Complete degradation of azo dye acid red 337 by Bacillus megaterium KY848339.1 isolated from textile wastewater

Ayman Y. I. Ewida, Marwa E. El-Sesy and Azza Abou Zeid

Central Laboratory for Environmental Quality Monitoring, National Water Research Center, Kalubiya, Egypt; Botany and Microbiology Department, Faculty of Science, Zagazig University, Sharqia, Egypt

ABSTRACT
Biodegradation has been proven as the most efficient, eco-friendly and cost-effective technique for the removal of complex organic matters such as textile dyes from wastewater effluents. Some environmentally friendly bacterial strains play an important role in such field. Acid red 337, an azo dye used extensively in textile industry, was reported as hazardous recalcitrant, when released into the aqueous environment. In the present research, a potential bacterial strain, capable of degrading acid red 337 (AR 337) dye was isolated from a textile wastewater effluent. Using 16S rRNA sequence analysis, the bacterium was identified as Bacillus megaterium KY848339.1. The decolorization capability of B. megaterium for AR 337 dye was optimized; the bacterium could remove 91% of dye concentration of 500 mg L\(^{-1}\) within 24 h when the inoculum size was 10% wt./v, solution pH was 7 and the incubation temperature was 30°C. The Liquid Chromatography-Mass Spectrum (LC-MS) analysis indicated the degradation of AR 337 azo dye by B. megaterium, to small aliphatic compounds and CO\(_2\). The application of B. megaterium on wastewater contaminated with red dyes using 10% wt./v of bacterial cells concentration was resulted in 98.9% removing of red color through 10 days.

Introduction
It is known that millions of liters of untreated textile wastewater are discharged daily into public drains that eventually empty into rivers (Dawkar, Jadhav, Jadhav, & Govindwar, 2008). The textile industry is consuming a high amount of water, where 100 L of water is used to process 1 kg of textile material, which generates a high amount of effluent (Verma, Raghukumar, Parvatkar, & Naik, 2012). Many of such dyes are hazardous and may affect aquatic life (Shaker, Zafarian, & Rao, 2014). Due to their chemical structures, dyes are resistant to fading on exposure to light, water and many chemicals and, therefore, are difficult to be decolorized once released into the aquatic environment (Sharma & Janveja, 2008). Azo dyes comprise a diverse group of synthetic chemicals that are widely used by the leather, textile, cosmetics, and paper product industries (Kalyani, Patil, Jadhav, & Govindwar, 2008). The general structure of azo dyes is comprising two or more of aromatic rings joined by one or more of azo groups (– N = N –), most of these dyes are toxic, recalcitrant and may be carcinogenic (Acuner & Dilek, 2004).

Acid red 337 is classified as a single azo dye which is extensively used in the textile industry. It was recorded by the European Chemical Agency (ECHA) as a harmful organic matter to the aquatic environment. A lot of physical and chemical treatment methods, such as oxidation, reduction, adsorption, chemical precipitation and flocculation, electrochemical treatment and ion-pair extraction, are used to remove dyes from wastewater effluents (Fan et al., 2009). These methods are attractive due to their efficiency, but they are complicated and expensive (Robinson, McMullan, Marchant, & Nigam, 2001). On the other hand, biological processes have received progressive attention due to their cost, effectiveness, ability to produce less sludge and environmental harmlessness (Chen, Wu, Liou, & Hwang, 2003); these processes can convert these complexed organic pollutants into water, carbon dioxide and inorganic salts (Daneshvar, Khataee, Rasoulifard, & Pourhassan, 2007). A wide variety of microorganisms can decolorize or even completely mineralize a wide range of dyes, including bacteria (Dave & Dave, 2008), fungi and algae (Ghanem, Al-Garni, & Biag, 2011; Ramya, Iyappan, Manju, & Jife, 2010). The most promising microorganisms for wastewater treatment are those isolated from sites contaminated with dyes (indigenous) because they have adapted to survive in adverse conditions (Dave & Dave, 2008; Myrna, Aida, Herminia, Norberto, & Maribel, 2012).

