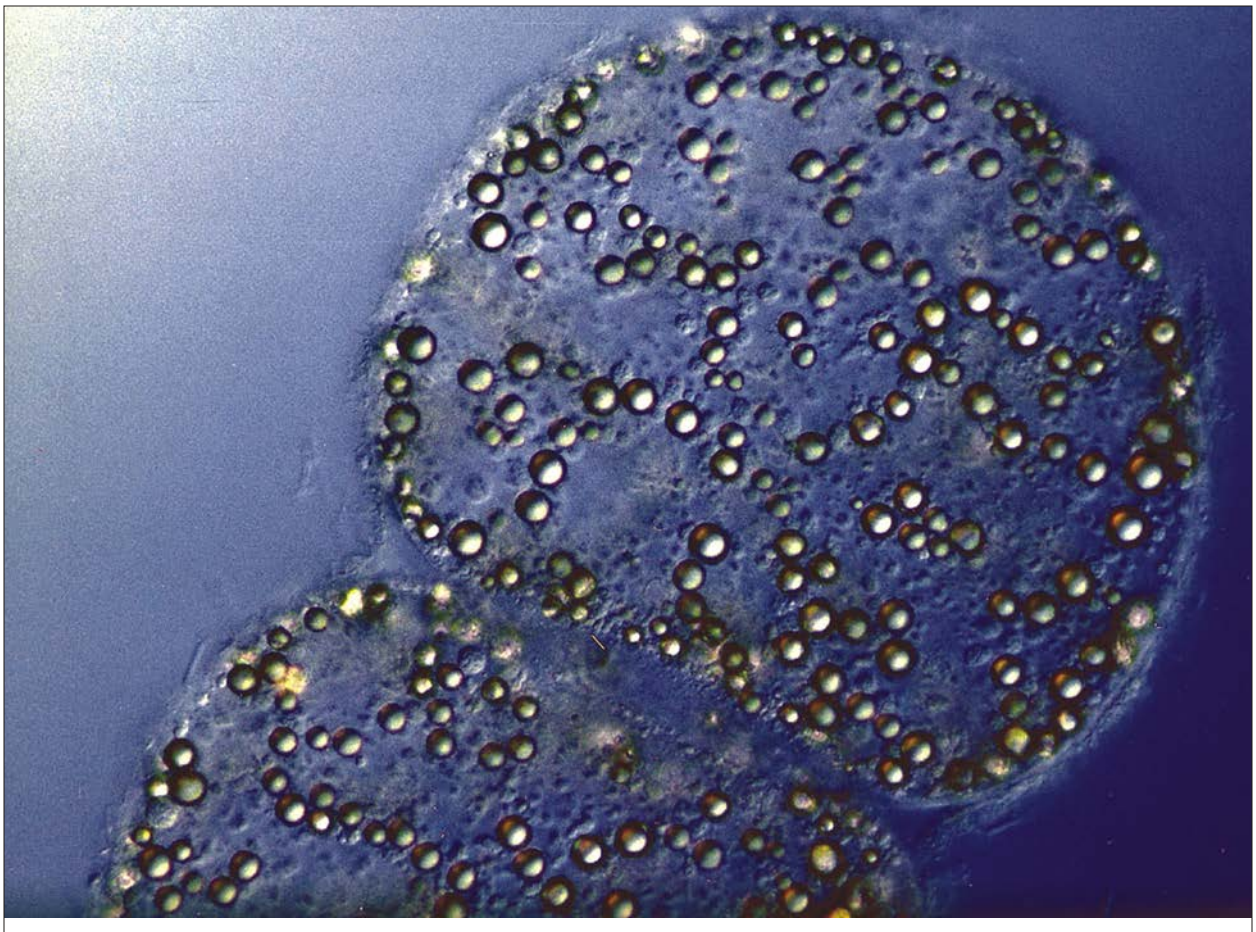

Microbiology

Journal

ISSN 2153-0696

Academic
Journals Inc.

