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Decolorization of azo dye methyl red by suspended and co-immobilized bacterial cells with mediators anthraquinone-2,6-disulfonate and Fe_3O_4 nanoparticles

Subed Chandra Dev Sharma [a, b], Qian Sun [a], Jiangwei Li [a], Yuwen Wang [a], Fidèle Suanon [a, b], Jinyan Yang [c], Chang-Ping Yu [a, d, *]

[a] Key Laboratory of Urban Pollutant Conversion, Institute of Urban Environment, Chinese Academy of Sciences, Xiamen 361021, China
[b] University of Chinese Academy of Sciences, Beijing 100049, China
[c] College of Architecture and Environment, Sichuan University, Chengdu 610065, China
[d] Graduate Institute of Environmental Engineering, National Taiwan University, Taipei 106, Taiwan

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ABSTRACT

In this study, the decolorization and degradation of methyl red (MR) by suspended and immobilized cells of *Aeromonas jandaei* strain SCS5 under anaerobic and aerobic conditions have been investigated. The complete decolorization of MR at a concentration of 100 mg L^{-1} by *A. jandaei* strain SCS5 was obtained within 6 h for both anaerobic and aerobic suspended cultures, where the decolorization rate was faster in acidic conditions than basic conditions. The decolorization efficiency under 6 h increased with increasing cell mass of inoculation and decreased with increasing initial dye concentrations. Immobilized cells of *A. jandaei* strain SCS5 could decolorize MR, and the decolorization rate was significantly enhanced by cells immobilized with mediators such as anthraquinone-2,6-disulphonate and magnetic Fe_3O_4 nanoparticles compared to immobilized cells only. Moreover, the immobilized bacterial beads with mediators retained high decolorization activity up to more than 10 repeating cycles. UV–visible spectra (200–800 nm) and gas chromatography-mass spectrometry analysis demonstrated that MR was degraded by *A. jandaei* strain SCS5 through reductive cleavage of azo bond. MR degradation products showed less phytotoxicity against *Triticum aestivum* and *Phaseolus mungo* compared to untreated MR. This study has demonstrated that *A. jandaei* strain SCS5 could be a promising microbiological agent for the removal of azo dyes from the environment.

1. Introduction

With the increasing use of a wide variety of dyes, water pollution by dye stuffs has become alarming day by day. As a consequence, more and more attention has been paid to the discharge of dye containing effluent (Miranda et al., 2013), and the removal of color from dyeing wastewater has raised a major scientific interest. Azo dyes characterized by the presence of one or more azo groups (−N=N−) are extensively used as coloring agents in textile, leather, additives, foodstuffs, cosmetics and paper industries where a significant proportion of used dyes is disposed into the environment through wastewater (Banat et al., 1996; Jadhav et al., 2007; Pandey et al., 2007; Stolz, 2001). The chemical stability of azo dyes makes them relatively resistant to biological and chemical degradation processes and therefore, causes disposal problems in dye industries (Banat et al., 1996; Ozkan-Yucel and Gokcay, 2010). The discharge of dye-containing wastewaters in the environment causes both environmental and public health risks because of the negative ecotoxicological effects and bioaccumulation in wildlife (Chung and Stevens, 1993). Additionally, the discharge of azo dye-containing wastewater into water bodies creates aesthetic problems and hampers aquatic ecosystem (Manu and Chaudhari, 2002). Importantly, many studies reported that most of the azo dyes are toxic and/or carcinogenic (Adedayo et al., 2004; Chung et al., 1981). Thus, the treatment of azo dye-containing wastewater has been a major environmental concern and must be well treated before it is discharged into the environment.

* Corresponding author. Key Laboratory of Urban Pollutant Conversion, Institute of Urban Environment, Chinese Academy of Sciences, Xiamen 361021, China.
E-mail address: cpyu@iue.ac.cn (C.-P. Yu).

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The physicochemical methods used to treat textile wastewater have some technical and economical limitations (dos Santos et al., 2007) because these techniques for dye-containing wastewater treatment require a large input of potentially hazardous chemicals and other more costly materials such as activated carbon, membrane etc. In addition, the physicochemical methods could generate high volumes of polluted sludge that need further treatment before discharge (Singh et al., 2015). On the contrary, microbial decolorization and degradation process does not need the inclusion of hazardous chemicals and produces less harmful sludge (Banat et al., 1996). Therefore, microbial decolorization and degradation of azo dye has been an environmentally friendly and cost effective alternative to physicochemical treatment approaches (e.g. UV/Fenton, adsorption) (Banat et al., 1996; van der Zee and Villaverde, 2005; Varma and Madamwar, 2003). Microbiological degradation of azo dyes can be carried out by anaerobic bacteria (Chung et al., 1978; Costa et al., 2010; Guo et al., 2007; Meng et al., 2014; Su et al., 2009; Toro et al., 2013) and also by aerobic bacteria (Ayed et al., 2011; Gomare and Govindwar, 2009; Jadhav et al., 2008; Wong and Yuen, 1996; Zhao et al., 2014). Methyl red (MR) is considered as one of the toxic azo dyes and has been recognized as mutagenic or cytotoxic in nature (Wong and Yuen, 1998). Most of the microbial MR degradation studies were carried out using only anaerobic or aerobic bacteria, but there is no report regarding MR degradation under both anaerobic and aerobic conditions by the same bacterial strain.

It has been reported that the decolorization rate of azo dyes could be increased by using redox mediators because they could speed up reaction rate by shuttling electrons from the electron donor to the electron acceptor (azo dyes) (Baeta et al., 2012; Costa et al., 2010; dos Santos et al., 2005; Rau et al., 2002). However, continuous dosing of redox mediators and bacteria represents continuous expenses as well as frequent discharge of sludge during operation (Li et al., 2013). As an alternative, immobilization of microorganism could be a better strategy for improving effectiveness in wastewater treatment (Moreno-Garrido, 2008). The application of immobilized microbial technique might reduce the repeated addition of bacteria and mediators to reduce costs and sludge discharges. However, currently only limited studies have been carried out on decolorization of azo dyes by immobilized bacteria under anaerobic conditions (Guo et al., 2007; Su et al., 2009). For better application of microbial approaches to treat azo dye-containing wastewater, it may be advantageous to immobilize bacteria with suitable redox materials.

Therefore, the present work has been planned firstly to study the effect of various physicochemical parameters on the decolorization of MR by a novel bacterial strain SCS5 phylogenetically related to *Aeromonas jandaei* and secondly to immobilize bacterial cells with redox mediators for the enhancement of MR decolorization and degradation under anaerobic and aerobic conditions. In addition, the degradation pathway and the phytotoxicity of the treated MR were investigated.

2. Materials and methods

2.1. Microorganism

The bacterial strain SCS5 used in this study was isolated from anodic biofilm of microbial fuel cell and identified as a novel strain of *Aeromonas jandaei* species by our previous work (Sharma et al., 2016). *A. jandaei* strain SCS5 was an exoelectrogen that could transfer electrons to extracellular materials.

2.2. Culture media and chemicals

The phosphate-buffered basal medium was used for the experiment, which contained (per liter of deionized water): 0.68 g KH_2PO_4, 3.58 g $Na_2HPO_4 \cdot 12H_2O$, 0.26 g NH_4Cl, 0.29 g NaCl, 10 mL trace metals solution and 1 mL vitamins solution as reported by Lovley and Phillips (Lovley and Phillips, 1988). The medium was supplemented with glucose (0.5 g), acetate (0.5 g) and 2.5% LB broth as substrate where MR was used as the target azo dye to be decolorized and degraded by bacteria. The culture medium for decolorization tests by suspended and immobilized cells contained the same compositions. For anaerobic culture, we put the medium into serum bottle and then passed 99.99% N_2 gas through sterilized filter (0.22 μm) into the medium to remove dissolved oxygen for 2 h. After that the bottle was sealed with butyl rubber stopper and aluminum crimp cap and then autoclaved. After autoclaving, further experimental steps such as inoculation and sample collection were conducted in the anaerobic chamber. Detailed description of all the chemicals used in this study is provided in the Supplementary Information (SI).

