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Analyzing Microbial Community Changes of Activated Sludge in Bioaugmentation Reactor by DGGE-PCR

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In this study, effluent of 4,5-Diamino-1-pyrazole sulfate were continuously monitored in the a strain L-1 bioaugmented SBR wastewater treatment reactor. It was found that the decolorization rate of the bioaugmented SBR reactor with strain was higher than that of the control, even those under long-time operation in the reactor B whithout adding degrading strain L-1 , at the first 2 weeks of almost no degradation during the operation 4,5-Diamino-1-pyrazole sulfate, but a slight increase in degradation rate after 21d. In the reactor A, number of DGGE profiles of activated sludge was found similar bands with L-1 strain's boands.

Key words: Bioaugmentation, Sequencing Batch Reactors, SBR,PCR-DGGE.

Gradient gel electrophoresis and sequencing technology is a viable method for the rapid analysis of samples of microbe in environment study. Gradient gel electrophoresis can be divided into two categories, denaturing gradient gel electrophoresis (Denaturing Gradient Gel Electrophoresis, DGGE) and temperature gradient gel electrophoresis (Temperature Gradient Gel Electrophoresis, TGGE). If DNA sequences of the same size contain different bases, the Tm of each is different. Even one different base-pair would give rise to different Tm. Based on these, DGGE and TGGE could distinguish different gene sequences by adding formamide to polyacrylamide to form a concentration gradient or temperature gradient from the cathode to the anode. When DNA in electrophoresis reaches its denatured site, the double-stranded part will unlock, resulting in changes in swimming speed, so as to achieve separation. The imaging system analysis of the staining gel can semi-quantitatively determine the concentration of the sample DNA and reflect changes in community composition. The DNA bands were sequenced and then blast with the gene library to determine the microbial species.

In this study, enhanced SBR reactor containing L-1 bio-degrading strain was conducted in simulated wastewater containing 4,5-Diamino-1-pyrazole sulfate, as an effort to explore the bioaugmentation process of wastewater containing 4,5-diamino pyrazole sulfate dye and analyze microbial community changes of activated sludge in bioaugmentation reactor by DGGE-PCR technology.

MATERIALS AND METHODS

Strains and synthetic wastewater: Candida rugopelliculosa L-1 strains

Synthetic waste water (mg / L): 4,5-Diamino-1-pyrazole sulfate 200, 407,KH_2PO_4 98,K_2HPO_4 33, $NaCl_3$0, $MgSO_4$ 200, glucose 1000, pH7.0.

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Inoculation and operation of the reactor

Preparation of response system

Take two 1000ml graduated cylinders as bio-enhanced SBR reactors, named A, B. Add 100ml activated sludge, which biomass was 120 mg /L dry matter. Add 95 ml the suspension of activated sludge and 5 ml the application folds plaque Candida embedded by sodium alginate to the reactor A. Meanwhile, add 95ml activated sludge with the concentration of 3000 mg /L to the reactor B as the control experiment.

Take the oxygen aeration pump with the model CX-0078 to provide oxygen, and the reaction time was 4 hours. Then, we would observe that dyes in reactor supernatant were almost removed. The period of a SBR process was 24 hours, which included 10 minutes of feeding, 23 hours of response, 4o minutes of still, 10 minutes of sampling. Supernatant in the reactor effluent was in the middle of the sampling, and the hydraulic retention time was 48 hours. Air was entering from the bottom of the reactor with the flow of 2.5l/min. The temperature in the reactor was about 25°C~ 32°C. The content of 4,5-Diamino-1-pyrazole sulfate and CODcr value of the sample were measured.

Microbial alginate embedding

The sodium alginate dissolved in hot water and then cooled. The sodium alginate solution mixed with the microbial cells with the final alginate concentration of 2% to 3%. The mixture of sodium alginate with cell was added to 5% to 10% $CaCl_2$ solution with a needle tube and placed 2 ~ 4 hours. Filter out particles and wash with saline.

Analysis method

Determination of cadmium by weight potassium CODcr;

The content of 4,5-Diamino-1-pyrazole sulfate was determined by spectrophotometry.

PCR

DNA extraction of activated sludge

First, suspend the bacteria in 200 μL ~ 400 μL lysis buffer, then place the tube in -80°C ultra-low temperature refrigerator (NU-6511E, NUAIRE, USA) for 5min till all frozen, and then quickly placed the tube in 95°C water for 2 min. Repeated freezing and thawing times were based on the experimental design. Then strongly vibrate the tube on vortex oscillator for 30 s; add 200 μL phenol - chloroform (1:1, V / V) and strongly vibrate for 2 min. Centrifuge at 16000 r / min for 3 min at room temperature. Aqueous phase was transferred to a clean centrifuge tube. Add ice ethanol to 1 mL. Natural deposit for 5 min at room temperature, then centrifuge at 16000 r / min for 5 min. Discard the supernatant carefully. Then add 0.5 mL 70% ethanol for washing. Add 20 μL TE to dissolve the DNA. Use electrophoresis (i-Mupid @ J, Japan) agarose gel electrophoresis to verify whether DNA was successfully extracted. DNA marker»DNA / Hind III was from Beijing Tiangen Biochemical Technology Co..