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OPEN ACCESS Microbiology Journal

ISSN 2153-0696
DOI: 10.3923/mj.2016.15.24

Research Article
Biodegradation and Detoxification of Azo Dyes by Some Bacterial Strains

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Abstract

Background and Objective: Azo dyes are the most widely used synthetic colorants in industry and they are regarded as pollutants. Bioremediation through microorganisms has been identified as an effective method. The objective of this study is to examine the potential of four bacterial strains for decolorization and degradation of azo dyes produced in the final effluent of textile dying industries. **Materials and Methods:** Bacterial isolates of *Bacillus subtilis*, *Bacillus cereus*, *Bacillus licheniformis* and *Pseudomonas* sp., isolated from dye contaminated sludge environment were investigated for degradation of various azo dyes. Nutritional and environmental parameters affecting dye decolorization were optimized. The degradation and detoxification was confirmed by UV-vis characterization, FTIR analysis and toxicity studies. **Results:** Glucose was the most effective carbon source for maximum decolorization efficiency of *Pseudomonas* sp., for black B and congo red accounting 49.46 and 75.31%, respectively and of *Bacillus subtilis* for black B reached 48.68%. However, the highest congo red decolorization percentages by *Bacillus cereus* and *Bacillus licheniformis* reached 72 and 80.32%, respectively were recorded in the presence of starch. Organic nitrogen sources peptone and yeast extract were the best inducers for decolorization of black B and congo red dyes, respectively by the four strains. The decolorization of reactive dyes by bacterial strains was efficient at pH 7, temperature of 37°C, with 200 mg L^{-1} dye concentration and 20% (v/v) inoculum size under static condition at 72 h. The UV-vis spectra of the decolorized dyes showed disappearance of peaks, which indicated that the decolorization is due to biodegradation, rather than inactive surface adsorption. **Conclusion:** The toxicity test concluded that the degradation products were less toxic compared to wild dyes. The phytotoxicity study showed good germination rate as well as significant growth of *Vicia faba* seeds observed in degraded metabolites as compared to control. This study recommended the application of the these bacterial strains in the decolorization of the azo dyes in the industrial effluents under all nutritional and environmental conditions in Egypt.

Key words: Azo dyes, decolorization, phytotoxicity, congo red dye, black B

Received: January 10, 2016 Accepted: February 18, 2016 Published: March 15, 2016

Citation: Eman Zakaria Gomaa, 2016. Biodegradation and detoxification of azo dyes by some bacterial strains. Microbiol. J., 6: 15-24.

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Competing Interest: The author has declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Azo dyes are the most widely used synthetic colorants in comparison to natural dyes because of their many advantages, namely the ease and cost-effectiveness of synthesis, stability and availability in a variety of colours. They are used in various industries such as pharmaceutical, food, brewing, textile dyeing, printing, cosmetic and other industries (Singh *et al*., 2012).

Presence of color and causative compounds has always been undesirable in water used for either industrial or domestic needs (Kiran *et al*., 2012). The inefficiency in dyeing processes has resulted in 10-15% of unused dye stuff entering the wastewater directly (Dwivedi *et al*., 2012). The effluents with high levels of Biochemical Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) values are highly toxic to biological life (Palamthodi *et al*., 2013). In textile industry, the process of dyeing results in the production of large amounts of wastewater exhibiting intense coloration that has to be eliminated before release into natural water streams. In addition, colored water is objectionable as it can spoil the beauty of water environments (Andleeb *et al*., 2010). Discharging of such wastewaters into receiving streams not only affects the aesthetic aspects but also interferes with transmission of sunlight into streams and therefore reduces photosynthetic activity. Without adequate treatment, such dyes will remain in the environment for an extended period of time. The existence of the colorless aromatic amines in the aqueous ecosystems is of a serious environmental and health concern, hence they are regarded as pollutants (Sahasrabudhe and Pathade, 2012; Celik *et al*., 2012).

Several physico-chemical techniques have been proposed for treatment of colored textile effluents (Dafale *et al*., 2010). These include adsorption on different materials, oxidation and precipitation by Fenton's reagent, bleaching with chloride or ozone photo degradation or membrane filtration (Robinson *et al*., 2001). All these physical or chemical methods are very expensive and result in the production of large amounts of sludge, which creates the secondary level of land pollution. Therefore, economic and safe removal of the polluting dyes is an important issue. Bioremediation through microorganisms has been identified as a cost effective and environment friendly alternative for disposal of textile effluent (Ponraj *et al*., 2011).