The present work was aimed to; i) isolate an indigenous bacterial strain from a textile effluent, being able to degrade AR 337 azo dye, ii) evaluate
and optimize the biodegradation capability of such strain for remediation purposes of AR 337 azo dye, and iii) Pilot scale laboratory application of using such strain to remove colors from a dye contaminated textile wastewater effluent.

**Materials and methods**

**Dye stuff, chemicals, and microbiological media**

AR 337 dye was kindly supplied by Moket Mac textile factory, Sharqia Governorate, Egypt. It is classified as azo acid dye with a Color Index (C.I.) acid red 337, and a chemical structure of (2-(Trifluoromethyl) benzene-diazonium and 6-amino-4-hydroxyn-naphthalene-2-sulfonic acid). A stock solution was prepared, filter sterilized and added to the medium at a known concentration. Sodium chloride, hydrochloric acid and other chemicals used were of analytical grade (Aldrich, USA). Nutrient broth and nutrient agar (Difco, USA) were used as enrichment and isolation media.

**Isolation of AR 337 dye-decolorizing bacteria**

Textile wastewater samples were collected from Moket Mac textile factory, Sharqia Governorate, Egypt. Enrichment culture technique was used to isolate AR 337 dye decolorizing bacteria as follows (Ewida, 2014); A 250 ml Erlenmeyer flask containing 100 ml of nutrient broth amended with 2.5 mg of AR 337 dye was inoculated with 10 ml of textile wastewater and incubated at 35°C for 24 h. Then 10 ml of the obtained consortium was used to inoculate another flask containing 100 ml nutrient broth amended with 5 mg of dye and incubated for 24 h. at 35°C. The enrichment continued by repeating the previous steps until the concentration of the dye reached 100 mg L⁻¹. One milliliter of each consortium was spread on nutrient agar plates amended with the same concentration of the dye. The predominated bacterial strains were selected to perform the rest of the research.

**Screening of high AR 337 dye-decolorizing bacteria**

Well-isolated colonies obtained on plates with halo zones were picked up, purified and used for decolorization assay with AR 337 dye concentration of 100 mg L⁻¹ using UV Spectrophotometer (Hach DR 6000TM, USA) at its respective λ max 500.4 nm. The percentage of decolorization is calculated using equation (1) (Ramya et al., 2010).

\[
\text{% of Decolorization} = \frac{(\text{Initial absorbance} - \text{Final absorbance}) \times 100}{\text{Initial absorbance}}
\]

**Identification of AR 337 dye-decolorizing bacteria using 16S rRNA technique**

The genomic DNA was extracted and cleaned up from the bacterial cells which showed high AR 337 dye – decolorization ability, using the PrepMan™ Ultra sample preparation reagent (PN 4322547). Universal primers 27F of (5’-AGA GTT TGA TCC TGG CTC AG-3’) and 1492R (5’-CGG YTA CCT TGT TAC GAC TT-3’) were used to amplify the 16S rRNA. The PCR reaction was run with the following thermal profile; initial denaturation for 10 min at 95°C, denaturation for 30 s at 95°C, annealing for 30 s at 60°C and extension for 45 s at 72°C and the final extension for 10 min at 72°C. These cycles were repeated for 30 times (Ewida, 2014). Nucleotide sequences were determined by a DNA sequencer, and the resulted sequence was deposited to Genbank (http://www.ncbi.nlm.nih.gov/Genbank).

**Optimizing the factors affecting decolorization of AR 337 dye by the isolated bacteria**

Various factors were optimized to achieve the highest decolorization capability of AR 337 by the best decolorizing bacteria in the consortium. The inoculum size, dye concentration, solution pH, incubation temperature and incubation time are the factors to be optimized. All the experiments were conducted in triplicates, in addition to control. To select the optimum inoculum concentration, a set of batch reactors in which some 250 ml Erlenmeyer flasks containing 100 ml of nutrient broth supplemented with 10 mg of AR 337 dye was inoculated with 2, 4, 6, 8 and 10 mg of bacterial cells. All batches were incubated at 35°C for 24 h, and the decolorization percentage was calculated. The optimum dye concentration selected by supplementing batches of 250 ml Erlenmeyer flasks containing 100 ml of nutrient broth with 50, 100, 200, 500 and 1000 mg L⁻¹ of AR 337 dye, inoculated with the optimum inoculum size of the isolated bacteria and the experiment was conducted as mentioned before.
Solution pH was adjusted at 5, 7 and 9, using hydrochloric acid and sodium hydroxide, the optimum concentration of dye was added, as well as, the optimum inoculum size of bacteria, other factors were kept as mentioned above. In order to detect the optimum incubation temperature, a wide range of temperatures 25°C, 30°C, 35°C and 40°C were tested, using the optimum inoculum size, optimum dye concentration, and optimum pH.

