precultured in BAA-Luria-Bertani medium for one day, and then the cells were washed twice with sterile water and re-suspended in ddH_2O. The cells suspension was mixed thoroughly with equal volume of sodium alginate solution. The mixture was extruded as drops into 5% $CaCl_2$ solution. The gauge number of the hypodermic needle used during extrusion controlled bead size. The gel beads were cross-linked for 4 h at 4 °C then washed twice and stored in ddH_2O using sealed vessels in refrigerator (4 °C) for subsequent use.

Effect of different environmental factors

Degradation of BAA by suspended and immobilized cells of strain QYY was carried out in BAA-Luria-Bertani medium with 100 mg l^{-1} BAA, different temperatures (20–40 °C), pH (5.0–10.0) and shaking speeds (0–200 rev min^{-1}).

Analytical methods

Cell concentration was measured spectrophotometrically at 660 nm. And the concentration of BAA was determined by monitoring the changes at maximum absorbance at 485 nm by Jasco UV–560 (Japan). Total organic carbon (TOC) was measured with a TOC analyzer (TOC 5050A, Shimadzu Co., Kyoto).

Genomic DNA extraction, PCR, DNA fingerprints

The genomic DNA was extracted from the sludge samples (without any pre-treatment) by the method described previously (Mufiel et al. 1999). The universal primers 8f and 1522r were used and the PCR amplification of 16S rDNA was conducted in a total volume of 50 μl containing 0.5 pmol of each primer per μl, 20 μM (each) dNTP, 1 × *LA-taq* buffer, and 0.5 μl *LA taq* (TaKaRa, Dalian Co., Ltd). The DNA templates were first subjected to an initial denaturation step for 2 min at 95 °C. The subsequent cycles consisted of a 1 min denaturation step at 94 °C, a 1 min annealing step at 55 °C, and 1 min extension step at 72 °C. A final 10 min extension at 72 °C was included after 30 cycles of PCR amplification.

After purification the resultant PCR products were digested with *Hin*f-I for 3 h, then the digestion fragments of 16S rDNA were resolved on native poly-acryl amide (6%) gels, which were stained with ethidium bromide and photographed. The cluster analysis of ARDRA was generated using UVI-Band-Map soft.

Results and discussion

Effects of different factors on BAA degradation

Some environmental factors (e.g. temperature, pH and oxygen supply) were reported to affect degradation activity of microorganisms (Pearce et al. 2003). Effects of temperature and pH on BAA degradation by both suspended and immobilized cells were shown in Figure 2 and Figure 3, respectively. The results suggested that the trends of temperature effect were similar, but it seemed that the immobilized cells showed higher capability than the suspended cells. The percentage of BAA degradation reached the highest value (98%) at 30 °C. It was obvious that either suspended or immobilized cells did not show high degradation percentage (only 20%) of BAA at 40 °C. According to Figure 3, immobilized cells could tolerate wider pH range (5.0–8.0) than that (6.0–7.0) of the suspended cells. It was concluded that immobilization could protect cells from losing activity. Thus, the optimal temperature and the appropriate pH range were 30 °C, 6.0–7.0, respectively.

Strain QYY was aerobic bacterium as described in the materials and methods. Therefore, the oxygen was an important factor to be considered. The effects of different shaking speeds were studied, which affected not only oxygen supply to BAA degradation but also agitation of immobilized cells. As shown in Figure 4, the immobilized cells exhibited lower BAA degradation percentage than that of suspended cells under each shaking speed. The possible explanation was that there might be some uncontrollable factors such as the quantity of bacterial cells in the immobilization, mass transfer, etc., which caused such experimental errors. However, these errors did not affect deciding the optimal shaking speed. It was obvious that the optimal shaking speed was 150 rev min^{-1}. In addition, the stability of immobilized cells was especially to be investigated. The results showed that the immobilized cells maintained extremely high BAA degradation percentage (more than 95% removal of BAA) after storage at 4 °C for 60 days (data not shown). Therefore, the immobilized cells demonstrated distinct superiority to BAA degradation.

