Nature Environment and Pollution Technology
An International Quarterly Scientific Journal

p-ISSN: 0972-6268 e-ISSN: 2395-3454	Vol. 17	No. 4	pp. 1405-1410	2018

Original Research Paper | Open Access

Decolorization and Mineralization of Azo Dye-Acid Orange 7 by Salt-tolerant Mixed Cultures Developed with Anaerobic and Aerobic Circle Method

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Nat. Env. & Poll. Tech.
Website: www.neptjournal.com

Received: 24-03-2018
Accepted: 04-06-2018

Key Words:
Decolorization
Mineralization
Azo dye wastewater
Acid Orange 7
Salt-tolerant mixed culture

ABSTRACT

Salt-tolerant mixed cultures proficient in complete decolorization and mineralization of azo dye - Acid Orange 7 (AO7) were developed through anaerobic and aerobic circle method. The salt-tolerant culture performed well in the medium containing no carbon source and 5 g/L yeast extract and could degrade 60 mg/L of AO7 within 48 h efficiently under static and shaking condition. The suitable pH range for the mixed cultures was 6-7. Decolorization and mineralization efficiency was found to be unchanged under salt concentration of 20 g/L NaCl. Microbial community composition based on 16S rDNA gene analysis showed that the dominated genera involved in the mixed cultures were *Lactococcus*, *Acinetobacter* and *Bacteroides*. With UV-Vis analysis, it is speculated that AO7 was degraded to sulfanilic acid and 1-amino-naphtol first. The two chemical intermediates were further mineralized to low-molecular-weight organic acids with the broken aromatic rings. The developed mixed cultures might be a promising alternative for treatment of saline azo dye wastewater.

INTRODUCTION

With the rapid development of printing and dyeing industry, dyes are widely used in textile, paper, food and cosmetic industry (Khalid et al. 2008). The printing and dyeing wastewater is known as one of the most difficult treated industrial effluents, because of its complex components, large volume of water quality, deep color, high concentration of toxic and refractory substances (Cui et al. 2012). There are numerous different kinds of dyes, in which azo dyes and anthraquinone dyes make up the first and second largest parts of textile dyes (González-Gutiérrez et al. 2009). Azo dyes consist of 60-70% of dye production containing -N=N- double bond, which is also the main chromophoric group (Xie et al. 2016a).

Currently, physical and chemical approaches such as adsorption, flocculation and photochemical oxidation are applied in treating azo dyes wastewater (Martínez-Huitle & Brillas 2009, Saratale et al. 2011). Although these methods are effective for the decolorization of the dye wastewater, the operating costs are usually higher and secondary pollution may be produced.

Compared to physical and chemical methods, biological method has the advantage on low cost and less secondary pollution (Schütte et al. 2008). Conventional biological approaches including aerobic, anaerobic and anaerobic-aerobic technologies are widely used in dye wastewater treatment, among which, anaerobic-aerobic process is the most popular (Silva et al. 2012). Screening of microbial azo dye decolorization strains, revealing of degradation characteristics and azo reductase mechanisms were extensively investigated (Garcia-Montano et al. 2008). However, there are still many problems in the degradation of azo dyes, such as the effective treatment of dye wastewater containing high concentration salt.

In dye bath, NaCl, Na_2SO_4 and NaOH are usually used to obtain maximal fixation effect in textile industry (Khalid et al. 2008). About 15-20% of salt was detected in dye-stuff industry wastewater (EPA 1997). Generally, in the high sodium concentration wastewater, the metabolism of conventional microorganisms is greatly inhibited, and the degradation efficiency is decreased. Thus, exploitation of salt-tolerant bacteria is of a significant importance for dye wastewater treatment in the industry.

Many salt-tolerant bacteria owning azo dye degradation ability were reported. For example, *Halomonas* sp. strain GTW isolated from coastal sediments contaminated by chemical wastewater could decolorize different azo dyes under high salt concentration conditions (Asad et al. 2007). The salt-tolerant *Staphylococcus cohnii* strain isolated from textile wastewater could decolorize azo dyes with different structures effectively (Amoozegar et al. 2010).

However, pure strain showed limited mineralization ability because of lacking catabolic pathway of aromatic amines. Degradation of azo dyes requires the catabolic and synthro-

phic interactions of several different species (Hau & Gralnick 2007). The synergistic effects of bacteria in the bacterial flora make them more adaptable. Thus, degradation of azo dye with mixed cultures may be more effective than that of single strains (Saroj et al. 2015).

