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Full Length Research Paper

Isolation and characterization of *Pseudomonas putida* WLY for reactive brilliant red x-3b decolorization

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Using the BMM medium containing 100 mg/L of reactive brilliant red X-3B, a decolorizing bacterium with higher decolorization activity was isolated and it showed a decolorization zone of 10 mm; this decolorizing bacterium was identified as *Pseudomonas putida* WLY based on physiological and biochemical characteristics as well as 16srRNA sequence. In this study, the effects of *P. putida* WLY's decolorizing ability, for example, glucose concentration, temperature, O_2, pH and dye concentration, were investigated. The results show that under 40°C, pH 7.0, glucose concentration 0.2%, O_2 absence and concentration of X-3B of 30 mg/L, the decolorization rate in 24 and 48 h was 86.3 and 93.2%, respectively. The azoreductase produced by *P. putida* WLY was extracellular and induced according to electrophoresis experiments and decolorization tests. After purification by ion exchange and gel chromatography, its molecular weight was estimated to be 28,000 Da by SDS-PAGE.

Key words: *Pseudomonas putida;* reactive brilliant red X-3B, biodecolorization, azoreductase.

INTRODUCTION

The synthetic dyes, especially azo dyestuff, have been produced and widely used in paper, textile and coating industries, which caused serious environmental pollution, especially in developing countries (Chen, 1989). Synthetic azo dyestuff is very difficult to remove and biodegrade due to its complex aromatic molecular structure, they are also highly colored, toxic and can heavily contaminate water source (Raffi et al., 1997; Zollinger, 1991). The dye annual production was about 800 to 900×10^3 t in China, with annual output has reaching 150×10^3 t (Zhou, 2001). About 10 to 15% of the dye is lost with the waste water; with the frequency of the water, it is directly discharged into the environment, and as such pose a serious threat of environmental pollution in China (Yu, 2004). Therefore, biological and chemical degradation of azo dyes have attracted much attention in recent years. Chemical degradation technologies, such as catalytic ozonation, photocatalysis, ultrasonic irradiation, and electrochemical oxidation, have been studied for the degradation of azo dyes in wastewater (Forgacs et al., 2004). Biological degradation technology used bacteria or fungi to decolor, which is kind to human and environment, nontoxic and no secondary contamination due to it's source (natural environment) (Brown and Stephen, 1993; Levine, 1991).

Many bacteria and fungi can efficiently biodegrade azo dyes and decolorized them, such as *Aeromonas*, *Pseudomonas*, *Bacillus*, *Rhodococcus*, *Shigella*, *Klebsiella*, *Rhizopus oryzae, Penicillium oxalicum* and *Phanerochaete chrysosporium* (Banat et al., 1996; Wong and Yuen, 1996; Zissi and Lyberatos, 2001; Chung et al. 1993; Chang et al., 2001). Bacteria could produce azo reductase, showing maximum decolori-zation activity while fungi could absorb the dying stuff (Fu and Viraraghavan, 2001; Zhang et al., 2003). Azo reductase has low substrate specificity and can break the dye molecules in the high-affinity electron azo bond, producing colorless and aromatic amines (Chen et al., 2003; Daeshwar et al., 2007). Bioremediation offers a cost-effective and environmentally friendly method for decolorization and degradation of dyes in industrial effluents and contaminated soil (Banat et al., 1996; Bhatt et al., 2000).

This study aimed to screen and isolates decolorizing bacteria with high efficiency and study azo enzyme

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characteristics. The decolorizing bacterium was employed for the biodegradation of reactive brilliant dye X-3B.

MATERIALS AND METHODS

Dyes and samples

The reactive brilliant dye X-3B used for this biodecolorization study was obtained from Light Chemical Engineering Laboratory from Changzhou University. Heavily dye contaminated soil and wastewater samples were obtained from Changzhou dye manufacturing industries and Qingtan wastewater treatment factory in Changzhou, respectively. All samples were collected in sterile plastic containers.

Isolation of X-3B resistant and decolorizing bacteria

Soil samples were used as inoculum and 1 g of soil was added into 50 ml of sterile modified base mineral medium (BMM) in flasks and X-3B was added as the sole carbon source to give a final concentration of 30 mg/L. One liter of BMM contained 5.17 g K_2HPO_4, 1.70 g KH_2PO_4, 1.63 g NH_4Cl, and 10 ml of a salt solution. One liter of the salt solution contained 8.5 g $MgSO_4$, 5 g $MnSO_4$, 5 g $FeSO_4$, 0.3 g $CaCl_2$. The initial pH value of media was 7.2 (Cai et al., 2008).

