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Biodecolorization of Azo Dye Acid Red B under High Salinity Condition

M. Salah Uddin · Jiti Zhou · Yuanyuan Qu · Jianbo Guo · Ping Wang · Li hong Zhao

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Abstract The study was conducted by a novel salt tolerant bacterium *Gracilibacillus* sp. GTY. The strain was identified on the basis of morphological and physio-biochemical characteristics, and 16S rDNA sequence analysis. Decolorization was performed by growing and resting cells, as well as by extracted azo reductase. Strain grown in the media containing 15% (w/v) of NaCl showed the best performance in decolorization. Decolorization was observed by the UV–visible absorbance spectra. The maximum absorption peak in the visible area decreased to a minimum level after 96 h of incubation. On the other hand, strain grown in very low, or high concentrations, of salt did not show good performance in decolorization; suggesting that salt concentrations in the surroundings control the production of azo reductase.

Keywords Salt tolerant · Decolorization · Azo reductase · *Gracilibacillus* sp. GTY

The presence of dye, the use of high concentrations of salt complicates the management of wastewater from reactive dye operations, which is currently a single most pressing environmental problem in the textile dyeing industry. Most of the textile manufacturers are located on the river banks and coastal areas because of low cost transportation and available source of water. Azo dyes discharge from their manufacturing processes and cause severe water pollutions around the located areas. In most cases, azo dyes are toxic and may cause serious chronic diseases of human beings as well as plants and animals. Some of azo dyes have been linked to bladder cancer in humans, to splenic sarcomas, hepatocarcinomas, and nuclear anomalies in experimental animals, and to chromosomal aberrations in mammalian cells (Medvedev et al. 1988; Percy et al. 1989). Some physical, chemical and biological methods have already been established for the treatment of azo dyes containing wastewater. Physical and chemical methods cannot be used always easily for wastewater treatment due to high cost, low efficiency and in-applicability to a wide variety of dyes. According to the biological method, many bacterial species non-specifically reduce azo compounds anaerobically to corresponding amines through the reductive cleavage of azo bonds, resulting in decolorization (Haugh et al. 1991; Weber 1991, Chung and Stevens 1993). Microbial decolorization is an environment-friendly and cost-competitive alternative to other treatment processes. But the presence of high amounts of salts (up to 10 g/L NaCl or Na_2SO_4) in dye containing wastewater often complicates this treatment process. Many microbial species are able to decolorize some azo dyes anaerobically within a certain limit of salts. But most of them are unable to decolorize azo dyes in high salinity conditions.

This paper deals with the biodecolorization of azo dye acid red B by a recently isolated highly salt tolerant bacterial strain *Gracilibacillus* sp. GTY, which is able to grow up to 2%–25% (w/v) of NaCl. In our study, at first the

M. S. Uddin (✉) · J. Zhou · Y. Qu · P. Wang · L. hong Zhao
School of Environmental and Biological Science and Technology, Dalian University of Technology, Dalian 116024, China
e-mail: salimench@yahoo.com

M. S. Uddin
Department of Genetics and Breeding, University of Rajshahi, Rajshahi 6205, Bangladesh

J. Guo
School of Environmental Science and Engineering, Hebei University of Science and Technology, Shijiazhuang 050018, China

characteristics and the phylogenetic analysis of this new strain were described and furthermore an attempt was made to optimize conditions for the efficient decolorization. The decolorization techniques were performed by growing and resting cells and by extracted azo reductase. In the literature, no report on the biodecolorization of azo dyes by *Gracilibacillus* sp. GTY was found.

Materials and Methods

The media used in this study were Luria–Bertani (LB) medium contained 5 g/L of yeast extract, 10 g/L of peptone and mineral-salt (MS) medium contained 1 g/L of $(NH_4)_2SO_4$, 1.4 g/L of K_2HPO_4, 0.6 g/L of KH_2PO_4, 1 g/L of $MgSO_4$, 0.1 g/L of $CaCl_2$, 0.1 g/L of $FeSO_4$ and 10 g/L of proline as carbon source. NaCl at the concentrations of 20–250 g/L (2%–25% w/v) were added in both LB and MS media. Both media were incubated at 30°C on a rotary shaker at 150 r/min and a standard pH was maintained 7.2.

Azo dye acid red B was collected from the Dye Synthesize Laboratory, Dalian University of Technology. The maximum absorbance wavelength of acid red B is at 515 nm. Figure 1 shows the chemical structure of this dye.

For the characteristics and phylogenetic analysis, genomic DNA was isolated using a modified technique used for the purification of DNA from Gram-negative bacteria (Neilan 1995). The 16S rRNA gene PCR amplifications were performed by using primers 8F and 1522R. The sequence of PCR product was done by TaKaRa Biotechnology (Dalian) Co. Ltd. 16S rDNA sequence analysis was performed with the aid of Clustal W 1.8 software package by using the neighbor-joining method and Jukes–cantor distance correction matrix method.