Finally, an experiment was conducted using the optimum inoculum size, optimum dye concentration, optimum pH and optimum incubation temperature for 7 days. The % of decolorization was measured daily.

Fate of the original AR 337 dye after decolorization using LC-MS

The decolorization experiment was conducted under the optimum conditions; samples were taken at various time intervals (0, 12 and 24 h) and used for LC-MS analysis (LC-MS-8030 triple quadruple Shimadzu, equipped with electrospray ionization (ESI) source and working in positive mode). Samples were filtered using a PVDF and nylon filters. Then, they were pre-concentrated (×100) with Strata™-X (Phenomenex) cartridges. The quantitative analysis was performed using the multiple reactions monitoring method. The parameters used were as follows: with C18 reverse phase column, capillary voltage of 4000 V, desolation line (DL) temperature of 250°C, interface temperature of 350°C, heat block temperature of 500°C, and nebulizing gas (N2) flow and drying gas (N2) flow 3 and 20 L min⁻¹, respectively. Argon was used as a collision gas. To study the degradation products, the samples were analyzed with an electrospray ionization (ESI) source (MicrOTOF-QII Bruker) in a positive mode. The following conditions were used: drying gas flow at 10 L min⁻¹, drying temperature at 200°C, ionization energy was 3 eV, the collision energy of the collision cell was 8 eV and the capillary voltage was 4500 eV. Nitrogen was used as both a collision and drying gas. The data were collected in the m/z range of 200–22000 at the speed of two scans per second, providing the resolution of 50,000 (FWHM). The best peak symmetry and resolution were obtained by using acetonitrile and water, both with 0.1% of formic acid, as mobile phase in gradient mode. Finally, compounds identification was corroborated based on the relative retention time and mass fragmentation pattern spectrums with those of standards and the NIST147 library database of the LC-MS system.

Application of decolorization technique on wastewater contaminated with red dye using Bacillus megaterium

Wastewater samples contaminated with red dye were collected from the oxidation pool of 10th of Ramadan city treatment station. To check the efficiency of the isolated bacteria in dye decolorization, a laboratory-scale experiment was set up using two 1 L Erlenmeyer flasks each containing 500 mL of such wastewater. One of them has been inoculated with the B. megaterium and incubated under the optimum conditions for decolorization, the other was left as control. The incubation time was extended to 14 days. The % of decolorization was measured daily guided by a spectral absorption curve made up for the wastewater sample before treatment.

Theory

Our environment is in danger due to the release of continuously emerging potential pollutants by human activities. Though many attempts have been made to remediate these noxious elements, every day thousands of xenobiotic of relatively new entities emerge, thus worsening the situation. Primitive microorganisms are highly adaptable to toxic environments and can reduce the load of toxic elements by their successful transformation and remediation. Some microorganisms have the ability to reduce the concentrations of a specific contaminant; others have a potential activity to completely remove it. The present work is considering the complete removal of a toxic azo dye acid red 337 using a newly isolated bacterial strain.

Results and discussion

Isolation of AR 337 dye-decolorizing bacteria from textile effluent wastewater

Three different bacterial colonies were grown with clear halo zones on nutrient agar supplemented with AR 337 dye after enrichment. They were isolated, purified and inventoried according to the morphological characteristics and given codes of 1A, 1B and 1C.

Screening of AR 337 high dye-decolorizing bacteria isolated from textile effluent wastewater

The selected bacterial isolates namely 1A, 1B and 1C were screened on nutrient broth supplemented with 100 mg L⁻¹ AR 337 dye, using the same bacterial cell concentration in each batch, and all incubated at 35°C for 24 h. The percentage of AR 337 dye decolorization was 22%, 27% and 73.5%, respectively. So, isolate 1C was selected as a strong AR 337 dye decolorizing bacterium and was used to perform the rest of the present research.
Identification of the AR 337 dye-decolorizing bacterial isolate

Based on 16 S rRNA sequence analysis, strain 1C was identified as Bacillus megaterium. The accession number given from GenBank nucleotide sequence database was KY848339.1.

