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Bioaugmentation on decolorization of C.I. Direct Blue 71 by using genetically engineered strain *Escherichia coli* JM109 (pGEX-AZR)

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ABSTRACT

The study showed that *Escherichia coli* JM109 (pGEX-AZR), the genetically engineered microorganism (GEM) with higher ability to decolorize azo dyes, bioaugmented successfully the dye wastewater bio-treatment systems to enhance C.I. Direct Blue 71 (DB 71) decolorization. The control and bioaugmented reactors failed at a around pH 5.0. However, the bioaugmented one succeeded at around pH 9.0, the influent DB 71 concentration was 150 mg/L, DB 71 concentration was decreased to 27.4 mg/L in 12 h. The 1–3% NaCl concentration of bioaugmented reactors had no definite influence on decolorization, DB 71 concentration was decreased to 12.6 mg/L in 12 h. GEM was added into anaerobic sequencing batch reactors (AnSBRs) to enhance DB 71 decolorization. Continuous operations of the control and bioaugmented AnSBRs showed that *E. coli* JM109 (pGEX-AZR) could bioaugment decolorization. The concentrations of activated sludge and GEM were still more than 2.80 g/L and 1.5×10^6 cells/mL, respectively, in the bioaugmented AnSBR. All the microbial communities changed indistinctively with time. The microbial community structures of the control AnSBR were similar to those of the bioaugmented one.

1. Introduction

Direct dye, most of which are di- or tri-azo dyes, is commonly used in the printing process of textile industry. Large amounts of wastewater were discharged during processing, especially in the coloring and washing steps. Chemical treatment processes, such as oxidation, photo catalysis, coagulation and adsorption, are traditionally used to remove color from the wastewater, especially in large factories. However, chemicals using and operating costs are quite high and a large amount of solid wastes are produced [1–4].

Nowadays, biological treatment processes, such as the activated sludge system and sequencing batch reactor (SBR) are used in textile factories [5,6]. The organic matter of textile wastewater can be removed by biological treatment systems, but the color-causing substances still remain in the treated wastewater [7,8]. It has been found that some bacteria with the aid of an azoreductase and an electron donor transform azo dyes into colorless products under anaerobic conditions [9–12]. They have potential advantages in developing bio-treatment methods of wastewater containing azo compounds.

Bioaugmentation can be explained as a process in which the application of indigenous, wild type or genetically modified organism to the bioreactor, or to the polluted sites in order to improve the performance of the on-going biological processes [13]. It has been reported to enhance the removal of specific pollutants, such as 2,4-dichlorophenol, resin acid and bromoamine acid [14–16]. Nowadays, genetically engineered microorganism (GEM) has received a lot of attention. It has become biotechnologically interesting microorganism for its degradation capabilities of xenobiotic compounds and been widely applied in bioaugmentation systems [17–19].

Bioaugmentation treatment systems with special bacteria host complex microbial communities. Some molecular techniques, such as denaturing gradient gel electrophoresis (DGGE), ribosomal intergenic spacer analysis (RISA) and amplified ribosomal DNA restriction analysis (ARDRA) are widely used to assess the persistence of added bacteria and the effects on indigenous population [14,20–23].

The objectives of this study were (a) to test the effects of bioaugmentation with *Escherichia coli* JM109 (pGEX-AZR) on C.I. Direct Blue 71 (DB 71) removal, (b) to investigate the persistence of *E. coli* JM109 (pGEX-AZR) in complex microbial communities of DB 71 bio-treatment systems using most-probable-number PCR (MPN-PCR) and (c) to assess the effects of bioaugmentation on indigenous microbial communities using ARDRA.

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2. Materials and methods

2.1. Microorganism, DB 71, DB 71 wastewater and anaerobic sludge

E. coli JM109 (pGEX-AZR) was a GEM with higher ability to decolorize azo dyes constructed in our laboratory [11]. The azoreductase gene (537 bp) was obtained by PCR amplification from *Rhodobacter sphaeroides* AS1.1737. It was then inserted in expression vector pGEX4T-1, under the control of a *lac* operon, transformed and expressed in *E. coli* JM109. *E. coli* JM109 (pGEX-AZR) demonstrated significantly more intracellular azoreductase activities than the other two bacterial strains, which was in line with our previous reports [11,24]. The cell was inoculated into nutrient broth medium (1 g/L beef extract, 2 g/L yeast extract, and 5 g/L NaCl).