Resting cells were prepared from the strain GTY grown in LB media supplemented with 5%, 15% and 25% (w/v) of NaCl. Strain was grown in three 250 mL flasks separately at 30°C, until $OD_{660\ nm}$ reaching 1.0 (about 0.9 g/L). The cells grown in various salt concentrations were harvested separately by centrifugation (8,000×*g*, 10 min at 40°C) and suspended in 0.1 M pH 8.0 phosphate buffers containing 15% (w/v) of NaCl. Then the cells were washed with the same buffer twice. Finally, the cells were resuspended separately to a final concentration of 10 mg/mL

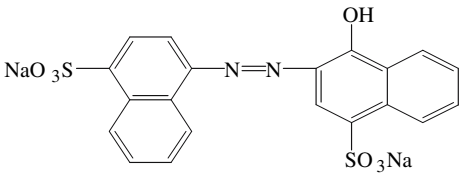

Fig. 1 Chemical structure of azo dye acid red B (λ_{max}, 515 nm)

(wet cells weight) in three sterilized serum bottles containing azo dye at the concentration of 100 mg/L and incubated them at 30°C. After successful decolorization, the end products in serum bottles were again centrifuged at 22,000×*g* for 20 min to see the presence of azo reductase. Centrifuged supernatant was used here as azo reductase for decolorization of azo dye acid red B. The assays were performed with 50 mg/L of azo dye, supernatant and NADH for the initialization of the reaction.

For the extraction of crude enzyme (azo reductase), the strain was harvested in LB liquid media containing 15% w/v of NaCl (optimum for the strain) and was incubated over night. Then the culture media were centrifuged twice at 8,000×*g* for 20 min and each time the cell pellets were washed by phosphate buffer containing 15% (w/v) of NaCl. The cells from 2,000 mL culture were preserved over night at –20°C. The cells were completely lysed by ultrasonic processor (Model cp ×750, USA) with 20 mM sodium-phosphate buffer (30 min, 225 W). Finally the lysed cells were centrifuged at 22,000×*g* for 20 min and the supernatant was used as intracellular enzyme for decolorization assays. The assays were performed with 100 mg/L of azo dye, enzyme in 20 mM sodium-phosphate buffer and NADH for the initialization of the reaction.

Absorbance of the dye-containing solutions were assayed by its λ_{max} value using an UV–visible spectrophotometer (JASCO, V-560, UV/VS spectrophotometer), and the assays were performed in duplicate.

Results and Discussion

The strain was obtained from the Dalian coastal area, Northern China as a mixed bacterial culture. Then its single colony was isolated and acclimatized in high salt containing LB liquid media. The strain was identified as *Gracilibacillus* sp. GTY on the basis of morphological and physio-biochemical characteristics and 16S rDNA sequence analysis, and was deposited as patent strain in China General Microorganism Culture Center with the accession number CGMCC 1527. The 16S rDNA sequence of the strain GTY was also submitted to GenBank with the number DQ 286727. It was Gram-negative, rod-shaped and strictly aerobic, 0.6–0.7 μm wide and 1.7–2.4 μm long, resistance to the antibiotics streptomycin and canamycin. Grown colonies were mucoid, rounded and white in color. The strain was oxidase-positive, catalyase-positive, but nitrification negative. Fructose and trehalose were hydrolyzed. Figure 2 shows the electron micrograph picture of the strain GTY.

Phylogenetically, the genus *Gracilibacillus* clusters within the alpha subclass of the *Firmicutes*. The strains selected including species names and accession numbers

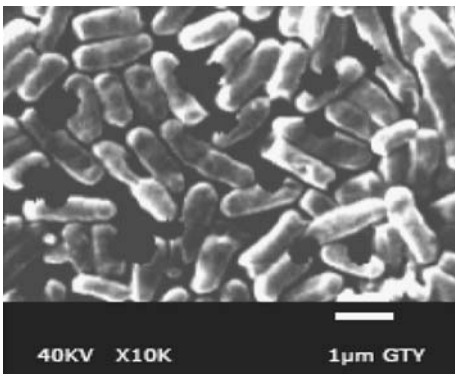

Fig. 2 The electron micrograph picture of *Gracilibacillus* sp. GTY, (0.6–0.7) μm × (1.7–2.4) μm