Optimizing the factors affecting decolorization of AR 337 by Bacillus megaterium

Factors like inoculum size, dye concentration, solution pH, incubation temperature and incubation time were optimized to get the maximum capability of AR 337 dye decolorization by B. megaterium. When the inoculum size of bacterial cells was 2, 4, 6, 8 and 10 mg per 100 mL of nutrient broth amended with 10 mg of AR 337 dye, the decolorization % within 24 h. was 17, 42, 72.8, 83.9 and 87, respectively. So, the optimum inoculum size is (10% wt/v), where the gap between the percentage of removal of the color is so close for cell inoculum of 8% and 10% wt/v. In accordance with such findings, Mohan and coworkers used 10% of cell concentration of Bacillus sp. and reported a maximum of 48.15% decolorization for Coractive Blue P-3R (Mohan, Madhumitha, & Menon, 2013).

Dye concentration in the solution is an important factor affecting decolorization. AR 337 dye concentrations used are 50, 100, 200, 500 and 1000 mg L\(^{-1}\), all inoculated with 10% wt./v of B. megaterium cells, for 24 h, the decolorization % was 75, 87, 89, 85 and 22, respectively. So, the maximum capability of B. megaterium to remove AR 337 dye from solutions is 85% of 500 mg L\(^{-1}\) dye concentration. Concerning this high capability for dye removing, Ramya et al. (2010) reported the decolorization of acid red dye by Acinetobacter radioresistens by 86% at a concentration of 400 mg L\(^{-1}\).

The maximum decolorization capability of AR 337 by B. megaterium was achieved at pH 7 within 24 h. of incubation, it was 85%, while at pH 5 and 9, the percentage of decolorization was 45% and 67 %, respectively. Chan and Kuo (2000) mentioned that the neutral pH would be more favorable for decolorization of azo dyes and is suitable for industrial applications.

Figure 1. LC-MS chromatogram of acid red 337 degradation at time 0.
The effect of incubation temperature and incubation time was studied. A wide range of incubation temperature (25°C, 30°C, 35°C and 40°C) was tested, using inoculum volume of *B. megaterium* cells 10% wt./v, AR 337 dye concentration of 500 mg L\(^{-1}\) and neutral pH. A maximum decolorization of 91% was achieved at 30°C. From the literature, it was recorded that *B. megaterium* could grow in a temperature range of 3°C to 45°C with the optimum around 30°C (Sneath, Mair, Sharp, & Holt, 1986). It has also been recognized as an important industrial organism (Sugumar & Berla, 2012; Varel, Hashimoto, & Chen, 1980; Vary et al., 2007).

The results obtained for decolorization with time factor showed that decolorization efficiency was not affected by incubation time after the first 24 h. There were slight changes in decolorization % from the 1\(^{st}\) day of incubation (91%) to the 7\(^{th}\) (91.7%). In contrast to these findings, Sandra et al. (2012) had stated that the length of contact time can influence the removing ability and it can be varied in accordance with properties of the dye and the activity of the microorganisms.