2.3. Immobilization

For the immobilization of bacterial cells, entrapment method in polyvinyl alcohol (PVA) was chosen for its higher stability. Prior to immobilization, bacterial cells were cultivated overnight in the medium supplemented with MR, which was used for the decolorization experiment at pH 7 and 30 °C. The cell pellets were collected through centrifugation at 6000 rpm for 10 min at 4 °C followed by washing with 50 mM phosphate buffer solution (pH 7.0) and suspended in the same buffer to adjust the optical density (OD) to 0.9 at 600 nm, which was equivalent to a cell concentration of 0.252 g L^{-1} (dry weight). The prepared cell suspension was homogenously mixed with PVA (10%, w/v), sodium alginate (0.5%, w/v) and kaolin (2.5%, w/v) (Cheng et al., 2012) supplemented with an appropriate amount of anthraquinone-2,6-disulfonate (AQDS) or Fe_3O_4 nanoparticles as mediators. Then the mixture was dropped through a syringe into $CaCl_2$ (4%, w/v) and boric acid (3%, w/v) solution and kept for 18 h at 4 °C to form gel beads (4 ± 0.1 mm in diameter). In all cases, the bead particles were prepared from 10 mL of the above PVA-SA-kaolin mixture for each 50 mL experimental medium solution where the dry weight of beads was around 1.30 ± 0.05 g. The beads contained 0.252 g L^{-1} bacterial cells (dry weight) and different concentrations of mediators with respect to medium volume. The formed beads were subsequently cultured in heterotrophic medium for 48 h to reactivate the cells followed by washing with distilled water and finally stored in 0.9% NaCl solution at 4 °C for further use. For control tests, beads without bacteria were prepared.

2.4. Methyl red degradation experiments

Methyl red degradation or decolorization experiments were conducted by bacterial cells of *A. jandaei* strain SCS5 in suspended and immobilized cultures. In suspended cultures, 50 mL sterilized medium containing 100 mg L^{-1} MR was inoculated with a bacterial cell concentration of 0.252 g L^{-1} (dry weight) in 100 mL serum bottle for anaerobic conditions and 250 mL conical flask for aerobic conditions without shaking at 30 °C and pH 7. The effects of different physiochemical parameters such as pH (4.0–9.0), temperature (25–50 °C), agitation, dye concentrations (50–1000 mg L^{-1}) and inoculated cell mass (0.033, 0.06, 0.118, 0.252, 0.376, 0.501 g L^{-1}) on decolorization of MR by suspended cells of *A. jandaei* strain SCS5 were investigated under both anaerobic and aerobic conditions. Equal concentrations of

inoculated cells (0.252 g L^{-1}) and MR (100 mg L^{-1}) were used for all experiments except for testing the effects of inoculated cell mass and initial dye concentrations. In immobilized cultures, bacterial cells immobilized with various amount of mediators such as AQDS (100, 300, 500 μM) or Fe_3O_4 nanoparticles (0.1, 0.25, 1.0 g 50 mL^{-1}) were examined to evaluate the performance of *A. jandaei* strain SCS5 on MR decolorization. The concentrations of mediators were calculated with respect to experimental medium volume. The beads formed from 10 mL of PVA-SA-Kaolin mixture with or without bacteria (0.252 g L^{-1}, dry weight) and/or mediators were aseptically added into the 50 mL experimental media containing MR (100 mg L^{-1}) and assayed under optimized temperature and pH 7 without shaking under anaerobic and aerobic conditions. To compare with immobilized cells, the same amount of suspended cells was also inoculated in parallel. The beads without bacteria were used as the blank control. All experiments were carried out in duplicate.

2.5. Repeated-batch operations

The immobilized bacterial beads were tested for evaluation of repetitive decolorization performance. The bead particles immobilized with AQDS (500 μM) and Fe_3O_4 nanoparticles (0.1 g 50 m L^{-1}) were placed into the 50 mL medium containing MR (100 mg L^{-1}) and statically incubated at 35 °C and pH 7 for decolorization. After complete decolorization, the immobilized beads were collected, rinsed twice with sterile deionized water and transferred into a fresh medium containing 100 mg L^{-1} MR for the second decolorization experiment. The same procedures were repeated several times up to the obtained degradation above 90%.

2.6. Analytical methods

The decolorization of the azo dye MR was monitored spectrophotometrically at the wavelength of 430 nm using a UV–visible spectrophotometer (UV-5200, METASH, Shanghai, China). The removal efficiency of MR was expressed in terms of percentage (%) and calculated as follows:

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

where A_0 is the absorbance at the maximum absorbance wavelength of MR before decolorization (time, 0) and A is the absorbance at the same wavelength after decolorization at time t.

Degradation of MR was qualitatively analyzed by an UV–visible scanning spectrophotometer (UV-2450, SHIMADZU, Kyoto, Japan) within a range of 200–800 nm. To further investigate and identify possible degradation byproducts, gas chromatography–mass spectrometry (GC-MS) analysis (Agilent 7890-5975c) was performed. After complete decolorization of MR, degradation byproducts were extracted from cultural supernatant with an equal volume of ethyl acetate. The extracts were evaporated and dried with gentle N_2 gas followed by dissolving in acetone. MR degradation products were analyzed by GC-MS under full scan mode equipped with Agilent 19091S-433 HP-5MS capillary column (30 m × 250 μm × 0.25 μm, 5% phenyl methyl siloxane). The oven temperature was programmed from 50 °C to 250 °C at 20 °C min^{-1} and then raised to 280 °C at 30 °C min^{-1} followed by 11 min hold at 280 °C. The injector temperature was kept at 250 °C and 1 μL sample was injected. Helium was used as the carrier gas at a flow rate of 1.0 mL min^{-1}. MS was operated under electron ionization mode at 70 eV with mass scan range of 40–300 amu. Scanning electron microscopy (SEM) was conducted using a Hitachi S-4800 scanning electron microscope (Hitachi Crop., Japan) as described in the previous study (Feng et al., 2014). In order to examine the viability of immobilized bacterial cells, confocal laser scanning microscopy (CLSM) was performed using a Zeiss LSM 710 laser scanning confocal microscope (ZEISS, CA, USA). Detailed description of the above analytical methods is provided in the SI.

2.7. Phytotoxicity

Phytotoxicity assays were conducted to evaluate the impact of MR (200 mg L^{-1}) and its metabolites against *Triticum aestivum* and *Phaseolus mungo* at room temperature (25 °C) (Gomare and Govindwar, 2009; Zhao et al., 2014). Prior to inoculation, the seeds of both crops were cleaned and surface sterilized with 3% hydrogen peroxide solution for 5 min and then washed three times using sterile distilled water. Ten healthy seeds of the each crop were separately grown in Petri-plates having a bed of filter paper with the daily supplement of 5.0 mL sample taken from untreated and treated MR. Medium without MR was used as control experiment. Percent germination (%), shoot and root length were recorded after 4 days of incubation at 25 °C. Each experiment was carried out in triplicate.

3. Results and discussion

3.1. Decolorization of MR by strain SCS5

The cell suspension of *A. jandaei* strain SCS5 was able to effectively decolorize MR under both anaerobic and aerobic conditions (Fig. 1). The MR decolorization rate was fast, and 77–80% decolorization was achieved within 2 h, while 100% decolorization was achieved within 6 h for both experimental conditions based on UV–visible spectrophotometric analysis. From Fig. 1, it is evident that, although the bacterial cell growth was somewhat higher under aerobic conditions, *A. jandaei* strain SCS5 was more effective to degrade MR in anaerobic culture than aerobic culture. This could be owing to the interference of azo bond reduction of MR by oxygen, because oxygen is a better electron acceptor than the azo dyes (dos Santos et al., 2007; Stolz, 2001). However, under anaerobic conditions, azo dyes could act as potent electron acceptors resulting in higher decolorization rates (dos Santos et al., 2005; Manu and Chaudhari, 2002; Mendez-Paz et al., 2005; Singh et al., 2007). It

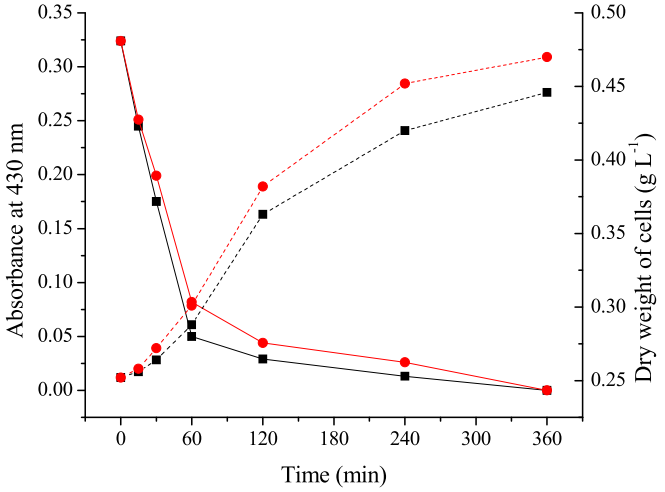

Fig. 1. MR decolorization by strain SCS5 with various times at a concentration of 100 mg L^{-1}. Solid lines reveal decolorization under anaerobic (black) and aerobic (red) conditions. Dash lines represent bacterial growth in dry cell mass under anaerobic and aerobic conditions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

has been reported that *Aeromonas* sp. could degrade azo dyes under either anaerobic or aerobic conditions (Du et al., 2015; Idaka et al., 2008; Shah, 2014). However, *A. jandaei* strain SCS5 could decolorize and degrade MR under both anaerobic and aerobic conditions suggesting that *A. jandaei* strain SCS5 would have high potential in the removal of azo dye MR.

