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PCR-DGGE Technique for Microbial Community Analysis in Activated Sludge Process

活性汚泥内の微生物群集解析のためのPCR-DGGE法の検討

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1 INTRODUCTION

Activated sludge process is using undefined bacterial community to treat a variety of influent wastewater. Usually, the operation in a reactor has been optimized to favor the growth of desirable organisms thus the integrity and stability of the microbial community of activated sludge is critical for the efficiency of wastewater treatment processes. A combined approach of PCR-DGGE (polymerase chain reaction-denaturing gradient gel electrophoresis) techniques have been extensively applied to the characterization of microbial diversity in various environments. In this approach, PCR (with one of the two universal primers bearing an apended GC-clamp) was used to amplify short 16S rDNA fragments of about 200-600 bp from total community DNA extracted from activated sludge samples. Among several methods of extracting nucleic acids from environmental samples for further PCR reamplification, salt concentration, humic acid and protein have been reported to interfere with PCR amplification. However, the study of factors affecting PCR by nucleic acid extraction from activated sludge samples remains incomplete.

In this study, two templates were selected with different nucleic acid and protein concentration. Their PCR products were compared by applying a low to high template concentration into PCR amplifications.

2. MATERIALS AND METHOD

2.1Sampling

1 ml of activated sludge was taken from an enhanced biological phosphate removal process (EBPR) reactor, on May 5 (sample 1,) and May 27 (sample 2), 1998 respectively. MLSS concentration was 2000 – 3000 mg/l during reactor operation period. The sample was washed with TE buffer, the pellet was harvested by centrifugation at the speed of 5000 rpm.

2.2 Nucleic acid extraction

Bacterial genomic DNA was obtained by a modified benzyl chloride extraction protocol. To each pellet, 250μl extraction buffer, 50μl 10% SDS and 150 μl benzyl chloride were added. The tube was vortexed and incubated at 53°C for 30 minutes. Then 150 μl 3M sodium acetate was added and the tube was kept on ice for 15 min. After two times centrifugation at 15000 rpm, DNA was precipitated with isopropanol, vaccum dried and dissolved in 100μl milliQ water. 10 times dilutions of sample 1 & 2 were prepared by mixing milliQ water at the ratio of 1: 9. Nucleic acid and protein concentrations were determined by Warburg-Christan Assay installed in Beckman DU Series 500 spectrophotometer. UV absorbtance at 260nm were measured by the same model spectrophotometer.

2.3 PCR amplification of rDNA fragments

Two primer sets were applied for PCR, 341fGC-534r primer covering the V3 region and 968fGC-1392r primer covering the V9 region of 16S rDNA. Incorporation of GC-clamp prevents the complete dissociation of a double-stranded DNA in the denaturant concentration, thus improves the ability to resolve separate DNA fragments by DGGE. PCR amplification was performed with a Perkin-Elmer PJ2000 DNA thermal cycler as follows: 1 μl of template, 50 pmol of each of the appropriate primers, 8 μl of dNTP mixture, 10μl of 10xPCRbuffer, 1μl Triton X-100 and 0.5 μl of Taq-Gold polymerase were added to a 0.5-ml-volume PCR test tube which was filled up to a volume of 100μl with sterile Milli-Q water and overlaid with 2 drops of mineral oil. Table1 shows the PCR reaction conditions for the V3 and V9 region primers.

Table 1 Primers and PCR working condition

Primer set	V9	V3
Position No. in 16S rRNA	968f-gc1392r	341f-gc534r
Denaturation	7min, 94°C	7min, 94°C
No. of PCR Cycles	35	35
Denature	1min, 94°C	1min, 94°C
Anneal	1 min, 54°C	1min, 53°C
Elongation	1min, 72°C	2min, 72°C
Final elongation	10min, 72°C	10min, 72°C
Amplified Length(bp)	500	200
Amount of primer used (100uM)	0.1 µl	1 µl

2.4 DGGE Analysis and Sequencing

DGGE was performed with BIORAD DCode DGGE system, 8% (wt/vol) polyacrylamide gel in 1x TAE with denaturing gradient ranging from 35-55% running for 4 h at constant voltage of 130V and

Table 2 Characteristics of 2 samples used as PCR templates

	nuclei c acid conc. µg /ml	protei n conc. µg /ml	E260	amount used as template in PCR
sample 1	99.6	142	0.747	2ul, 1ul, 5 µl of 1/10 solution, 2µl 1/10, 1µl 1/10, 0.5µl of 1/10 solution
sample 2	48.5	–7.2	1.77	same as above

