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PCR–DGGE as a Supplemental Method Verifying Dominance of Culturable Microorganisms from Activated Sludge

Zhou, Sheng[1,2], Chaohai Wei[1]*, Lin Ke[1], and Haizhen Wu[3]

[1]*College of Environmental Science and Engineering South China University of Technology, The Key Lab of Pollution Control and Ecosystem Restoration in Industry Clusters, Ministry of Education, Guangzhou 510006, P. R. China*
[2]*Guangxi Yulin Normal College, Yulin 537000, P. R. China*
[3]*School of Bioscience and Bioengineering, South China University of Technology, Guangzhou 510640, P. R. China*

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To verify the dominance of microorganisms in wastewater biological treatment, PCR–DGGE (denaturing gradient gel electrophoresis) was performed as a supplementary support method for screening of the dominant microorganisms from activated sludge. Results suggest that the dominant microorganisms in activated sludge are primarily responsible for strengthening its effectiveness as a biological treatment system, followed by the non-main dominant microorganisms, whereas the non-dominant microorganisms showed no effects. The degree of microbial abundance present on the profile of PCR–DGGE was in line with the treatment efficiency of augmented activated sludge with isolated cultures, suggesting that PCR–DGGE can be used as an effective supplementary method for verifying culturable dominant microorganisms in activated sludge of coking wastewater.

Keywords: Biological treatment, dominant microorganisms, non-dominant microorganisms, PCR–DGGE, non-main dominant microorganisms

In conventional screening for dominant microorganisms, the target bacteria in certain habitats such as activated sludge are enriched and screened by culturing on selective media that contain restrictive nutrients [3, 4, 6, 10, 17]. Alternatively, the dominant microorganisms can be isolated on a selective medium without enrichment. Specifically, different media such as TYG (tryptone, yeast extract, glucose), Gause 1, and beef-cream peptone containing target contaminants, restrictive nutrients [1], and high levels of nutrients are used to recover as many culturable microorganisms as possible. The target bacteria are then acclimated in wastewater that contains the target contaminants for 2–6 months, and then added directly to the activated sludge. Measurements will be then conducted to determine if the degradability of the activated sludge is enhanced by augmentation during the wastewater treatment processes.

Recently, the application of molecular [8, 13, 23] techniques such as PCR–DGGE [5, 7, 12] to ecological studies has provided valuable information on changes in the community structure of activated sludge before and after acclimation to a target contaminant. Indeed, the profile provided by PCR–DGGE enables the investigator to verify the dominance of the screened microorganisms in the original activated sludge prior to conducting additional experiments. In this study, coking wastewater-degrading microorganisms in activated sludge were screened using the two above-mentioned conventional methods (*i.e.*, enrichment isolation and direct isolation) and identified using PCR–DGGE as a supplementary means to determine their theoretical functions. The results revealed that PCR–DGGE could avoid the time-consuming and labor-intensive drawbacks of the conventional techniques when screening for target microorganisms for use in the acclimation of sludge. Indeed, screening and identifying microorganisms based on their theoretical functions provided by PCR–DGGE could serve as a guide for acclimating, testing, and strengthening the target microorganism-containing activated sludge.

The coking wastewater-degrading microorganisms were isolated from activated sludge at a coking wastewater treatment plant (Shaogang coking plant in Guangdong Province, China) after enrichment. The enrichment culture medium was composed of the following (g/l): phenol, 0.5; K_2HPO_4, 2.24; KH_2PO_4, 2.75; $(NH_4)_2SO_4$, 1; $MgCl_2 \cdot 6H_2O$, 0.2; NaCl, 0.1; $FeCl_3 \cdot 6H_2O$, 0.02; $CaCl_2$, 0.01; pH 7.0. A 10% (w/v) suspending coking wastewater activated sludge sample was used as the inoculum. Five hundred mg/l of

*Corresponding author
Phone: +86-20-3938-0502; Fax: +86-20-3938-0588;
E-mail: cechwei@scut.edu.cn

phenol (the coking wastewater consisting of 80% phenol [14]) was added to the enrichment culture medium and then incubated at 28°C. The phenol concentration was checked at every four hours until it decreased to zero, at which point 800 mg/l of phenol was added to the enrichment culture medium. The above process was repeated by successively adding 1,000, 1,200, 1,500, and 2,000 mg/l of phenol to the enrichment culture medium. Next, enrichment cultures containing 2,000 mg/l of phenol were diluted serially to 10^{-8}. The last four dilutions were then used to inoculate (1 ml) agar plates containing the enrichment culture medium, after which the samples were incubated at 28°C for one day. Well-separated colonies were then subcultured in plates containing the same enrichment culture medium. A total of four strains (A, B, C, and D) were isolated.