The inoculated flasks were agitated on an orbital shaker, at 120 r.p.m at 30℃ for 72 h. 2 ml of the culture medium was transferred to another 50 ml of fresh culture medium and cultivation was carried out under the same condition for 2 to 3 times. The obtained suspensions were streaked on the nutrient agar plates. The medium composition is BMM, 30 mg/l of X-3B and 1.5% of agar. All the plates were incubated at 30℃ for 48 h. The morphologically distinct bacterial isolates, showing a clear decolorization zone of were selected and preserved on X-3B dye containing nutrient agar slant at 4℃

Identification of *Pseudomonas putida*

The isolate with the largest decolorization zone for the dye, was selected and streaked on X-3B containing nutrient agar for further purification and study. The purified isolate was used to inoculate on several media for biochemical testing. The physiological and biochemical characteristics of the X-3B decolorization organisms were examined using standard procedures (Dong and Cai, 2001). Gram stain, catalase- and oxidase-activities, methyl-red, V-P, citrate, gelatin agar, indol and starch dydrolysis tests were investigated. Motility was assessed by testing the ability of the strains to migrate from the point of inoculation through semisolid agar plate (Wang et al., 2009). The utilization of some substrates as carbon sources and nitrogen sources by the isolate was performed based on Bergey's Manual of Determinative Bacteriology (Buchanan et al., 1984).

Genomic DNA was isolated from the pure culture pellet using consensus primers and partial 16s rRNA genes were amplified by PCR using forward primer (5'–GAGCGGATAACAATTTCACACAGG-3'), reverse primer (5'-CGCCAGGGTTTTCCCAGTCACGAC-3') and internal primer (5'-CAGCAGCCGCGGTAATAC- 3'). The amplified 16s rRNA gene was sequenced by Takara Bio (Dalian, China). The obtained sequence data was aligned and analyzed for identification and finding the closest homology for the isolate. The sequence was deposited in GenBank (GenBank accession: JN002065), the next closet homology was found with *Pseudomonas putida* and it was designated as *P. putida* WLY.

X-3B decolorization quantification

The dye decolorizing quantification studies were performed in cultivated medium. The cultivation was centrifuged at 10,000 g for 15 min; the supernatant was scanned for absorbance maxim on UV-VIS spectrophotometer (Gold Spectrumlab 53, Shanghai, China).

Decolorization study in aqueous medium was performed by inoculating 15% of (v/v) actively growing strain WLY to another fresh 100 ml of medium containing X-3B dye in 250 ml flask. All the tests are three replicates. One set of the flasks without inoculation was kept as control. After incubation, 10 ml of the inoculated and un-inoculated samples were centrifuged at 10.000 g for 15 min and supernatant was analyzed at 540 nm wavelength and decolorization rate was calculated. Viable cell counts during X-3B decolorization was followed previous report (Kim et al., 2005).

Decolorization experiments (single factor experiments for determining the optimal range of seven factors)

The X-3B stock solution of 3000 mg/L was prepared and using the required concentrations of dye solution was diluted. To study the effect of temperature (15 to 50℃), pH (3.0 to 10.0), dye concentration (15 to 500 mg/L), glucose concentration (0 to 0.5%) and O_2 (presence and absence), in each experiment, one factor was changed, with the other factors remaining constant. The effects of these factors were evaluated by measuring the X-3B decolorization rate and in triplicate.

Azo reductase preparation

In order to test if azo reductase was an induced or a constitute enzyme, the purified strain WLY was inoculated in three different media: one contained 30 ppm X-3B as the carbon source, another without the dye, and the third glucose as the carbon source. The incubation conditions were: 30℃, pH 7.0, with shaking velocity 100 rpm for 48 h. Crude enzyme was obtained by centrifuging the cultivation medium solution at 8000 g for 15 min at 4℃. The supernatant was stored at 4℃ for further study and the cells were frozen at -80℃ until use.

The cold ethanol (-20℃) was added into the supernatant, mixed entirely and then the mixture is kept in a fridge (-20℃) for overnight. The mixture was centrifuged at 8000 g, 4℃ for 20 min; the deposit was dissolved in phosphate buffer for further study.

The cells were resuspended in phosphate buffer and were disrupted by ultrasonication three times for 25 s each time and spun at 8000 g for 20 min at 4℃. The supernatant fraction was used as crude cell extracts.

Molecular weight for protease was determined through the casein zymography gel electrophoresis experiment (Raser et al., 1995). The zymography gel electrophoresis assay was developed for proteases in non-denaturing casein-containing polyacrylamide gels. Samples of crude enzyme were run into the polyacrylamide gels by electrophoresis. Upon the electrophoresis end, the gels were washed and incubated in an activation buffer containing 1 mM calcium and 10 mM dithiothreitol for 20 h. After staining of the casein gels with Coomassie blue G250, the protease showed up as clearing bands.