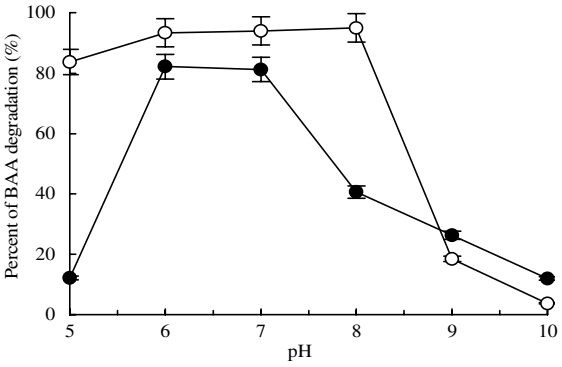

Figure 3. Effect of pH on BAA degradation (Temperature 30 °C; shaking speed 150 rev min^{-1}; ○: immobilized cells; ●: suspended cells).

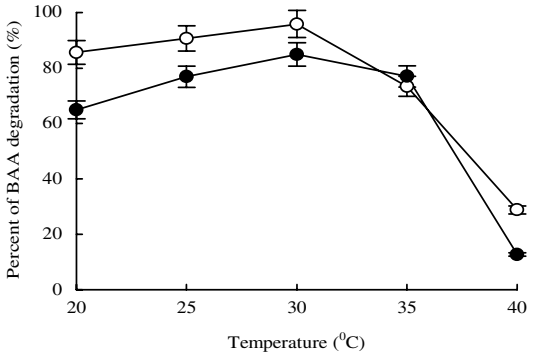

Figure 2. Effect of temperature on BAA degradation (pH 7.0; shaking speed 150 rev min^{-1} ; ○: immobilized cells; ●: suspended cells).

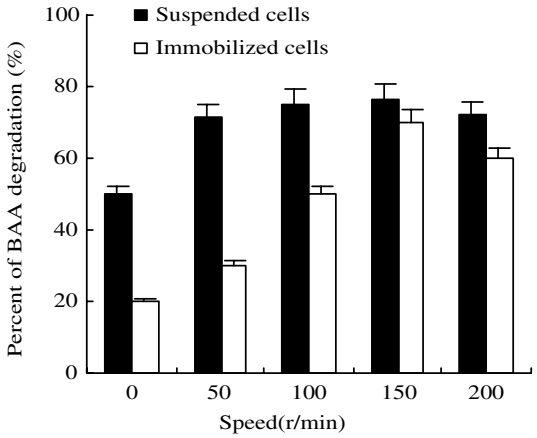

Figure 4. Effect of shaking speed on BAA degradation (Temperature 30 °C; pH 7.0; □: immobilized cell; ■: suspended cells).

UV–VIS and TOC changes during BAA degradation

The specific wavelengths of BAA are 235, 250 and 485 nm. Figure 5 showed the UV–VIS absorbance spectra during BAA degradation. A maximum absorbance was observed at 485 nm, and this peak decreased as a function of time and disappeared completely after 120 h cultivation. Meanwhile, there was a significant decrease at 250 nm. And it can be easily seen that the peak of 235 nm could not be detected also. All the great changes occurred both in UV and VIS fields indicated that the molecular structure of BAA had been destroyed during degradation, simultaneously, the solution containing BAA changed from red to colorless. During the degradation process, TOC of BAA decreased gradually, and then maintained stable removal percentage. As shown in Figure 6, more than 50% TOC of BAA was removed. Combined analysis of UV–VIS spectra with TOC removal, it was concluded that the structure of BAA was substantially changed and the anthraquinone ring should be destroyed.