Target coking wastewater-degrading microorganisms were also obtained by direct isolation using TYG culture medium, beef-cream and peptone culture medium, and Gause 1 culture medium to screen different kinds of microorganisms to the greatest extent. The pH of all media was adjusted to 7.2 with 1 M NaOH and 1 M HCl. TYG culture medium contained (g/l) glucose, 1; yeast extract, 3; dextrose, 5; K_2HPO_4, 3; agar, and 20; peptone, 1. The beef-cream and peptone culture medium contained (g/l) beef cream, 3; peptone, 10; NaCl, 5; and agar, 20. The Gause 1 culture medium contained (g/l) starch, 20; KNO_3, 1; NaCl, 0.5; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.5; $FeSO_4$, 0.01; and agar, 20. One ml of liquid activated sludge was collected, diluted, and spread on agar plates containing each of the aforementioned culture media. After incubation for 24 h at 30°C, 15 individual colonies (A1, A2, A3, A4, B1, B2, B3, C1, C2, C3, D1, D2, E1, E2, and F1) were selected and isolated as pure cultures by repeated streaking.

Screening and identification of the culturable microorganisms in coking wastewater activated sludge were confirmed using PCR–DGGE. Firstly, the genomic DNA of the 19 target bacterial strains obtained from both enrichment isolation (A, B, C, and D) and direct isolation methods (A1, A2, A3, A4, B1, B2, B3, C1, C2, C3, D1, D2, E1, E2, and F1) were extracted using a protocol previously described [22]. Briefly, 1.5 ml of liquid sample was added to an Eppendorf tube containing 200 µl of extraction buffer [100 mM Tris, 100 mM EDTA, 200 mM NaCl, 1% (w/v) polyvinylpyrrolidone, 2% hexadecylmethylammonium bromide (CTAB); pH 8.0] and then suspended by vortexing for 2 min. Next, 200 µl of sodium dodecyl sulfonate (SDS) buffer [2% (w/v) SDS, 10 mM Tris, 200 mM NaCl; pH 8.0] was added, after which the sample was immediately placed on ice and incubated for 10 min with gentle mixing by inverting the tube several times. After incubation, the tube was centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant was transferred to a new Eppendorf tube and then extracted sequentially with equal volumes of phenol, phenol–chloroform–isoamyl alcohol [25:24:1 (v/v/v)], and chloroform–isoamyl alcohol [24:1 (v/v)]. Next, the aqueous phases were precipitated with two volumes of ethanol at −20°C overnight, after which they were centrifuged at 15,000 rpm for 20 min at 4°C to collect the DNA. Next, the pellet was washed with 70% ethanol once and then vacuum-dried. Finally, the dried DNA pellets were dissolved in 30 µl of TE buffer (pH 8.0). The activated sludge genomic DNA was extracted using the following protocol based on the method described by Zhou *et al*. [24], with some modifications. Briefly, 2 g of sample was added to 13.5 ml of autoclaved extraction buffer (pH 8.0, containing 100 mM Tris-HCl, 100 mM disodium phosphate, 1.5 M NaCl, and 1% CTAB). The samples were then incubated on −20°C ice for at least 20 min, after which they were placed in 100°C water. This step was repeated 3–5 times, and then 30 µl of 10 mg/ml Proteinase K was added. The mixture was then incubated at 37°C for 60 min while being shaken horizontally at 150 rpm. Next, 3 ml of 10% SDS was added, and each tube was then incubated at 65°C in a water bath for 3 h with gentle mixing every 20 min. The mixture was then centrifuged at 2,500 rpm for 5 min, after which the supernatant was collected. The debris was extracted twice and the supernatants were combined. Next, the proteins were denatured by the addition of chloroform–isoamyl alcohol [24:1 (v/v)] and the DNA was then precipitated in 0.6 volume of isopropanol overnight at room temperature. Finally, the DNA was pelleted by centrifugation, washed twice with 5 ml of cold 75% ethanol, dissolved in 60 µl of sterile deionized water, and stored at 4°C. To remove the inhibitors, heavy metals, and humic acids, the DNA was size fractionated by agarose gel (0.8%) electrophoresis, and the DNA fragments ≥20 kb were recovered using a Geneclean spin kit (Sangon Biological Company, Shanghai, China) according to the manufacturer's instructions. Then PCR was conducted using the protocol described by Yan *et al.* [22]. Primers used for amplifying the V3 region of the 16S rRNA genes of the 19 target strains (A, B, C, D, A1, A2, A3, A4, B1, B2, B3, C1, C2, C3, D1, D2, E1, E2 and F1) were F357GC (5'-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCCCCTACGGGAGGCAGCAG-3'), which contains a GC-rich clamp and is specific for most bacteria, and a universal primer, R518 (5'-ATTACCGCGGCTGCTGG-3')[10]; primers used for amplifying the 16S rRNA genes of the target strains were 27F (5'-GAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). PCR was conducted using 50-µl reaction mixtures composed of 1× PCR buffer, 2 mM $MgCl_2$, 3.0 U of *Taq* DNA polymerase, 80 mM of each deoxynucleotide (Fermentas Inc., Hanover, USA), 0.3 mM of each primer, and approximately 40 ng of template DNA. PCR was conducted on a GeneAmp PCR System 9600 thermal cycler (Perkin Elmer Cetus, USA) using the following

