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Isolation of Different Azo Dye Decolorizing Bacteria and Their Decolorization Mechanisms

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ABSTRACT

Four strains, which can decolorize acid orange, congo red, methyl orange and amino black respectively, were isolated from anaerobic activated sludge using the enrichment, acclimation and purification methods. With 16S rDNA sequencing analysis, they were identified as *Serratia nematodiphila* subsp. sakuensis st, *Morganella morganii* subsp. morganii str, *Lysinibacillus* sp. BAB-5845 and *Bacillus* sp. NJSN49. The decolorization conditions were studied and results demonstrated that the optimal conditions for the four strains were starch of 4 g/L, peptone of 8 g/L, pH 7.5; sucrose of 2 g/L, yeast powder of 2 g/L, pH 7.5; yeast powder of 2 g/L, pH 7.0 and sucrose of 8 g/L, urea of 4 g/L, pH 6.0. UV-Vis, FTIR and GC-MS analysis showed that, with the four strains, -N=N- double bond in acid orange, congo red, methyl orange and amino amine were broken effectively and the related aromatic amino were yielded. Our research can provide efficient strains and technical parameters for azo dye wastewater treatment industry.

INTRODUCTION

With the development of printing and dyeing industry, preparation and application of dyes have been expanded rapidly (Cui et al. 2014). Dye is one of the major pollutants in dyeing and printing, textile, and paper making industrial wastewater (Khalid et al. 2008). About 300-400 tons of printing and dyeing wastewater (PDW) per year is discharged in China and causes severe pollution in aquatic environment (Xie et al. 2016a). The composition of printing and dyeing wastewater (PDW) is complex and contains high concentrations of toxic and refractory substances (González-Gutiérrez et al. 2009). There are numerous different forms of dyes, and the three most widely used are azo dyes, anthraquinone dyes and methane dyes (Maljaei et al. 2009).

Azo dye, containing one or more -N=N- double bond, constitute up to 60-70% of dye production (Cui et al. 2012, Xie et al. 2016b, Köchling et al. 2017). Currently, physical, chemical and biological methods were applied for PDW wastewater treatment. Physical and chemical methods such as adsorption, flocculation, and photochemical oxidation are effective methods in the decolorization of PDW (Martínez-Huitle & Brillas 2009, Saratale et al. 2011). However, the running costs are usually higher and secondary pollutants might be produced. Compared with physical and chemical methods, biological approach has the advantages of low cost and convenient management, now is widely used in PDW treatment plant (Schütte et al. 2008).

Usually, azo dyes are decolorized under anaerobic conditions and the yielded aromatic amines are degraded in aerobic situation (Silva et al. 2012). Thus, the efficiency of decoloration was important for the whole degradation. At present, most of the isolated bacteria could degrade azo dyes under anaerobic conditions (Garcia-Montano et al. 2008). The reduction of azo dyes is nonspecific, a great deal of intermediate mediators can provide electrons for the reaction.

A variety of bacteria, which can effectively degrade azo dyes under anaerobic conditions, have been reported recently, such as *Bacteroides*, *Eubacterium* and *Clostridium* (Rasool et al. 2016, Vijaykumara et al. 2007, Matthies et al. 2007). The biological decolorization process can be affected by various factors (Liu et al. 2007, Feng et al. 2012). Therefore, researches on the factors such as culture medium, fermentation conditions have been done (Seesuriyachan et al. 2007, You & Teng 2009, Xie et al. 2016b). However, the structure of azo dye is complicated; the functional group involved in azo dye might affect the performance of bacteria. Thus, selection of different azo dye decolorization bacteria was important.

In this research, the strains, which can decolorize different azo dyes with high efficiency, were isolated from anaerobic sludge, the influence of various factors on the decolorization efficiency were investigated, and the decolorization mechanism was also studied, providing the technical parameters for the industrial application.