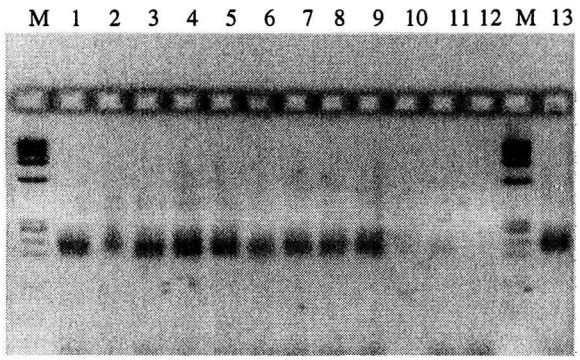

Fig.1 V3 region PCR product amplified from sample 1 and sample 2 with varying dosages, 0.5, 1, 2 and 5 µl of 1/10 solutions (lane 1,2,3 &4, lane 7,8,9&13), 1 and 2 µl of original extract solutions (lane 5&6, lane 11 &12), M-Marker

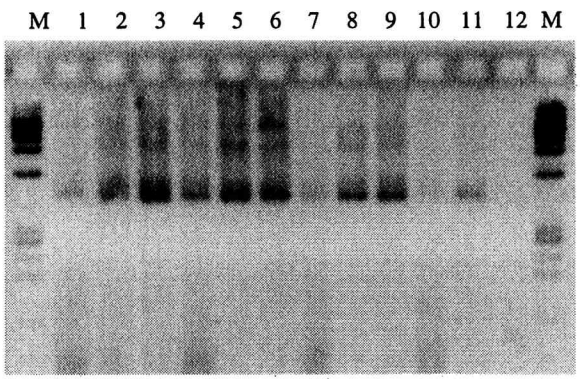

Fig.2 V9 region PCR product amplified from sample 1 and sample 2 with varying dosages, 0.5, 1, 2 and 5 µl of 1/10 solutions (lane 1,2,3 &4, lane 7,8,9&10), 1 and 2 µl of original extract solutions (lane 5&6, lane 11 &12), M-Marker

temperature of 60°C. After the electrophoresis, the gels were incubated for 20 min in Milli-Q water containing 1x Vistra green solution, photographed with FluorImager 595 (Molecular Dynamics).

3. RESULT AND DISCUSSION

3.1 Template concentration and PCR amplification

Nucleic acid and protein concentration of extracts from two activated sludge samples were shown in Table 2. In case of V3 region primer, the change of sample 1's template concentration did not interfere with PCR. Only the highest dosage (2 µl of extracts solution) showed slightly lower band intensity (Fig.1, lane 6). On the other hand, the increase of sample 2's template concentration greatly deteriorate the PCR amplification resulting in poor or no PCR products. (Fig.1, lane 11& 12).

When the V9 region primer set was applied, good PCR was obtained by using 10 times diluted extracts (1/10 solution, Fig., 2, lane 3, 8 & 9), Lower (Fig.2, land 1,2,7) or higher (Fig.2, lane 4,5,6,10,11&12) dosage gave rise to poor PCR amplification.

The result shows that though the protein concentration in sample 2 was measured much lower than sample1 (Table 2), the interference from sample 2 was observed more significant than that from sample1, which reveals that protein contamination from activated sludge sample was not the main cause of the PCR failure. The absorbtance at 260nm implies that sample 2 contained much higher amount of humic acid than sample 1.

3.2 DGGE analysis of V3 region PCR products

Four PCR products (Fig1, lane 1, 6, 7 & 13) were selected for DGGE analysis to investigate the effect of different template concentration on DGGE result. Fig.3 shows the DGGE banding pattern was almost same between lane 1 and 2 of sample 1 as template, and between lane 3 and 4 of sample 2 as template. This indicates that once PCR product is obtained, the major DGGE band pattern is not influenced by a diluted DNA extract as template.

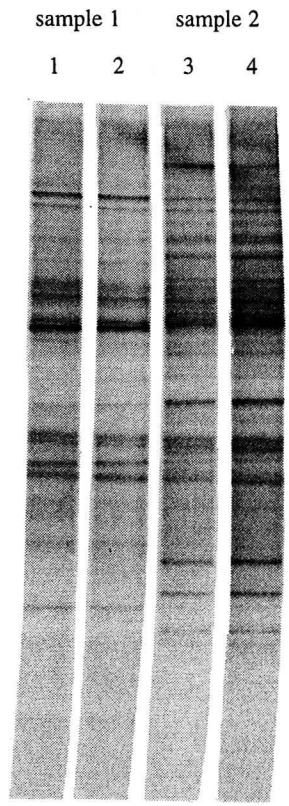

Fig.3 DGGE analysis of V3 region PCR products with varying amount of template of sample 1 (0.5 µgl of 1/10 solution (lane 1), 2 µl of original NA extract solution (lane 2)) and sample 2 (o.5 & 5µl of 1/10 solution (lane 3&4)

4. CONCLUSIONS

The effect of dilution of DNA extract from activated sludge was studied. Result of this study shows that protein content of nucleic acid extract from activated sludge sample was not the main cause to PCR failure. Humic acid contamination should be put into further consideration. To try to avoid the interference from highly microbial diversed sample, eg. activated sludge, dilution of DNA extracts was recommended and was shown to be effective in improving PCR product quality and obtaining reliable DGGE banding pattern.

5. REFERENCES

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