DB 71, a typical azo dye used in dyeing textiles, was of commercial quality. It was purchased from a textile factory in China.

The DB 71 wastewater used in this study was synthetic wastewater medium (SWM), which contained: mineral media, 150–550 mg/L DB 71, 500 mg/L glucose and 200 mg/L lactose. Glucose was used as co-substrate to provide the electrons for the reductive cleavage of the azo dyes. Lactose was used as inducer to induce expression of the GEM's azoreductase.

The mineral media contained (g/L): 3 $(NH_4)_2SO_4$, 4.26 $Na_2HPO_4{\cdot}12H_2O$, 2.65 $KH_2PO_4{\cdot}12H_2O$, 0.002 $MnSO_4{\cdot}7H_2O$, 0.01 $FeSO_4{\cdot}7H_2O$, 0.2 $MgSO_4{\cdot}7H_2O$, 0.02 $CaCl_2$, pH 7.5.

The anaerobic sludge (AS) was taken from our own laboratory and used as the indigenous populations.

2.2. Effects of initial pH on the bioaugmented reactor start-up phase

The effects of initial pH was tested under anaerobic conditions. The anaerobic reactor was carried out at 30 °C in 500 mL serum bottles (sealed with rubber septa) containing 500 mL SWM (final concentrations of 1 g/L dry AS, 150 mg/L DB 71).

Six reactors were used to study the effects of initial acid on the start-up. First, second and third reactors were used as control ones, their pH was adjusted to 7.5, 5.0 and 9.0 using 1N HCl and NaOH, respectively. The other three reactors were bioaugmented with 10% GEM (the dry weight of the GEM to that of AS), and their pH was like adjusted to 7.5, 5.0 and 9.0, respectively. Each experiment was conducted in triplicates.

2.3. Effects of initial salt concentration on the bioaugmented reactor start-up phase

Four reactors were used to study the effects of salt concentration. Experimental methods were similar to those of the acid shock. First and second reactors were used as control ones, their salt concentration was adjusted to 1 and 3%, respectively, using NaCl. The other two reactors were bioaugmented with 9.9% GEM, their salt concentrations were like adjusted to 1 and 3%, respectively.

2.4. Construction of AnSBRs and operation of bioaugmented AnSBRs

Anaerobic sequencing batch reactors (AnSBRs) were 2500 mL flasks containing 2000 mL of DB 71 SWM with 2.5 g/L AS. The anaerobic conditions were achieved by pumping nitrogen into the flask and topping the mouth with rubber stopper. The AnSBRs were operated at a constant temperature of 30 ± 2 °C.

Two AnSBRs were used to study the effects of GEM inoculation. The first reactor was a control one without bioaugmentation. The second reactor was a bioaugmented one inoculated with 10.2% GEM. These two AnSBRs were continuously operated for 66 d. Samples were taken once a day for both DB 71 concentration and molecular fingerprint analysis.

The AnSBRs were operated on a 24-h cycle, and each cycle consisted of 20-min fill, 22-h reaction, 80-min settle and 20-min decant.

The experimental process could be divided into four stages. The first stage was day 1–30, the AnSBRs were cultivated. The second stage was day 31–45, the response and stability of bioaugmented and control AnSBRs under concentration shock loading conditions were compared. The third stage was day 46–56, a continuous operation without DB 71 in the influent, survival and maintenance of the added GEM were investigated in the bioaugmented AnSBR. The final stage was day 57–66, during which DB 71 appeared in influent again and the AnSBRs was restarted up.

2.5. Analytical method

Samples (10 mL) were withdrawn and centrifuged at $10{,}000 \times g$ for 10 min to sediment the suspended particles. The DB 71 concentration (after appropriate dilution) was measured photometrically by Jasco UV-560 spectrophotometer (Japan).

2.6. DNA extraction, MPN-PCR and ARDRA

DNA was extracted by the method described previously [23].