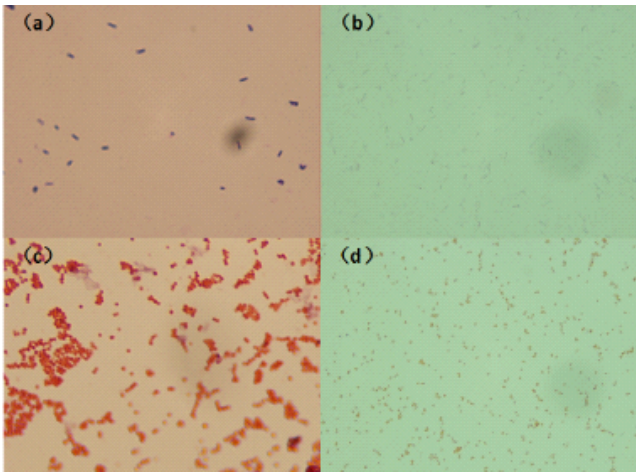

Fig. 1: Gram staining of the four strains (a: AO7; b: CR; c: MO; d: AB).

MATERIALS AND METHODS

Screening of decolorizing bacteria: Activated sludge from anaerobic tank in Shaoxing wastewater treatment plant (Shaoxing, Zhejiang, China) was used for screening of the bacteria. One mL of the sludge was inoculated to 100 mL nutrient medium containing 200 mg/L of different dyes (acidic orange II, methyl orange, congo red, and amino black), respectively. The Erlenmeyer flasks were incubated at 30°C under static as well as shaking (150 rpm) conditions. The bacteria demonstrating decolorization under static condition were transferred from decolorized flask to the fresh medium. The acclimatization was repeated for five times. Then, with diluting method, pure culture was obtained. The pure culture was inoculated to the broth medium containing different dyes, again to investigate its decolorization ability. The bacteria owning decolorizing ability were diluted until a single colony was obtained. The single decolorizing bacterial colonies were stored on agar slants at 4°C.

Identification of decolorizing bacteria: 16S rDNA sequence analysis was carried out to identify the screened strains. Primers BSF8/20(27f): AGAGTTTGATCCTGGCTCAG and BSR1510/1492(1492R): TACGGYTACCTTGTTACGACTT were used. The results were compared with the homology in GenBank with BLAST, and MEGA 7.0 was used to build the phylogenetic tree.

Influencing factors of decolorization efficiency: ***Influence of carbon source*:** The effects of carbon on the decolorization efficiency were evaluated. Four kinds of carbon were studied, no addition, glucose, sucrose and starch. Then the optimal carbon concentration (2, 4, 6, 8, 10 g/L) was determined.

***Influence of nitrogen source*:** The influence of nitrogen on the decoloration efficiency was determined. Four kinds of nitrogen source were studied, no addition, peptone, yeast powder, urea and NH_4Cl. The optimal nitrogen concentration (2, 4, 6, 8, 10 g/L) was then evaluated.

***Influence of pH value*:** With best carbon and nitrogen

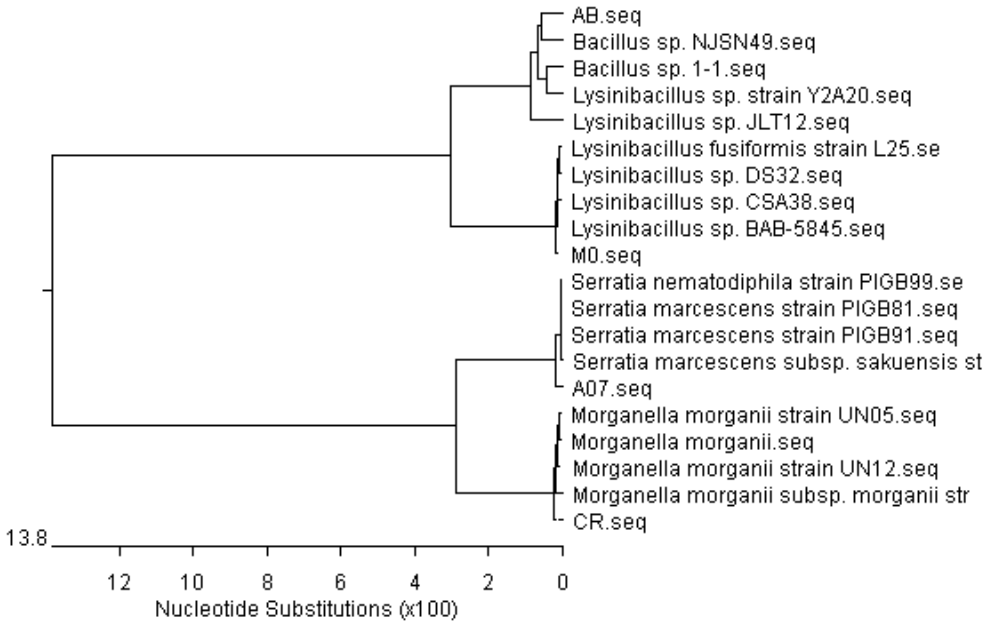

Fig. 2: Phylogenetic positions of isolated strains based on 16S rDNA gene sequence comparisons.

