



# Exploration of the key functional strains from an azo dye degradation microbial community by DGGE and high-throughput sequencing technology

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## Abstract

This study investigated a previously developed thermophilic microbial community with the ability to effectively degrade azo dyes. To identify the key microbes of this microbial community, a dilution-to-extinction approach was combined with polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) and Illumina high-throughput sequencing technology (HTST). Strains belonging to *Tepidiphilus* sp. almost disappeared from the degradation solution at dilution ratios above  $10^{-7}$ ; furthermore, at this ratio, the diluted microbial community almost lost their decolorization ability, indicating this ratio as the critical point for effective azo dye decolorization. Strains belonging to *Tepidiphilus* sp. were indicated as possible key functional microbes of this microbial community for effective azo dye decolorization. Moreover, the synergistic action of other microbes, such as *Anoxybacillus* sp., *Clostridium* sp., and *Bacillus* sp., was suggested to further promote the decolorization process by secreting azoreductase and laccase. *Caloramator* spp. were found have the ability to degrade proteins and amino acids, which might promote the degradation process with further degradation microbes. The loss of these bacteria might diminish the synergistic relationships among different strains, which further results in the failure of efficient azo dye decolorization and degradation by this microbial community.

**Keywords** Azo dyes · Thermophilic microbial community · Microbial degradation · Dilution-to-extinction · PCR-DGGE · High-throughput sequencing

## Introduction

Azo dyes are the largest class of synthetic dyes and are extensively used within the food, textile, paper, and leather industries (Michaels and Lewis 1986). However, even a very low concentration of azo dyes in water bodies can cause human visual discomfort upon exposure, and affect the normal function of the

receiving waters. Additionally, azo dyes weaken the transmission of light in water, which in turn inhibits the photosynthetic abilities of aquatic plants and thus decreases the diversity of aquatic ecosystems. In addition, the degradation products of azo dyes are mostly aromatic compounds (e.g., benzidine), which are often toxic, mutagenic, and carcinogenic (Levine 1991). The discharge of wastewater containing these pollutants results in significant threats to human health and the environment (Nigam et al. 1996). Therefore, the wide usage of azo dyes generated a severe disposal problem.

Physical and chemical methods have previously been used for the treatment of water contaminated with azo dyes and obtained positive effects (Hao et al. 1999). However, these methods have not been widely applied due to high cost, low efficiency, and intensive energy requirement (Tan et al. 2013). Compared to physicochemical methods, biodegradation was considered as the most efficient and environmentally sustainable method, and has therefore attracted increasing attention (Bent and Forney 2008).

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In recent years, many biological systems (including bacteria, fungi, algae, and plants) that can degrade azo dyes have been documented (Saratale et al. 2011). Among these systems, treatment with mixed microbial communities was regarded as more effective to remove azo dyes compared to pure cultures (Khehra et al. 2005). These mixed microbial communities have therefore received more attention from the current scientific community. It has been suggested that the dynamic composition structure and diversity of the microbial community affects the degradation efficiency of azo dyes. Therefore, it is essential to explore the microbial community and diversity in several azo dye degradative systems, to eventually clarify the key microbes involved in the degradation process.

However, it is very complicated and time-consuming to separate functional bacteria via traditional plate separation technology, because only a small portion of the existing microbes in a natural microbial community can be cultivated *in vitro*. However, for this study the function of an entire microbial community is of particular interest; therefore, clarification of the mechanism corresponding to a specific biological process is especially important. Moreover, several key functional strains in the natural microbial community remain uncultured and the plating technique is not practically feasible; therefore, a simple and rapid approach for the screening of the functional strains of a microbial consortium is desirable. Recently, a novel dilution-to-extinction approach has been proposed to identify the key functional strains from natural microbial consortiums (Wang et al. 2010; Zhang et al. 2015a). This approach offers the advantage to not require isolation of pure strains for testing. Instead, it depends on retaining the functional strains while non-functional strains are eliminated via serial dilution. This method offers comprehensive application prospects for the investigation of natural functional microbial communities. Furthermore, recent significant improvements in the dilution-to-extinction method offer the potential to isolate novel bacterial strains with low abundance (Yang et al. 2015b). Moreover, the utilization of modern molecular biology technology has greatly promoted the development of microbiology for the exploration of the community structure and species diversity of environmental microorganisms. Among these technologies, PCR-DGGE has been extensively applied for the evaluation of microbial community dynamics in a number of azo dye contaminated wastewater treatment systems due to its virtues of low cost, good repeatability, and less intensive data analysis (Cui et al. 2017; Khan et al. 2009; Walvekar et al. 2017). Furthermore, this method can provide a more intuitive analysis of the microbial diversity and composition (Bagwell et al. 2009). However, most of the organisms with low abundance in microbial community cannot be identified, which results in the loss of rare species in samples (Chen et al. 2013). Recently, Illumina

HTST has become the most popular technology to investigate the microbial community and its diversity (David et al. 2014; Gangadoo et al. 2017; Sun et al. 2016; Zhang et al. 2015b). Illumina HTST generated sufficient DNA data and sequencing depth to cover a complex microbial community (Polka et al. 2015). Therefore, a combination of PCR-DGGE and Illumina HTST for the investigation of natural microbial communities could enable a large-scale and intuitive analysis of microbial communities, and significantly contribute to an improved overview of the function of microbial communities. However, the combined usage of Illumina HTST and PCR-DGGE technology to explore the species diversity in azo dye degradation systems has not been reported to date and neither was the dilution-to-extinction method combined with PCR-DGGE and Illumina HTST.

A thermophilic microbial community that can effectively degrade azo dyes was successfully isolated in our previous investigation. This microbial community could completely decolorize 600 mg L<sup>-1</sup> of Direct Black G within 8 h of incubation at 55 °C, revealing great industrial application potential (Chen et al. 2018). However, the key functional strains for an efficient degradation of azo dye of this microbial community still remain unclear. Therefore, in the present study, a dilution-to-extinction approach was combined with PCR-DGGE and Illumina HTST for the first time to explore the dynamic changes in microbial compositions and to gain further insight into the key microbes of the microbial community. The results of this study describe this thermophilic microbial community, and provide technical support for the investigation of other natural functional microbial communities.

## Materials and methods

### Dyes and chemicals

Direct Black G (C<sub>34</sub>H<sub>27</sub>N<sub>13</sub>Na<sub>2</sub>O<sub>7</sub>; CAS No. 6428-31-5; CI No. 35255; purity > 98%) used in the study was purchased from Xiya Chemical Co., Ltd., Shandong Province, China. All chemicals used were of analytical grade or the highest quality available.

### The thermophilic microbial community and basal medium

The microbial community utilized in the present study was isolated and developed from azo dye-contaminated soil (Chen et al. 2018). The liquid basal medium (PDS) used for the cultivation of this microbial community had the following composition (g L<sup>-1</sup>): beef extract, 3.00; glucose, 5.00; peptone, 2.00; KH<sub>2</sub>PO<sub>4</sub>, 1.80; NaH<sub>2</sub>PO<sub>4</sub>, 3.50; FeCl<sub>3</sub>, 0.01; MnSO<sub>4</sub>, 0.02; MgSO<sub>4</sub>, 0.20 (pH 8.0).