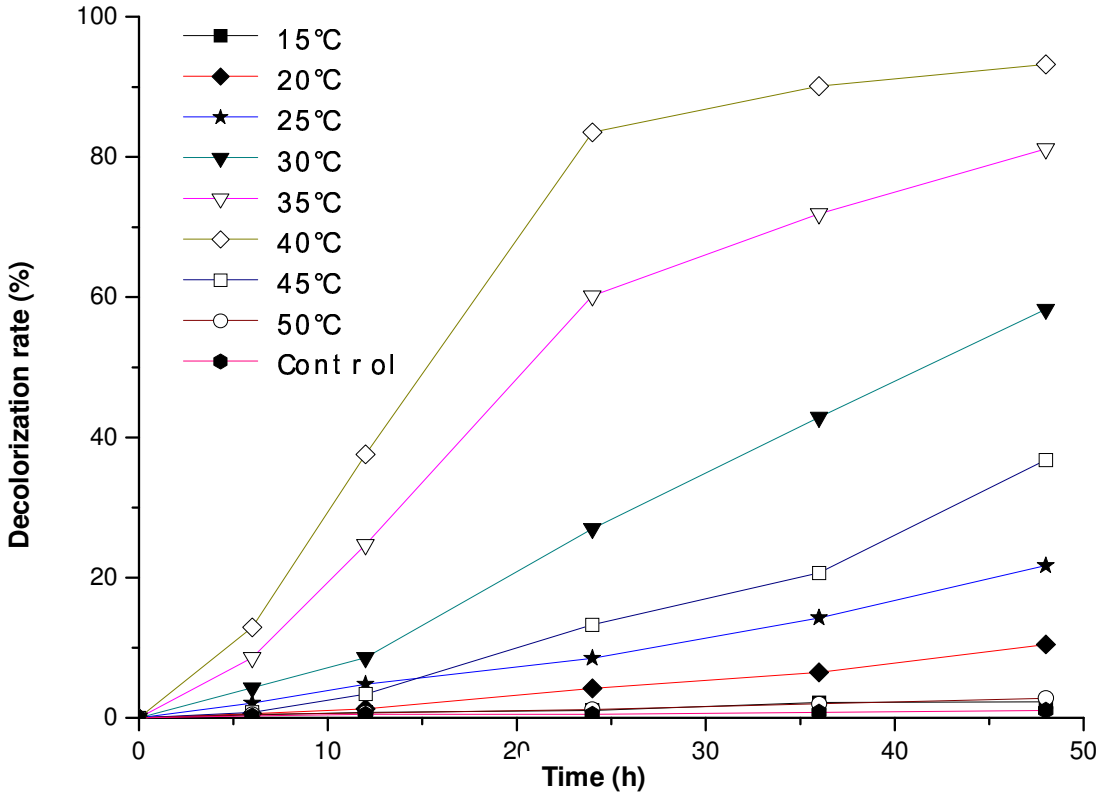

Figure 1. Effect of temperature on decolorization of X-3B.

RESULTS

Isolation of X-3B decolorization bacteria

There were seven morphologically distinct isolates showing decolorization zones from 4 to 10 mm on nutrient agar containing 30 ppm X-3B. The isolate 1, designated as WLY, was the one with a larger decolorized zone (10 mm) for a colony size of 4 to 5 mm, and was selected for further studies. The other 6 isolates showed decolorization zones between 4 to 7 mm and were resistant to 150 mg/L of X-3B , which may be the results of heavy contamination with dyes due to the consequence of natural adaptation of the organism as the samples from which the bacterial isolate was obtained were contaminated heavily with dyes . The strain WLY had higher efficient decolori-zation activity and could degrade the synthetic dye X-3B.

Identification of the WLY isolate

The X-3B decolorization strain WLY was determined to be pure by visual observation of the colonies on nutrient agar, after purification procedures and confirmation by microscopic observation, which demonstrated that both colonies as cells presented uniform morphology.

The strain WLY was a Gram-negative, rod-shaped bacterium, facultative aerobe and motile. The colonial morphology on the agar nutrient plate was smooth, wet and orange, after 1 to 2 days of incubation.

Substrate-utilization experiments were performed and showed that the strain WLY was capable of using a variety of substrates, including phenol, alcohols, carbo-xylic acids, aromatic compounds and most inorganic nitrogen sources. The 16s rRNA gene sequences were compared by using BLAST similarity searches, and the closely related sequences were obtained from GenBank. On the basis of morphological and biochemi-cal analysis in combination with phylogenetics analysis, the strain WLY was identified as *P. putida* and designated as *P. putida* WLY (GenBank Accession: JN002065).