Therefore, the aim of this study is isolation and characterization of salt-tolerant mixed cultures, which could degrade azo dyes under high salt concentration conditions. A new anaerobic and aerobic circle method was explored to develop the mixed cultures which could decolorize azo dye under static circumstance and mineralize the produced intermediates in shaking environment.

MATERIALS AND METHODS

Materials: Acid orange II (AO7) used in this study was industrial grade and purchased from Jinsui Biological Technology Co. Ltd. (Shanghai, China). Other chemicals of analytical grade were procured from Sinopharm Chemical Regent Co., Ltd. of industrial grade.

Development of salt-tolerant mixed cultures: The salt-tolerant mixed cultures were isolated from activated sludge in Shaoxing wastewater treatment plant. Anaerobic sludge (5 mL) was inoculated into a 500 mL flask containing 200 mL enrichment medium (20 mg/L AO7, 5.0 g/L glucose, 5.0 g/L yeast extract, 20 g/L NaCl). The medium was incubated at 30°C under static condition until the color was faded. Then aerobic sludge (5 mL) from the wastewater treatment plant was transferred into the mixture and shaken at 150 rpm at 30°C. 5 mL of the solution was taken out each 12 h, centrifuged and scanned with UV-Vis spectrophotometer from 200-800 nm. The shaking experiment was stopped until the peaks assigned to aromatic amines were disappeared in UV-Vis spectrum. Then the mixed cultures (5 mL) combined with anaerobic sludge (5 mL) were inoculated to 200 mL fresh medium and repeated the static and shake experiments for four times. The salt-tolerant mixed cultures which could decolorize and mineralize AO7 effectively were developed.

Effect of different environmental conditions: Decolorization and mineralization of AO7 with the salt-tolerant mixed cultures was studied at different carbon sources (no carbon, glucose, sucrose, starch), carbon nitrogen sources (no nitrogen, peptone, yeast exact, urea and NH_4Cl), different pH (5-9), different NaCl concentrations (0-40 g/L) and different initial AO7 concentrations (20-100 mg/L). 10 mL of the mixed cultures was inoculated into the medium and incubated at 30°C under static condition for 24 h, then shaken at 150 rpm for another 24 h. Decolorization efficiency was detected at 484 nm. Mineralization efficiency was determined at 309 nm and 250 nm with UV-Vis scanning.

Microbial community analysis: In order to analyze the microbial community of the salt-tolerant mixed cultures, Illumina Miseq analysis was carried out. The mixed culture was cultivated for 48 h (static for 24 h and shaking for another 24 h), then centrifuged at 10000 rpm at 4°C. The solid was collected and extracted with Powersoil DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA) following the manufacture's instruction to obtain DNA. The V3 and V4 variable regions of the extracted DNA were PCR-amplified targeting with primers 27F (5'-AGAGTTTGATC CTGGCTCAG-3') and 533R (5'-TTACCGCGGCTGCTG GCAC-3') in an Genemap R 9700 PCR System (ABI company, USA). Illumina Miseq analysis was carried out on the platform in Sangon Biotech (Shanghai, China) Co., Ltd.

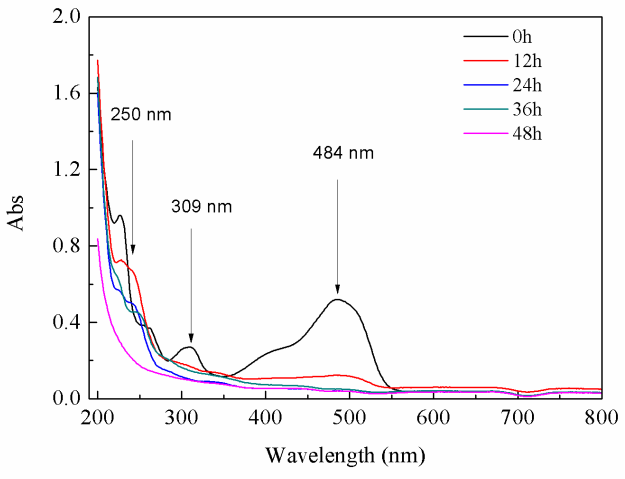

Fig. 1: UV-Vis spectra of AO7 and degraded products at varied time intervals under static and shaking condition at 30°C by mixed cultures.