**Fate of the original AR 337 dye after decolorization using LC-MS**

The products of degradation were followed up using LC-MS. Samples were taken at zero time, after 12 and 24 h of incubation of solutions containing 500 mg L\(^{-1}\) of AR 337 dye, inoculated with 10% (wt./v.) of cell concentration of *B. megaterium*. The chromatogram of Acid red 337 at 0 time showed a peak at a retention time (RT) of 6.20 which is corresponding to (2-(Trifluoromethyl) benzeneamine diazo and 6-amino-4-hydroxynaphthalene-2-sulfonic acid) [Acid Red 337]. The mass spectrometric peak at this retention time showed m/z ratio of 500.01 (Figure 1). After 12 h. of biodegradation two peaks were obtained, the first was at RT 4.52 with 181 m/z corresponding to 2-Trifluoro methyl 5-[(aminoxy) phenyl]. The second was at RT 6.23 with 277 m/z corresponding to 7,
8-diamino-3-{(aminoxy) sodium sulfonyl) naphthalene}−1-ol (Figure 2). Such findings ensure the cleavage of azo bond of the original compound. At the end of biodegradation time (24 h) four peaks were obtained, the 1st was at 5.51 RT with 44.2 m/z likely similar to Carbon dioxide, the 2nd was at RT 7.32 with 60.03 m/z likely similar to acetic acid, the 3rd was at RT 8.44 with 63.2 m/z likely similar to methyl formate, and the 4th at RT 9.33 with 87.11 m/z likely similar to methyl propionate (Figure 3). These results could give evidence that B. megaterium had completely degraded Acid red 337 dye and converted it to small and simple aliphatic compounds. The suggested degradation pathway (Figure 4) could be; cleavage of the azo bond resulting in the formation of two intermediate degraded products 2-Trifluoro methyl 5-{(aminoxy) phenyl) and 7, 8-diamino-3-{(aminoxy) sodium sulfonyl) naphthalene}-1-ol, which further degraded to methyl propionate, methyl formate and acetic acid. Then all intermediate by-products, finally, have converted to carbon dioxide.

Application of decolorization technique on wastewater contaminated with red dye using Bacillus megaterium

Decolorization of red-colored wastewater, collected from the oxidation pool of 10th of Ramadan city wastewater treatment plant by B. megaterium was observed within 10 days, in vitro. The decolorization % was assayed by UV Spectrophotometer (Hach DR 6000™, USA) at its respective λmax 550 nm according to the obtained spectral absorption curve. The efficacy of B. megaterium in color removing was slowed down at the beginning of the experiment, but it reached 98.9% after 10 days of incubation (Figure 5). Vary et al. (2007) recommended the use of such bacterium in protein industry, and the authors of the present work are also recommending Bacillus megaterium KY848339.1 as a promising and suitable microorganism to be used in the treatment of dye contaminated wastewater effluents.
Conclusion

*Bacillus megaterium* KY848339.1 is a bacterial strain isolated from a textile wastewater effluent. It could remove 91% of AR 337 azo dye in a concentration of 500 mg L\(^{-1}\) within 24 h. when the bacterial inoculum size was 10% wt./v, the solution pH was 7 and the incubation temperature was 30°C. The Liquid Chromatography-Mass Spectrum (LC-MS) analysis indicated the complete degradation of AR337 azo dye, where the final products of biodegradation were small aliphatic compounds and CO\(_2\). The application of *B. megaterium* on wastewater contaminated with red dyes using 10% wt./v of bacterial cells concentration resulted approximately in a complete removing of the red color (98.9%) through 10 days.

ORCID

Ayman Y. I. Ewida [http://orcid.org/0000-0001-6913-6748](http://orcid.org/0000-0001-6913-6748)
Marwa E. El-Sesy [http://orcid.org/0000-0002-9984-9383](http://orcid.org/0000-0002-9984-9383)

References

Acuner, E., & Dilek, F. B. (2004). Treatment of tectilon yellow 2G by *Chlorella vulgaris*. *Journal of Process Biochemistry*, 39, 623–631.
Chan, J., & Kuo, T. (2000). Kinetics of bacterial decolorization of azo dye with *Escherichia coli* NO3. *Journal of Bioresource Technology*, 75(2), 107–111.
Chen, C., Wu, J. Y., Liou, D. J., & Hwang, S. C. (2003). Decolorization of the textile dyes by newly isolated bacterial strains. *Journal of Biotechnology*, 101(1), 57–68.
Daneshvar, N., Khataee, A. R., Rasoulifard, M. H., & Pourhassan, M. (2007). Biodegradation of dye solution containing Malachite Green: Optimization of effective parameters using Taguchi method. *Journal of Hazardous Materials*, 143, 214–219.

Dave, S. R., & Dave, H. R. (2008). Isolation and characterization of *Bacillus thuringiensis* for Acid red 119 dye Decolourisation. *Journal of Bioresource Technology*, 100, 249–253.