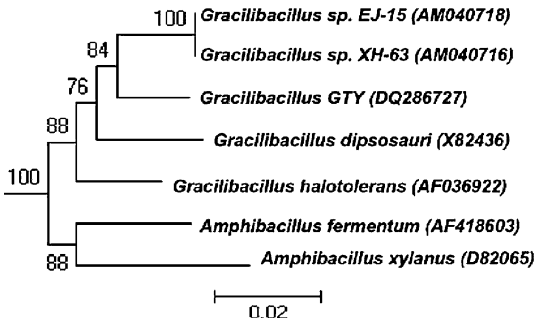

Fig. 3 Phylogenetic tree of *Gracilibacillus* species

were as follows: *Gracilibacillus* sp. *EJ-15* (*AM040718*); *Gracilibacillus* sp. *XH-63* (*AM040716*); *Gracilibacillus GTY* (*DQ286727*); *Gracilibacillus dipsosauri* (*X82436*); *Gracilibacillus halotolerans* (*AF036922*); *Amphibacillus fermentum* (*AF418603*); *Amphibacillus xylanus* (*D82065*). Figure 3 shows the phylogenetic tree of *Gracilibacillus* species.

We cultivated this strain in different NaCl concentrated LB liquid media to observe its decolorization performance. Among all of the concentrations, strain grown in media containing10%–15% (w/v) NaCl showed the best performance of azo dye acid red B decolorization. Dye was almost completely decolorized (nearly 100%) after 96 h of treatment (Fig. 4a). Figure 4b shows the UV–visible absorbance of acid red B. According to the UV–visible absorbance, a maximum absorbance was observed at 515 nm and this peak decreased as a function of time.

The absorbance peak at 515 nm decreased to a minimum level after 96 h of cultivation. The significant changes occurring both in UV and visible spectra indicate that the conjugated bonds (–N–N–) of dye acid red B might be destroyed and that the molecules are broken into small ones after decolorization. They are possibly be oxidized to nitrate.

On the other hand, strain grown in the media with lower percentages (2%–5%, w/v) of NaCl showed very poor decolorization ability where bacterial growth rates were very poor also. Strain grown in the media with the highest percentage (25%, w/v) of NaCl showed moderate decolorization ability (Fig. 4a). Strain grown in lower percentages of NaCl could not show good performance of decolorization because of low growth rates. Decolorization was not satisfactory also in the media containing 25% (w/v) of NaCl, because the inhibition to microorganisms by high salt concentration, which may cause plasmolysis and/or loss of activity of cells (Panswad and Anan 1999). After completely decolorization of dye acid red B, an UV–visible intermediate product probably an amino derivative with λ_{max} of 340 nm (Fig. 4b), was produced (O'Neill et al. 2000).

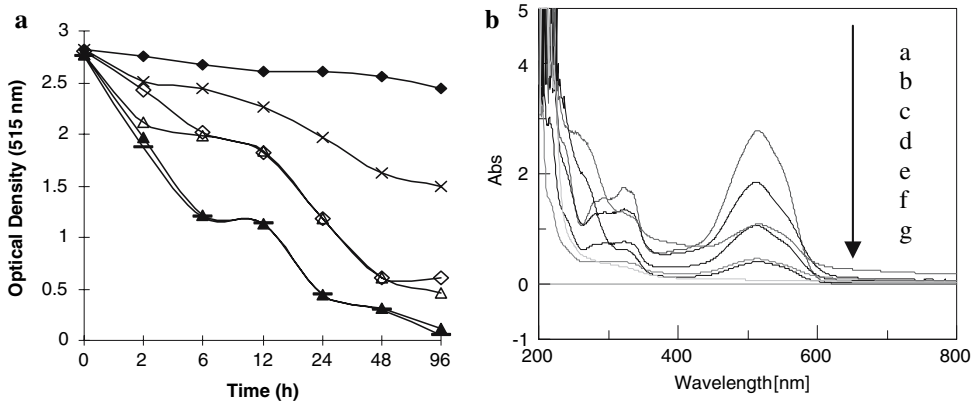

Fig. 4 **a** Decolorization of azo dye acid red B by growing cells of GTY grown in the media containing different concentrations of NaCl (*filled diamonds* 2%; *crosses* 5%; *filled triangles* 10%; *solid lines* 15%; *open triangles* 20%; *open diamonds* 25% w/v of NaCl). Concentration of dye was kept constant at 100 mg/L. **b** UV–visible absorbance spectra of acid red B decolorized by GTY grown in the media containing 15% w/v of NaCl. Observations were taken place at regular time intervals (*a* Abs of acid red B 100 mg/L; *b* 2 h; *c* 6 h; *d* 12 h; *e* 24 h; *f* 48 h; *g* 96 h)