PCR reagents

Lysis buffer: 2% Triton X-100, 1% SDS, 100 mmol / L NaCl, 10 mmol / L Tris-HCl (pH 8.0), 1 mmol / L EDTA (pH 8.0), phenol - chloroform (1:1), 100% ethanol, TE: 10 mmol / L Tris, 1 mmol / L EDTA (pH 8.0).

PCR instruments and primers

Instrument: PCR amplification (Icycler Thermal Cycler, Bio-rad, USA).Primers: Primers of the first round of PCR:

NL-1 (5'-GCATATCAATAAGCGGA GGAAAAG-3 ‘) and NL-4 (5'-GGTCCGTGT TTCAAGACGG-3').

Primers of the second round of PCR:

NL1-GC (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGG-3') and LS2 (5'-ATTCCCAAACAACTCGACTC-3 ‘).

Primers were synthesized by Shanghai Sangon Biotechnology Co..

PCR operating conditions

Add 10×PCR buffer 5 μL, 10 nmol DNTP 4 μL, 10 pmol of each primer, DNA template1.5 μL, Taq enzyme(2.5 U/ul)1 ul to a tube, and finally make up 50 μL by adding double-distilled water.

PCR conditions: denaturation at 95°C 5 min; denaturation 95°C 45 s, annealing 55°C 45 s, extension 72°C 50 s; 36 cycles; final extension of 72°C for 10 min.

PCR product detection

The target product size of the first round and second round PCR amplification were approximately 600 bp and 300 bp. Test them by electrophoresis. 100 bp DNA ladder marker was from Beijing Tiangen Biochemical Technology Co.

DGGE

Reagents

The DGGE gel concentration for yeast is

8% (acrylamide / bisacrylamide 37.5:1, V / V). The denaturing gradient is 30% to 50% (100% denaturant contains 7 mol / L urea and 40% deionized formamide).

Operating Conditions

Add 10 µL 6 × loading buffer to 50 µl the second round of PCR amplification products. Hit several times with a pipette. Use U.S. DcodeTM Bio-Rad gene mutation detection system for electrophoresis. Electrophoresis conditions were 60 °C, 140V for 4 h ~ 6 h. After electrophoresis, the gel solution was stained in 1% EB for 20 min and then washed with deionized water 2 times, each time 5 min. Place gel under imaging system (UNIVERSAL HOOD-SN75S/02564, Bio-Rad, USA) for observation and photograph[3,4]. PCR products were sequenced by Shanghai Invitrogen Biotechnology Development Company.

DGGE positive reference system

Take 26S rRNA D1/D2 gene universal primers of Candida L-1 strain as PCR amplificating primers. They were 5'-gcatatcaat aagcggaggaaaag-3 '(NL-1) and 5'-ggtccgtgtttcaagacgg-3' (NL-4). PCR products of 10 strains were mixed. Take 1 µL of the mixture as the template for the second round of the PCR protocol. The product was the positive reference sample of DGGE[3,4]. In the DGGE experiments, three in the outer lane were positive reference samples, which were helpful to identify specific strains of Candida.

PCR and the product sequencing

The product of the first round PCR amplification was stained by ethidium bromideing after DGGE. After de-ionized water rinsed it, more than eight bands on DGGE gel were cut off under 354nm long-wave UV. For each band selected, choose the middle part for cutting with a sterile scalpel and put it into a 2mL centrifuge tube. Then add 100µL TE buffer. The gel was crushed and stayed in - 20°C freezer overnight. The gel was incubated in 60 ~ 100°C until the gel melted. Centrifuge in 5000rpm/min for 5min. Take 6µL samples as the template for the second round PCR amplification. Conditions were the same. Check the product with agarose gel and take 20µL of the PCR products for DGGE under the same conditions. The separation results of the gel showed that a number of DGGE bands could be seen and some of the DNA samples were still mixed. For the mixed samples, the target band should be cut and take the second round of PCR by the above method. This kind of rotation was finished until satisfactory amplification results were got.