Performance of bioaugmented SBRs

Bioaugmentation is a procedure adding a selected species to a complex ecosystem in order to improve the ecosystem's capability (van Limbergen et al. 1998). In our study, the non-bioaugmented AS system together with ASSC and ASIC systems were investigated under the same conditions. However, the results indicated that the AS system

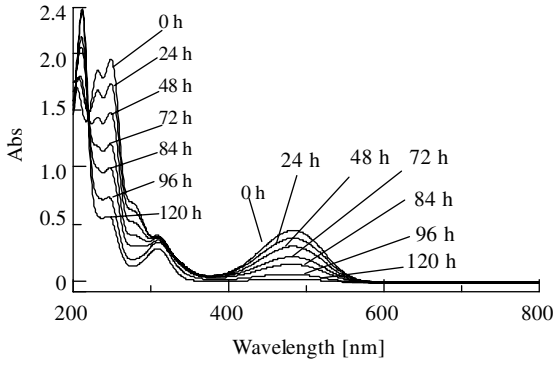

Figure 5. Variation of UV–VIS spectra during BAA degradation (pH 7.0; temperature 30 °C; shaking speed 150 rev min^{-1}).

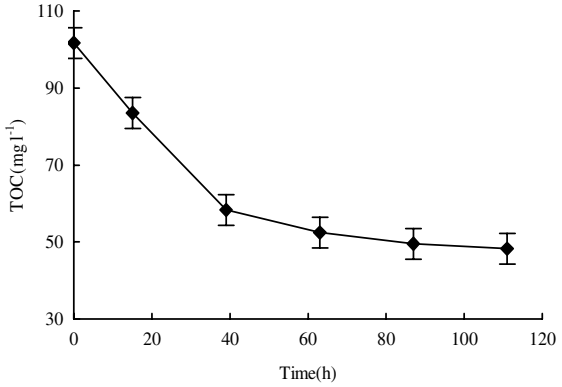

Figure 6. TOC removal of BAA (pH 7.0; temperature 30 °C; shaking speed 150 rev min^{-1}).

hardly remove BAA during the experiment process (data not shown). It could be presumed that there were no BAA-degrading indigenous populations in AS, or such specialized organisms were not abundant in AS system.

Bioaugmentation of BAA with 9.07% inocula, which exhibited the greatest ability in the initial tests, was shown in Figure 7. Meanwhile two operation conditions were marked out in Figure 7. According to ASSC, BAA degradation was relatively stable during the first BAA shock loading. However, the degradation efficiency of ASSC became worse after 12 days operation. When BAA was increased to 220 mg l^{-1}, the ASSC systems appeared upset. In order to remedy the upset system, suspended cells were inoculated once more just on day 18. Although the system was remedied,

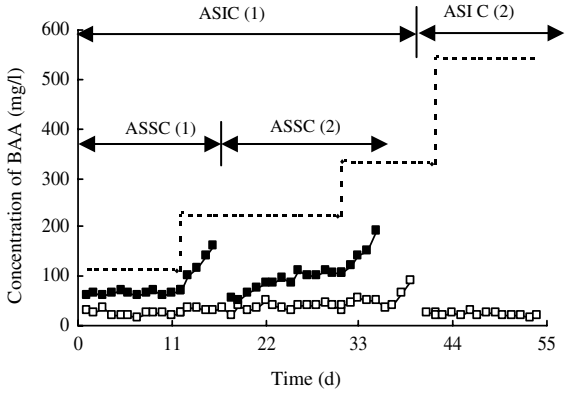

Figure 7. BAA degradation in augmented SBRs. (--- : influent; □: performance of ASSC systems; ■: performance of ASIC systems).

the performance was not good, only 70% BAA degradation.

Compared with ASSC system, the ASIC system demonstrated stronger ability to cope with higher BAA shock loading and successfully dealt with BAA up to 520 mg l^{-1}. Under the second operation condition, the cycle time was changed to 24 h. It was concluded that ASIC systems could improve the buffering capability and prevent the inocula from being grazed or washed out. Therefore, bioaugmentation with immobilized cells of strain QYY was potentially useful in practical treatment processes.