RESULTS AND DISSCUSSION

Decolorization and mineralization of AO7 by mixed cultures: The changes of UV-Vis spectra of AO7 solution before and after treatment with the mixed cultures are shown in Fig. 1. Before treatment, one peak at 484 nm assigned to -N=N- double bond and two peaks at 309 nm and 250 nm corresponding to naphthalene and benzene structures were detected. After 12 h, it was clearly observed that the peaks at 484 nm and 309 nm decreased sharply, implying the -N=N- azo linkage was destroyed and the naphthalene ring was degraded. However the absorbance at 250 nm was increased, which indicated that chemical intermediates containing benzene structure were produced. Peak of 250 nm was minimized at 24 h and disappeared at 48 h, indicating the destruction of benzene ring.

It can be seen that in the biotransformation process, degradation of AO7 happened by cleavage of -N=N- bond first,

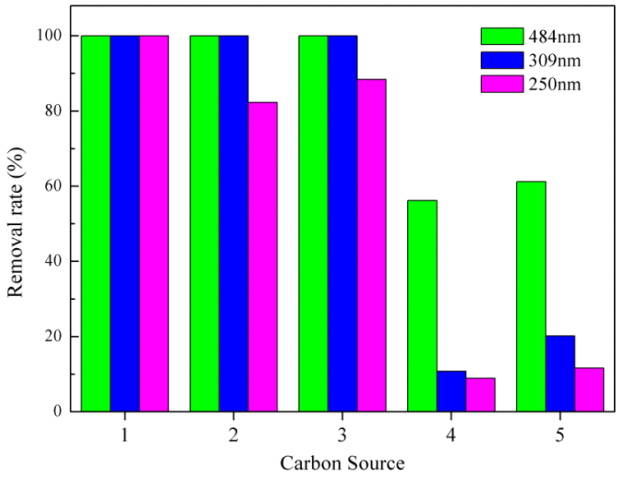

Fig. 2: Effect of various carbon sources on degradation of AO7 (20 mg/L) along with 5 g/L yeast extract. 1: no carbon source; 2: glucose; 3: sodium acetate; 4: sucrose; 5: starch

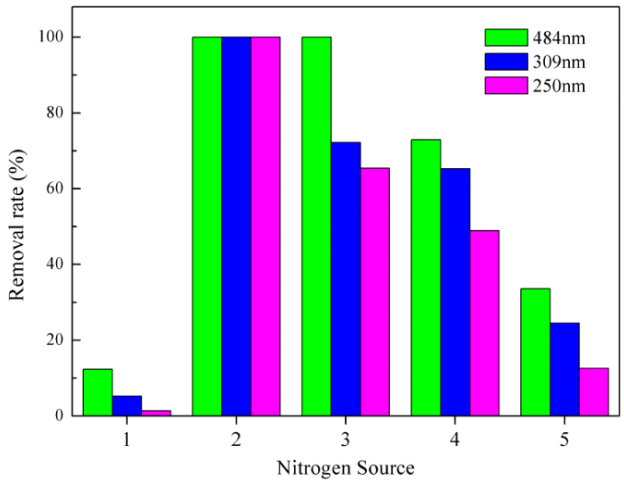

Fig. 3: Effect of various nitrogen sources on degradation of AO7 (20 mg/L). 1: no nitrogen source; 2: yeast extract; 3: peptone; 4: urea; 5: ammonium nitrate.

with the generation of sulfanilic acid and 1-amino-naphtol (Xu et al. 2008). Subsequently, the two chemical intermediates underwent further deamination and desulfonation reaction to yield phenol and naphthol intermediates. Cleavage of naphthalene and benzene rings occurred, leading to the formation of low-molecular-weight organic acids such as aliphatic acids, which were further degraded to CO_2 (Zhao et al. 2010). The results demonstrated that the mixed culture could decolorize and mineralize AO7 effectively under high salt condition.

Effect of carbon source: The influence of different carbon sources on AO7 decolorization and mineralization was studied along with 5 g/L yeast extract (Fig. 2). When no carbon source was added in the medium, the mixed culture could decolorize and mineralize AO7 completely. When glucose and sodium acetate were used as carbon source, decoloration and naphthalene removal efficiency could reach 100% within 48 h, while the benzene removal efficiency was 82.3% and 88.4%, respectively. Decoloration in the presence of sucrose and starch was only 56.2% and 61.2%. In the two media, the naphthalene removal efficiency was 10.8% and 20.2%, respectively. And only 8.9% and 11.6% of benzene was removed. Thus, no carbon source was suitable for biodegradation of AO7 with mixed culture. The selected mixed culture could use AO7 as carbon source. Hence, the mixed culture was of significant importance for it provided an economical alternative for the treatment of azo dye (Rani et al. 2012, Tan et al. 2013).