The principal aim of this study is to demonstrate decolorization ability of *Bacillus subtilis*, *Bacillus cereus*, *Bacillus licheniformis* and *Pseudomonas* sp., isolated from dye contaminated sludge environment. Dye decolorization by bacterial cultures was optimized with respect to various nutritional sources and environmental parameters which are thought to affect decolorization process. The degradation and detoxification was confirmed by UV-vis characterization, FTIR analysis and toxicity studies.

MATERIALS AND METHODS

Dyes and chemicals: Six azo dyes, namely reactive black B, methyl red, orange G, green B, bromophenol blue and congo red were purchased from Sigma-Aldrich CO. Chemical compounds constituting BHM and used in all other experiments were also of analytical grade.

Isolation, screening and identification of dye degrading bacteria: Microorganisms with efficient decolorization potential of textile dyes particularly reactive azo dyes were isolated from soil collected from effluent channel of a local textile industry in El-Mahalla Elkobra. The media used for isolation and growth of microbial strains (and for decolorization of dyes under study) was Bushnell and Hass Medium (BHM) containing (g L^{-1}): Glucose 10, yeast extract 5, $MgSO_4$ 0.2, $CaCl_2$ 0.02, K_2HPO_4 1.0, NH_4NO_3 1.0 and $FeCl_3$ 0.05. The final pH was adjusted at 7.0. Reactive dyes selected for this study were sterilized by passing it through a 0.45 µm pore size filter and added to sterilize BHM from their respective filter sterilized stock solutions (Khalid *et al*., 2008). Dye was added at a concentration of 100 mg L^{-1}.

For enrichment study, 1 g soil was added to BHM containing 6 different dyes separately and incubated at 37°C. The decolorized cultures were transferred to the fresh media to obtain successive pure strains by streak plate technique in agar medium (Supaka *et al*., 2004). The pure bacterial strains were inoculated in BHM amended dyes and the potential degrader showing maximum decolorization was isolated for further degradation study.

The strains were identified by the API 50 CHL kit system (bioMérieux, Marcy l'Etoile, France) according to the manufacturer's instructions (Ghanbari *et al*., 2009) at the microbial culture collection center (MIRCIN), Faculty of Agriculture, Ain Shams University. The isolates were identified by using the API WEB software version 5.0 from bioMérieux and Bergey's Manual of Determinative Bacteriology for comparison of assimilation and/or fermentation pattern (Buchanan and Gibson, 1974). All identified isolates were kept at -80°C with glycerol (30% v/v). The strains were serially transferred at least three times prior to use.

Decolorization assay: Decolorization activity was expressed in terms of decolorization percentage and was determined by monitoring the decrease in absorbance at absorption maxima (λ max) of respective dyes (i.e., 597 nm for reactive black B, 430 for methyl red, 480 for orange G, 634 for green B , 533 for bromophenol blue and 490 for congo red) (Sugiura *et al*., 2006). The culture suspension was centrifuged at 10,000×g for 20 min for removal of the biomass. The degree of decolorization of the tested dye was measured at its respective maximum absorbance wavelength by UV-visible spectrophotometer (1800, Shimadzu, Japan). The uninoculated dye free medium was used as blank. All assays of dye decolorization were performed in triplicate and the decolorization efficiency was expressed as (Telke *et al*., 2010):

$$\text{Decolorization (\%)} = \frac{\text{Initial absorbance} - \text{Observed absorbance}}{\text{Initial absorbance}} \times 100$$

UV-vis characterization: UV-visible spectra of culture supernatants of 0 and 72 h were compared between 400-800 nm and possible degradation products were speculated.

Toxicity and phytotoxicity assays: The biodegraded products were tested for their toxic effect on the agriculturally important soil bacterial flora (Mali *et al*., 2000). Several bacterial strains were inoculated on nutrient agar medium. Wells were made on the respective media containing plates and filled with decolorized centrifuged broth. The plates were incubated at 37°C for 48 h. Zone of inhibition surrounding the wells represented the index of toxicity.

The phytotoxicity was carried out at room temperature using *Vicia faba* plant seeds. The plant seeds were tested with the wild dyes and the dyes degraded metabolites and their phytotoxic natures were analyzed. The control was carried out using distilled water at the same time. Experiments were carried out in triplicates. Germination (%), length of plumule (shoot) and radicle (root) was recorded after 7 days (Phugare *et al*., 2011).