Dawkar, V. V., Jadhav, U. U., Jadhav, S. U., & Govindwar, S. P. (2008). Biodegradation of disperse textile dye Brown 3REL by newly isolated *Bacillus* sp. VUS. *Journal of Applied Microbiology*, 105(1), 14–24.

Ewida, A. Y. I. (2014). Biodegradation of Alachlor and Endosulfan using environmental bacterial strains. *World Applied Sciences Journal*, 32(4), 540–547.

Fan, H. J., Huang, S. T., Chung, W. H., Jan, J. L., Lin, W. Y., & Chen, C. C. (2009). Degradation pathways of crystal violet by Fenton and Fenton-like systems: Condition optimization and intermediate separation and identification. *Journal of Hazardous Materials*, 171, 1032–1044.

Ghanem, K. M., Al-Garni, S. M., & Biag, A. K. (2011). Statistical optimization of cultural conditions for decolorization of methylene blue by mono and mixed bacterial culture techniques. *African Journal of Microbiology Research*, 5(15), 2187–2197.

Kalyani, D. C., Patil, J. P., Jadhav, S. U., & Govindwar, S. P. (2008). Biodegradation of reactive textile dye Red BLI by an isolated bacterium *Pseudomonas* sp. SUK1. *Journal of Bioresource Technology*, 99, 4635–4641.

Mohan, V., Madhumitha, M., & Menon, S. (2013). Isolation and screening of potential dye decolorizing bacteria from textile dye effluents in Tamil Nadu, India. *JAIR*, 2(2), 74–79.

Myrna, S., Aida, S., Herminia, I. P., Norimoto, M., & Maribel, F. (2012). Microbial decolorization of azo dyes: A review. *Journal of Process Biochemistry*, 47, 1723–1748.

Ramya, M., Iyappan, S., Manju, A., & Jiffe, J. S. (2010). Biodegradation and decolorization of acid red by *Acinetobacter radioreisistens*. *Journal of Bioremediation & Biodegradation*, 1, 105–111.

Robinson, T., Mcmullan, G., Marchant, R., & Nigam, P. (2001). Remediation of dyes in textile effluents: A critical review on current treatment technologies with a proposed alternative. *Journal of Bioresearch Technology*, 77(3), 247–255.

Sandra, T. A., Jose, C. V., Carlos, A. A., Kaoru, O., Aline, E. N., Ricardo, L. L., & Galba, M. C. (2012). A biosorption isotherm model for the removal of reactive azo dyes by inactivated mycelia of *Cunninghamella elegans* UCP542. *Journal of Molecules*, 17, 452–462.

Shaker, S., Zafarian, S., Chidurala, S., & Rao, K. V. (2014). Size control synthesis of magnetite nanoparticles for purification of wastewater, 5th International conference of nanostructures. Iran: Kish Island.

Sharma, J., & Janeva, B. (2008). A study on removal of Congo red from the effluents of textile industry using Rice Husk carbon activated with steam. *Rasayan Journal of Chemistry*, 1(4), 936–942.

Sneath, P. H., Mair, N. S., Sharp, E., & Holt, J. G. (1986). *Bergey’s manual of systematic bacteriology* (Vol. 2). Baltimore, MD: Williams and Wilkins.

Sugumar, S., & Berla, T. E. (2012). Biodegradation and decolorization of reactive orange 16 by *Nocardiopsis alba* soil isolate. *Journal of Bioremediation & Biodegradation*, 3, 155–161.

Varel, V. H., Hashimoto, A. G., & Chen, Y. R. (1980). Effect of temperature and retention time on methane production from beef cattle waste. *Journal of Applied and Environmental Microbiology*, 40, 217–222.

Vary, P. S., Biedendiek, R., Fuerch, T., Meinhardt, F., Rohde, M., Deckwe, W. D., & Jahn, D. (2007). *Bacillus megaterium* - from simple soil bacterium to industrial protein production. host. *Journal of Applied Microbiology and Biotechnology*, 76, 957–967.

Verma, A. K., Raghukumar, C., Parvatkar, R. R., & Naik, C. G. (2012). A rapid two-step bioremediation of the anthraquinone dye, reactive blue 4 by a marine-derived fungus. *Water, Air, & Soil Pollution*, 223, 3499–3509.