For the analysis of MPN-PCR, serial 10-fold dilutions of the DNA were made. The primers (P1 and P2) for azoreductase gene were designed based on its open reading frame (GenBank database, Accession Number AY150311).

P1, 5′-TCACTCCACTCCTAGTTGT-3
P2, 5′-ATGTCCCCTATACTAGGTTATTG-3′

The PCR amplification of azoreductase gene was conducted in a total volume of 25 μL containing 1 μL DNA templates, 2 μL of each primer (10 pmol/L), 2 μL dNTP (2.5 mmol/L), 2.5 μL 10× PCR buffer, 0.25 μL *Taq* DNA polymerase (5 U/μL, TaKaRa, Dalian) and 15.25 μL ddH_2O. The DNA templates were first subjected to an initial denaturation step for 5 min at 94 °C. The subsequent cycles consisted of a 30 s denaturation step at 94 °C, a 30 s annealing step at 55 °C and a 1 min extension step at 72 °C. A final 7 min extension at 72 °C was included after 30 cycles of PCR amplification. The PCR amplified fragments were separated in agarose gel (1.5%) electrophoresis.

For the ARDRA, the primers were FD1 (5′-CCGAATTCGTCGACAACAGAGTTTGATCCTGGC-3′), RD1 (5′-C CCGGGATCCAAGCTTAAGGAGGTGATCCAGCC-3′). The PCR amplification of 16S rDNA was conducted in a total volume of 50 μL containing 2 μL DNA templates, 0.5 μL of each primer (20 pmol/L), 4 μL dNTP (2.5 mmol/L each), 5 μL 10× PCR buffer, 0.25 μL *Taq* DNA polymerase (5 U/μL, TaKaRa, Dalian) and 37.75 μL ddH_2O. The DNA templates were first subjected to an initial denaturation step for 1.5 min at 94 °C with a hot start. The subsequent cycles consisted of a 1 min denaturation step at 94 °C, a 1 min annealing step at 48 °C and a 1 min extension step at 72 °C. A final 5 min extension at 72 °C was included after 30 cycles of PCR amplification. 16S rDNA templates were digested with 2 U of restriction enzyme HaeIII (which was optimized in previous studies, data not shown) in a total volume of 25 μL at 37 °C for 3 h [24]. The PCR amplified fragments were separated in 8% native polyacrylamide gel, and stained with ethidium bromide and photographed for ARDRA analysis.

3. Results and discussion

3.1. Effects of initial pH on the bioaugmented reactor start-up phase

The effects of initial pH on DB 71 decolorization during the start-up phase of anaerobic reactor bioaugmented with GEM were illustrated in Fig. 1.

The influent DB 71 concentration was 150 mg/L. In the control reactor (pH 7.5), DB 71 concentration was decreased to 48.5 mg/L until 6 h, and steadily to 16.7 mg/L in 12 h. In the bioaugmented reactor (pH 7.5), DB 71 concentration was decreased to 29.2 mg/L until 6 h, and steadily to 12.6 mg/L in 12 h. The specific decolorization rate was 15.3 and 18.0 mg/(g h), respectively, in the control and bioaugmented reactors. The results showed that the bioaugmentation improved the removal of the target compounds. In the control reactor (pH 5.0), DB 71 concentration was decreased to 79.9 mg/L until 12 h. The system could not decolorize DB 71 completely, and its decolorization rate was lower than that of the pH 7.5 control reactor. In the bioaugmented reactor (pH 5.0), DB 71 decolorization rate was similar to that of control one.

The alkali and acidic conditions affected bioaugmentation differently. The bioaugmented reactors also decolorized DB 71 faster than the control ones under alkali conditions. In the control reactor (pH 9), DB 71 concentration was decreased to 65.9 mg/L until 6 h, and steadily to 27.4 mg/L in 12 h. The specific decolorization rate was 13.9 and 16.5 mg/(g h), respectively, in the control and bioaugmented ones. In the bioaugmented reactor (pH 9.0), DB 71 decolorization rate was similar to that of the bioaugmented reactor (pH 7.5).