3.2. Effect of pH, temperature and agitation on MR decolorization

Different parameters such as pH, temperature and agitation were evaluated on MR decolorization since these factors may affect decolorization activity of microorganisms. *A. jandaei* strain SCS5 could decolorize MR (100 mg L^{-1}) completely within the pH range of 5–7 under both anaerobic and aerobic conditions (Fig. 2a). At pH 5 and 6, MR was completely degraded within 2 and 4 h, respectively. However, at pH 7, 100% decolorization occurred within 6 h under both conditions. On the contrary, under basic condition, MR was decolorized partially within 6 h, but not completely. Around 60% and 40% of MR were decolorized in 6 h at pH 8 and pH 9, respectively (Fig. 2a). Therefore, the decolorization rate was much higher in acidic than alkaline pH. The results are consistent with those of a previous study which reported that MR decolorization by *Galactomyces geotrichum* increased for the acidic pH of 3–5 and decreased under alkaline conditions (Jadhav et al., 2008). However, one study showed that MR degradation by *Bacillus* sp. strain UN2 was higher in a pH range of 7–9 than acidic conditions (Zhao et al., 2014).

As shown in Fig. 2b, MR was decolorized completely within 6 h of incubation at the temperature ranging from 25 to 45 °C and 25 to 40 °C under anaerobic and aerobic conditions, respectively where the optimum temperature for MR decolorization was 35 °C under both conditions. At higher temperature (50 °C), the decolorization rate was found to be lower but about 90% and 80% were still removed in anaerobic and aerobic cultures respectively. This decline in decolorization activity at higher temperature may be due to the partial denaturation or inactivation of responsible enzymes or proteins related to MR decolorization. Thus, *A. jandaei* strain SCS5 will be an effective MR decolorizing agent within a temperature range of 25–50 °C. Zhao et al. reported that *Bacillus* sp. strain UN2 could degrade more than 90% of MR (100 mg L^{-1}) in a temperature range of 20–40 °C within 6 h, while 45% degradation was achieved at 45 °C (Zhao et al., 2014).

Under both static and shaking (150 rpm) conditions, 100% decolorization was achieved within 6 h (Fig. S1 in SI), where the

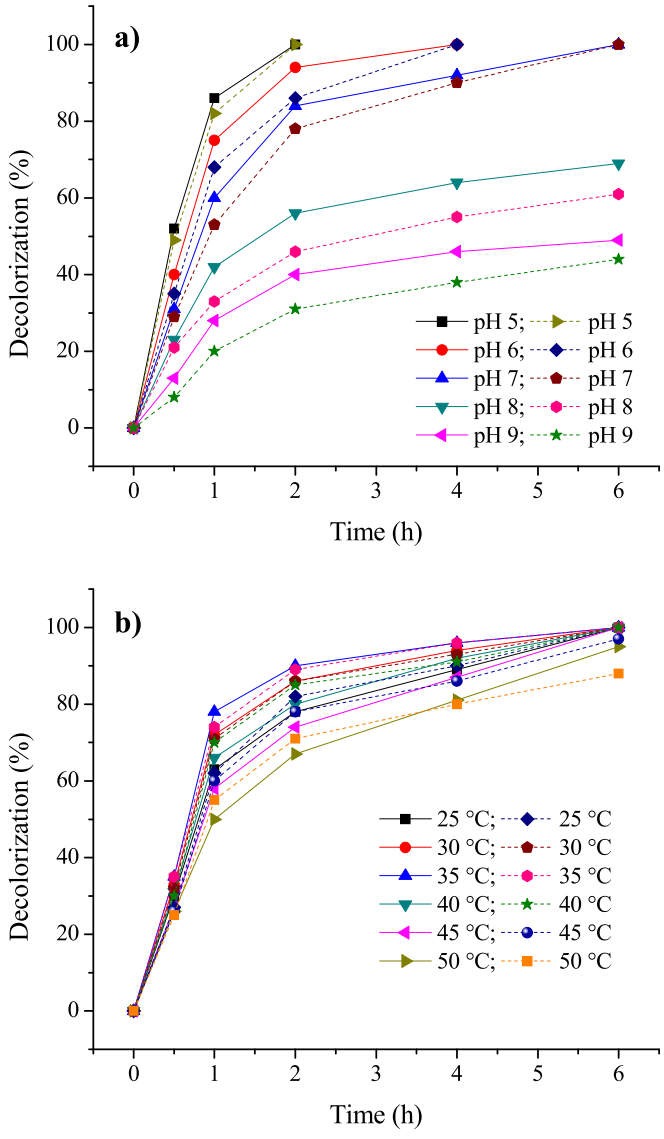

Fig. 2. Effect of pH (a) and temperature (b) on MR decolorization under anaerobic (solid line) and aerobic (dash line) conditions.

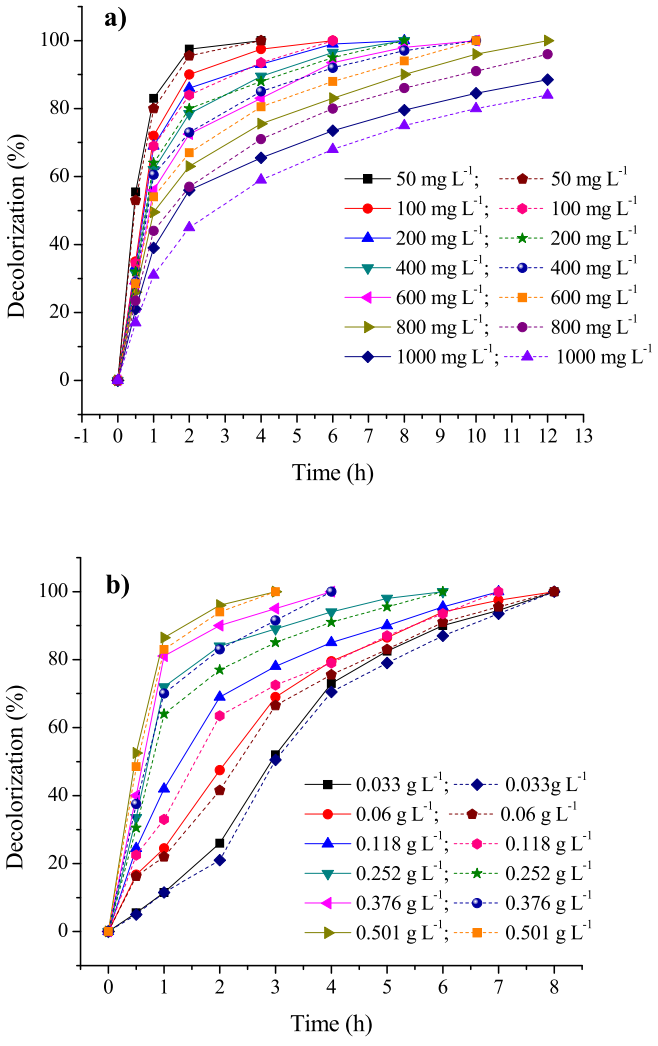

Fig. 3. Effect of initial dye concentration (a) and inoculating cell mass (b) on MR decolorization under anaerobic (solid line) and aerobic (dash line) conditions.