Effect of nitrogen source: The efficiency of the mixed culture in the presence of different nitrogen source was studied without any carbon source (Fig. 3). Only 12.3% of decoloration was achieved with no nitrogen source. Whereas urea and ammonium nitrate demonstrated 72.9% and 33.6% decoloration of AO7, and the naphthalene removal efficiency of 65.3% and 24.5% were obtained with 48.9% and 12.6% of benzene removed in 48 h. In the presence of peptone, 100% of AO7 was decolorized. 72.2% of naphthalene and 65.4% of benzene were degraded. While, the best results were obtained when yeast extract was used as nitrogen source. The mixed cultures could completely decolorize and mineralize AO7 in the presence of yeast extract. In accordance with previous studies, yeast extract was usually reported as the best nitrogen source for decoloration and mineralization of azo dyes (Kunal et al. 2012).

With increase of yeast extract concentration from 2 to 10 g/L, decoloration and mineralization efficiency also increased (Fig. 4). Complete decoloration and mineralization was detected in the presence of 4 and 6 g/L yeast extract. However, further increase in yeast extract concentration to 8 and 10 g/L resulted in decreased degradation efficiency. 88.4% and 65.4% of AO7 was decolorized in 48 h, respectively. Therefore, 4 g/L yeast extract concentration was applied as an optimal nitrogen source. It is reported that yeast extract is considered as electron donor in azo dye degradation.

Effect of pH: The effect of pH on the decoloration and mineralization of AO7 was studied in a range of 5-9 (Fig. 5). Complete degradation was obtained at pH 6.0 and 7.0. However, decrease in decoloration and mineralization was observed in alkaline environment. 66.3% and 32.4% of AO7 was decolorized at pH 8.0 and 9.0, respectively. 56.2% and 10.4% of naphthalene and 42.8% and 8.6% of benzene were

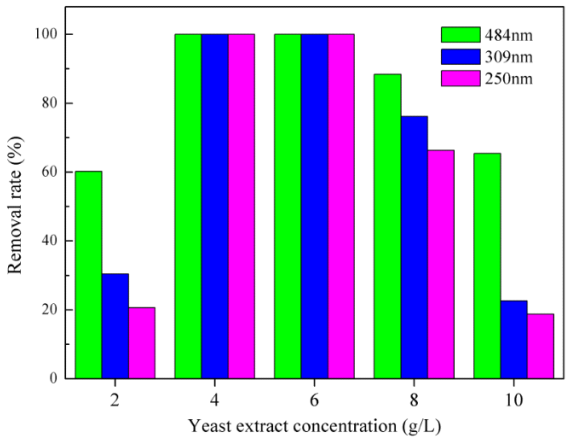

Fig. 4: Effect of yeast extract concentration on AO7 (20 mg/L) degradation.

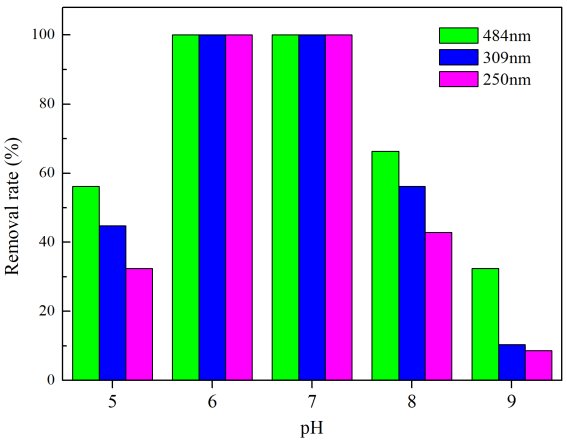

Fig.5. Effect of pH on AO7 degradation.

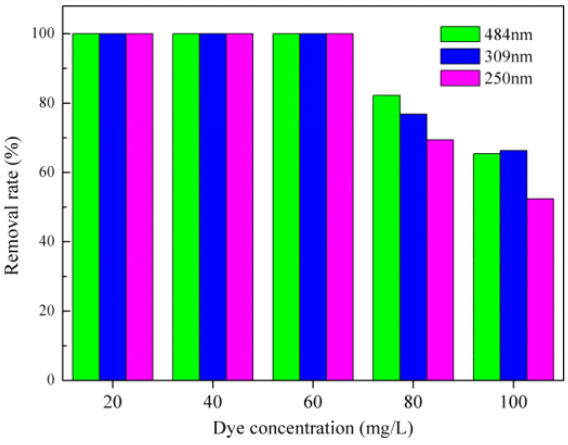

Fig. 6: Effect of initial dye concentration on AO7 degradation.