## Investigation of the thermophilic microbial community using the dilution-to-extinction approach

The dilution-to-extinction approach was used to investigate the microbial community, where 5 mL of the activated seed broth was inoculated into a 150-mL flask containing 45 mL of sterilized PDS medium and then incubated at 55 °C under static conditions. When the azo dye was totally decolorized, the dye solution in the flask was used as the original microbial community, 5 mL of which was inoculated into another flask with 45 mL PDS medium. After thorough mixing, the liquor in the flask was used as inoculum with dilution ratio of  $10^{-1}$ . Correspondingly, the inoculum with dilution ratios of  $10^{-3}$ ,  $10^{-5}$ , ...  $10^{-11}$  were obtained in the same way. Afterwards, 10% (v/v) of these different diluted microbial communities were separately inoculated into the PDS medium containing  $600 \text{ mg L}^{-1}$  of Direct Black G. All cultures were incubated at 55 °C under static conditions until dyes were decolorized or still could not be decolorized even after 8 days of incubation. The microbial diversity of the dye solutions pretreated by the microbial community at different dilution ratios were investigated with both PCR-DGGE and Illumina HTST. In addition, the related enzymatic activities (azoreductase activities, laccase activities, lignin peroxidase activities, and manganese peroxidase activities) and the decolorization efficiency in these different dye solutions were also measured. The decolorization efficiency was calculated via the following equation:

$$\text{Decolorization}(\%) = (A_0 - A) / A_0 \times 100\%$$

where  $A_0$  represents the initial absorbance and  $A$  represents the absorbance of the decolored medium.

### Enzymatic assays

When azo dyes were wither completely decolorized or after 8 days of incubation (in cases, where decolorization was incomplete), the cell biomass was harvested via centrifugation (8000 r/min for 15 min) and suspended in potassium phosphate buffer (pH 7.2). The cells were then disrupted by sonication (output at 50 amp), with 10 strokes every 20 s, with a 10-s interval for 15 min at 4 °C. The processed homogenate was centrifuged again at 8000 r/min for 15 min, and the supernatant was used for the analysis of enzymatic activities.

All enzymes were spectrophotometrically assayed, and laccase activity was determined in a 4-mL reaction mixture containing 1 mL ABTS (1 mM) in sodium acetate buffer (100 mM, pH 4.0) by monitoring the increase in optical density at 420 nm ( $420 \text{ nm} = 36,000 \text{ (mol/L)}^{-1} \text{ cm}^{-1}$ ) (Wolfenden and Wilson 1982); lignin peroxidase activity was measured at 310 nm ( $310 \text{ nm} = 93,000 \text{ (mol/L)}^{-1} \text{ cm}^{-1}$ ) by monitoring the formation of veratraldehyde in a 3-mL reaction mixture, containing 10 mM resveratrol, 10 mM  $\text{H}_2\text{O}_2$ , and 100 mM tartaric

acid (pH 3.0) (Kalme et al. 2007); manganese peroxidase activity was assayed at 240 nm ( $240 \text{ nm} = 6500 \text{ (mol/L)}^{-1} \text{ cm}^{-1}$ ), where the volume of reaction mixture contained 10 mM  $\text{H}_2\text{O}_2$  and 15 mM  $\text{MnSO}_4$  in 50 mM succinate buffer (pH 4.5), kept at 3 mL (Martinez et al. 1995); the azo reductase activity was assayed by monitoring the absorbance decrease of methyl red at 430 nm ( $430 \text{ nm} = 23,360 \text{ (mol/L)}^{-1} \text{ cm}^{-1}$ ) according to the method reported by Zimmerman et al. (1982) with minor modification: methyl orange was replaced by methyl red. One unit (U) of azoreductase activity was defined as the amount of enzyme required to reduce  $1 \mu\text{M}$  of azo dye  $\text{min}^{-1} \text{ mL}^{-1}$  under assay conditions. One unit of laccase, lignin peroxidase, and manganese peroxidase activity was defined as a change in absorbance units  $\text{min}^{-1} \text{ mg protein}^{-1}$  (Lade et al. 2015). The protein content was determined according to Lowry using bovine serum albumin as standard (Lowry et al. 1951).

### DNA extraction and PCR

After inoculation of azo dyes with the original or diluted microbial community, the genomic DNA of cells in the original and diluted samples was extracted using a DNA extraction kit (TaKaRa, Dalian, China) according to a previously published protocol (Lade et al. 2015). The DNA was used as template for the following polymerase chain reaction (PCR) amplification of the 16S rRNA by using the primers 357F (5'-CCTA C G G G A G G C A G C A G -3') and 518R (5'-ATTA C C G C G G C T G C T G G -3'). The following thermal cycling conditions were used: initial denaturation for 2 min at 94 °C followed by 28 cycles of 30 s, denaturation at 94 °C, 30 s annealing at 56.4 °C, and 30 s extension at 72 °C, ending with a final extension at 72 °C for 5 min. The amplification products were purified with the PureLink PCR Purification Kit (TaKaRa, Dalian, China) and investigated via agarose gel electrophoresis. The DNA was stored at  $-20 \text{ }^\circ\text{C}$  for the following PCR-DGGE and HTST analyses.

### PCR-DGGE analysis

PCR-DGGE was performed on 8% (w/v) polyacrylamide gels (37.5: 1, acrylamide/bisacrylamide) with a denaturing gradient ranging from 40 to 75% (100% denaturant was defined as 7 M urea and 40% (v/v) formamide) using a Bio-Rad Model 475 Gradient Delivery System (Bio-Rad, Hercules, CA, USA). Electrophoresis was proceeded at 70 V and 60 °C for 16.5 h. Afterwards, the DGGE gel was stained with Biolinker DNA Red, and then photographed using a Bio-Rad system. Prominent bands from the gel were excised, and deposited overnight in 30  $\mu\text{L}$  of sterilized distilled water at 4 °C. DNA was then extracted and re-amplified as described above. Subsequently, DNA was purified using the gel extraction kit (Axygen, Union City, CA, USA) and cloned using the pEDT

cloning vector (Biolinker, Shanghai, China). Sequencing of PCR products was performed by Tiny Gene Biotech Co., Ltd. (Shanghai, China). The thus obtained sequences were compared against the current database of nucleotide sequences using the BLAST program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

### Illumina high-throughput sequencing analysis

The total DNA from 21 samples was sent to TinyGene Bio-Tech Co., Ltd. (Shanghai, China) for Illumina high-throughput sequencing with Illumina MiSeq reagent kit v3. Raw sequence reads were filtered by Trimmomatic 0.35, and low-quality sequences were removed. FLASH V1.2.11 was used to make contigs (Magoc and Salzberg 2011). To obtain more detailed biological information, the quality of reads after merging should be quality controlled. PCR amplification likely produces non-specific amplification, which can be removed by specific primers; the sequence may contain ambiguous bases, homologous single bases, and chimeras generated in the PCR process. Inclusion of these sequences into the analysis range will decrease the quality of analysis. Therefore, an optimized sequence can be obtained for accurate analysis by removing this part of the sequence, which was conducted by MOTHUR V.1.33.3. Subsequently, the sequences were clustered into operational taxonomic units (OTUs) by USEARCH V8 at a 97% sequence similarity threshold. Chimera sequences, generated during PCR amplification, were removed by UCHIME V8, and the diversity indexes including Chao1 (species richness), Shannon and Simpson diversity indexes (species diversity), and Good's coverage (percentage of the total species) were calculated using the MOTHUR program. The alpha diversity was calculated to obtain the rarefaction curves.

### Accession numbers

The 16S rRNA gene sequences of the DGGE bands have been deposited in the DDBJ database under accession numbers LC469718–LC469743. The raw Illumina read data was deposited in the NCBI Sequence Read Archive (SRA) under accession number SRP149305.

### Statistical analysis

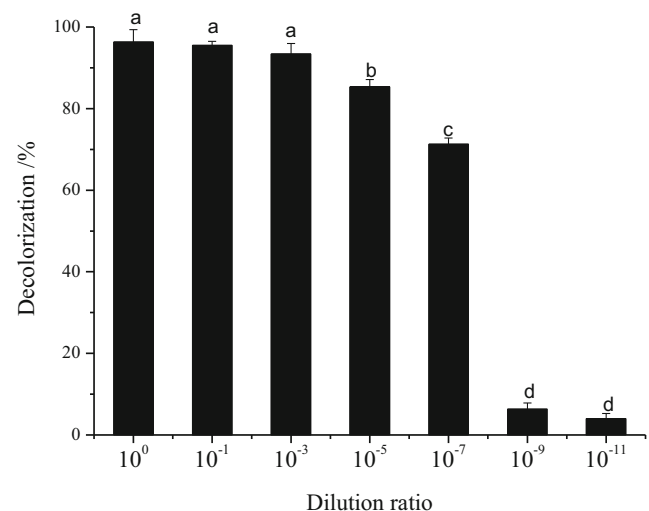
Triplicate data were expressed as means  $\pm$  SD. SPSS 17.0 (SPSS, Chicago, IL, USA) was used to perform statistical analysis by means of independent one-way ANOVA tests. Differences with  $P < 0.05$  were considered statistically significant.