Statistical analysis: All experiments were done in triplicate and the results were presented as Mean±standard deviation. The experimental data were analyzed by using SPSS. Statistical significance was accepted at a level of $p<0.05$.

RESULTS AND DISCUSSION

Removal of colored compounds from textile industry effluents by physico-chemical and biological methods is currently available. Biological decolorization of dye effluent is receiving much consideration due to cost effective and less regeneration by microorganisms such as bacteria, fungi, actinobacteria, yeast, algae and plants (Ramachandran *et al*., 2013). In the present study, four bacterial strains with efficient decolorization potential of textile dyes particularly reactive azo dyes were isolated from soil collected from effluent channel of a local textile industry at El-Mahalla Elkobra. It was expected that sites near textile industries contaminated with dyes harbor several microorganisms which are capable to coexist with higher toxic levels of pollution. These microorganisms adapt to the polluted environment thus that they can play an important role in clearance of this environment through their growth and function.

Screening experiments assessed the potential of 23 bacterial isolates from enrichment cultures for decolorizing different industrial dyes (100 mg L^{-1}) viz., reactive black B, methyl red, orange G, green B, bromophenol blue and congo red under static conditions in liquid medium. Out of these strains, the most efficient bacterial strains were selected and tentatively identified with an API 50CHL kit system as *Bacillus subtilis*, *Bacillus cereus*, *Bacillus licheniformis* and *Pseudomonas* sp. A screening test for the ability of these isolates to utilize azo dyes as a sole carbon source was established to select beside effective organisms, the dyes which would be used to complete the study. Reactive black B and congo red were also selected because they gave the highest color removal in screening experiments and represent the most usable groups of azo dyes. All isolates decolorize both dyes with different capacity ranging from 13.89-23.29% for black B and 10-25.98% for congo red. The difference in decolorization pattern is due to the dissimilarity in specificities, structure and complexity, particularly on the nature and position of substituent in the aromatic rings and the interaction with azo bond with different dyes (Vijaykumar *et al*., 2007).

Optimization of dye decolorization: Effectiveness of biological treatment system was greatly influenced by nutrients and the operational parameters. The influence of each parameter on the color removal process must be optimized to increase dye reduction.

Effect of carbon sources: It is clear from the results presented in Fig. 1a and b that all the isolated bacterial strains were able to grow on chosen additional carbon sources and showed higher decolorization percent than control indicating that all the tested sugars could be utilized effectively as carbon source by these isolates. The increase in decolorization percentage after addition of carbon sources is attributed to