Though bioaugmentation is powerful in wastewater treatment, its effectiveness remains controversial. Various possible explanations have been proposed to explain these experimental failures: problems related to the adaptation of the inoculated microorganisms, substrate limitations, competition between the introduced species and the indigenous biomass, or grazing by protozoa (Eberl et al. 1997). However, these studies have been hampered due to limited ecological data, which are about activity and fate of the inoculated organisms as well as about their interaction with the indigenous microbial community (Rittmann & Whiteman 1994). So far, only limited studies have been reported to use molecular tools to monitor the inoculation of pure cultures in activated sludge (Eberl et al. 1997; Oerther et al. 1998; Bouchez et al. 2000) or adapted microbial communities (Oerther et al. 1998; Dabert et al. 2001). Therefore, it is necessary to study microbial community dynamics of bioaugmented systems.

Microbial community dynamics revealed by ARDRA

Amplified ribosomal DNA restriction analysis (ARDRA) was usually used for assessing the microbial community dynamics (Elsas et al. 1998; Lionel et al. 2000). It is a DNA fingerprint technique based on PCR amplification of 16S rDNA using primers for conserved regions, followed by restriction enzyme digestions and electrophoresis. In this study, ARDRA technique was adopted to monitor the effect of bioaugmentation on the microbial community dynamics of AS systems. The restriction fragments produced were between 100 bp and 1500 bp in size (Figure 8a). As expected, the AS systems had similar fingerprint patterns (Figure 8a), and the samples of AS systems clustered together on the basis of similarity (Figure 8b). The percent similarity among the AS samples was about 80%.

In Figure 8a, band Q belonged to strain QYY (16S rDNA GenBank accession number AY611716). It was obvious that there were some

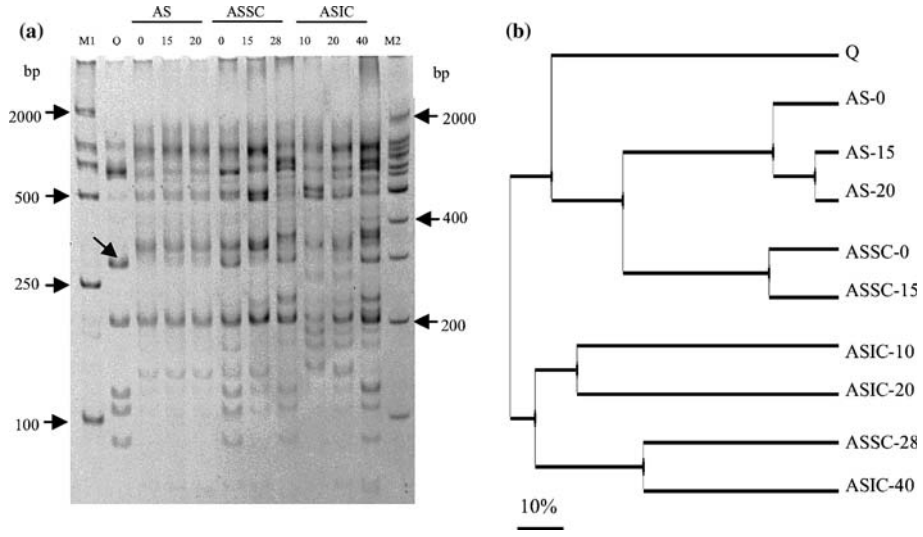

Figure 8. ARDRA fingerprints (a) and clusters analysis. (b). M1: DL2000 DNA marker; M2: 100 bp ladder DNA marker; Q: Strain QYY; AS: activated sludge alone; ASSC: AS with suspended cells of strain QYY; ASIC: AS with immobilized cells of strain QYY. The numbers 0, 15, 20, and 40, etc., indicates the sampling time (days). Bar indicates 10% similarity.