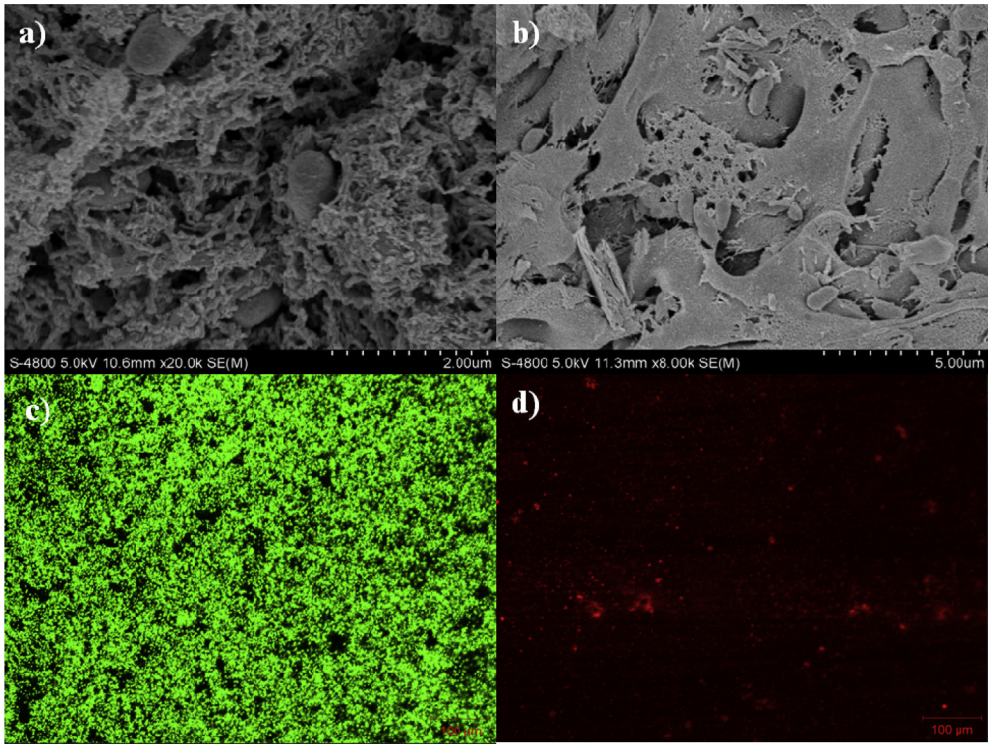

Fig. 4. Microscopic image of bacteria immobilized bead. SEM image of outer surface (a) and inside (b). CLSM image for live (c) and dead (d) bacterial cells.

decolorization rate was slightly higher around 2 h of incubation under shaking conditions than static conditions. After that period the decolorization rate under shaking conditions became gradually lower than static conditions and was almost similar at 6 h when 100% decolorization achieved. Overall there was no significant difference on the rate of MR decolorization under static and shaking conditions like previous report (Moutaouakkil et al., 2003). However, the shaking effect seemed to vary with different strains. One report showed that MR degradation using *Klebsiella pneumoniae* RS-13 increased under shaking conditions compared to static conditions (Wong and Yuen, 1996). Another report showed that degradation of sulphonated diazo dye C.I. Reactive Green 19A using *Micrococcus glutamicus* NCIM-2168 was more rapid under static conditions than shaking conditions (Saratale et al., 2009).

3.3. Effect of initial dye concentrations and cell mass on MR decolorization

It was obvious that 100% decolorization could be achieved at 4 h and 6 h for the initial dye concentrations of 50 mg L^{-1} and 100 mg L^{-1} under both aerobic and anaerobic conditions (Fig. 3a). However, when the initial concentration of MR was in the range of 200–600 mg L^{-1}, the complete decolorization required 8–10 h in both anaerobic and aerobic conditions. In addition, 100% and around 90% decolorization was found within 12 h at a dye concentration of 800 mg L^{-1}, and about 80% and 70% of color could be removed within 12 h at 1000 mg L^{-1} dye concentration under anaerobic and aerobic conditions respectively. This result suggested that *A. jandaei* strain SCS5 could effectively resist the toxic effect of MR at 1000 mg L^{-1}. Previous study found that *Bacillus* sp. strain UN2 could completely degrade MR within a range of 100 mg L^{-1} to 400 mg L^{-1} where the degradation rate decreased with the increasing initial concentration of MR (Zhao et al., 2014). Ayed et al. also showed the percentage of MR decolorization by *Sphingomonas paucimobilis* decreased with the increase in the initial concentration of 750–1000 mg L^{-1} (Ayed et al., 2011).

In the cases of various initial cell concentrations (0.501, 0.376, 0.252, 0.118, 0.06, 0.033 g L^{-1}; dry weight) used for MR (100 mg L^{-1}) decolorization, it was observed that increase in cell concentrations reduced the time required for 100% decolorization of MR (Fig. 3b). At a cell concentration of 0.501 g L^{-1} and 0.376 g L^{-1} (dry weight), 100% decolorization was achieved at 3 h and 4 h in anaerobic and aerobic cultures respectively, and the time was shorter when compared with 0.252 g L^{-1} cell concentration, which required 6 h for complete decolorization. However, at low cell concentrations such as 0.033 g L^{-1} and 0.06 g L^{-1}, the time for complete decolorization was found to be the same at 8 h. The time needed for 100% decolorization was almost similar under anaerobic and aerobic cultures.

3.4. SEM and CLSM analysis

The PVA-SA-Kaolin immobilized beads (Fig. S2 in SI) were used for MR degradation or decolorization experiments due to good mechanical strength. SEM analysis of immobilized bacterial beads showed that there were a lot of bacteria entrapped inside and on the outer surface of the beads (Fig. 4a, b). The beads also possessed many internal porous structures which facilitated diffusion and mass transfer. As shown in Fig. 4c, d, it is obvious that most of the bacteria were active in immobilized beads, but a few of them were likely killed during immobilization.

3.5. AQDS and Fe_3O_4 immobilization

Fig. 5 depicts the decolorization of MR by bacteria immobilized with different concentrations of AQDS, immobilized bacteria without AQDS, immobilized bacteria with free AQDS, suspended bacteria, suspended bacteria with free AQDS and control beads

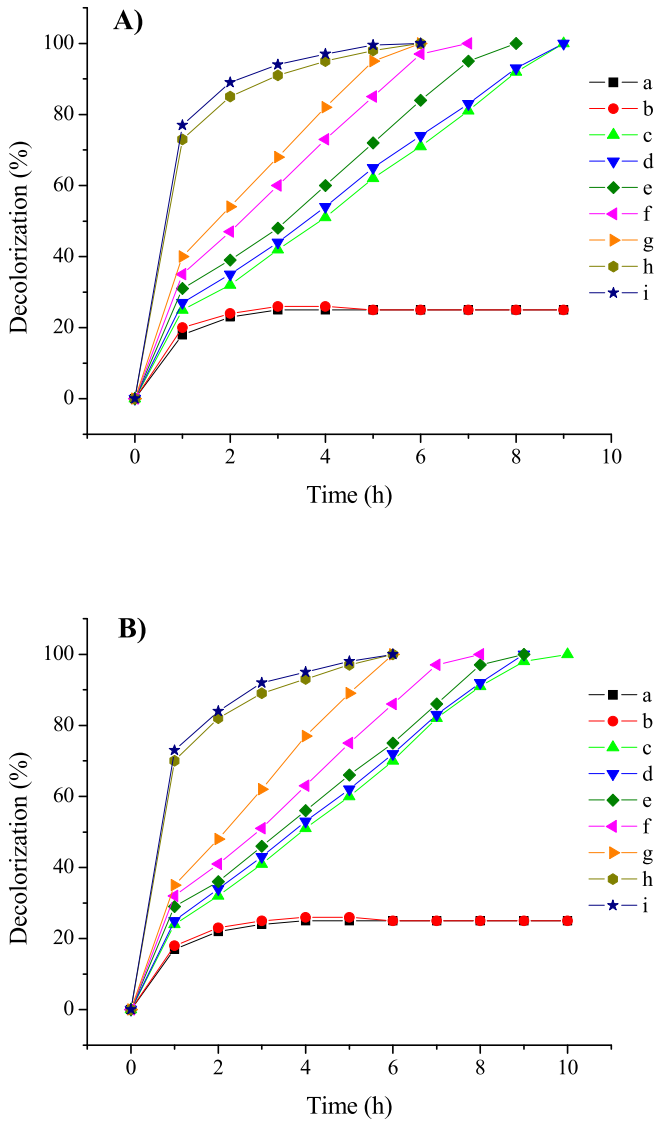

Fig. 5. Decolorization of MR by AQDS immobilized bacteria under anaerobic (A) and aerobic (B) conditions. a, Only the bead particles; b, Only AQDS immobilized beads; c, Only bacteria immobilized beads; d, Bacteria immobilized beads + free AQDS (100 μM); e, Immobilized bacteria and AQDS (100 μM); f, Immobilized bacteria and AQDS (300 μM); g, Immobilized bacteria and AQDS (500 μM); h, Suspended free cell; i, Suspended free cell + free AQDS (100 μM).

