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Characterization of functional microbial communities involved in diazo dyes decolorization and mineralization stages

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ABSTRACT

A new sequencing anaerobic-oxic-sedimentation (AOS) bioreactor was constructed to characterize the functional microbial communities involved in different diazo dye transformation stages. Two typical refractory diazo dyes, Congo red (CR) and Amino Black (AB), were treated in the two AOS bioreactors as model contaminants. Chemical oxygen demand (COD), dissolved oxygen (DO), pH and diazo dye intermediates in each compartment in the two bioreactors were distinct, leading to the difference in the corresponded microbial communities. High-throughput sequencing was applied to study the microbial community structure. *Bacteroides, Parabacteroides, Chryseobacterium* and *Comamonas* were dominant genera. Linear discriminant analysis (LDA) effect size (LEfSe) was employed to characterize significantly different genera involved in anaerobic and oxic zones. Multiple linear regression was employed to further evaluate the relationship between azo dye intermediates and bacterial community compositions, and the results were demonstrated in t-value biplots. Functional genera corresponding to azo bond reduction and aromatic amines mineralization were characterized and the mechanism of biotransformation of two diazo dyes to CO_2 was proposed. These findings were beneficial for process optimization and efficiency improvement.

1. Introduction

Azo dyes, which are characterized by azo bonds (-N=N-) as chromophore, are widely used in textile-processing and other industries. Large volumes of dye wastewater with high concentration of azo dyes (between 100 and 250 mg L^{-1}) are daily disposed by many factories throughout the world (Garcia-Segura and Brillas, 2016). If the wastewater can not be completely treated, it will not only cause aesthetic problem, but also affect human health due to the carcinogenic, mutagenic and toxic effects of azo dye breakdown products (González-Gutiérrez et al., 2009). Treatment of azo dye wastewater has been extensively studied. Several physical and chemical techniques such as adsorption, coagulation-flocculation, Fenton's oxidation, ozonation and polycatalytic oxidation have been applied (Martínez-Huitle and Brillas, 2009; Saratale et al., 2011). These methods can remove color effectively, but the high operational costs hinder their application in a large scale.

For dye wastewater treatment plant, biological method is a better choice (Maljaei et al., 2009). Aerobic, anaerobic, or a combination have been employed. Among which combined anaerobic-aerobic treatments have been most widely used (Muda et al., 2011; Sarayu and Sandhya, 2012; Spagni et al., 2010). Azo dyes are decolorized in anaerobic situation and the break down products (aromatic amines) are degraded under aerobic condition (Silva et al., 2012). The efficiency of the biological process is very important for removing azo dye from wastewater. Microorganisms involved in the two biological operators play key roles in decolorization and degradation of azo dyes. It is generally believed that the microbial community structure and diversity can affect the performance and stability of the biological process (Ye et al., 2017). Qualitative and quantitative analysis of bacterial community will provide information for optimization of the treatment process (Xie et al., 2016; Köchling et al., 2017).

In the previous study, various conventional molecular ecological methods, involving terminal-restriction fragment length polymorphism (T-RFLP), denaturing gradient gel electrophoresis (DGGE), real-time PCR (qRT-PCR), and fluorescence in *situ* hybridization (FISH) have been applied in analyzing the microbial community of activated sludge in biological operators. However, DGGE and T-RFLP only identify specific bacteria with high abundance but do not detect the overall diversity of microbial community (Schütte et al., 2008). qRT-PCR and FISH can

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only quantitative analyze target microbes (Cottrell and Kirchman, 2000; Amann and Fuchs, 2008). Recently, high-throughput sequencing technology, which can identify and quantify most of the species in the sample, has been used in analyzing the microbial community in activated sludge (Kumaraswamy et al., 2014; Polka et al., 2015). It overcomes the limitations of conventional methods. For example, Illumina MiSeq sequencing analysis was applied in investigating the microbial community in the anaerobic process under different dye conditions. The dominate species which might play important roles in Reactive Black 5 and Remazol Brilliant Blue R degradation were identified (Xie et al., 2016). However, decolorization and degradation of azo dye into CO_2 and H_2O is a complicated process. Several reactions were happened and different bacterial types were involved (Xu et al., 2008). The evaluation of microbial community structure in response to different dye breakdown products can help identify the crucial steps and key microorganisms in the transformation process. Unfortunately, this work has seldom been reported, and little is known about the functional microorganisms and microbial communities in different azo dye degradation steps. Recently, statistical model such as principal component analysis (PCA) and redundancy analysis (RDA) based on multiple linear regression is usually used to estimate the correlation between microorganisms and environmental variables (Leps and Smilauer, 2006; Gao et al., 2016). While, with the model, only the positive or negative relationship between the two variables can be identified. Their statistically significance was failed to establish. In other words, the most responsible microorganisms for specific step of transformation were not elucidated.