## Results

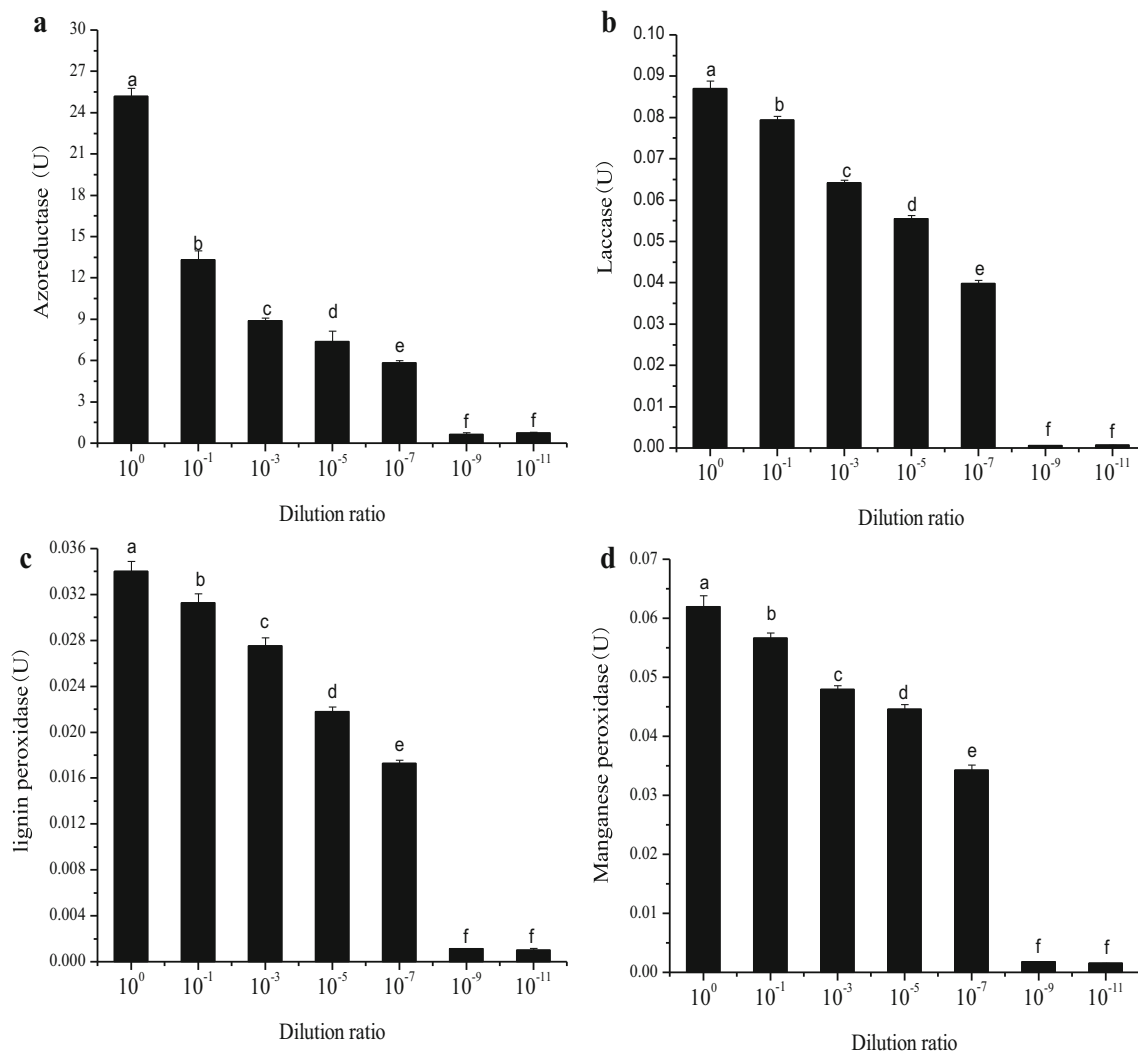
### Critical dilution point for effective azo dye degradation of the microbial community

The azo dye decolorization abilities of both the original and diluted microbial communities (with dilution ratios of  $10^0$ – $10^{-11}$ ) were tested. Decolorization efficiencies (Fig. 1) and related enzymatic activities (Fig. 2) in the degradative solutions were analyzed. The decolorization efficiencies were affected by increasing dilution ratios of the microbial community (Fig. 1). The decolorization efficiencies in the decolorized dye solutions by the original and diluted microbial community  $\leq 10^{-3}$  retained over 93.0%, and these results showed no significant difference ( $P > 0.05$ ). The decolorization efficiencies for dye solutions with dilution ratios of  $10^{-5}$  and  $10^{-7}$  retained 85% and 71%, respectively, which were both slightly lower than that in the solution after degradation by the microbial community with a dilution ratio of  $10^{-3}$ . A significant difference was found among these three groups ( $P < 0.05$ ). However, only 6% and 4% of the decolorization efficiencies ( $P > 0.05$ ) were found in the dye solutions after degradation by the microbial community at dilution ratios of  $10^{-9}$  and  $10^{-11}$ , while a highly significant difference ( $P < 0.01$ ) was found between solutions after degradation at dilution ratios of  $10^{-7}$  and  $10^{-9}$ . This result indicated a sharp decrease in the decolorization efficiencies when the dilution ratios increased beyond  $10^{-7}$ .

In addition, the changing trends of the related enzymes (azoreductase, laccase, lignin peroxidase, and manganese peroxidase) in the dye solutions after degradation showed similar tendencies, i.e., the enzymatic activities in the degraded



**Fig. 1** Change in decolorization efficiencies of the dye solutions pretreated by the original or diluted microbial community ( $10^0$ ,  $10^{-1}$ ,  $10^{-3}$ ,  $10^{-5}$ ,  $10^{-7}$ ,  $10^{-9}$ ,  $10^{-11}$ ). Data of triplicate trials are expressed as mean  $\pm$  SD. Different letters above the bars indicate significant difference ( $P < 0.05$ )



**Fig. 2** Change in the related enzymatic activities of the dye solutions pretreated by the original or diluted microbial community ( $10^0$ ,  $10^{-1}$ ,  $10^{-3}$ ,  $10^{-5}$ ,  $10^{-7}$ ,  $10^{-9}$ ,  $10^{-11}$ ); **a** azoreductase activities, **b** laccase

activity, **c** lignin peroxidase activity, **d** manganese peroxidase activity. Data of triplicate trials are expressed as mean  $\pm$  SD. Different letters above the bars indicate significant difference ( $P < 0.05$ )

solutions with dilution ratios  $\leq 10^{-7}$  decreased with the increment of dilution ratios; however, further increase in the dilution ratios to  $10^{-9}$  and  $10^{-11}$  retained the enzymatic activities in the solutions at a very low level (Fig. 2). No significant difference ( $P > 0.05$ ) was found between these two groups ( $10^{-9}$  and  $10^{-11}$  dilutions). As shown in Figs. 1 and 2, although the related enzymatic activities in the dye solutions significantly decreased with dilution ratios from  $10^0$  to  $10^{-3}$ , the decolorization efficiencies of these solutions had no significant difference ( $P > 0.05$ ). This indicated that the decrease in enzymatic activities in these decolorized dye solutions (the original and diluted microbial community  $\leq 10^{-3}$ ) exerted little negative effects on the decolorization efficiencies. Moreover, a further increase in the dilution ratios from  $10^{-3}$  to  $10^{-7}$  significantly reduced the decolorization efficiencies and the related enzymatic activities in these dye solutions, indicating that this further decrease in enzymatic activities

affected the decolorization of azo dyes. However, increasing the dilution ratios beyond  $10^{-7}$  led to sharp decreases in the decolorization efficiencies and enzymatic activities of the dye solutions after degradation at dilution ratios of  $10^{-9}$  and  $10^{-11}$ . No significant difference ( $P > 0.05$ ) was found between both groups.