Effect of the temperature on X-3B decolorization by *P. putida* WLY

Temperature plays an important role in microbial growth and enzyme activity and is one of the most important parameters taken into consideration in the development of biodecolorization processes. The temperature range was 15 to 50℃. The results (Figure 1) reveal a high decolorization percentage at 35 and 40℃. At 40℃ and after 48 h cultivation, the decolorization percentage was 93.2%, while it was only 81.2 and 58.3% at 35 and 30℃, respectively.

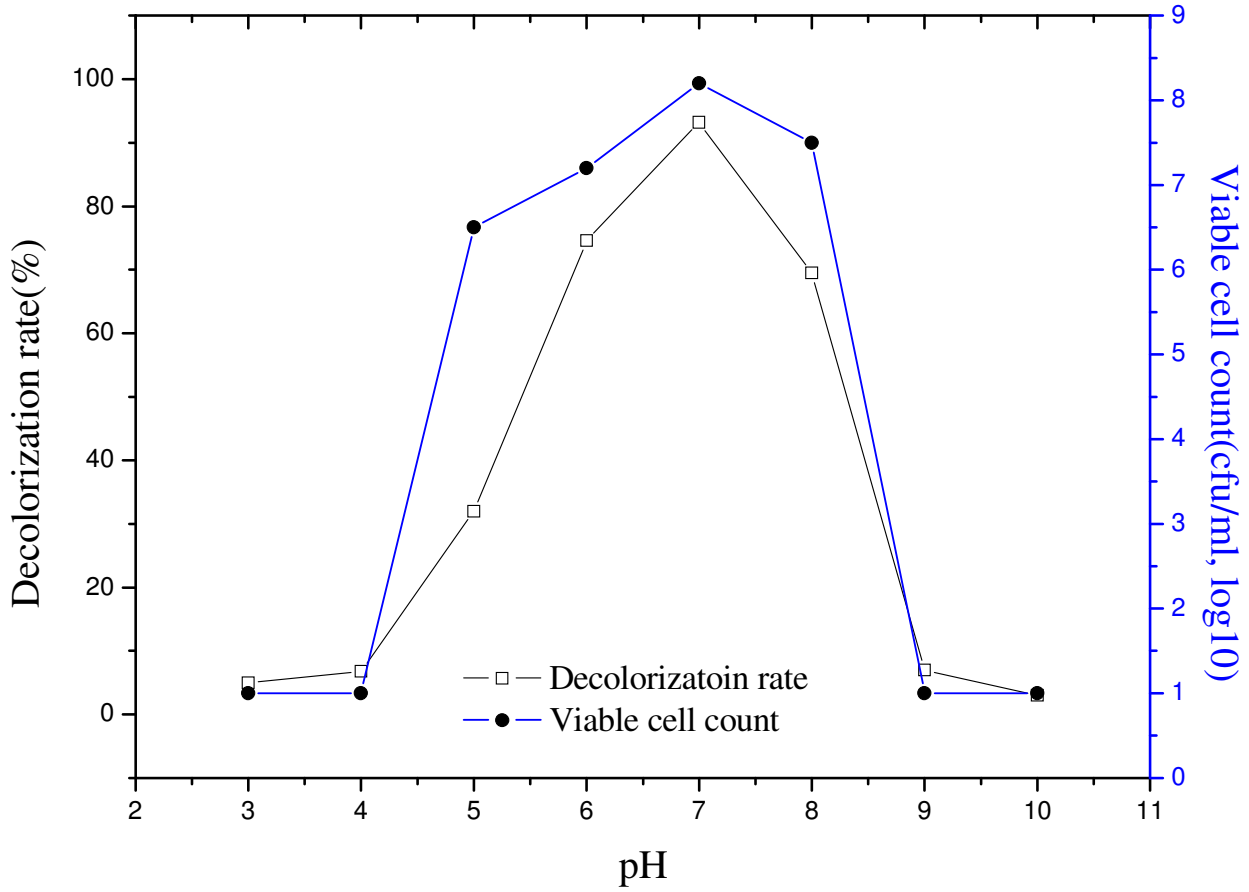

Figure 2. Effect of pH on decolorization of X-3B.

Viable cell count was evaluated during X-3B decolorization. Cell density was about 10^6cfu/ml at the beginning of the tests. At 40℃ cell density increased rapidly, and alive bacteria amount in medium could reach 10^8 CFU/ml, more than that under the condition at 35℃ (1.3×10^7cfu/ml) and 45℃ (1.1×10^7 cfu/ml). WLY strain could not stay alive when temperature exceeded 50℃, and the X-3B was not decolorized because of that. Therefore, the strain WLY was a facultative heat-resistant bacteria and the optimum decolorization temperature could be determined as 40℃.