conditions: initial denaturation at 94°C for 6 min, followed by 10 cycles of 1 min at 94°C, annealing at 65–55°C (in the first cycle annealing was conducted at 65°C, after which the temperature was decreased by 1°C during each cycle) for 1 min, and extension at 72°C for 1 min. This procedure was followed by 20 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C. Finally, a primer extension at 72°C for 6 min was conducted. Similarly, PCR amplification of the 16S rRNA genes of the target strains was conducted under the following conditions: initial denaturation at 94°C for 6 min, followed by 30 cycles of 1 min at 94°C, annealing at 55°C for 1 min, and extension at 72°C for 1.5 min. Finally, a primer extension at 72°C for 6 min was conducted. Sequencing of the PCR products was commercially available by the Sangon Biological Company, Shanghai, China. The sequences were submitted to the GenBank database (http://www.ncbi.nlm.nih.gov), and the BLAST software was used to search for similar sequences in the database. Lastly, DGGE was conducted with an INGENYphorU-2 system (INGENY International BV, Leiden, The Nether- lands) using a 9% (w/v) polyacrylamide gel (acrylamide: bisacrylamide ratio of 37.5:1) in 1× TAE buffer (40 mM Tris acetate, 40 mM acetic acid, and 1.0 mM EDTA, pH 7.6). The protocol has been described elsewhere [22]. In brief, PCR products containing approximately equal amounts of DNA of similar sizes were separated on a gel containing a linear gradient of the denaturants (urea and formamide) increased from 40% at the top of the gel to 60% at the bottom. Electrophoresis was conducted at 60°C by applying 120 V to the gel for 6 h. After electrophoresis, gels were stained in 1× TAE buffer containing 1× SYBR Gold (Molecular Probes Europe BV, Leiden, The Netherlands) and then photographed using a UVP Imaging System (UVP Inc., CA, USA). The gel images were further processed using Adobe Photoshop 8.01 to maximize the image contrast.

Fig. 1 shows the PCR–DGGE profile of the 19 candidate strains A, B, C, D, A1, A2, A3, A4, B1, B2, B3, C1, C2, C3, D1, D2, E1, E2, and F1, and the activated sludge before and after acclimation. The bands of A1, A2, A3, and A4 were at the same position (Fig. 1), indicating that they may be from the same species. This was confirmed by sequencing analysis of the 16S rRNA gene of A1–A4 (Access No. HM560951), which revealed that they were identical to *Microbacterium* sp. PHD-5 (Access No. DQ227343), a phenol-degrading strain. Similarly, sequencing analysis of the 16S rRNA genes of C1–C3 (Access No. HM560952), B1–B3 (Access No. HM560953), D1–D2 (Access No. HM560954), and E1–E2 (Access No. HM560955) revealed that B1–B3 were identical to *Cellulosimicrobium cellulans* (Access No. FJ793202), which is a phenol- and chromium (VI)-reducing strain; C1–C3 were identical to *Pseudomonas* sp. PND-1 (Access No. DQ227339), which is a phenol-degrading strain;