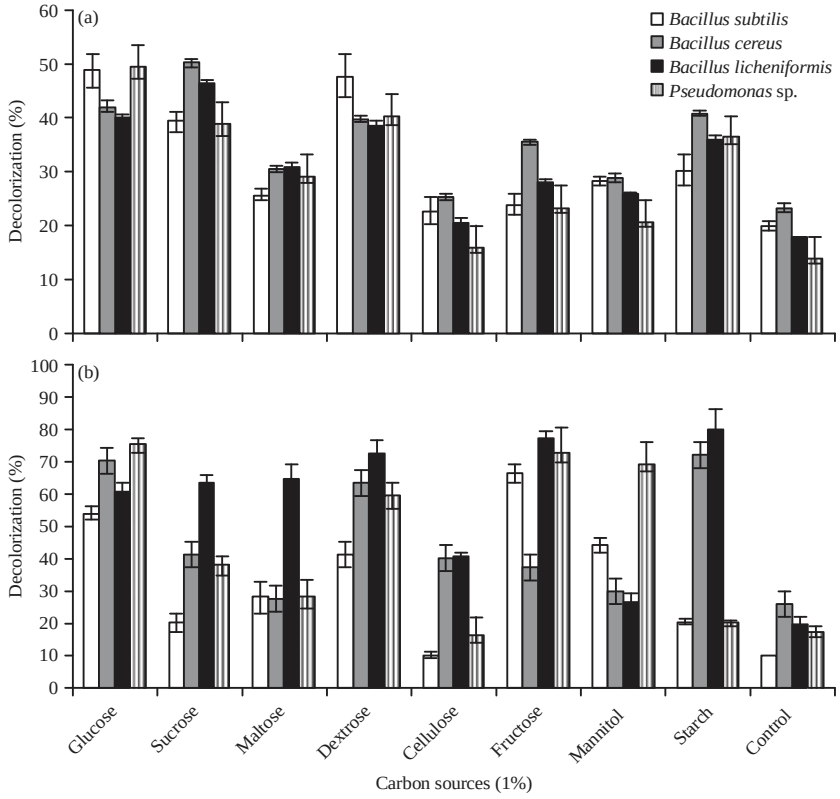

Fig. 1(a-b): Effect of carbon sources on decolorization activity of (a) Black B and (b) Congo red by *Bacillus subtilis*, *Bacillus cereus*, *Bacillus licheniformis* and *Pseudomonas* sp., values shown are the mean of three independent experiments, error bars represent the standard deviation

the fact that the dyes are deficient in carbon content and biodegradation without any extra carbon sources is difficult (Padamavathy *et al*., 2003).

Glucose was the most effective carbon source for maximum decolorization efficiency of *Pseudomonas* sp., for black B and congo red accounting 49.46 and 75.31%, respectively and of *Bacillus subtilis* for black B reached 48.68%. However, glucose is not commonly used in waste water treatment since it is an expensive carbon source. Therefore, various other inexpensive carbon sources such as starch, molasses and fructose have been applied in decolorization (Lucas *et al*., 2006). The highest congo red decolorization percentages by *Bacillus cereus* and *Bacillus licheniformis* reached 72 and 80.32%, respectively were recorded in the presence of starch. It can be concluded that in start metabolism of glucose or starch resulted production of nucleotides (NADH and FADH) which in turn lead to increase decolorization efficiency (Khehra *et al*., 2005). These nucleotides in reduced forms are recorded to act as redox mediators.

Effect of nitrogen sources: Concerning the effect of using different nitrogen sources for the purpose of decolorization of the azo dyes black B and congo red by the tested strains *Bacillus subtilis*, *Bacillus cereus*, *Bacillus licheniformis* and *Pseudomonas* sp., it was found that organic nitrogen sources peptone and yeast extract were the best inducers for decolorization of black B and congo red dyes, respectively by the four strains (Fig. 2a, b). Metabolism of peptone and yeast extract is considered essential for regeneration of NADH, which is the electron donor for azo bond reduction (Asad *et al*., 2007). Azoreductase catalyzes the NADH-dependent reduction of azo compounds to the corresponding amines, which involves cleavage of the azo linkages (-N=N-), resulting in azo dye degradation (Chen, 2006).

Addition of urea caused a decrease in decolorization potential of all tested microbial strains. Urea when dissolved in liquid culture causes a shift of pH more towards acidic side, which decreased the color removal, growth as well as enzyme activity of strains. The lowest black B and congo red removal