RESULTS

Reactor operation conditions

Activated sludge had been suspended up in the reactors by aeration air after inoculation. Waste water has become cleaner. In the first few days of starting, due to the adsorption of the sludge and the entire system was in the adaptation period, dye degradation efficiency was not counted until 12 days later. Then the normal operation of the reactor reached. Under the condition of continuing to increase dye concentrations, in the reactor A which had dosing L-1 degradation strain, the

Table 1. Sequences analysis of DNA recovered from single band in DGGE fingerprints of SBR

Clone No.	Closest species in Genbank	Similarity (%)
DG1	Carchesium polypinum	87
DG2	Rhodotorula mucilaginosa	100
DG3	Halicephalobus gingivalis	94
DG4	Candida rugopelliculosa	99
DG5	Candida rugopelliculosa	99
DG6	Carchesium polypinum	83
DG7	Carchesium polypinum	83
DG8	Phialemonium curvatum	99

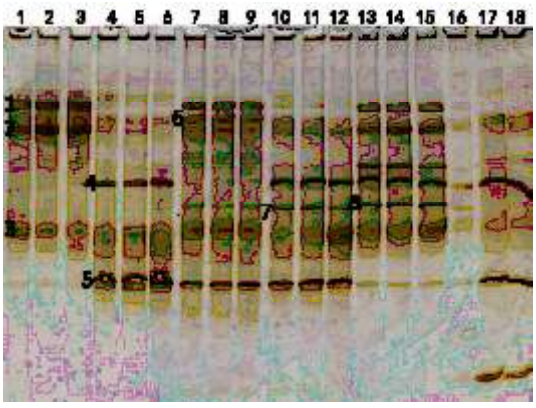

Fig. 1. DGGE profiles of Candida rugopelliculosa L-1 bioaugmentation SBR reactor sludge DGGE profiles 1-3,4-6 is for Reactor B running 0 day and 45days, DGGE profiles 7-9010-12013-15 is for ReactorA running 0 day025days and 45days, DGGE profiles 16-18 is for Candida rugopelliculosa L-1

concentration of 4,5-Diamino-1-pyrazole sulfate decreased rapidly. In the following 37 days of operating, with the addition of 200 mg/L waste water, the effluent dye concentration could be maintained at a lower level after a few days processing. The 4,5-Diamino-1-pyrazole sulfate content dropped to 66.1mg/L and 57.4 mg/L. 4,5-Diamino-1-pyrazole sulfate removal rate in reactor A reached 75% or more. To the reactor B which had no dosing of L-1 degrading strain, the dye degradation was weak during operation. The 4,5-Diamino-1-pyrazole sulfate content in the efflux was about 165.8 ~ 173.8 mg/L with the addition of 200 mg/L waste water. When added a concentration of 300 mg/L and 400 mg/L waste water, the effluent of a 4,5-Diamino-1-pyrazole sulfate in reactor B were high, which was significantly different from reactor A.

Microbial community

PCR-DGGE analysis was a repeatable, fast and easy operation technology. It has been widely used in molecular microbial research in recent years. For example: exploring complex microbial communities, observing population dynamics , tracking the expression of related gene in the environment, separating of the bacteria samples and checking its cultivation efficiency in a rich medium. The applications of PCR-DGGE method in wastewater treatment made a new understanding of the changes in microbial community in sewage treatment process . In order to detect degradation of L-1 strain on the diversity of microbial communities, take the pure L-1 bacteria 26S rDNA D1/D2 sequence as a control, make the total DNA extraction from activated sludge the when the A reactor running 0,25,45 days, meanwhile, make the total DNA extraction when the B reactor running 0,45 days. Take the total DNA of pure L-1and activated sludge bacteria as template for 26S rDNA D1/D2 amplification. PCR products were analyzed by DGGE.

Activated sludge samples in the reactor A showed the same bands corresponding to the positive control in the DGGE and the banks were wider. This indicated the presence of L-1 strains in activated sludge in reactor A. Activated sludge samples in the reactor B working for 45 days showed the same bands corresponding to the positive control in the DGGE, but the concentrations of banks were weak. This indicated that activated sludge in reactor B could produce L-1 strain under long-period domestication.

The PCR-DGGE bands of reactor biofilm samples were marked with DG1 to DG8. Recycle the bands which represented the dominant bacteria, clone and sequence them. Alignment analysis of the sequences was shown in Table 1.

DISCUSSION

The fast-degrading capacity of microbial had very important significance in dealing with the changes of the cyclical nature in wastewater and contaminant leakage. Compounds made things difficult for the degradation, and most of them had carcinogenic, teratogenic and mutagenic effects. Now in our country a lot of small and medium-sized chemical companies are processing enterprises, and products are constantly changing so the types of compounds in wastewater changes frequently as well. Indigenous microbial communities in the processing system have difficulties to quickly adapt and degradate the toxic refractory organics. Adding fast-degrading microbial could enhance the ability of degradation of dyes and other toxic. Adding fast-degrading microbial could also improve the processing efficiency of the system, reduce the processing time, avoid security problems and economic losses due to efflux and spread of toxic and refractory organics.

Survival and maintain (colonization) of dosing microbial in the system was the basis for long-term degradation. DGGE results of activated sludge samples running 3 weeks showed that activated sludge samples in reactor A had the same bands to pure strain L-1. This evidence indicated that degrading strain L-1 had been colonized in the activated sludge. L-1 has not been washed away by water or dead. This might be related to the high concentration of 4,5-Diamino-1-pyrazole sulfate, which provided a selective pressure for the survival of degrading strain L-1. Another reason might be that the wastewater type was similar to the source water environment of original degrading strain.

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