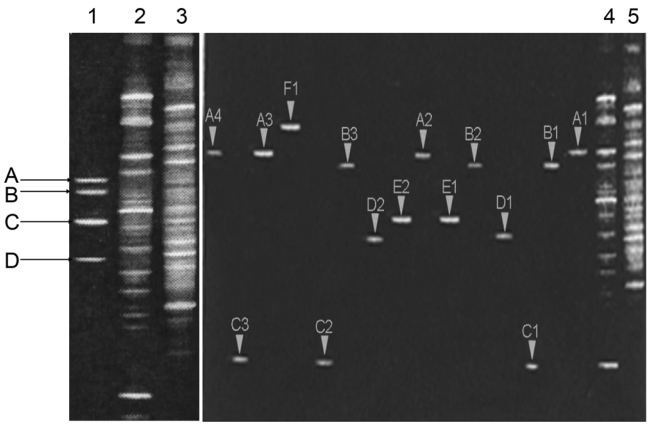

Fig. 1. PCR–DGGE profiles of the isolated strains and activated sludge.
1, PCR–DGGE profile of four species of microbes, A, B, C, and D; 2, PCR–DGGE profile of activated sludge after acclimation; 3, PCR–DGGE profile of activated sludge before acclimation; 4, PCR–DGGE profile of activated sludge after acclimation; 5, PCR–DGGE profile of activated sludge before acclimation; individual PCR–DGGE profiles of 15 strains, A1, A2, A3, A4, B1, B2, B3, C1, C2, C3, D1, D2, E1, E2, and F1 are shown.

D1–D2 were identical to *Alcaligenes* sp. (Access No. AJ002815), which is a hydroxybenzene-degrading; and E1–E2 were identical to *Rhodococcus baikonurensis* (Access No. FJ796416), which is a hydroxybenzene-degrading strain. Taken together, these findings indicate that there were four different groups of similar microorganisms in the sludge: A1, A2, A3, and A4; B1, B2, and B3; D1 and D2; and E1 and E2. Therefore, to evaluate the coking wastewater-degradation activity, 10 candidates bacterial strains (A, B, C, D, A1, B1, C1, D1, E1, and F1) were selected as the representatives.

The degrading abilities of selected target strains including A, B, C, D, A1, B1, C1, D1, E1, and F1 were further confirmed using COD content as an index for checking treatment efficiency. Acclimation processes including aeration, precipitation, and removal of supernatant were applied to both original and augmented activated sludges. The freshly collected activated sludge and isolated target strains (*i.e.*, A, B, C, D, A1, B1, C1, D1, E1, and F1) were aerated for 6 h in 10% (v/v) coking wastewater, followed by precipitation for 1 h. The COD of the coking wastewater was then determined. This step was repeated until the COD of the coking wastewater decreased to a constant value, when the supernatant was removed and replaced by a new batch of coking wastewater. The strength of coking wastewater was gradually increased to 100% (v/v) by repeating the acclimation processes for 2 months, and the acclimated activated sludge and bacterial cultures were obtained. To evaluate the augmentation effects, each of the acclimated candidate strains (A, B, C, D, A1, B1, C1, D1, E1, and F1) was mixed with the

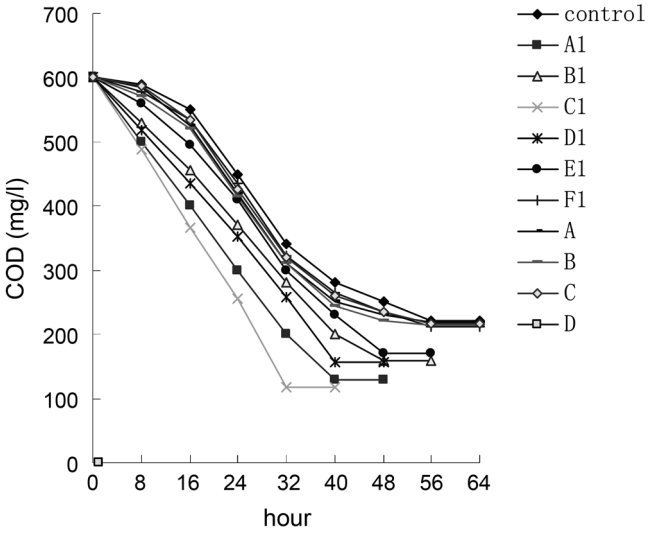

Fig. 2. Effects of bioaugmentation in the sludge with isolated strains.