Resting cells mixed with sodium-phosphate buffer were inoculated separately in three sterilized serum bottles containing azo dye at the concentration of 100 mg/L and incubated them at 30°C. Cells were used to a final concentration of 10 mg/mL (wet cells weight) in these three bottles. Observations took place at different time intervals. Cells grown in the medium containing 15% (w/v) of NaCl decolorized dye within 72–96 h of incubation. Decolorization rate was distinctly faster than other two concentrations. Slow and poor azo dye decolorization was observed by both resting cells grown in the media containing 5% and 25% (w/v) of NaCl (Fig. 5). This finding suggests that NaCl in very lower or higher concentrations can inhibit or decrease the production of azo reductase. Biodecolorization by resting cells is a scientifically advantageous and comparatively easy process. The resting cells can be repeatedly utilized and the cells can be used in high concentration. This process can be run under non-sterile condition. Those phenomenon advocates that the protocol can be utilized in large-scale biodecolorization.

After successful decolorization, we used decolorized end products for further decolorization of azo dye. Enzyme in resting cells could decolorize azo dye for the second time also. Figure 6a shows the time course of azo dye acid red B. This result indicates that the enzymes in resting cells catalyze the products for further dye degradation (Zhilong et al. 2006).

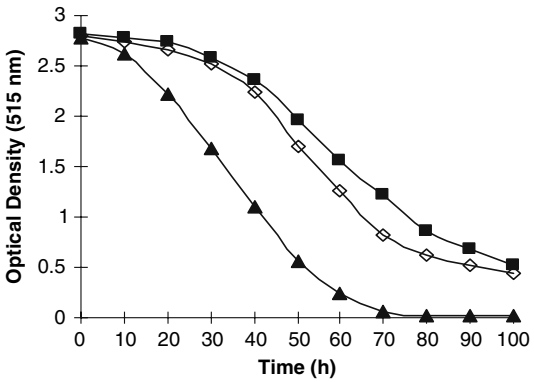

Fig. 5 Decolorization of azo dye acid red B (100 mg/L) by resting cells of the strain GTY. Resting cells were prepared from GTY grown in the media containing different concentrations of NaCl (*open diamonds* 5%; *filled triangles* 15%; *filled squares* 25% w/v of NaCl)

Azo reductase (crude enzyme) was extracted by the method described above. Extracted enzyme was non-purified and non-characterized. The assays were performed with 100 mg/L of azo dye, enzyme in 20 mM sodium-phosphate buffer and NADH for the initialization of the reactions. The decolorization of dye was conducted in 3 ml of reaction mixture at 30°C in 700 s. Reaction rate obtained from time course spectra measurement analysis was comparatively slow. The percentage of dye removal was about 10% in the reaction mixture (Fig. 6b).

Cellular enzyme is located in the cytoplasm and extracellular secretion of this enzyme immediately follows its synthesis without accumulation in the cell (Rafii and Cerniglia 1993). Extracted azo reductase was not purified. A disadvantage of whole-cell biocatalysis is the possible formation of by-products because whole cells contain many other enzymes that can catalyze undesired reactions (Hamid-Reza et al. 2007). So the decolorization rate might be slow in the reaction mixture.

Microbial decolorization of azo dye acid red B under high salinity conditions by a novel bacterium *Gracilibacillus* sp. GTY was studied here. The present study confirmed that growing and resting cells as well as extracted azo reductase of the strain GTY decolorized used dye successfully in optimum conditions (temperature ±30°C, pH 7.2, and 10%–15% w/v of NaCl). Strain grown in very low or high concentrations of salt could not show good performance in decolorization. This finding suggests that azo reductase of this strain is controlled by the concentrations of salt in their surroundings. However, our established protocol can be utilized in large-scale biodecolorization. Dye-polluted wastewater containing high concentrations of salt can be treated by this new isolated strain. As a new isolated strain, other characteristics such as molecular and genetic characteristics will be carried out in our further study.

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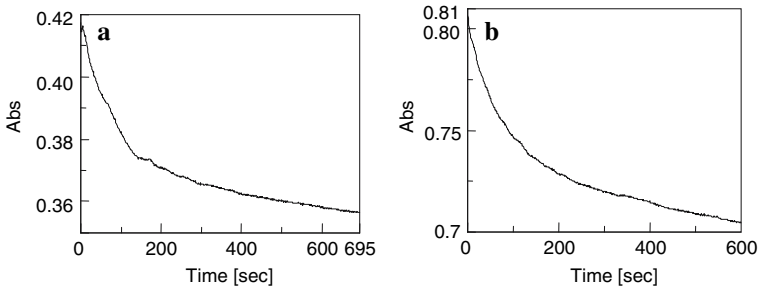

Fig. 6 **a** Time course of acid red B decolorized by crude enzyme produced from resting cells. Dye was used at the concentration of 50 mg/L. **b** Time course of acid red B decolorized by extracted enzyme. Dye was used at the concentration of 100 mg/L

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