specific bands of strain QYY compared with those from indigenous populations in AS, e.g. the band approximately 300 bp, and other three bands around 100 bp. Such bands appeared in the samples of ASSC-0, ASSC-28 and ASIC-40 (Figure 8a). Therefore, bioaugmentation had great impacts on community fingerprints. In each augmented system, temporal changes in community composition were apparent. Several other bands entirely disappeared or appeared in the fingerprints of the augmented systems. The reason was that inoculation of strain QYY led to large decrease or increase of certain populations in the community, which is different from the earlier study (Yu & Mohn 2001b).

The ASSC-15 fingerprints appeared anomalous, which was extremely different from the samples ASSC-0 and ASSC-28 (Figure 8a). The densities of some bands (approximately 1500 bp, 500 bp, 350 bp and 200 bp) were increased. Such phenomena were not detected in other systems. As mentioned above, ASSC system showed worse performance in enhancing BAA degradation on day 15. Accordingly, such irregular changes of microbial communities would result in the function loss or decrease. Some studies have concluded that there was no, or only transient, improvement of process performance and it always correlated with the disappearance of the inoculated microorganisms (Bouchez et al. 2000). However, ASSC-0 and ASSC-15 were clustered together with 70% similarity (Figure 8b), whereas ASSC-28 and ASIC-40 were gathered in the same cluster with relative low similarity (40%), and this was not surprising due to similar function of these two systems.

According to the ASIC system, the fingerprints of sample ASIC-40 were different from others. There were specific restriction bands belonging to strain QYY in ASIC-40. It could be explained that with the growth of strain QYY and carriers being decomposed, the introduced cells entered the system. Therefore, the densities of specific bands belonging to QYY increased greatly after long operation (Figure 8a). It is interesting that the immobilized cells could persist well in ASIC systems. Possible explanation of this phenomenon include (a) *S. xenophaga* QYY possesses the ability to produce unique exopolysaccharide (David et al. 1996), which can incorporate itself into sludge floc, therefore, improving the settle ability of strain QYY, or (b) after long interaction, the indigenous and leakage QYY cells may develop so relative stable community structure that strain QYY can survive.

Although ARDRA method is available to reveal microbial community dynamics of augmented systems in our study, it may not be predicted to use elsewhere. Whatever the approach chosen, either ARDRA or other DNA fingerprinting, these techniques always describe the dominant species of microbial communities. They cannot detect a population that represents less than 1/100 of the ecosystem. Sub-dominant species, which are probably also very important for an ecosystem's adaptability, remain undetectable (Fisher & Triplett 1999; DeEtta et al. 2003).

Conclusion

This research demonstrates that BAA can be degraded by *S. xenophaga* QYY with the anthraquinone ring cleavage, and both suspended and immobilized cells of strain QYY can enhance BAA degradation. The degradation conditions were optimized as follows: temperature 30 °C, pH 6.0–7.0 and 150 rev min^{-1}. Based on our results, it is evident that ASIC system can tolerate high BAA shock loading than ASSC system, and will be potentially useful in practical wastewater treatments. Also, the microbial community dynamics was examined, which was substantially changed after bioaugmentation, and the DNA fingerprint patterns of augmented systems are absolutely different from the non-augmented one. Finally, it should also be recognized that ARDRA, like all other DNA fingerprint methods, might fail for various reasons to detect certain organisms, even if they are in high abundance (Amann et al. 1998; Dabert et al. 2002). The biases must be kept in mind, but they do not preclude useful comparisons of communities based on detectable phenotypes. Otherwise, the genus *Sphingomonas* is one kind of novel and unique microorganism resource (David et al. 1996; Busse et al. 1999). Therefore, bioaugmentation of BAA degradation with bacteria of such genus (e.g. strain QYY) may be authentically feasible in practical wastewater treatments.

Acknowledgements

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