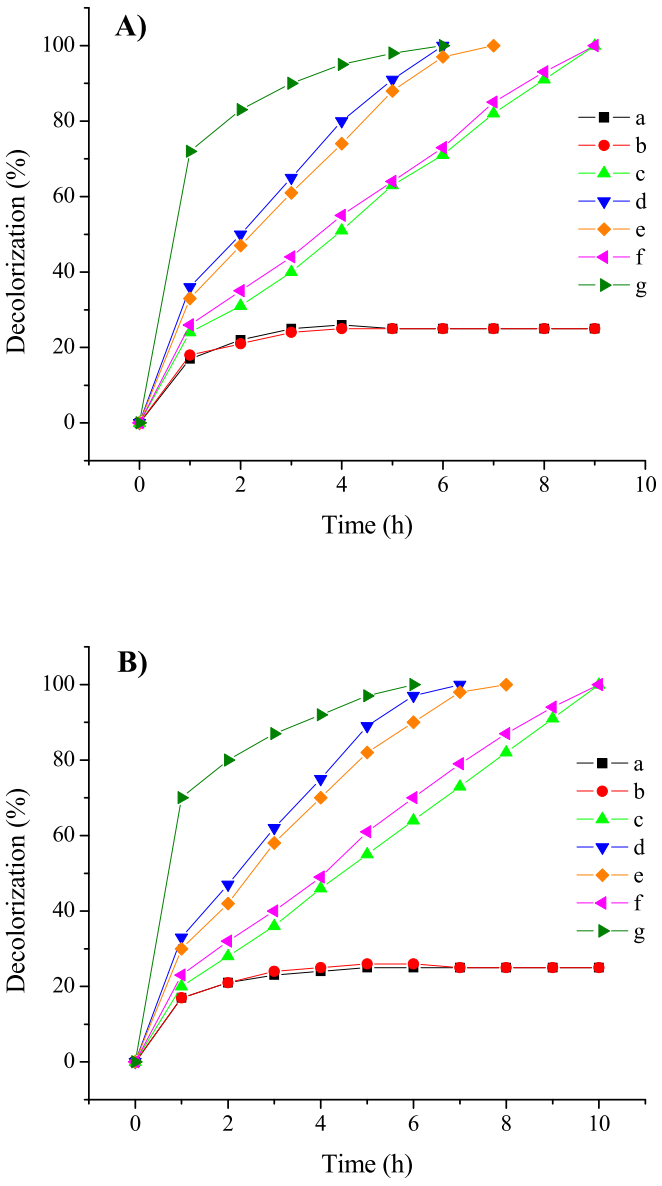

Fig. 6. Decolorization of MR by Fe_3O_4 magnetically immobilized bacteria under anaerobic (A) and aerobic (B) conditions. a, Only the bead particles; b, Only Fe_3O_4 immobilized beads; c, Only bacteria immobilized beads; d, Immobilized bacteria and Fe_3O_4 (0.1 g 50 mL^{-1}); e, Immobilized bacteria and Fe_3O_4 (0.25 g 50 mL^{-1}); f, Immobilized bacteria and Fe_3O_4 (1.0 g 50 mL^{-1}); g, Suspended free cell.

(beads without bacteria and AQDS as well as beads with AQDS and no bacteria). From the results it is evident that AQDS can act as a catalyst in the reductive decolorization of MR by *A. jandaei* strain SCS5. The decolorization of MR by immobilized bacterial beads increased with time, but complete decolorization required longer incubation time (9 h) compared to suspended bacteria when equal amount of cells were used. However, the decolorization rate of MR by immobilized bacteria accelerated when AQDS was added to the immobilized beads. The time requirement for 100% decolorization by bacteria immobilized with a concentration of 100, 300, 500 μM AQDS was 8 h, 7 h and 6 h under anaerobic conditions as well as 9 h, 8 h and 6 h under aerobic conditions respectively (Fig. 5). Low absorption capability of PVA-SA-Kaolin beads for MR dye was observed because only about 16% MR could be removed by immobilized AQDS control beads. The reason to take longer time for complete decolorization by immobilized bacteria compared to suspended cells may be due to the fact that during immobilization, some bacterial cells died as shown in CLSM analysis (Fig. 4). The other possible reason could be the limitation of mass transfer and availability of MR inside the beads in contrast to that in the bulk liquid. However, the presence of AQDS helps to increase the decolorization rate compared with the mediator free immobilized bacterial beads. With high concentrations of AQDS such as 500 μM AQDS in immobilization, 100% decolorization achieved within 6 h (Fig. S3 in SI) which was the same time also required for suspended bacterial cells to decolorize MR completely under both anaerobic and aerobic conditions. It has been reported that redox mediators can enhance azo dye reduction by accepting electrons from electron donor (bacteria) first and then transferring them to azo dye as the terminal electron acceptor (Keck et al., 1997). Thus the redox mediator AQDS could increase the MR color removal efficiency of immobilized bacterial beads by accelerating the electron transfer through the cell membrane. Consequently, the above results

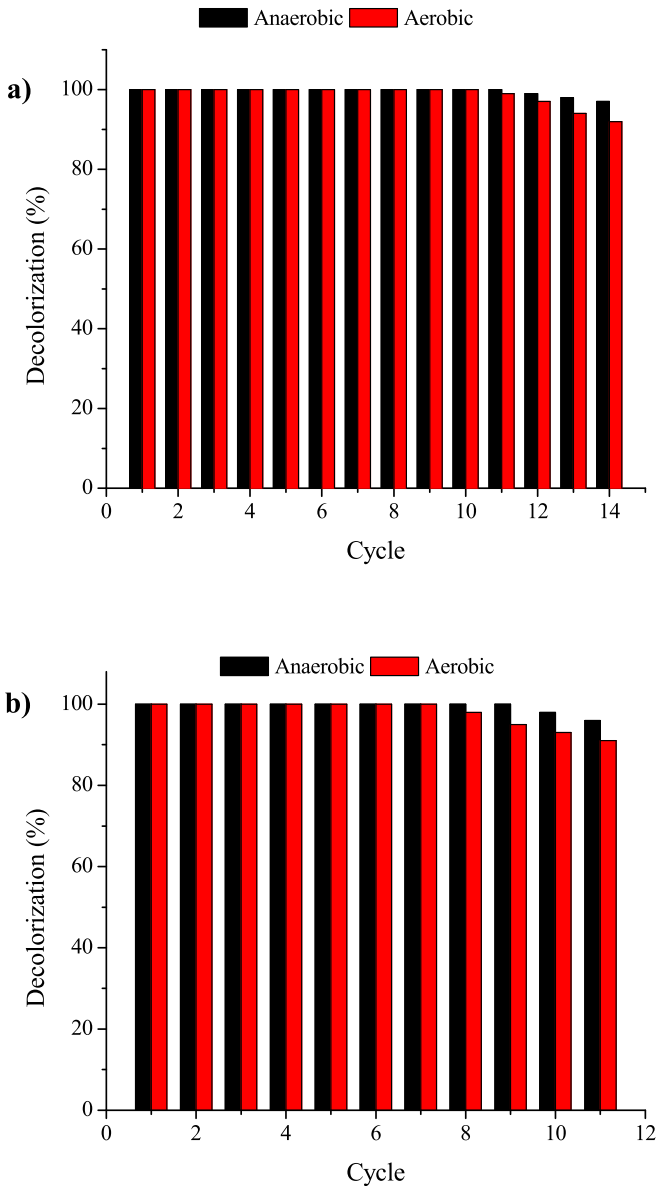

Fig. 7. Repeated batch operation for MR decolorization by bacteria immobilized bead particles having AQDS (a) and Fe_3O_4 nanoparticle (b).

indicated that *A. jandaei* strain SCS5 immobilized with AQDS was feasible and potentially useful to enhance reductive azo dye decolorization under anaerobic and aerobic conditions. Previous studies that reported on immobilization of AQDS for enhancing decolorization of azo dyes such as Acid Red 3R, Reactive Brilliant Red K-2BP, Reactive Red 2 and Congo Red only under anaerobic condition (Costa et al., 2010; Guo et al., 2007; Su et al., 2009), but not in aerobic condition. It would also be possible to increase the color removal efficacy of immobilized bacterial beads by increasing the amount of bacterial cells into the beads because high concentrations of initial cell inocula of *A. jandaei* strain SCS5 could remove MR faster and take shorter time for complete decolorization.