Table 1: Best fermentation conditions of four azo dye degradation bacteria.

	Carbon		Nitrogen		pH
	Type	Concentration	Type	Concentration	
AO7	Starch	4 g/L	Peptone	8 g/L	7.5
CR	Sucrose	2 g/L	Yeast powder	2 g/L	7.5
MO	No added	Yeast powder	2 g/L	7	
AB	Sucrose	8 g/L	Urea	4 g/L	6.0

source, the pH of the medium was adjusted to 5, 6, 7, 8, 9 and 10 respectively, with 1 M HCl or NaOH. The optimal pH was chosen for dye decolorization.

Analysis: Samples were taken from the transformation flasks every 24 h and then centrifuged at 110000 g for 10 min. Ten mL of the supernatant was scanned from 200-800 nm with UV-Vis spectrophotometer. Twenty mL of the supernatant was frozen dried and analyzed with Fourier Transform Infrared spectroscopy (FTIR). One hundred mL of the supernatant was extracted with 10 mL dichloromethane at pH 2, 7 and 13 respectively. The extraction was combined, dehydrates by Na_2SO_4 and dried with nitrogen. The residue was dissolved in 1.0 mL of CH_2Cl_2 (chromatogram pure grade, Fisher, USA) and analyzed by GC-MS using the method proposed by Xu et al. (2008) and Zhao et al. (2010). According to the change of dye molecular structure in the degradation process, the decolorization mechanism of dyes was analyzed in the aspect of reduction of nitrogen-nitrogen double bond, the degradation of aromatic amines and the oxidation decomposition of thiol groups.

RESULTS AND DISCUSSION

Screening of different azo dye decolorizing bacteria: It was found that the medium containing azo dyes could be obviously decolorized after incubating the screened bacterium from anaerobic activated sludge. After screening the mixed strains repeatedly, four strains with the best degradation effect on acid orange II, congo red, methyl orange and amino black were selected under anaerobic condition. They were named as AO7, CR, MO and AB respectively. The single colony of AO7 strain was irregular round, irregular edge, rough surface and opaque white. AO7 was stained with gram and observed by microscope. The stained colour was purple, which demonstrated that AO7 was a Gram positive bacterium (Fig. 1a). The cell morphology was granular and existed alone. The single colony of CR strain had regular tiny origin, neat edges and opaque white. After Gram staining, CR was purple, long and rod shaped. It is a Gram positive bacterium (Fig. 1b). MO colony was white, irregular, and had rough surface and irregular edge. After Gram staining, MO bacteria was red and granular, it is Gram nega-