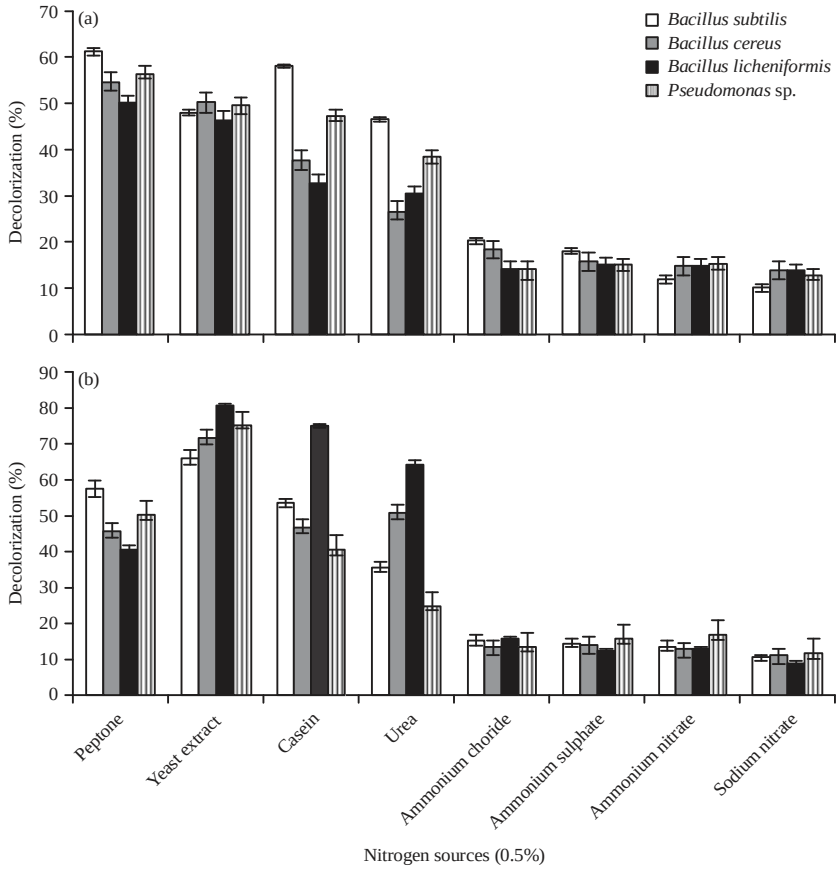

Fig. 2(a-b): Effect of nitrogen sources on decolorization activity of (a) Black B and (b) Congo red by *Bacillus subtilis*, *Bacillus cereus*, *Bacillus licheniformis* and *Pseudomonas* sp., values shown are the mean of three independent experiments, error bars represent the standard deviation

were attained by using sodium nitrate as a nitrogen source by the four strains. Presence of nitrate in culture media might slow down the process of color removal, because it serves as electron acceptor which could interfere the first step in dye decolorization (Panswad and Luangdilok, 2000).

Effect of dye concentrations: The decolorization performance of black B and congo red by the four strains was also studied at various dye concentration (50, 100, 150, 200 and 250 mg L^{-1}). Rate of decolorization increased with increase in initial dye concentration up to 200 mg L^{-1} (Fig. 3a, b). Further increase in dye concentration resulted in reduction in decolorization rates. This might be attributed to the toxicity of dye to bacterial cells through the inhibition of metabolic activity, saturation of the cells with dye products, inactivation of transport system by the dye or the blockage of active site of azoreductase enzymes by the dye molecules (Mabrouk and Yusef, 2008), so the effect of dye concentration on growth of organisms is an important consideration for its field application.

Effect of inoculum concentrations: The optimum inoculum size needed for higher decolorization percentage by the four isolates was tested at different inoculum concentrations starting from 5-30% (v/v). The decolorization rate increased with increase in the inoculum size, reaching maximum at 20% (v/v). However, beyond 20% (v/v) inoculum size, rate of decolorization did not vary significantly (Fig. 4a, b).

Effect of pH: The pH of the medium is also an important factor with regards to decolorization. The rate of color removal is higher at the optimum pH and tends to decrease rapidly at strongly acid or strongly alkaline pH. It is thought that the effect of pH may be related to the transport of dye molecules across the cell membrane, which is considered as the rate limiting step for decolorization (Kodam *et al*., 2005). In the present study, the maximum decolorization of black B and congo red were observed with all isolates at pH 7. At lower pH values, the H^+ ions compete effectively with dye cations, causing a decrease in color removal efficiency. Furthermore, at high pH, the surface of biomass gets negatively charged,