The results showed that the bioaugmentation improved the removal of the target compound. The bioaugmented reactor also demonstrated faster DB 71 decolorization rate than the control one. After a longer incubation, the control reactor also removed 90% DB 71, indicating the existence and survival of the indigenous DB 71-degrading populations. The control and bioaugmented reactors failed at around pH 5.0, suggesting that exposure to pH 5.0 affected the indigenous DB 71-degrading populations and *E. coli* JM109 (pGEX-AZR). The bioaugmented reactor succeeded at around pH 9.0, suggesting that exposure to pH 9.0 did not affect activity of *E. coli* JM109 (pGEX-AZR). *E. coli* JM109 (pGEX-AZR) inoculation enhanced biodegradability of the high-alkali dyeing wastewater.

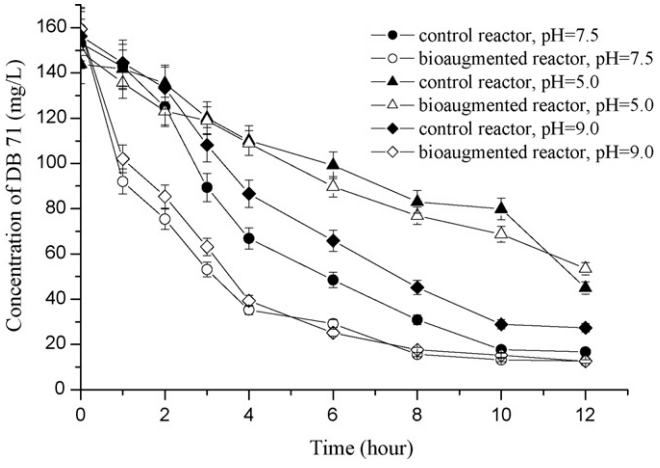

Fig. 1. Effects of initial pH on DB 71 decolorization during the start-up phase bioaugmented with GEM. All values are means ($n = 3$).

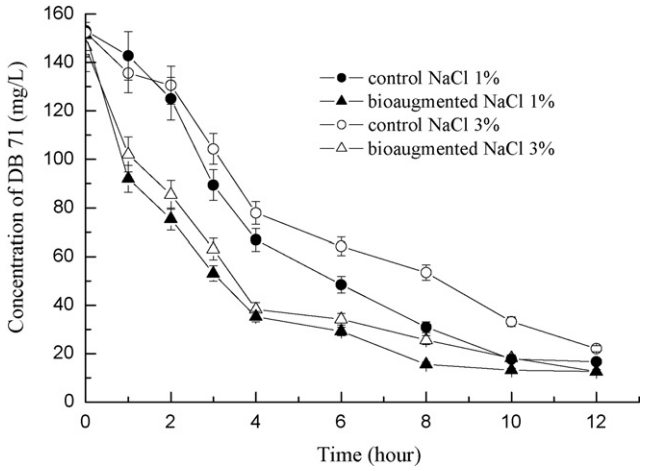

Fig. 2. Effects of NaCl concentration on DB 71 decolorization during the start-up phase bioaugmented with GEM. All values are means ($n = 3$).

3.2. Effects of salt concentration on the bioaugmented reactor start-up phase

Usually there is a salting out process in the production of dye. Thus the salt concentration is generally high in dyeing and printing wastewater. This requires the bio-treatement system to have outstanding salt tolerance ability. The effects of NaCl concentration on the start-up phase of bioaugmented reactor decolorizing DB 71 were illustrated in Fig. 2.

In the bioaugmented reactor (NaCl, 3%), DB 71 decolorization rate was similar to that of bioaugmented reactor (NaCl, 1%). DB 71 concentration was decreased to approximately 30 mg/L until 6 h, and steadily to 12.6 mg/L in 12 h. The concentration of salinity had not obvious influence on decolorization.

3.3. Long operation of bioaugmented and control AnSBRs

The retention of degradation properties and cell maintenance of the bioaugmented system are usually concerns of bioaugmentation study. It has been reported that the periodic change of selected environmental conditions is an effective means to control the abundance and activity of the introduced microorganisms in multi-species microbial communities [15,25,26]. Sequencing batch reactor is operated in such a periodic mode. Its environment conditions can be changed periodically, which is useful to facilitate the survival and growth of the specific species and keep their activities. On the other hand, entrapment of the specific culture is an efficient way to prevent microbes from grazing by protozoa, thus reducing biomass loss caused by washout and increasing its ability to cope with high-organic loading. It was reported that appropriate inoculation amount is a key factor for successful bioaugmentation [27]. The decolorization rate of azo dye was affected by the amount of *E. coli* JM109 (pGEX-AZR) inoculated and the best amount of inoculation was 10% [28].