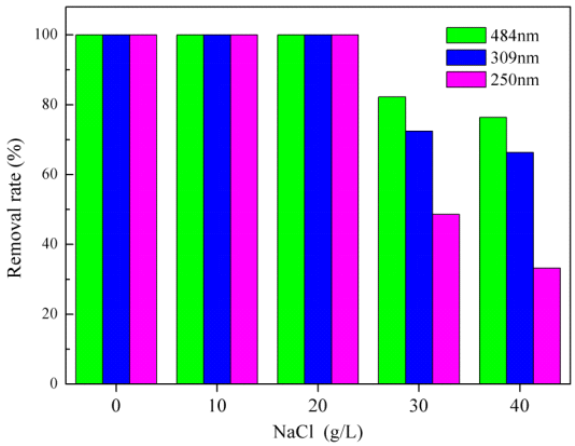

Fig. 7: Effect of NaCl concentration on AO7 degradation.

degraded in 48 h. Further, pH at 5.0 exhibited an inhibitory effect for the mixed cultures during decoloration and mineralization of AO7. Many reported researches also showed that neutral pH was optimal for bacterial decolorization of azo dyes (Rasool et al. 2016, You et al. 2009).

Effect of initial dye concentration: Decoloration and mineralization of various initial AO7 concentrations exhibited different patterns of dye degradation (Fig. 6). 20, 40 and 60 mg/L of AO7 could be completely metabolized within 48 h. When the initial concentration increased to 80 mg/L, the decoloration efficiency was decreased to 82.2%, with 76.8% of naphthalene and 69.4% of benzene removing from the medium. Thus, the optimal initial dye concentration is 60 mg/L AO7.

Effect of NaCl concentration: Chloride salts of sodium and potassium, which are applied for salting out of dyes, are usually detected in dye wastewater along with unused dyes. Hence, assessment of the decoloration and mineralization efficiency of the mixed cultures under high salt condition is essential. The experiment was carried out over a range of NaCl concentrations (0 to 40 g/L) (Fig. 7). It can be seen that no inhibitory effects were observed upto 20 g/L of NaCl and complete decoloration and mineralization were noticed within 48 h. At 30 g/L of NaCl, 82.2% of decoloration was achieved and 72.4% of naphthalene and 48.6% of benzene were removed. It was reported that sodium concentration more than 3 g/L will lead to inhibitory effects on the microbial metabolism (De Baere et al. 1984). While, in our study, it was observed that 100% decoloration and mineralization efficiency was achieved at 10 g/L and 20 g/L NaCl within 48 h. Hence, the mixed cultures we explored were effective in treatment of textile effluent containing high concentration salt.

Microbial community analysis: Illumina Miseq analysis

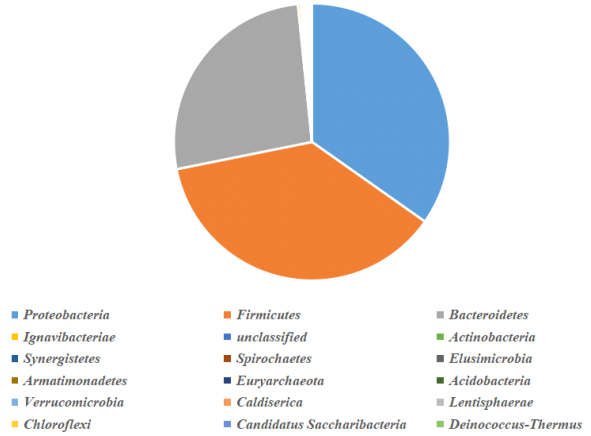

Fig. 8: Microbial community structures in salt-tolerant cultures at the phylum level.

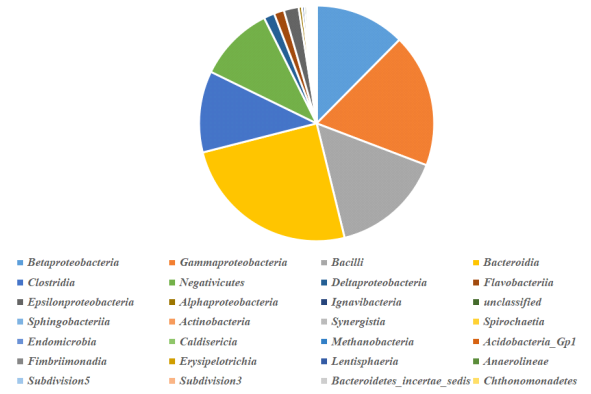

Fig. 9: Microbial community structures in salt-tolerant cultures at the class level.