In summary, the related enzymatic activities in these dye solutions ( $\leq 10^{-7}$ ) reached relatively high levels to decolorize azo dye: azoreductase (5.844–25.195 U), laccase (0.0398–0.0870 U), lignin peroxidase (0.0173–0.034 U), and manganese peroxidase (0.0342–0.062 U). Furthermore, the decolorization efficiencies could reach 71.3–96.3% in these decolorized dye solutions (original and diluted microbial communities  $\leq 10^{-7}$ ). However, according to the decolorization, the activated original and diluted microbial communities ( $\leq 10^{-7}$ ) could decolorize azo dyes within 1 to 4 days of incubation (data not shown), and the liquid media at dilutions above

$10^{-7}$  still remained black even after 8 days of incubation (data not shown). At the same time, the related enzymatic activities in these dye solutions ( $10^{-9}$  and  $10^{-11}$  dilutions) always retained very low levels throughout the experiment, which only accounted for a small percentage of the original and diluted microbial communities at dilutions  $\leq 10^{-7}$ . These results indicate that the dilution ratio of  $10^{-7}$  was the critical dilution point for effective azo dye decolorization of this microbial community.

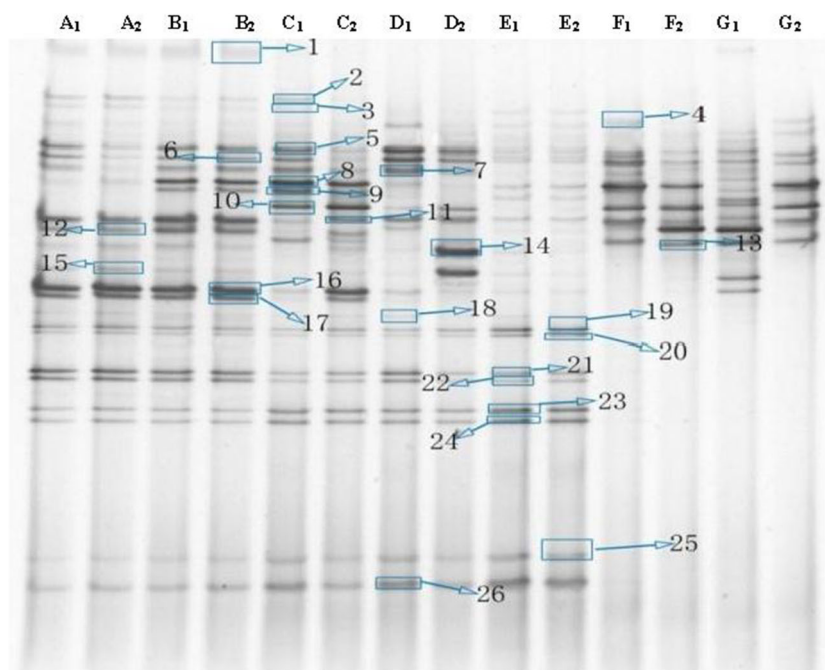
All these results further demonstrated that during the dilution process, the decrease in enzymatic activity and decolorization efficiency might be related to the loss or reduction of a number of bacterial species. The decolorization efficiencies gradually decreased as dilution ratios increased from  $10^{-3}$  to  $10^{-7}$ . There was a noticeable decrease in the decolorization efficiency when the dilution ratios increased beyond  $10^{-7}$ ; this change indicated that key functional strains of the microbial community might be washed out or that their amount decreased to a relatively low level. This was further accompanied by a sharp decrease in the related enzymatic activities to extremely low levels that could not effectively decolorize azo dyes at a visual level during the dilution process.

### PCR-DGGE analysis

The microbial distribution in samples after degradation by the original and diluted microbial communities ( $10^0$ – $10^{-11}$ ) are illustrated in Fig. 3. Fourteen samples were divided into seven groups, i.e., A–G ( $A_1, A_2$  represents  $10^0$ ;  $B_1, B_2$  represents  $10^{-1}$ ;.....  $F_1, F_2$  represents  $10^{-9}$ ;  $G_1, G_2$  represents  $10^{-11}$ ). Twenty-six distinctive bands appeared on the DGGE

fingerprint patterns, indicating the microbial diversity of the broths pretreated with the original and diluted microbial communities. Sequences with a similarity exceeding 99% are presented in Table 1. The microbes in these broths can be classified into nine species including *Caloramator* sp., *Anoxybacillus* sp., *Tepidiphilus* sp., *Brevibacillus* sp., *Bacillus* sp., *Desulfotomaculum* sp., *Clostridium* sp., *Thermoanaerobacterium* sp., and *Ureibacillus* sp. The distribution of gel bands from broths pretreated at  $\leq 10^{-3}$  almost remained constant. Figures 1 and 2 show that the decolorization efficiencies of these solutions ( $10^0$ – $10^{-3}$ ) remained above 93.0%, which indicated that the dilution ratios affected the decolorization efficiencies in these decolorized dye solutions little (the original and diluted microbial communities  $\leq 10^{-3}$ ), while the related enzymatic activities in these dye solutions significantly decreased with increasing dilution ratios. This phenomenon might be attributed to the increased abundance of certain strains such as *Brevibacillus aydinogluensis* strain PDF25, corresponding to bands 8 and 10, where the existence of a large volume of these strains might pose negative effects on the secretion of related degradative enzymes. In addition, the decolorization efficiencies in the dye solutions at dilution ratios from  $10^{-3}$  to  $10^{-7}$  decreased from 93.0 to 71.3%, and the related enzymatic activities in these solutions significantly decreased with increasing dilution ratios. Moreover, bands 2, 11, and 16–17, corresponding to *Anoxybacillus flavithermus* strain WK1, *Clostridium thermopalmarium* strain BVP, and *Caloramator proteoclasticus* strain Uruguayensis, disappeared from gels with increasing dilution ratios from  $10^{-3}$  to  $10^{-7}$ . This illustrates that these strains might be positively involved in the azo dye degradation by the microbial

**Fig. 3** DGGE profiles of amplified 16S rDNA from the samples pretreated by the original or diluted microbial community ( $10^0, 10^{-1}, 10^{-3}, 10^{-5}, 10^{-7}, 10^{-9}, 10^{-11}$ ) (The microbial community were divided into seven groups, i.e., A–G, where  $A_1, A_2$  represents  $10^0$ ;  $B_1, B_2$  represents  $10^{-1}$ ;.....  $F_1, F_2$  represents  $10^{-9}$ ;  $G_1, G_2$  represents  $10^{-11}$ )