The MR decolorization activity of immobilized cells with magnetic Fe_3O_4 nanoparticles was investigated and also compared with Fe_3O_4–free immobilized and suspended cells. Fig. 6 shows that 100 mg L^{-1} azo dye MR could be decolorized within 9 h and 10 h by Fe_3O_4–free immobilized cells under anaerobic and aerobic environments. However, the equivalent amount of MR could be decolorized within a time shorter than the above mentioned periods by immobilized cells with Fe_3O_4 nanoparticles. Furthermore, the beads without bacteria showed around 16% MR removal (with or without Fe_3O_4 nanoparticles) in the control experiment. Therefore, the complete decolorization or removal of MR by immobilized bacterial beads with Fe_3O_4 nanoparticles was due to biodegradation of MR by *A. jandaei* strain SCS5, but not due to adsorption. The effects of different concentrations of Fe_3O_4 nanoparticles (0.1, 0.25, 1.0 g 50 mL^{-1} medium) on the activity of immobilized cells were studied. Fig. 6 shows that the complete decolorization of MR (100 mg L^{-1}) by immobilized cells with Fe_3O_4 required different time periods when different concentrations of Fe_3O_4 nanoparticles were immobilized. Among the different concentrations of Fe_3O_4 nanoparticles, the immobilized cells with Fe_3O_4 showed the highest decolorization activity for MR at a Fe_3O_4 nanoparticle concentration of 0.1 g 50 mL^{-1} and the complete decolorization was achieved at 6 h and 7 h under anaerobic and aerobic conditions respectively. However, the bacterial beads with a Fe_3O_4 nanoparticle concentration higher than 0.1 g 50 mL^{-1} exhibited lower activities than that of 0.1 g 50 mL^{-1} concentration, but still higher than that of beads without Fe_3O_4 nanoparticles. Thus, these results revealed that the decolorization activity of the immobilized cells of *A. jandaei* strain SCS5 was enhanced by adding Fe_3O_4 nanoparticles, and the possible reason could be because Fe_3O_4 nanoparticles have the ability to facilitate microbial extracellular electron transfer to electron acceptors (Liu et al., 2015). More importantly, the immobilized beads containing Fe_3O_4 could be easily separated and recycled by an external magnetic field due to the super paramagnetic properties of Fe_3O_4 nanoparticles (Fig. S3 in SI).

3.6. Repeated-batch experiments

In the case of immobilized bacteria-AQDS beads, complete decolorization was observed up to 11 and 10 cycles under anaerobic and aerobic conditions, respectively (Fig. 7a). Immobilized bacteria-Fe_3O_4 nanoparticles beads showed complete decolorization up to 9 and 7 cycles under anaerobic and aerobic conditions, respectively (Fig. 7b). After the above mentioned cycles, the decolorization rate reduced gradually, but more than 90% decolorization was still found in the following several cycles for immobilized bacterial beads with AQDS and Fe_3O_4 nanoparticles as reported in previous studies (Guo et al., 2007; Wang et al., 2007). Thus the immobilized bacteria-mediators beads exhibited good reusability for MR decolorization. The mechanical strength of the beads for both cases remained almost unchanged during the experiments.

3.7. Analysis of degradation products

The UV–visible scanning (200–800 nm) spectra of the cultural supernatant at pH 7 of different time intervals showed decolorization of MR (Fig. S4 in SI). Peaks for MR at 430 nm decreased gradually with increasing incubation time up to complete decolorization of MR within 6 h under anaerobic and aerobic conditions (Fig. S4 in SI). However, UV–visible spectra in both cultural conditions revealed that some new peaks appeared within the 200–300 nm range while the MR peak diminished, suggesting that biodegradation of MR occurred by *A. jandaei* strain SCS5.

GC-MS was applied to investigate the MR degradation products. One predominant peak with retention time around 6.56–6.58 min was observed in ethyl acetate extract of both anaerobic and aerobic cultures. The mass spectrum showed that the most abundant peak had m/z value of 136.1 for both anaerobic and aerobic conditions (Fig. 8). The peak with m/z value of 136.1 was identified as *N, N′* dimethyl-*p*-phenylenediamine (DMPD) by comparing with GC-MS library. Thus GC-MS analysis suggested that the biodegradation of

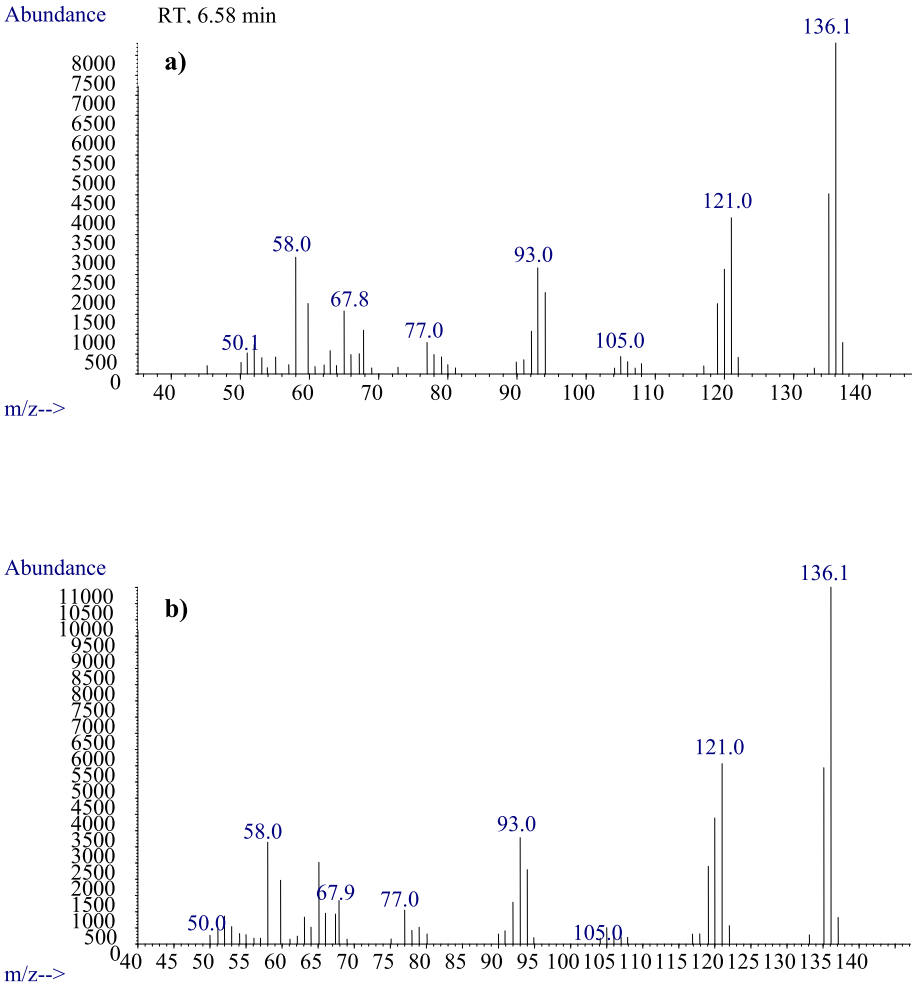

Fig. 8. Mass spectra of *N,N′*-dimethyl-*p*-phenylenediamine, a MR degradation product, identified by GC-MS analysis under anaerobic (a) and aerobic (b) conditions.

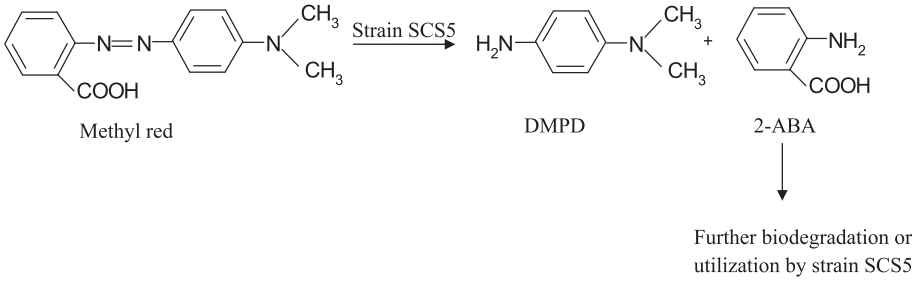

Fig. 9. Predicted MR degradation pathway by strain SCS5.