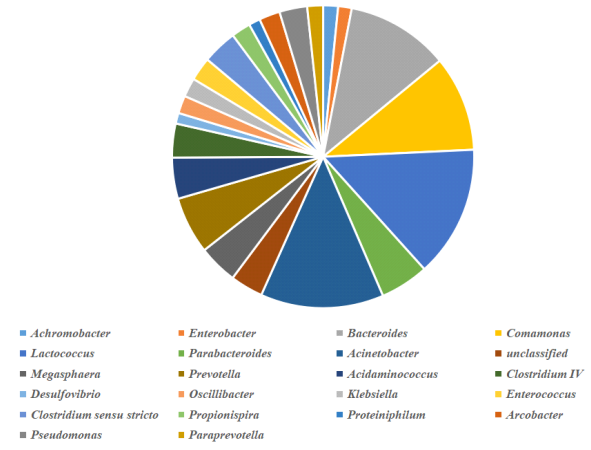

Fig. 10: Microbial community structures in salt-tolerant cultures at the genus level.

was applied to study the microbial community involved in the selected salt-tolerant mixed cultures. DNA sample extracted from the mixed cultures yield 37585 raw number of sequences. The raw sequence was qualified and clustered taxonomically with RDP's classifier resulting in 3796 OTUs. The sample showed a Shannon index of 4.77, a ACE index of 106293.92 and Chao 1 index of 38836.4.

To classify the microbial community, the bacterial sequence was assigned to phylum, class, order, family and genus at 50% threshold. The relative abundance of the bacterial community at phylum level is demonstrated in Fig. 8. Total of 18 phyla were detected. It can be seen that the predominant phyla of the effective sequence were *Proteobacteria* (34.75%), *Firmicutes* (37.1%) and *Bacteroides* (26.52%). The three phyla were commonly found in dye wastewater treatment activated sludge. Lee et al. (2016) investigated the microbial community in a full scale digester. It was found that *Proteobacteria*, *Bacteroidetes* together with *Firmicutes* were the three dominant phyla.

There were 28 classes determined in the total bacterial population (Fig. 9). The dominant classes detected in the salt-tolerant mixed cultures were *Bacteroidia* (24.86%), *Gammaproteobacteria* (18.33%), *Bacilli* (15.39%), *Betaproteobacteria* (12.44%), *Clostridia* (11.17%) and *Negativicutes* (10.43%).

The overall most dominant genus determined in the mixed culture was *Lactococcus* (12.31%), followed by *Acinetobacter* (11.55%) and *Bacteroides* (9.64%) (Fig. 10). *Lactococcus* was a genus of lactic acid bacteria and widely detected in printing and dyeing wastewater treatment process under anaerobic and aerobic conditions (Seesuriyachana et al. 2007). Besides, it was also reported that *Lactococcus* could decolorize AO7 effectively. In this study, *Lactococcus* may be the main bacteria involved in mixed culture and play important roles in decoloration and mineralization of AO7. Bacteria in *Acinetobacter* genus possess a kind of key enzyme named lignin peroxidase which was capable of oxidizing textile dyes of various groups (Yang et al. 2012). *Bacteroides* species were commonly detected in bioreactors in the presence of azo dyes (Xie et al. 2016b). Previous researches also demonstrated that azoreductase which could effectively hydrolyze -N=N- double bond under anaerobic situation were purified from *Bacterroides* sp. (Rafii et al. 1990).

CONCLUSION

With the anaerobic and aerobic circle method, salt-tolerant mixed cultures which could decolorize and mineralize AO7 under high salt condition were developed. No carbon source and 5 g/L yeast extract were suitable for degradation of

AO7. The optimal pH for decoloration and mineralization of AO7 was in a range of 6-7. Complete degradation was detected under high AO7 and salt concentration where 60 mg/L of AO7 was decolorized and mineralized in presence of 20 g/L NaCl. Several genera such as *Lactococcus*, *Acinetobacter* and *Bacteroides* may play important roles in degradation of AO7.

ACKNOWLEDGEMENT

This study was supported by Shaoxing science and technology project [2017B70057], Innovation and Entrepreneurship Training Program in Zhejiang [2017R428001] and National Undergraduate Training Program for Innovation and Entrepreneurship [201710349006].

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