**Table 1** Sequence identity of the DGGE bands

| Strain no. | Band no.              | Name of strain  | Similarity (%) |
|------------|-----------------------|---|----------------|
| 1          | 1, 15, 16, 17         | <i>Caloramator proteoclasticus</i> strain Uruguayensis            | 100            |
| 2          | 2                     | <i>Anoxybacillus flavithermus</i> WK1 strain WK1                  | 99             |
| 3          | 3, 19, 21, 22, 23, 24 | <i>Tepidiphilus succinatimandens</i> strain JHK30                 | 99             |
| 4          | 4, 8, 10              | <i>Brevibacillus aydinogluensis</i> strain PDF25                  | 100            |
| 5          | 5, 20, 25, 26         | <i>Tepidiphilus margaritififer</i> strain N2-214                  | 100            |
| 6          | 6, 7, 18              | <i>Bacillus thermoamylovorans</i> strain DKP                      | 100            |
| 7          | 9                     | <i>Desulfotomaculum nigrificans</i> strain NCIMB 8395             | 100            |
| 8          | 11                    | <i>Clostridium thermopalmarium</i> strain BVP                     | 99             |
| 9          | 12, 14                | <i>Thermoanaerobacterium thermosaccharolyticum</i> strain DSM 571 | 99             |
| 10         | 13                    | <i>Ureibacillus suwonensis</i> strain 6T19                        | 100            |

community. However, with further increasing dilution ratios to  $10^{-9}$  and  $10^{-11}$ , the decolorization efficiencies in these dye solutions decreased to 6% and 4%, respectively, accompanied by a sharp decrease in the related enzymatic activities to an extremely low level. The sum of gel bands in these broths significantly decreased, specifically band 3 and bands 19–26, corresponding to *Tepidiphilus succinatimandens* strain JHK30 and *Tepidiphilus margaritififer* strain N2-214, which disappeared from the gels as the dilution ratios increased beyond  $10^{-7}$ . These dilutions lost their decolorization abilities. This phenomenon indicated that the strains belonging to

*Tepidiphilus* sp. might play an essential role in the efficient degradation of azo dye in the original microbial community.

## HTST analysis

### Overall analysis of Illumina high-throughput sequencing

Illumina high-throughput sequencing analysis was conducted to identify the microbial composition and diversity of the

**Table 2** Alpha diversity indices of the microbial community of samples

| Dilution ratio | Sample name | Sequence number | OTUs | Chao 1 | Shannon | Simpson | Coverage |
|----------------|-------------|-----------------|------|--------|---------|---------|----------|
| $10^0$         | A1          | 23213           | 24   | 24     | 1.79    | 0.2171  | 1        |
|                | A2          | 30144           | 25   | 25     | 1.59    | 0.2807  | 0.999967 |
|                | A3          | 30783           | 26   | 36     | 1.65    | 0.2580  | 0.999838 |
| $10^{-1}$      | B1          | 26197           | 22   | 22     | 2.06    | 0.1587  | 0.999962 |
|                | B2          | 32995           | 24   | 26     | 2.00    | 0.1763  | 0.999879 |
|                | B3          | 30481           | 23   | 29     | 1.82    | 0.2126  | 0.999869 |
| $10^{-3}$      | C1          | 27781           | 15   | 16     | 1.74    | 0.1991  | 0.999892 |
|                | C2          | 21112           | 16   | 18     | 1.73    | 0.2025  | 0.999858 |
|                | C3          | 33381           | 12   | 12     | 1.68    | 0.2387  | 0.99997  |
| $10^{-5}$      | D1          | 37396           | 17   | 18     | 1.25    | 0.3265  | 0.99992  |
|                | D2          | 30761           | 13   | 13     | 0.80    | 0.6431  | 0.999935 |
|                | D3          | 35691           | 10   | 11     | 1.41    | 0.2815  | 0.999944 |
| $10^{-7}$      | E1          | 35126           | 19   | 21     | 0.78    | 0.5022  | 0.999915 |
|                | E2          | 31737           | 17   | 17     | 0.77    | 0.4986  | 0.999937 |
|                | E3          | 28405           | 18   | 19     | 0.81    | 0.4919  | 0.99993  |
| $10^{-9}$      | F1          | 37010           | 21   | 26     | 0.42    | 0.8017  | 0.999865 |
|                | F2          | 29134           | 18   | 19     | 0.63    | 0.6607  | 0.999931 |
|                | F3          | 34339           | 20   | 22     | 0.53    | 0.7416  | 0.999913 |
| $10^{-11}$     | G1          | 39425           | 8    | 8      | 0.01    | 0.9985  | 0.999949 |
|                | G2          | 30513           | 12   | 17     | 0.02    | 0.9951  | 0.999803 |
|                | G3          | 28795           | 10   | 16     | 0.02    | 0.9959  | 0.999861 |

degradation dye solutions pretreated by the microbial community at different dilution ratios ( $10^0$ – $10^{-11}$ ). Twenty-one samples were divided into seven groups (A–G), and their sequencing indices are summarized in Table 2. After applying quality control procedures, the number of effective sequences obtained from each sample ranged from 43,607 to 56,000. A total of 32 OTUs were detected in these samples and rather high coverage values were achieved at 0.999 for each sample.

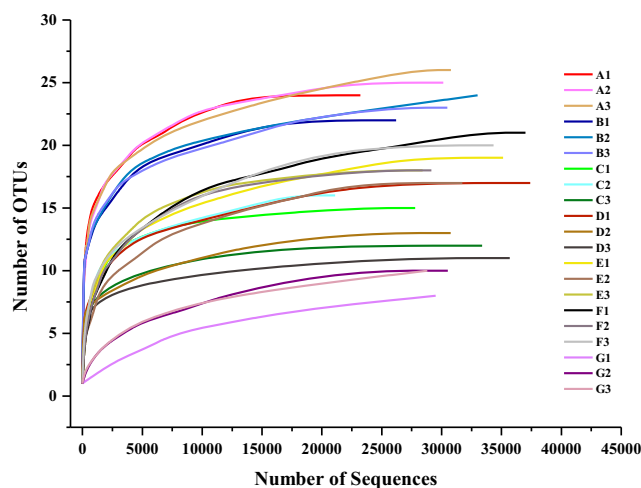
A rarefaction curve describes the variation in the number of OTUs, identified as a function of the number of sequence reads. When the curve tends to a plateau, the number of samples is reasonable, which indicates that increasing the number of reads does not change the number of OTUs that can be determined. In this study, rarefaction curves of the 21 samples followed a similar pattern (Fig. 4). These curves plateaued as the number of analyzed sequences increased, indicating that the sequencing depth was sufficient to cover the entire diversity. Chao1, Shannon, and Simpson indexes were used to evaluate the alpha diversity of the dye solutions pretreated by microbial communities at different dilution ratios. The Chao1 index represents the community richness, while Shannon and Simpson indexes reflect bacteria diversity (Yang et al. 2015a). The Chao1 estimator in samples at dilution ratios between  $10^0$  and  $10^{-7}$  (11–36) was larger than those at dilution ratios between  $10^{-9}$  and  $10^{-11}$  (8–26), indicating that the dye solutions pretreated by microbial communities at lower dilution ratios ( $\leq 10^{-7}$ ) had greater richness. In addition, the Shannon index of samples at dilution ratios between  $10^0$  and  $10^{-7}$  (0.77–2.06) was also much larger than those at dilution ratios between  $10^{-9}$  and  $10^{-11}$  (0.01–0.63). Thus, the microbial diversities of microbial communities at dilution ratios of  $10^0$ – $10^{-7}$  were higher than those of microbial communities at higher dilutions ( $10^{-9}$  to  $10^{-11}$ ). This conclusion was also demonstrated by the Simpson indexes (where a smaller

Simpson index value indicates higher Alpha diversity). Combining with the analysis shown in Figs. 1 and 2, the community richness and diversity significantly decreased at high dilutions ( $10^{-9}$  and  $10^{-11}$ ), and the corresponding microbial communities lost their decolorization abilities.