To study the differences between a bioaugmented and a control AnSBRs under long time operation, two AnSBRs were operated for about 2 months (Fig. 3). In the first stage, the bioaugmented AnSBR achieved a steady state after 11 d with 71.2 mg/L influent DB 71, while 17 d were needed for the control AnSBR. The bioaugmented AnSBR also remained higher DB 71 decolorization efficiency than the control one during the stable period, DB 71 decolorization rate was above 90 and 95% in the control and bioaugmented AnSBRs, respectively.

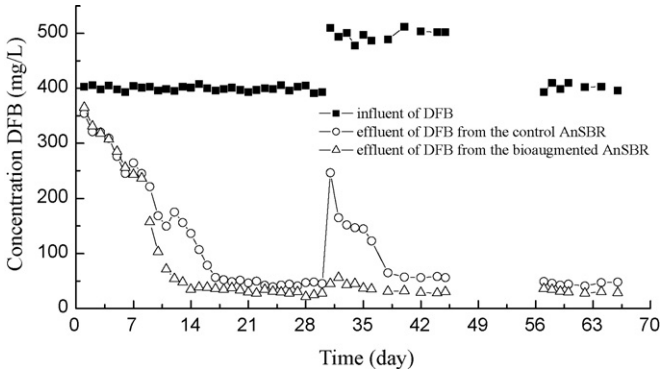

Fig. 3. Performance of the control and bioaugmented AnSBRs over long time operation.

In the second stage, the DB 71 concentration in the influent increased abruptly to 500 mg/L. The control AnSBR could not decolorize DB 71 completely. The effluent DB 71 concentration climbed up to 246 mg/L, and then dropped gradually to 56–65 mg/L in 7 d. However, the bioaugmented AnSBR could cope with the increase of concentration successfully, demonstrating a steady effluent DB 71 concentration of 28–35 mg/L.

One concern on bioaugmentation method was whether occasional changes of carbon sources, especially from a less bio-available substance to a more bio-available substance, would cause the supplemented specific culture to lose its specific ability. Glucose was known as a more bio-available carbon source and it was served as sole carbon source in feed for about 10 d (day 46–56). When influent DB 71 concentration increased to 400 mg/L, the bioaugmented and control AnSBRs still demonstrated strong decolorization ability. The above results suggested that for both the bioaugmented and the control AnSBRs, the ability to decolorize DB 71 could not be easily lost due to the lack of selective target compound occasionally. The bioaugmented AnSBR exhibited higher tolerance to shock loading of DB 71 than the control system.

The concentration changes of the sludge in two AnSBRs were illustrated in Fig. 4. The changes of control AnSBR were similar to those of the bioaugmented one. In the control AnSBR, the concentration of the sludge was increased to 3.02 g/L until 6 d, which might be due to the supplementation of nutrients. It was found that activated sludge was able to adapt to the variation during operation. The concentration of sludge decreased after day 6. The azo dye and its intermediates were toxic substance, which would inhibit the activated sludge multiplication. The control AnSBR reached a new stable state in 12 d, the concentration of activated sludge was still more than 2.30 g/L. The bioaugmented AnSBR reached a new stable state in 14 d, the concentration of activated sludge was still more than 2.80 g/L.

It is worth pointing out that it is difficult to enumerate a bacterial population in a complex microbial community like activated sludge using conventional methods. In this study, species-specific MPN-PCR assays, which are culture-independent, specific, and sensitive, were used to monitor the population dynamics of the GEM in bioaugmented AnSBR.

The survival and maintenance of the added bacteria in the system is a prerequisite to a successful bioaugmentation. After being introduced, the GEM was persistent in the sludge microbial community (Fig. 5). These results indicate that GEM was competitive in the sludge microbial community and were retained well during settling of the biomass. Initially the GEM was more than 1.8×10^6 cells/mL in the bioaugmented AnSBR. During 3 d, the GEM populations increased slightly, to more than 2.1×10^6 cells/mL in the bioaugmented AnSBR. It was found that nutrients supplementation increased amounts of GEM. The GEM populations decreased slightly after day 7, the GEM was still more than 1.5×10^6 cells/mL in 14 d. This decrease may be attributed to their adaptation to the new experimental conditions.