Effect of initial pH on X-3B decolorization *P. putida* WLY

To test the effect of pH on X-3B decolorization by *P. putida* WLY, the temperature was maintained constant (40℃), for 48 h and pH varied from 3 to 10. Figure 2 shows that decolorization percentage of X-3B was higher at pH 6 to 8. The highest decolorization rate could be obtained at pH 7 for 48 h, and the decolorization rate also reached over 65% at pH 6 and 8, while the decolorization rate was 32.5% at pH 5 and was almost zero at lower (3 and 4) or higher (9 and 10) pH values. The variation of the amount of viable bacteria under different pH was also tested and the results show (Figure 2) that there was no bacteria growth at pH values under 6 and upper 9, and the highest densities of viable bacteria were obtained between the pH range (6 to 8.5).

Effect of the glucose concentration on X-3B decolorization *P. putida* WLY

To test the effect of glucose concentration on X-3B decolorization by WLY isolate, temperature and pH were kept constant at 40℃ and pH 7, for 48 h, and glucose concentration varied from 0 to 0.5%. Even though, *P. putida* WLY had the ability to use X-3B as the sole carbon source, it grew slowly. But, when glucose was added the growth rate increased as well as the decolorization rate. Figure 3 shows that decolorization rate of X-3B was higher at the range 0.2 to 0.3%. The highest decolorization rate is 92.5% when the glucose concentration is 0.2%, while the rate is 59.8 and 82.6% when the glucose concentration is 0.1 and 0.3% respectively. When the glucose concentration was over

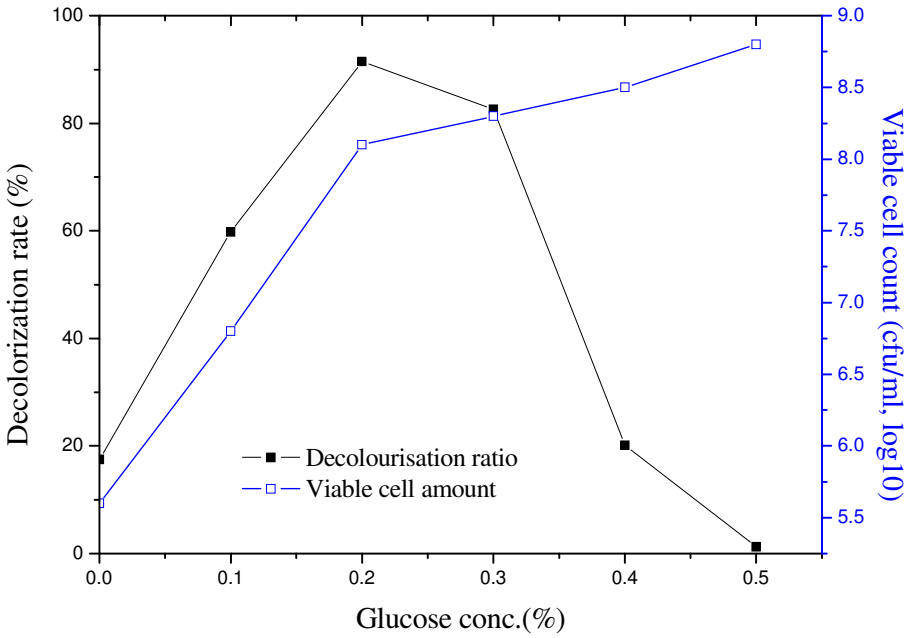

Figure 3. Effect of glucose concentration on X-3B-decolorization.

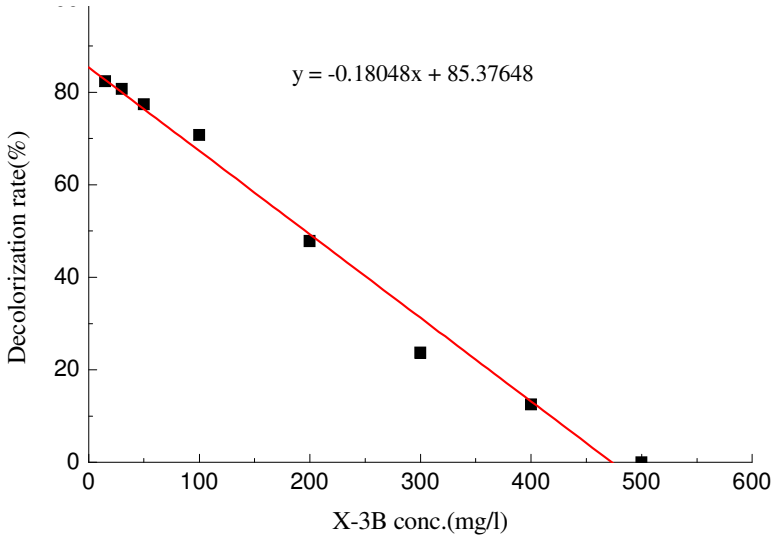

Figure 4. Effect of X-3B concentration on decolorization of X-3B.