### Taxonomy and dominant genera composition

The relative bacterial community abundance at the phylum level is illustrated in Fig. 5a, where five phyla including *Firmicutes*, *Verrucomicrobia*, *Proteobacteria*, *Bacteroidetes*, and *Synergistetes* were identified. In total, *Proteobacteria* and *Firmicutes* were dominant phyla in all samples, while *Verrucomicrobia*, *Bacteroidetes*, and *Synergistetes* accounted for less than 0.08% of the total reads. Higher percentages of *Proteobacteria* and *Firmicutes* were observed in dye solutions after degradation by microbial communities at dilution ratios  $\leq 10^{-7}$ , and *Proteobacteria* was the absolutely dominant phylum in this broth. However, with the further increment of dilution ratios to  $10^{-9}$  and  $10^{-11}$ , *Proteobacteria* almost completely disappeared from the serial dilution process, while the *Firmicutes* population increased significantly. Combined with the analysis shown in Figs. 1 and 2, the disappearance of *Proteobacteria* coincided with the dramatic decrease in the decolorization ability of the microbial community at dilution ratios above  $10^{-7}$ . Therefore, it could be concluded that the functional microbial community for effective azo dye decolorization is principally composed of *Proteobacteria*. This finding coincided with a previous report, which declared that *Proteobacteria* widely existed in effluent treatment systems (Yang et al. 2015a), and furthermore, *Proteobacteria* played an important role in a number of dye degradation systems (Köchling et al. 2017). In addition, *Proteobacteria* was the absolutely dominant phylum in the broth after being degraded by microbial communities at dilution ratio of  $10^{-7}$ , while the decolorization efficiency and related enzymatic activities in the broth at a dilution ratio of  $10^{-7}$  were lower than in the dye solutions at dilution ratios of  $10^0$ – $10^{-5}$ . This indicates that strains belonging to *Firmicutes* promote the decolorization ability of azo dyes by *Proteobacteria*.

In addition, the relative bacterial community abundance at the class level are shown in Fig. 5b, and 10 class were identified. Larger percentages of *Betaproteobacteria*, *Bacilli*, and *Clostridia* were found in the dye solutions pretreated by microbial communities at dilution ratios  $\leq 10^{-7}$ , and *Betaproteobacteria* was still the absolutely dominant class in this broth. However, with a further increasing dilution ratio to  $10^{-9}$  and  $10^{-11}$ , *Betaproteobacteria* almost disappeared from the serial dilution process, and *Bacilli* became the dominant class in these solutions. Moreover, the disappearance of *Betaproteobacteria* coincided with the dramatic decrease in the decolorization ability of the microbial community when dilution ratios increased to  $10^{-9}$  and  $10^{-11}$ . This result demonstrates that strains belonging to the



**Fig. 4** Rarefaction curves for total high-quality 16S rDNA from the samples pretreated by the original or diluted microbial community (A<sub>1</sub>, A<sub>2</sub> A<sub>3</sub> represents  $10^0$ ; B<sub>1</sub>, B<sub>2</sub>, B<sub>3</sub> represents  $10^{-1}$ ;..... F<sub>1</sub>, F<sub>2</sub>, F<sub>3</sub> represents  $10^{-9}$ ; G<sub>1</sub>, G<sub>2</sub>, G<sub>3</sub> represents  $10^{-11}$ )



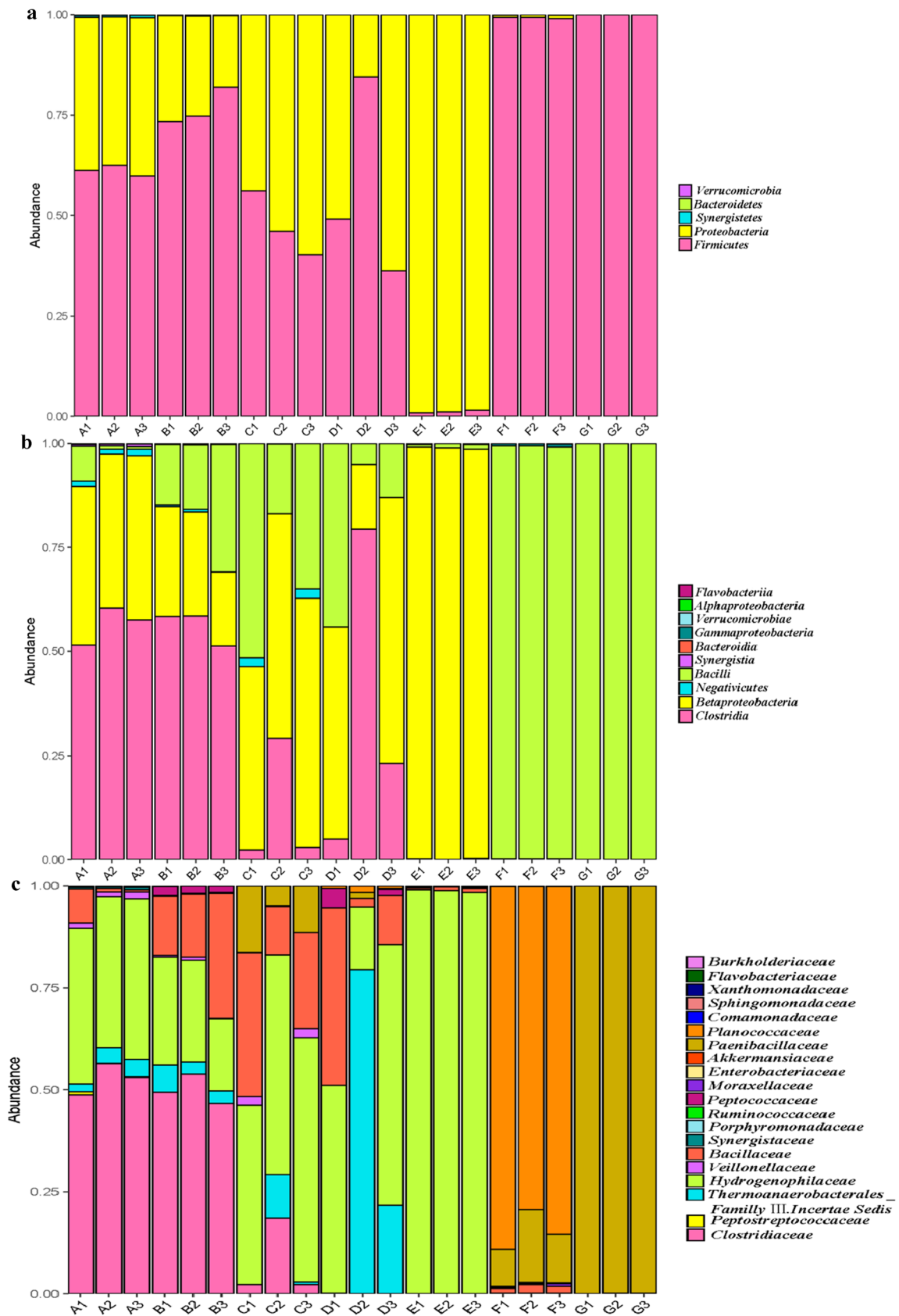
class *Betaproteobacteria* might play an important role in the decolorization of azo dye. This finding was consistent with a previous report that declared that *Betaproteobacteria* play an important role in mixed bacterial consortia capable of efficient degradation azo dye (Miran et al. 2015). In addition, the decolorization efficiencies of these solutions ( $10^0$ – $10^{-3}$ ) showed no significant difference, while the related enzymatic activities decreased significantly with increasing dilution ratios. This phenomenon might be caused by the inhibiting effect of several bacteria that belong to *Bacilli*, which inhibited the secretion of related enzymes by competing with other bacteria (that would otherwise have contributed to the decolorization and degradation of azo dyes) for nutrients, such as *Betaproteobacteria*. In addition, *Betaproteobacteria* was the dominant class in the broth with dilution ratio of  $10^{-7}$ , while the decolorization efficiency and enzymatic activity in this broth were lower than those in the dye solutions with dilution ratio of  $10^0$ – $10^{-5}$ . This indicated that several bacteria, such as *Clostridia*, might be able to promote the decolorization of azo dyes by *Betaproteobacteria*.