The bioaugmented AnSBR with the suspended GEM could cope with a stepwise increase of the azo dye concentration successfully. It is interesting that although the GEM is not a flock-forming bacterium, it was well retained by the settling procedure used for the AnSBR. Possible explanations of this phenomenon include (1) activated sludge harbors numerous flock-forming bacteria (e.g., *Zoogloea* spp.), which can incorporate other non-flocculating bacteria including the GEM into sludge flocks, thus improving the settleability of the GEM, or (2) some members of the sludge community may have provided beneficial effects to GEM so that it grew better. These results showed that even non-flocculating bacteria can be used as inocula for bioaugmentation of activated sludge systems. It is also interesting that *E. coli* JM 109 (pGEX-AZR) was not indigenous to the sludge sample used to inoculate the SBRs. However, after introduction, the GEM could compete well with the indigenous bacteria in the sludge community and persist. This indicates that the inoculation for bioaugmentation may not necessarily have to be from the microbial community to be bioaugmented. Therefore, the

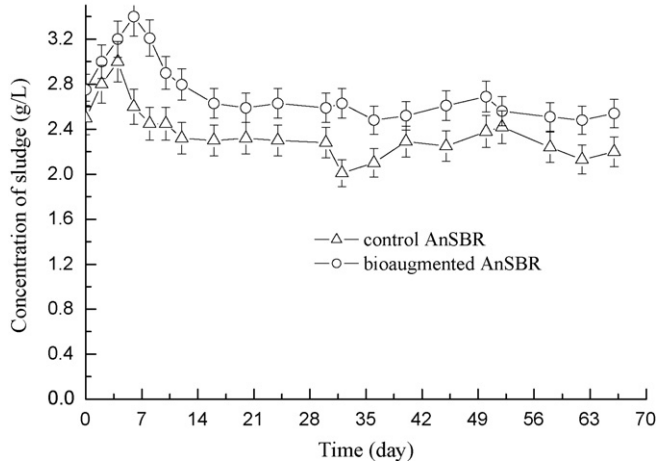

Fig. 4. Changes of the concentration of the sludge in bioaugmented and control AnSBRs.

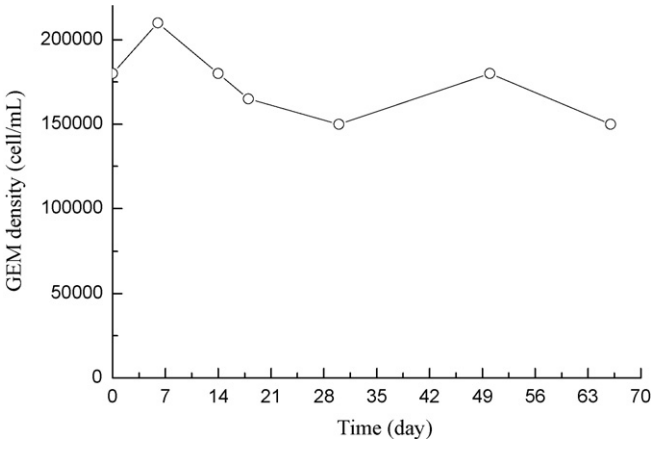

Fig. 5. Changes of the concentration of *E. coli* JM109 (pGEX-AZR) in the bioaugmented AnSBR.

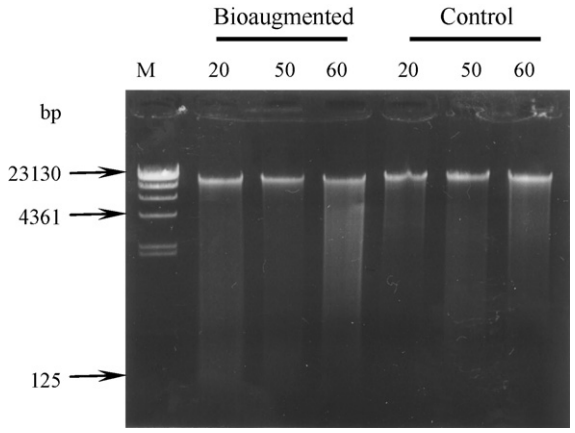

Fig. 6. Agarose gel (1.5%) electrophoresis of genomic DNA of activated sludge in the control and bioaugmented AnSBRs. M: λ-HindIII DNA marker; numbers of 20, 50 and 60 indicated the sampling time (days).