0.3%, the decolorization X-3B rapidly decreased. Bacteria densities, for a determined glucose concentration (Figure 3) varied greatly in the range 0 to 0.5%, with the highest value reached at 0.5% (10^9cfu/ml). *P. putida* WLY can use glucose as carbon and energy sources and thus, provided sufficient electrons for reductive conditions through the cleavage of the azo bond of X-3B. The increase of glucose concentration, decrease the decolorization rate of X-3B and it could be assumed that when there is enough glucose in medium, *P. putida* WLY prefer to use glucose instead of dye X-3B.

Effect of X-3B concentration on decolorization *P. putida* WLY

For the study of the effect of X-3B concentration on decolorization, temperature, pH and glucose concentration were constant at 40°C, 7 and 0.2%, respectively, for 48 h, while X-3B varied from 15 to 500 mg/L. Figure 4 shows that decolorization rate decreases with the increasing X-3B concentration. This relation can be explained by the linear equation y = -0.18x + 85.38 (R^2=0.98).

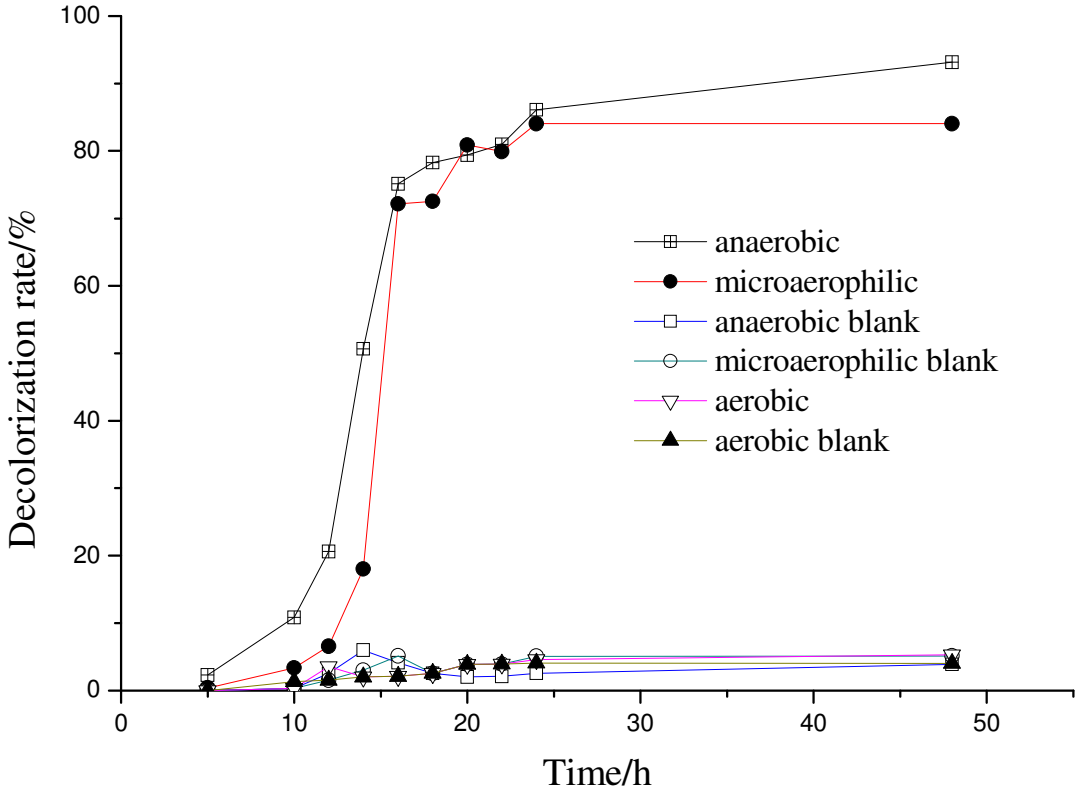

Figure 5. Effect of O_2 on decolorization of X-3B.

Effect of O_2 on decolorization of X-3B

Figure 5 presents color removal efficiencies throughout 48 h of incubation, under anaerobic, aerobic and microaerophilic conditions. More than 81% of the color was removed within 24 h under anaerobic conditions, while at the same time 15 and 67% decolorization was observed under aerobic and microaerophilic incubations, respectively which meant that the efficiency of color removal with *P. putida* WLY in anaerobiosis was faster than that of in aerobiosis.