Table 1
Phytotoxicity of MR (200 mg L^{-1}) and its degradation products against *T. aestivum* and *P. mungo*.

Parameters studied	*T. aestivum*				*P. mungo*			
	Medium solution	Untreated MR	Treated MR		Medium solution	Untreated MR	Treated MR	
			Anaerobic	Aerobic			Anaerobic	Aerobic
Germination (%)	100	53 ± 5	100	100	100	100	100	100
Shoot (cm) (±SD)	4.9 ± 0.20	0.49 ± 0.02*	2.8 ± 0.20§	1.9 ± 0.17§	6.8 ± 0.15	2.0 ± 0.15*	4.0 ± 0.05§§	2.8 ± 0.05§
Root (cm) (±SD)	5.3 ± 0.20	0.20 ± 0.01*	2.4 ± 0.29§	1.5 ± 0.15§	5.4 ± 0.15	1.7 ± 0.05*	2.1 ± 0.10 §	1.7 ± 0.05

Values are mean of the triplicate experiments; SD (±), standard deviation. Shoot and root lengths of the seeds germinated in MR are significant different from seeds germinated in medium at $^{*}P \leq 0.001$. Shoot and root lengths of the seeds germinated in MR degradation products are significantly different from seeds germinated in MR at $^{\S}P \leq 0.01$ and $^{\S\S}P \leq 0.001$.

MR should undergo the reductive cleavage of azo bond (−N=N−) by *A. jandaei* strain SCS5. In this study, the occurrence of the other possible product 2-amino benzoic acid (2-ABA) produced by reductive degradation (Wong and Yuen, 1996; Zhao et al., 2014) was not detected from GC-MS analysis probably because of its further degradation or utilization by *A. jandaei* strain SCS5. This result was in agreement with other previous studies which reported MR degradation via the reductive cleavage of azo bond to produce colorless aromatic amines (Moutaouakkil et al., 2003; Wong and Yuen, 1996; Zhao et al., 2014). Jadhav et al. reported that 2-ABA and DMPD were detected after degradation of MR by *G. geotrichum* only at 5 °C and 50 °C respectively, while both metabolites were not found in the sample (due to further degradation) incubated at 30 °C, indicating that the MR degradation could be influenced under different experimental conditions (Jadhav et al., 2008). Consequently, it is predicted that the biodegradation of MR by *A. jandaei* strain SCS5 first produces DMPD and 2-ABA as main metabolic by-products, whereas 2-ABA undergoes further rapid degradation or utilization by *A. jandaei* strain SCS5 as shown in Fig. 9. To elucidate the exact mechanism of MR degradation by *A. jandaei* strain SCS5 in detail, more research will be needed.

3.8. Phytotoxicity

The phytotoxic effect of untreated and treated azo dye MR on *T. aestivum* and *P. mungo* were assessed in this study. The relative sensitivity of these two important cereal seeds, *T. aestivum* and *P. mungo*, against the untreated and treated MR (200 mg L^{-1}) after complete decolorization were presented in Table 1, which illustrated the germination (%), shoot length and root length of *T. aestivum* and *P. mungo*. The untreated dye at 200 mg L^{-1} showed 47% germination inhibition in *T. aestivum*, whereas no germination inhibition was found in *P. mungo*, but significant inhibition on the growth of root and shoot was observed in both plants compared to control medium solution. However, there was 100% germination of *T. aestivum* obtained when treated MR (200 mg L^{-1}) solution was applied. The treated MR solution showed a slight inhibition on the growth of shoot and root of these two plants, which was significantly less than the parent MR solution. Therefore, the phytotoxicity results implied that biodegradation of MR by *A. jandaei* strain SCS5 could detoxify azo dye MR in aqueous solution. The anaerobically treated MR was found to be less toxic than aerobically treated one. Overall, microbial degradation is effective to reduce the toxicity of azo dyes as shown in previous studies (Ghodake et al., 2009; Lim et al., 2014; Moawad and El-Rahim, 2003).

4. Conclusions

The complete decolorization of MR (100 mg L^{-1}) by *A. jandaei* strain SCS5 was achieved within 6 h in both anaerobic and aerobic conditions. The acidic condition was more effective for decolorization of MR with an optimum temperature of 35 °C. *A. jandaei* strain SCS5 was active in immobilized beads, and the decolorization rate by immobilized bacteria was enhanced when AQDS and Fe_3O_4 nanoparticles were co-immobilized into the beads. The degradation of MR by *A. jandaei* strain SCS5 may occur through the reductive cleavage of azo bond. The treated MR solution exhibited less phytotoxicity against *T. aestivum and P. mungo*. Thus, *A. jandaei* strain SCS5 can act as a potential microbial agent for the treatment of azo dye MR containing wastewater.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibiod.2016.04.035.

References

Adedayo, O., Javadpour, S., Taylor, C., Anderson, W.A., Moo-Young, M., 2004. Decolourization and detoxification of methyl red by aerobic bacteria from a wastewater treatment plant. World J. Microbiol. Biotechnol. 20, 545–550.
Ayed, L., Mandhi, A., Cheref, A., Bakhrouf, A., 2011. Decolorization and degradation of azo dye methyl red by an isolated *Sphingomonas paucimobilis*: biotoxicity and metabolites characterization. Desalination 274, 272–277.
Baeta, B.E., Aquino, S.F., Silva, S.Q., Rabelo, C.A., 2012. Anaerobic degradation of azo dye Drimaren blue HFRL in UASB reactor in the presence of yeast extract a source of carbon and redox mediator. Biodegradation 23, 199–208.
Banat, I.M., Nigam, P., Singh, D., Marchant, R., 1996. Microbial decolorization of textile-dye-containing effluents: a review. Bioresour. Technol. 58, 217–227.
Cheng, Y., Lin, H.Y., Chen, Z.L., Megharaj, M., Naidu, R., 2012. Biodegradation of crystal violet using *Burkholderia vietnamiensis* C09V immobilized on PVA-sodium alginate-kaolin gel beads. Ecotoxicol. Environ. Saf. 83, 108–114.
Chung, K.T., Fulk, G.E., Andrews, A.W., 1981. Mutagenicity testing of some commonly used dyes. Appl. Environ. Microbiol. 42, 641–648.
Chung, K.T., Fulk, G.E., Egan, M., 1978. Reduction of azo dyes by intestinal anaerobes. Appl. Environ. Microbiol. 35, 558–562.
Chung, K.T., Stevens, S.E., 1993. Degradation of azo dyes by environmental microorganisms and helminths. Environ. Toxicol. Chem. 12, 2121–2132.
Costa, M.C., Mota, S., Nascimento, R.F., Dos Santos, A.B., 2010. Anthraquinone-2,6-disulfonate (AQDS) as a catalyst to enhance the reductive decolourisation of the azo dyes reactive red 2 and congo red under anaerobic conditions. Bioresour. Technol. 101, 105–110.
dos Santos, A.B., Cervantes, F.J., van Lier, J.B., 2007. Review paper on current technologies for decolourisation of textile wastewaters: perspectives for anaerobic biotechnology. Bioresour. Technol. 98, 2369–2385.
dos Santos, A.B., Traverse, J., Cervantes, F.J., van Lier, J.B., 2005. Enhancing the electron transfer capacity and subsequent color removal in bioreactors by applying thermophilic anaerobic treatment and redox mediators. Biotechnol. Bioeng. 89, 42–52.
Du, L.N., Li, G., Zhao, Y.H., Xu, H.K., Wang, Y., Zhou, Y., Wang, L., 2015. Efficient metabolism of the azo dye methyl orange by *Aeromonas* sp. strain DH-6: characteristics and partial mechanism. Int. Biodeterior. Biodegrad. 105, 66–72.
Feng, C.J., Li, J.W., Qin, D., Chen, L.X., Zhao, F., Chen, S.H., Hu, H.B., Yu, C.P., 2014. Characterization of exoelectrogenic bacteria *Enterobacter* strains isolated from a microbial fuel cell exposed to copper shock load. PLoS One 9.
Ghodake, G., Jadhav, S., Dawkar, V., Govindwar, S., 2009. Biodegradation of diazo dye Direct brown MR by *Acinetobacter calcoaceticus* NCIM 2890. Int. Biodeterior. Biodegrad. 63, 433–439.
Gomare, S.S., Govindwar, S.P., 2009. *Brevibacillus laterosporus* MTCC 2298: a potential azo dye degrader. J. Appl. Microbiol. 106, 993–1004.
Guo, J.B., Zhou, J.T., Wang, D., Tian, C.P., Wang, P., Uddin, M.S., Yu, H., 2007. Biocalalyst effects of immobilized anthraquinone on the anaerobic reduction of azo dyes by the salt-tolerant bacteria. Water Res. 41, 426–432.
Idaka, E., Ogawa, T., Horitsu, H., Tomoyeda, M., 2008. Degradation of azo compounds by *Aeromonas hydrophila* var. 24B. Color. Technol. 94, 91–94.
Jadhav, J.P., Parshetti, G.K., Kalme, S.D., Govindwar, S.P., 2007. Decolourization of azo dye methyl red by *Saccharomyces cerevisiae* MTCC-463. Chemosphere 68, 394–400.
Jadhav, S.U., Kalme, S.D., Govindwar, S.P., 2008. Biodegradation of methyl red by *Galactomyces geotrichum* MTCC 1360. Int. Biodeterior. Biodegrad. 62, 135–142.
Keck, A., Klein, J., Kudlich, M., Stolz, A., Knackmuss, H.J., Mattes, R., 1997. Reduction of azo dyes by redox mediators originating in the naphthalenesulfonic acid degradation pathway of *Sphingomonas* sp. strain BN6. Appl. Environ. Microbiol. 63, 3684–3690.
Li, C., Li, Y., Cheng, X., Feng, L., Xi, C., Zhang, Y., 2013. Immobilization of *Rhodoccocus rhodochrous* BX2 (an acetonitrile-degrading bacterium) with biofilm-forming bacteria for wastewater treatment. Bioresour. Technol. 131, 390–396.
Lim, C.K., Aris, A., Neoh, C.H., Lam, C.Y., Majid, Z.A., Ibrahim, Z., 2014. Evaluation of