GEM meets the second criterion for successful bioaugmentation: competitive and persistent in sludge microbial communities [14].

3.4. Effects of bioaugmentation on community structure

Bio-treatment systems host complex microbial communities. Such complex communities cannot be comprehensively characterized with available methods. In this study, ARDRA was used to study the change of microbial community structure after bioaugmentation.

The community genomic DNA of control and bioaugmented AnSBRs in 20, 50, 60 d were extracted and determined by 1% agarose gel electrophoresis (Fig. 6). The acquired results showed DNA with the length of 23 kb.

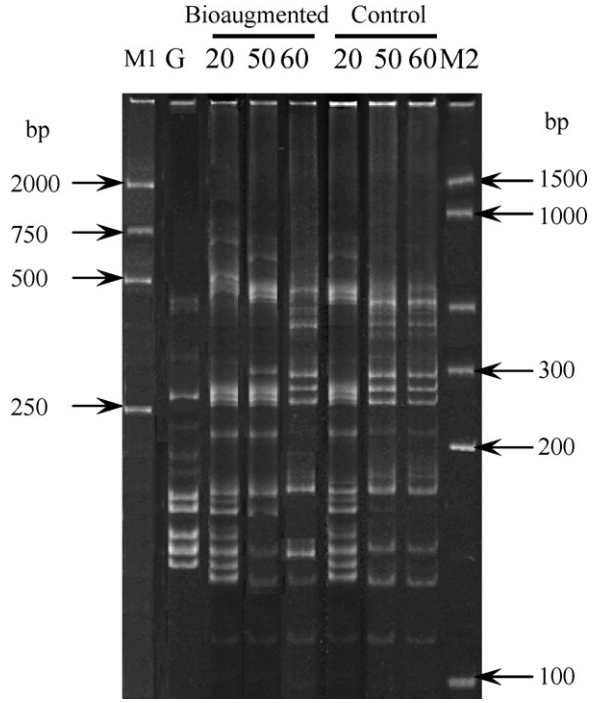

Fig. 7. Analysis of ARDRA fingerprints of the control and bioaugmented AnSBRs. M1: DNA marker DL2000; M2: 100 bp DNA ladder marker; G: *E. coli* JM109 (pGEX-AZR); numbers of 20, 50 and 60 indicated the sampling time (days).

The bioaugmentation affected greatly the ARDRA banding patterns of the sludge community (Fig. 7). All the microbial communities changed indistinctively with time. The microbial community structures of the control AnSBR were similar to that of the bioaugmented one, though demonstrating lower intension. The application of GEM could increase the biodiversity of the sludge community.

4. Conclusions

E. coli JM109 (pGEX-AZR) was a GEM with higher ability to decolorize azo dyes stored in our laboratory. The bioaugmented reactor failed at around pH 5.0. The bioaugmented reactors succeeded at around pH 9.0 and at the salinity of 1–3%. *E. coli* JM109 (pGEX-AZR) inoculation enhanced biodegradability of dyeing wastewater with high alkalinity and salinity and was effective in the removal of organic pollutants in wastewater. *E. coli* JM109 (pGEX-AZR) was tested in AnSBRs in order to enhance DB 71 decolorization. For 66 d continuous operation in the AnSBRs, both the tolerance to DB 71 concentration shock and the decolorization rate in the bioaugmented AnSBR were higher than those of the control one. The concentration of activated sludge was still more than 2.80 g/L and GEM was still more than 1.5×10^6 cells/mL in the bioaugmented AnSBR after 14 d. ARDRA revealed that the introduced *E. coli* JM109 (pGEX-AZR) was persistent in the bioaugmented systems and maintained higher metabolic activity.

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