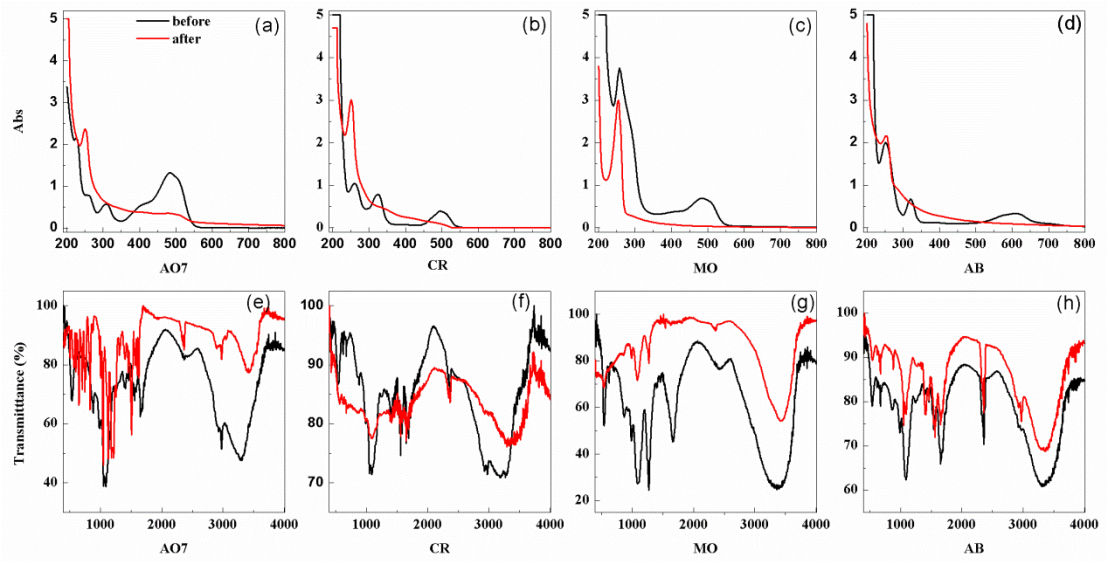

Fig. 3: The UV-vis spectra of degradation of azo dyes (a-d), FTIR spectra of degradation of azo dyes (e-h).

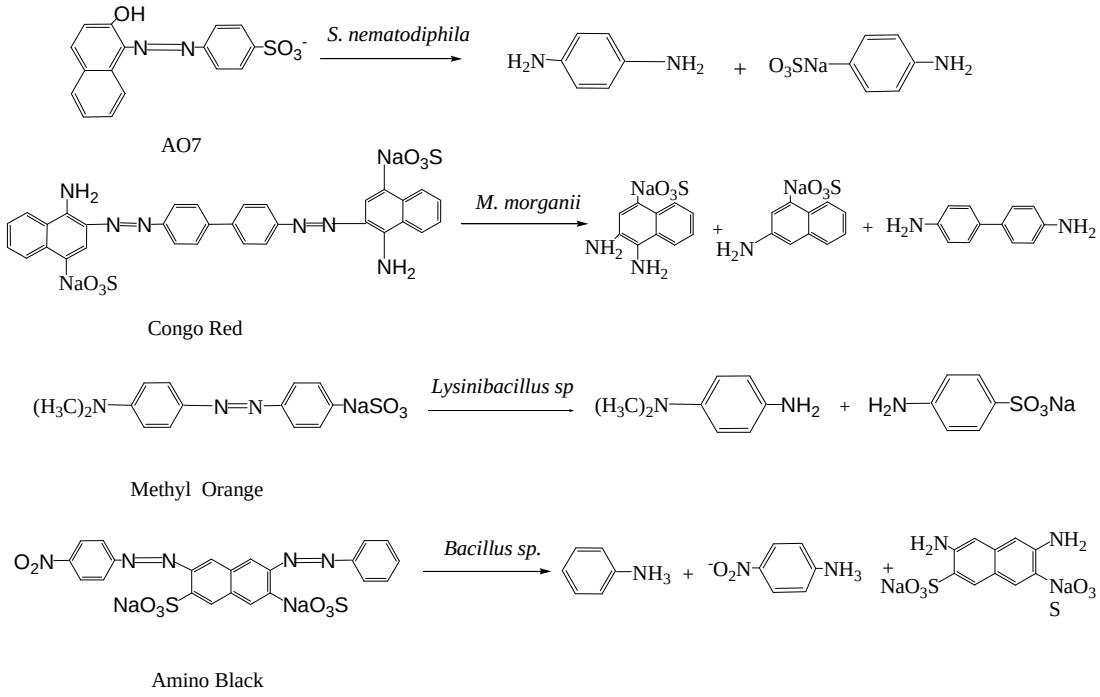

Fig. 4: Decolorization reactions of azo dyes.

tive (Fig. 1c). AB colony is transparent white, regular and smooth surface. After Gram staining, AB bacteria was red granular and negative (Fig. 1d). Four strains showed uniform morphology, and no other individual bacterium was found. It can be seen that the characters of the four strains are different from each other.