The relative bacterial community abundance at the family level is shown in Fig. 5c. Higher percentages of *Hydrogenophilaceae*, *Bacillaceae*, *Thermoanaerobacteriales\_Familly\_III\_Incertae\_Sedis*, and *Clostridiaceae* were found in dye solutions at dilution ratios  $\leq 10^{-7}$ ; especially, *Hydrogenophilaceae* still remained the dominant family in this broth. The quantity of *Hydrogenophilaceae* decreased significantly with increasing dilution ratio to  $10^{-9}$  or above, and both *Planococcaceae* and *Paenibacillaceae* were the dominant families in these solutions. The disappearance of *Hydrogenophilaceae* coincided with a sharp decrease in the effective azo dye degradation ability, demonstrating that strains belonging to the family of *Hydrogenophilaceae* might play an important role in the decolorization of azo dye. In addition, according to the combined observation of the decolorization efficiencies and the related enzymatic activities at different dilution ratios, the decolorization efficiencies of these solutions ( $10^0$ – $10^{-3}$ ) were not significantly different ( $P > 0.05$ ), while the related enzymatic activities in the dye solutions decrease significantly with increasing dilution ratios. This change might be caused by the inhibiting effect of specific bacteria belonging to *Bacillaceae*, which inhibited the secretion of related enzymes by competing with other bacteria for nutrients, such as *Hydrogenophilaceae*. In addition, *Hydrogenophilaceae* was the dominant family in the broth with a dilution ratio of  $10^{-7}$ , while the decolorization efficiencies and enzyme activities in this broth were lower than those in the dye solutions with dilution ratios of  $10^0$ – $10^{-5}$ . This indicated that a number of bacteria such as *Clostridiaceae* and *Thermoanaerobacteriales\_Familly\_III\_Incertae\_Sedis* might be able to promote the decolorization of azo dyes by *Hydrogenophilaceae*.

Furthermore, the heat map clearly shows the profiles of the 21 most abundant genera in the 21 investigated samples at different dilution ratios (Fig. 6). As shown in Fig. 6, a

large variation of relative bacteria community abundance was observed at dilution ratios above  $10^{-7}$ , which indicated that different dilution ratios exert a significant effect on the microbiome structure at the genus level. Higher percentages of *Tepidiphilus* sp., *Caloramator* sp., *Thermoanaerobacterium* sp., *Bacillus* sp., *Brevibacillus* sp., *Thermobrachium* sp., *Clostridium* sp., *Anoxybacillus* sp., and unclassified strains were found in dye solutions at dilution ratios  $\leq 10^{-7}$ , and *Asaccharospora* sp., *Fonticella* sp., *Proteiniphilum* sp., and *Thermanaerovibrio* sp. were also identified and accounted for less than 5.51%. *Tepidiphilus* sp. still remained the dominant genus in the broth with dilution ratio of  $10^{-7}$ . However, with further increasing dilution ratios to  $10^{-9}$  and  $10^{-11}$ , *Tepidiphilus* sp. was almost eliminated from the serial dilution process, and populations of *Brevibacillus* sp. and *Ureibacillus* sp. significantly increased, which coincided with a sharp decrease in their effective azo dye degradation ability. In the present study, *Tepidiphilus* sp. was found to be the dominated genus in the dye solution at a dilution ratio of  $10^{-7}$ , and the decolorization efficiencies in the dye solution of  $10^{-7}$  reached 71.3%, indicating that the genus has an azo dye degradation ability. According to the analysis of the decolorization efficiencies and the related enzymatic activities under different dilution ratios (Figs. 1 and 2), the decolorization efficiencies of these solutions ( $10^0$ – $10^{-3}$ ) was not significantly different ( $P > 0.05$ ), and the decolorization efficiencies of these solutions exceeded 93.0%, while the related enzymatic activities in these dye solutions significantly decreased with increasing dilution ratios. This phenomenon might be caused by the increased abundance of bacteria such as *Brevibacillus* sp., which inhibited the secretion of related enzymes by competing with other bacteria for nutrients. The decolorization efficiencies in the dye solutions with dilution ratios from  $10^{-3}$  to  $10^{-7}$  decreased from 93.0 to 71.3%, and the related enzymatic activities in the same dye solutions reduced significantly with increasing dilution ratios. *Anoxybacillus* sp., *Clostridium* sp., *Caloramator* sp., *Thermobrachium* sp., and *Thermoanaerobacterium* sp. disappeared from the systems when the dilution ratios increased from  $10^{-3}$  to  $10^{-7}$ , which illustrated that these strains might be involved in the azo dye degradation of the microbial community. *Bacillus* sp. was always found in the whole serial dilution process, which indicates that this genus might aid in the decolorization of azo dyes by other bacteria.

**Fig. 5** Microbial composition structures of the samples pretreated by the original or diluted microbial community at the **a** phylum level, **b** class level, and **c** family level ( $A_1, A_2, A_3$  represents  $10^0$ ;  $B_1, B_2, B_3$  represents  $10^{-1}$ ;  $\dots, F_1, F_2, F_3$  represents  $10^{-9}$ ;  $G_1, G_2, G_3$  represents  $10^{-11}$ ). The abundance was presented in terms of a percentage of the total effective bacterial sequences in each sample