Control: no functional bacteria; A1: augmented with bacterium A1; B1: augmented with bacterium B1; C1: augmented with bacterium C1; D1: augmented with bacterium D1; E1: augmented with bacterium E1; F1: augmented with bacterium F1; A: augmented with bacterium A; B: augmented with bacterium B; C: augmented with bacterium C; D: augmented with bacterium D.

acclimated activated sludge at a ratio 1:9 to accomplish a "new" activated sludge, which was then added to coking wastewater at 1% (v/v). One percent inoculation of the acclimated activated sludge was treated as the control. Each treatment was then aerated at 60 ml/s and the COD of the supernatant of each treatment was measured every 8 h [15]. The initial value of the coking wastewater COD was 600 mg/l. All experiments were conducted three times and variations between the replicated measurements and their means were less than 5%. Therefore, the average values are reported (Fig. 2).

To adapt themselves to a new and contaminated habitat such as coking wastewater, the microbial population as well as the community structure in activated sludge changes with acclimation duration. Some endurable microorganisms can use the contaminants as nutrients to support their growth, concomitantly with the decrease of the target contaminants. These microorganisms become the dominant group in this new habitat. Theoretically, in the profile of PCR–DGGE, the number of DNA bands can reflect the degree of diversity of the microbial community in general, and its relative quantity can be reflected by the signal intensity [19–21]. Larger numbers of microbes produce brighter abundant bands (V3 region of the 16S rRNA genes of the "main dominant" microorganisms) on the PCR–DGGE profile [18–22]. This information can be used to identify the microorganisms that exert the greatest enhancing effects during biological treatment of wastewater. Specifically, if the bands of some microorganisms on the PCR–DGGE profile are relatively dim, they are non-main dominant microorganisms in the habitat that the samples were collected from and therefore have relatively weak effects [18–22]. If there are no bands for some microorganisms evaluated by PCR–DGGE, then they are not dominant microorganisms in the habitat that the samples were collected from and therefore provide no enhancement to the treatment of wastewater.

As shown in Fig. 1, the bright bands of cultures A1 and C1 on the PCR–DGGE profile, selected from the acclimated activated sludge, were level with the bright band on the PCR–DGGE profile of acclimatized activated sludge. It indicates that they were abundant species, whereas those of B1, B2, D1, D2, E1, and E2 were level with the dim band on the PCR–DGGE profile of acclimatized activated sludge and strains A, B, C, D, and F1 level with no band on the PCR–DGGE profile of acclimatized activated sludge. Therefore, microorganisms A1 and C1 were the "main dominant" microorganisms in acclimatized activated sludge, whereas bacteria B1, B2, D1, D2, E1, and E2 were non-main dominant bacteria, and bacteria A, B, C, D, and F1 were not dominant microorganisms in acclimatized activated sludge. These findings indicate that the use of PCR–DGGE as a supplementary tool for screening enables the identification of dominant bacteria.

Activated sludge augmented with the dominant microorganisms A1 and C1 resulted in the fastest decrease in the COD of coking wastewater from 600 mg/l to 128 mg/l after 40 h, and to 118 mg/l after 32 h of treatment, respectively, with an improvement efficiency of 41.81% and 46.36% for A1 and C1, respectively (Fig. 2). Similarly, augmentation with the non-main dominant microorganisms B1, D1, and E1 resulted in the COD of coking wastewater decreasing from 600 mg/l to 156–170 mg/l after 40–48 h of treatment, and the time required to reach a stable COD decreased by 8–16 h (Fig. 2). As expected, augmentation with non-dominant microorganisms F1, A, B, C, and D had no effect on the COD. Overall, these results are in good agreement with the PCR–DGGE results.

Some commonly found disadvantages using DGGE to analyze the dominant microorganisms [2, 11, 16] were also identified in this study. For instance, there were differences in the genome size and the number of 16S rRNA gene copies in different bacteria, the lysis efficiency of cells during the extraction of total DNA, the extraction and purification of DNA, and the processes used to conduct PCR amplification. Since only bacteria that accounted for 1% of the total microorganisms in the sample can be detected by DGGE profile [9], bands observed on the DGGE profile should represent bacteria present in greater number (*i.e.*, main and non-main dominant species) rather than bacteria that do not produce bands in the DGGE profile.

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