macrocomposite based sequencing batch biofilm reactor (MC-SBBR) for decolorization and biodegradation of azo dye acid orange 7. Int. Biodeterior. Biodegrad. 87, 9–17.
Liu, F., Rotaru, A.E., Shrestha, P.M., Malvankar, N.S., Nevin, K.P., Lovley, D.R., 2015. Magnetite compensates for the lack of a pilin-associated c-type cytochrome in extracellular electron exchange. Environ. Microbiol. 17, 648–655.
Lovley, D.R., Phillips, E.J., 1988. Novel mode of microbial energy metabolism: organic carbon oxidation coupled to dissimilatory reduction of iron or manganese. Appl. Environ. Microbiol. 54, 1472–1480.
Manu, B., Chaudhari, S., 2002. Anaerobic decolorisation of simulated textile wastewater containing azo dyes. Bioresour. Technol. 82, 225–231.
Mendez-Paz, D., Omil, F., Lema, J.M., 2005. Anaerobic treatment of azo dye acid orange 7 under fed-batch and continuous conditions. Water Res. 39, 771–778.
Meng, X.M., Liu, G.F., Zhou, J.T., Fu, Q.S., 2014. Effects of redox mediators on azo dye decolorization by *Shewanella algae* under saline conditions. Bioresour. Technol. 151, 63–68.
Miranda, R.C., Gomes Jr., E.B., Pereira, N., Marin-Morales, M.A., Machado, K.M., Gusmao, N.B., 2013. Biotreatment of textile effluent in static bioreactor by *Curvularia lunata* URM 6179 and *Phanerochaete chrysosporium* URM 6181. Bioresour. Technol. 142, 361–367.
Moawad, H., El-Rahim, W.M.A., 2003. Bioremediation of irrigation water contaminated with textile dyes. Fresenius Environ. Bull. 12, 786–792.
Moreno-Garrido, I., 2008. Microalgae immobilization: current techniques and uses. Bioresour. Technol. 99, 3949–3964.
Moutaouakkil, A., Zeroual, Y., Dzayri, F.Z., Talbi, M., Lee, K., Blaghen, M., 2003. Bacterial decolorization of the azo dye methyl red by *Enterobacter agglomerans*. Ann. Microbiol. 53, 161–169.
Ozkan-Yucel, U.G., Gokcay, C.F., 2010. Effect of initial azo dye concentration and biomass acclimation on sludge digestion and dye co-treatment. Clean. Soil Air Water 38, 387–393.
Pandey, A., Singh, P., Iyengar, L., 2007. Bacterial decolorization and degradation of azo dyes. Int. Biodeterior. Biodegrad. 59, 73–84.
Rau, J., Knackmuss, H.J., Stolz, A., 2002. Effects of different quinoid redox mediators on the anaerobic reduction of azo dyes by bacteria. Environ. Sci. Technol. 36, 1497–1504.
Saratale, R.G., Saratale, G.D., Chang, J.S., Govindwar, S.P., 2009. Ecofriendly degradation of sulfonated diazo dye C.I. Reactive Green 19A using *Micrococcus glutamicus* NCIM-2168. Bioresour. Technol. 100, 3897–3905.
Shah, M., 2014. Evaluation of *Aeromonas* spp. in microbial degradation and decolorization of reactive black in microaerophilic – aerobic condition. J. Bioremediat. Biodegrad. 5, 246.
Sharma, S.C.D., Feng, C., Li, J., Hu, A., Wang, H., Qin, D., Yu, C.P., 2016. Electrochemical characterization of a novel exoelectrogenic bacterium strain SCS5, isolated from a mediator-less microbial fuel cell and phylogenetically related to *Aeromonas jandaei*. Microbes Environ. http://dx.doi.org/10.1264/jsme2.ME15185.
Singh, P., Sanghi, R., Pandey, A., Iyengar, L., 2007. Decolorization and partial degradation of monoazo dyes in sequential fixed-film anaerobic batch reactor (SFABR). Bioresour. Technol. 98, 2053–2056.
Singh, R.L., Singh, P.K., Singh, R.P., 2015. Enzymatic decolorization and degradation of azo dyes - a review. Int. Biodeterior. Biodegrad. 104, 21–31.
Stolz, A., 2001. Basic and applied aspects in the microbial degradation of azo dyes. Appl. Microbiol. Biotechnol. 56, 69–80.
Su, Y.Y., Zhang, Y.F., Wang, J., Zhou, J.T., Lu, X.B., Lu, H., 2009. Enhanced biodecolorization of azo dyes by co-immobilized quinone-reducing consortium and anthraquinone. Bioresour. Technol. 100, 2982–2987.
Toro, E.E.R.D., Celis, L.B., Cervantes, F.J., Rangel-Mendez, J.R., 2013. Enhanced microbial decolorization of methyl red with oxidized carbon fiber as redox mediator. J. Hazard. Mater. 260, 967–974.
van der Zee, F.P., Villaverde, S., 2005. Combined anaerobic-aerobic treatment of azo dyes - a short review of bioreactor studies. Water Res. 39, 1425–1440.
Varma, P., Madamwar, D., 2003. Decolorization of synthetic dyes by a newly isolated strain of *Serratia marcescens*. World J. Microbiol. Biotechnol. 19, 615–618.
Wang, X., Gai, Z.H., Yu, B., Feng, J.H., Xu, C.Y., Yuan, Y., Lin, Z.X., Xu, P., 2007. Degradation of carbazole by microbial cells immobilized in magnetic gellan gum gel beads. Appl. Environ. Microbiol. 73, 6421–6428.
Wong, P.K., Yuen, P.Y., 1996. Decolorization and biodegradation of methyl red by *Klebsiella pneumoniae* RS-13. Water Res. 30, 1736–1744.
Wong, P.K., Yuen, P.Y., 1998. Decolourization and biodegradation of N,N′-dimethyl-p-phenylenediamine by *Klebsiella pneumoniae* RS-13 and *Acetobacter liquefaciens* S-1. J. Appl. Microbiol. 85, 79–87.
Zhao, M., Sun, P.F., Du, L.N., Wang, G., Jia, X.M., Zhao, Y.H., 2014. Biodegradation of methyl red by *Bacillus* sp strain UN2: decolorization capacity, metabolites characterization, and enzyme analysis. Environ. Sci. Pollut. Res. 21, 6136–6145.