Thus, in this study, to identify the key microorganisms in the crucial steps in azo dye degradation process, a multiple linear regression model combined with t-value biplot is proposed. Two batched reactors fed with two typical refractory diazo dyes Congo red (CR) and Amino Black (AB) were established respectively. Each reactor was composed of six compartments, degradation intermediates inflowed into each compartment are varied, which may affect the microbial communities. The aims of the study were (1) to investigate the transformation reactions in each compartment; (2) to evaluate the corresponded microbial communities (3) to reveal the relationship between azo dye degradation and microorganisms with multiple linear regression model; (4) to identify the key species involved in crucial azo dye degradation steps. The proposed model will provide a new method for the identification of functional microorganisms involved in wastewater treatment.

2. Materials and methods

2.1. Dyes and chemicals

The diazo dyes used in this study were purchased from Jinsui Biological Technology Co. Ltd. (Shanghai, China). All the chemical used are analytical grade.

2.2. AOS bioreactors

In order to reveal the relationship between microbial community and azo dye degradation intermediates, two corridor style AOS reactors were established (Fig. 1). The AOS bioreactor was made of plexiglass, which involves three anaerobic compartments (1#, 2#, 3#), two oxic compartments (4#, 5#) and one sedimentation compartment (6#). All of the compartments were divided by baffles with dimensions L 13 cm × W 15 cm × H 55 cm.

2.3. Source of inoculated sludge

The anaerobic zones were inoculated with anaerobic sludge from a hydrolysis acidification tank at Shaoxing wastewater plant, Zhejiang Province, China. The aerobic zones were inoculated with aerobic activated sludge from aerobic tank at the same plant. The mixed liquor suspended solids (MLSS) was maintained at 10–16 g L^{-1} in the first five compartments.

The sludge was acclimated with synthetic azo dye wastewater consisting 30 mg azo dye, 2 g glucose, 1 g $NaHCO_3$, 0.015 g KH_2PO_4 and 1 L water. The synthetic wastewater was pumped into the reactor with an inflow rate of 10 r min^{-1}. The reactor was run at room temperature with a HRT of 24 h. When the dye removal efficiency became stable, the azo dye concentration was increased to 45 mg L^{-1} and then to 60 mg L^{-1}.

2.4. Chemical measurements

pH and DO were measured with pH and DO meter, respectively. COD was measured following the standard methods. To identify the degradation products, samples were analyzed by GC/MS and LC/MS. For GC/MS analysis, 100 mL of sample was taken out from each compartment, centrifuged and extracted with 10 mL of CH_2Cl_2 three times at pH 2, 7, 13 respectively. The extract was combined, dehydrated with Na_2SO_4 and concentrated using rotary evaporator. The residue was dissolved in 1.0 mL of CH_2Cl_2 (chromatographically pure grade) and filtrated with 0.22 μm organic filter membrane. GC/MS analysis was performed on Agilent-6890/5975 MSD with the method demonstrated by Zhao et al. (2010). For LC/MS analysis, 100 mL of the centrifuged sample was frozen dried, dissolved in 5.0 mL methanol, filtered and dried with nitrogen. The residue was dissolved in 1.0 mL CH_3OH (chromatographically pure grade) and filtrated with 0.22 μm organic filter membrane. A Thermo Fisher Scientific LCQ Fleet with C_{18} inverse phase column (4.6 mm × 250 mm, particle size 5 μm) was used. The gradient elution of CH_3OH/H_2O ranged from 0:100 to 100:0 in 30 min at a flow rate of 1.0 mL min^{-1}. The MS spectra was set from 100 to 500 m/z, and analyzed in both positive and negative ion mode.

2.5. DNA extraction, polymerase chain reaction (PCR) and illumina sequencing analysis

In this study, microbial community in each compartment was determined with 16S rDNA gene Illumina Miseq. The sludge sample was collected and centrifuged at 10,000 rpm at 4 °C, and DNA in the pellets was extracted with Powersoil DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA) with the manufacture's instruction, then analyzed by 1% agarose gel and quantified with NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). Total extracted DNA was PCR-amplified targeting the 16S rDNA variable regions 3 and 4 with primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 533R (5′-TTACCGCGGC TGCTGGCAC-3′) by Geneamp R 9700 PCR System (ABI company, USA). The purified PCR amplicons were determined with Illumina Miseq platform in Sangon Biotech (Shanghai, China) Co. Ltd. To enable multiplex sequencing, samples were individually barcoded in the study. The raw sequences were deposited in the NCBI Sequence Read Archive with the accession number SRP119807.

2.6. Bioinformatic analysis

After MiSeq sequencing, the obtained reads > 8-bp homopolymers or shorter than 200 bp were removed to ensure high quality sequence data for further analysis. The primers, barcodes and adapters achieved with default parameters were also deleted. The raw data were then denoised with the Mothur implementation of PyroNoise algorithm and QIIME (Schloss et al., 2009; Caporaso et al., 2010). The sequences in each sample were clustered into operational taxomic units (OTU) using Ribosomal Database Project (RDP) with a sequence similarity level of 97%, individually. The number of OTUs, Chao 1 and ACE richness, Simpson and Shannon diversity were calculated with a dissimilarity level of 3% using the Mothur software (Schloss et al., 2009). Taxonomic classification of OTUs was conducted with RDP Classifier (http://rdp.cme.msu.edu/) via SILVA database at 80% confidence threshold.