UV-V is absorption spectra

The azo dye of X-3B has four structure units: benzene, triazine, naphthalene rings and a conjugated system linked by two azo groups. In the UV region, the absorbance bands at 234, 280 and 330 nm are attributed to the benzene ring, triazine ring and naphthalene ring, respectively whereas the visible band at 540 nm is designated to the long conjugated π system linked by two azo groups (Silverstein et al., 1991). It was shown in Figure 6 that main visible absorption peaks of X-3B decreased after 24 h and almost disappeared at 48 h, which indicated that the azo groups were broken. And it was also concluded that the naphthalene ring was opened during the decolorization of X-3B, because its absorbance value at 330 nm was decreased at 24h and almost disappeared at 48h.

It has been reported that the oxidation of azo bonds led to decolorization of dye solutions with the formation of primary aromatic amines and the formation of aromatic amines has also been reported in the natural anaerobic degradation of azo dyes (Weber and Adams, 1995). The azo bond of X-3B was broken and the naphthalene ring was opened during decolorization of X-3B by *P. putida* WLY in this study, which would lead to form aromatic amines in the medium. This is in accordance with previous report.

Protease released by *P. putida* WLY

The *P. putida* WLY in the biodecolorization experiments could decolorize the azo dye of X-3B and after decolorization the vis absorption bands of azo dye X-3B disappeared, which showed that the azo group was broken, thus, azoreductase might be released. In order to study the feasibility of azoreductase production by *P. putida* WLY and to test the azoreductase in extracellular or intracellular enzyme, different conditions were controlled in this study. It was shown from Figure 7 that the azoreductase produced by *P. putida* WLY was an extracellular enzyme. The cultivation supernatant could decolorize the dye X-3B. The azoreductase was purified

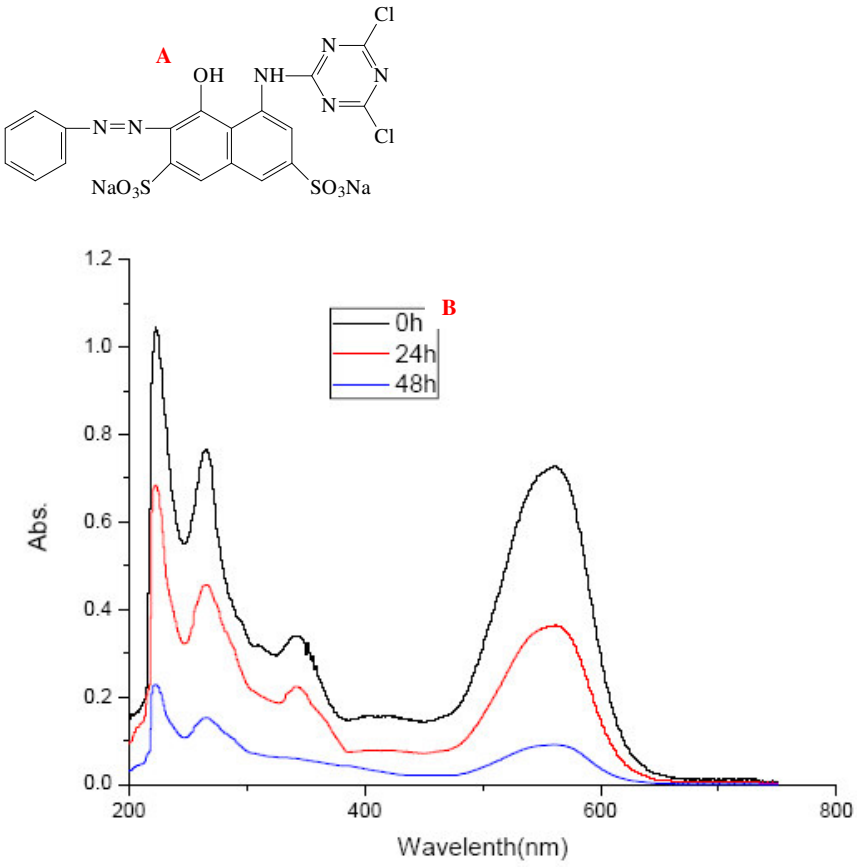

Figure 6. (A): Chemical structure of reactive brilliant red X-3B and (B): its UV-vis absorption spectrum.