Identification of decolorizing bacteria: The DNA of AO7, CR, MO and AB strains were extracted respectively. The target fragments were linked with PMD 18-T plasmid and then transformed into JM109. After PCR, the positive clones were sequenced. The sequencing results showed that the length of 16S rDNA sequences of the four strains were 1362bp, 1367bp, 1403bp and 1383bp, respectively. Compared with GeneBank database, it was found that the sequence homology was the highest among the genera of *Serratia*, *Morganella*, *Lysinibacillus* and *Bacillus*, respectively. After the primary analysis at NCBI, the relevant sequences were compared and phylogenetic analysis was carried out. The system tree was constructed using the adjacency method in MEGA 7 (Fig. 2). From the system tree, it can be seen that the four strains were clustered with *Serratia nematodiphila* subsp. sakuensis st (*S. nematodiphila*); *Morganella morganii* subsp. morganii str (*M. morganii*), *Lysinibacillus* sp. BAB-5845 (*Lysinibacillus* sp.); *Bacillus* sp. NJSN49 (*Bacillus* sp.), respectively. They were supported by the bootstrap value of 99%. *S. nematodiphila* and *M. morganii* are reported that have the azo dye degradation ability for the first time, which demonstrates that our study has a significant importance. While, in previous study, it is reported that isolated *Lysinibacillus* sp. RGS can cause decolorization and detoxification of sulphonated azo dye C.I. Remazol Red in textile effluent efficiently. While, a newly isolated salt-tolerant strain *Bacillus circulans* BWL1061 can simultaneous decolorize sulphonated azo dyes and reduce hexavalent chromium under high salt condition (Liu et al. 2017). And, *Bacillus* sp. strain CH12 isolated from alkaline lake was applied in biodecolorization of textile azo dye (Awoke et al. 2017).

Influence factors on decolorization efficiency: As given in Table 1, the type and concentration of carbon and nitrogen had different effects on the degradation efficiency of four azo degrading bacteria. The best decolorization condition of AO7 degrading bacteria was starch of 4 g/L, peptone of 8 g/L and pH 7.5. The optimum condition of CR degrading bacteria was sucrose of 2 g/L, yeast of 2 g/L and pH 7.5. The optimum decolorization condition of MO degrading bacteria was yeast of 2 g/L and pH 7. The optimum decolorization condition of AB degrading bacteria was 8 g/L of sucrose, urea of 4 g/L and pH 6.0. The optimal carbon and

nitrogen source for the four strains were different, which implied that the decolorization mechanisms may be different. It needs to be further studied.

Analysis of decolorizing products of decolorizing bacteria: As shown in Fig. 3, UV-Vis were used to analyse the chemical structure changes of four azo dyes during the degradation process. Before degradation, the absorption peak at 484 nm was detected in AO7, which was caused by the vibration of -N=N- double bond. The two absorption peaks were also detected at 254 and 310 nm which were caused by the vibration of benzene ring and naphthalene ring respectively. After degradation, the absorption peak at 484 nm could not be detected, but the absorption peak of 254 nm remained, indicating the existence of benzene ring. While in IR spectra, non-uniform vibration of 1584 cm^{-1} characterization of azo dye -N=N- double bond; 1450 and 1510 cm^{-1} characterization of benzene skeleton vibration; 1180 cm^{-1} characterization of benzene ring in plane bending vibration; 698 and 536 cm^{-1} characterize the outward flexural vibration of benzene ring were detected. After AO7 were degraded, many peaks were weaken or disappeared, and the weakening of absorption peak of -N=N- double bond at 1584 cm^{-1} indicated the occurrence of decolorization reaction.

The structural changes of congo red, methyl orange and amino black during degradation process were similar to those of AO7. Combining the results from H^1NMR and GC-MS, the decolorization pathway of AO7, congo red, methyl orange and amino black were shown in Fig. 4. Incubated with the four strains, reduction reactions happened first, -N=N- double bond was broken and the aromatic amines were yielded. Results also showed that the decolorizing bacteria cannot further degrade the aromatic amines. The biodegradation strains need to be screened again.

CONCLUSION

Four decolorization strains were selected from anaerobic activated sludge and identified as *S. nematodiphila*, *M. morganii*, *Lysinibacillus* sp. and *Bacillus* sp., respectively. They own strong abilities for decolorizing AO7, CR, MO and AB dyes. The best decolorization condition of AO7 degrading bacteria was starch of 4 g/L, peptone of 8 g/L, pH 7.5; the optimum condition of CR degrading strain was sucrose of 2 g/L, yeast powder of 2 g/L, pH 7.5; the optimum decolorization conditions of MO degrading bacteria was yeast powder of 2 g/L, pH 7.0 and the optimum decolorization conditions of AB degrading bacteria is sucrose of 8 g/L, urea of 4 g/L, pH 6.0. Under optimized conditions, the four strains could decolorize AO7, CR, MO and AB effectively.

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