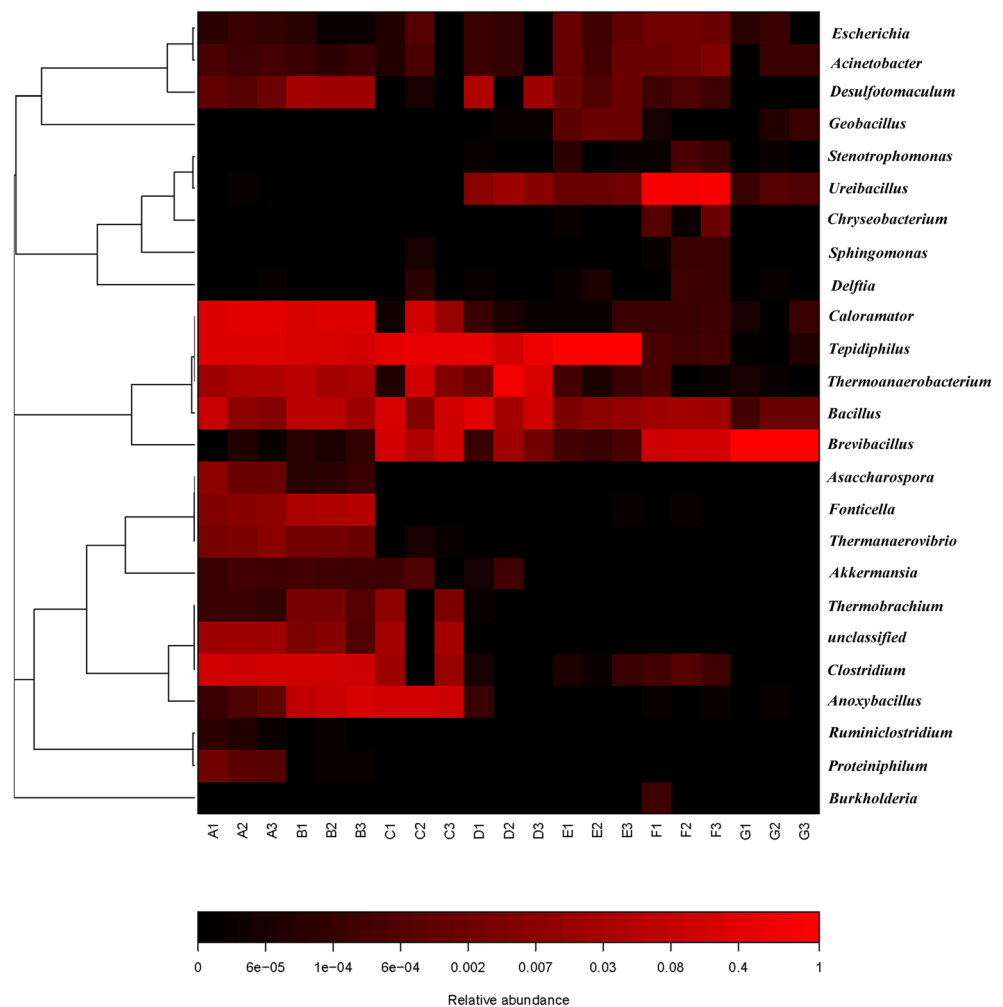


## Discussion

A dilution-to-extinction approach has been suggested as a relatively simple method for the investigation of natural microbial communities, where no complex and time-consuming strain isolation process is required (Wang et al. 2010; Zhang et al. 2015a). In this study, a dilution-to-extinction approach was combined with PCR-DGGE and HTST to explore the functional microbes from the microbial community. Based on the analysis of DGGE fingerprint patterns, the microbial composition of the original microflora comprises *Caloramator* sp., *Anoxybacillus* sp., *Tepidiphilus* sp., *Brevibacillus* sp., *Bacillus* sp., *Desulfotomaculum* sp., *Clostridium* sp., *Thermoanaerobacterium* sp., and *Ureibacillus* sp. According to decolorization efficiencies and related enzymatic activities (azoreductase, laccase, lignin peroxidase, and manganese peroxidase), the dilution ratio of  $10^{-7}$  was considered to be a critical point for effective azo dye decolorization of the microbial community. Furthermore, strains belonging to *Tepidiphilus* sp. were dominant in the degradation liquor with dilution ratio of  $10^{-7}$ . Similar to the

DGGE results, the Illumina HTST investigation further showed that the strains belonging to *Tepidiphilus* sp. played an essential role in the efficient degradation of azo dye in the original microbial community. *Tepidiphilus* sp., which can utilize  $H_2$  and sulfide as electron donors, commonly exists in cellulose-degrading microbial communities (Manaia et al. 2003). According to the complete genome sequences analysis of *Tepidiphilus* sp., part of the gene fragments of this strain is related to 3-hydroxyacyl-CoA dehydrogenase and acetyl-CoA acetyltransferase. According to a previous investigation, 3-hydroxyacyl CoA dehydrogenase was classified as an oxidoreductase and indicated to be actively involved in fatty acid metabolic processes (Qi et al. 1999). Acetyl-CoA acetyltransferase, a mitochondrial enzyme involved in both amino acid degradation and fatty acid oxidation, was suggested to play an important role in the metabolic process of organisms and the energy supply for living organisms (Green et al. 1954). Therefore, combined with the findings of the present investigation, it could be deduced that both enzymes in *Tepidiphilus* sp. might also play an important role in the metabolic process of azo dyes. However, little information about the

**Fig. 6** Heat map of the 21 most abundant genera of the samples pretreated by the original or diluted microbial community ( $A_1$ ,  $A_2$ ,  $A_3$  represents  $10^0$ ;  $B_1$ ,  $B_2$ ,  $B_3$  represents  $10^{-1}$ ; .....  $F_1$ ,  $F_2$ ,  $F_3$  represents  $10^{-9}$ ;  $G_1$ ,  $G_2$ ,  $G_3$  represents  $10^{-11}$ ). The color intensity in each cell showed the abundance of a genus in a group, and the abundances were displayed as percentage of the total sequences in each group



biodegradation of azo dyes by strains of this genus could be found in recent literatures. In addition, *Tepidiphilus* sp. was found to be the dominated genus in the functional microbial community ( $10^{-7}$ ) in this study, and the decolorization efficiencies in dye solutions could reach 71.3%. However, the related enzymatic activities and decolorization efficiencies in these broths were not better than those in the broths with dilution ratios below  $10^{-7}$ . This result further indicated that the highly efficient decolorization of azo dyes might result from the synergistic action of *Tepidiphilus* sp. with other bacteria.

In total, the decolorization efficiencies of these solutions ( $10^0$ – $10^{-3}$ ) have no significant difference ( $P > 0.05$ ), and the decolorization efficiencies of these solutions all exceeded 93.0%, while the related enzymatic activities in the dye solutions significantly decreased with increasing dilution ratios. This phenomenon might be caused by the increased abundance of bacteria such as *Brevibacillus* sp., which can inhibit the secretion of related enzymes via competition with other bacteria for nutrients. The decolorization efficiencies in the dye solutions after pretreatment by this microbial community ( $10^{-3}$ – $10^{-7}$ ) decreased from 93.0 to 71.3%, and the related enzymatic activities in these dye solutions reduced significantly with increasing dilution ratios. *Anoxybacillus* sp., *Clostridium* sp., *Caloramator* sp., *Thermobrachium* sp., and *Thermoanaerobacterium* sp. disappeared from the dye solutions after pretreatment by the microbial community ( $10^{-3}$ – $10^{-7}$ ). This phenomenon illustrated that strains belonging to *Anoxybacillus* sp., *Clostridium* sp., *Caloramator* sp., *Thermobrachium* sp., and *Thermoanaerobacterium* sp. might be positively involved in the azo dye degradation of the microbial community. *Bacillus* spp. were always found in the whole serial dilution process; this genus might be helpful to enhance the decolorization of azo dyes with other bacteria.

In addition, according to previous research, many genera including *Anoxybacillus* sp. and *Clostridium* sp. were reported to have the capability to degrade azo dyes. For example, *Clostridium perfringens* was reported to release azoreductase to break azo bonds (Morrison and John 2015); *Anoxybacillus* sp. with hyperthermostable alkaline laccase activity was successfully isolated, which has been reported to have excellent removal ability for Reactive Black 5 (Deive et al. 2010; Alkahem Al-balawi et al. 2017). *Bacillus* sp., a facultatively anaerobic genus, which has wide antimicrobial activity and strong resistance to adversity (Wungsintaweekul et al. 2010), was reported to possess the ability to decolorize azo dyes under static or anoxic conditions (Jain et al. 2012), and the azoreductase secreted from this genus could catalyze the degradation of dyes by breaking N=N double bonds (Schmitt et al. 2012). Bacteria belonging to this genus were also reported to have the ability to metabolize toxic compounds (Banerjee and Ghoshal 2010). These strains might create an optimum condition by consuming toxic intermediate

metabolites, such as intermediate degradation products (aromatic compounds), and thus facilitate the removal of azo dyes by other dye-decolorizing microbes (Bengtsson and Carlsson 2001). In addition, *Caloramator* sp. was reported to have the ability to degrade proteins and amino acids, which might further promote the degradation process with other textile dye degradation microbes (Tarlera and Stams 1999). The genera *Thermoanaerobacterium* sp. and *Thermobrachium* sp. are strictly anaerobe and can produce  $H_2$  using cellulose or other wastes as substrate (O-Thong et al. 2008; Zhao et al. 2013). Bacteria of these genera might be able to use azo dyes or their intermediates as substrates for hydrogen production.

Therefore, it could be preliminarily concluded that the synergistic action of *Anoxybacillus* sp., *Clostridium* sp., *Bacillus* sp., and *Caloramator* sp. can promote the decolorization process with the key functional strains *Tepidiphilus* sp. The loss of these bacteria during the serial dilution processes might deteriorate the synergist relationships among different strains and thus decrease the ability of this microbial community to decompose azo dye.

## Conclusions

In conclusion, the dilution ratio of  $10^{-7}$  was found to be the critical dilution point for effective azo dye decolorization of the microbial community via decolorization situation and enzymatic activities analyses during the serial dilution process with a dilution-to-extinction approach. Further analysis of the results obtained from the combination investigation of PCR-DGGE and HTST suggested that the strains belonging to *Tepidiphilus* sp. were key functional strains of the microbial community, and the synergistic action of other microbes (such as *Anoxybacillus* sp., *Clostridium* sp., *Bacillus* sp., and *Caloramator* sp.) was suggested to further promote the decolorization process of these key functional strains.

**Author contributions** Yan Chen and Lizhen Zhang contributed equally.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

**Informed consent** Informed consent was obtained from all individual participants included in the study.

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