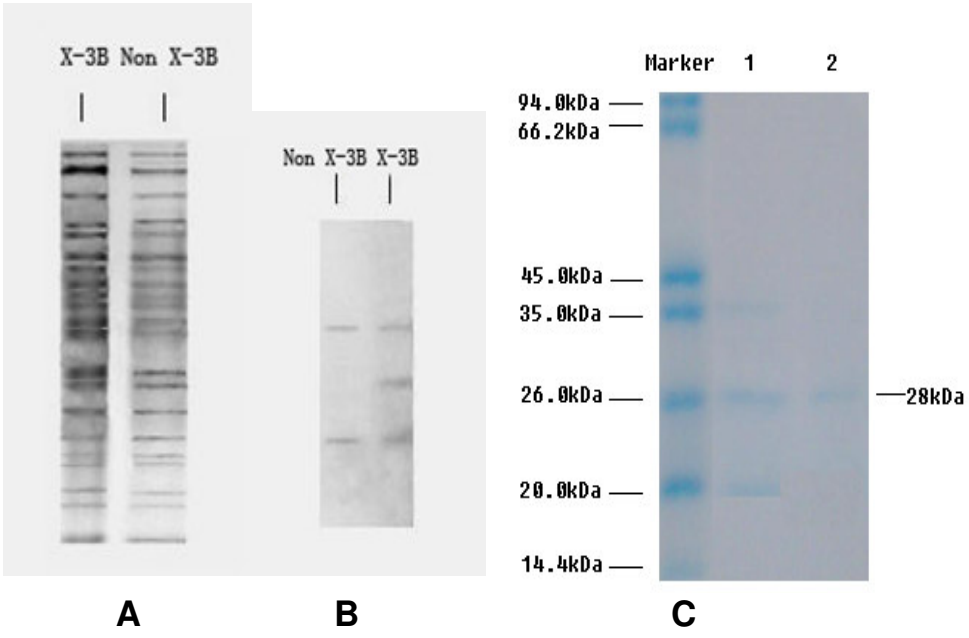

Figure 7. Electrophoresis image of protease produced by *P. putida* WLY (A: the image of all intracellular protein; B: the image of all extracellular protein; C: the molecular weight of azoreductase produced by *P. putida* WLY).

through ion exchange chromatography and the purified azoreductase had high enzyme activity to decolor the dye X-3B, it's MW (molecular weight) is 28 kDa.

DISCUSSION

Azoreductase can catalyze the reductive cleavage of azo bond, which is the key enzyme expressed in azo dyes degrading bacteria. In the last decade, enzymes with azoreductase activity have been identified and characterized from many bacteria, such as *Pseudomonas chrysosporium, Xenophilus azovorans* KF46F, *Pigmentiphaga kullae* K24, *Enterococcus faecalis*, *Staphylococcus aureus* (Blümel et al., 2002; Blümel and Stolz, 2003; Chen et al., 2004, 2005; Olfat et al., 2000). Azoreductase has low substrate specificity and can break the dye molecules in the high-affinity electron azo bond, producing a colorless aromatic amines utilizing NAD(P)H as electron donor *in-vitro* (Chen et al., 2003; Daeshwar et al., 2007). However, the involvement of intracellular azoreductase in bacterial decolorization has been doubted in recent years due to their high polarities and complex structures, many azo dyes are difficult to diffuse through cell membrane. In this present study, the azo dye X-3B was degraded by *P. putida* WLY, which was isolated from sludge. The other six isolates showed decolorization zone of 4 to 7 mm and they were resistant to 150 ppm X-3B concentration. The azoreductase from *P. putida* WLY is an induced extracellular enzyme, and was successfully purified by chromatographic methods. The purified azoreductase shows a single band on SDS-PAGE, its molecular weight is 28,000 Da, which differ from those described for azoreductase from *Pseudomonas* sp., *S. dysenteriae* type 1, *Escherichia coli* K12 and *Escherichia coli* SS125 (Zimmermann et al., 1982; Ghosh et al., 1992, 1993; Sandhya et al., 2008; Wang et al., 2007).

The temperature, pH, concentration of X-3B, glucose content and O_2 could change the strain WLY biodecolorization rate and its rate reached 93.2% in 48 h, under the condition of 40°C, pH 7.0, glucose concentration 0.2% and anaerobic culture, which is one of the fastest reported biodecolorization organisms for X-3B. The results also show that O_2 could inhibit the production of azoreductase, which could break the azo groups. All these results showed that *P. putida* WLY had higher activity of the azo dye decolorization than previous reports (Ola et al., 2010; Saratale et al., 2009; Sandhya et al., 2008; Chang et al., 2001). Therefore, *P. putida* WLY is a potential candidate for degrading and bioremediating azo dye waste water and polluted soil.

The next step we will purify its azoreductase and test its physicochemical properties of the purified azoreductase, which may open new possibilities for its biotechnological applications and allow the use of *P. putida* WLY in the treatment of azo dyes in industrial effluents. And we will also construct gene engineering bacterium to be used in a continuous process for the degradation of azo dyes in industrial effluents.

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