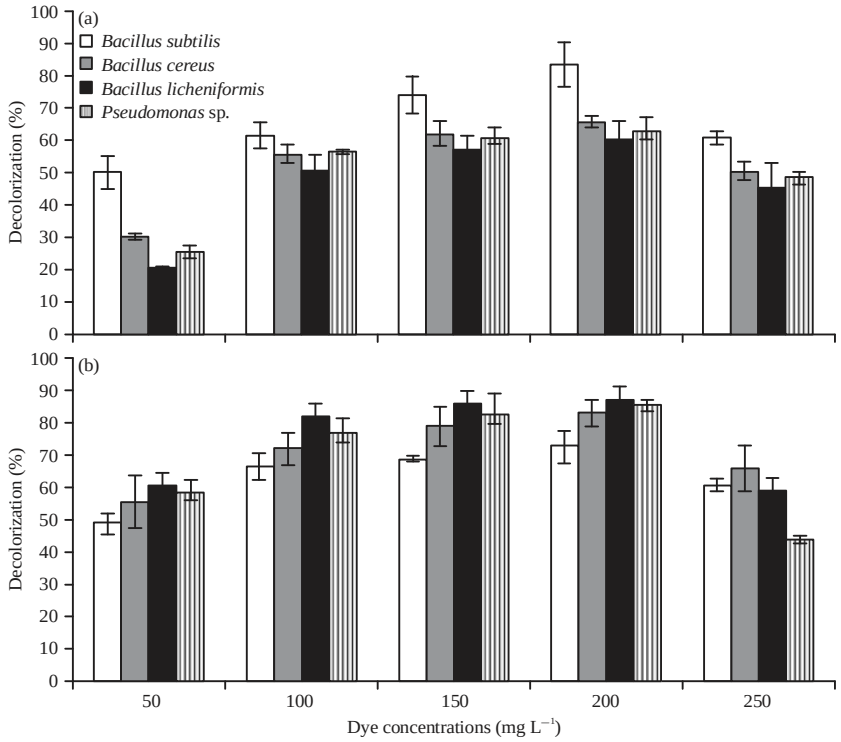

Fig. 3(a-b): Decolorization performance of *Bacillus subtilis*, *Bacillus cereus*, *Bacillus licheniformis* and *Pseudomonas* sp., at different dye concentrations, (a) Black B and (b) Congo red, values shown are the mean of three independent experiments, error bars represent the standard deviation

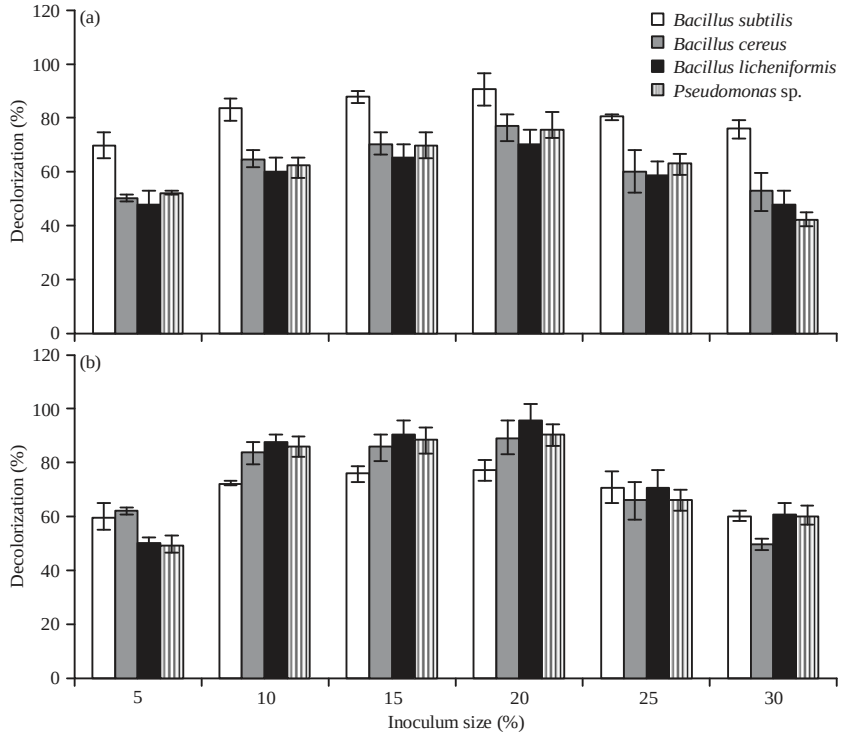

Fig. 4(a-b): Effect of inoculums size on decolorization activity of (a) Black B and (b) Congo red by *Bacillus subtilis*, *Bacillus cereus*, *Bacillus licheniformis* and *Pseudomonas* sp., values shown are the mean of three independent experiments, error bars represent the standard deviation

which enhance the positively charged dye cations through electrostatic force of attraction (Ayed *et al*., 2009).

Effect of temperature: Temperature is the most critical parameter for dye decolorization. Growth of microorganism is a cumulative activity of a large number of reactions mediated by enzymes. Therefore a direct relationship is observed between rate of microbial growth and these enzymatic reactions. These enzymatic reactions are thus directly influenced by temperature. In most of cases growth increases with increase in temperature but it represses sharply and abruptly at extreme upper and lower limits of temperature. In the present study, the dye decolorization activity of all strains was found to increase with increase in incubation temperature from 20-37°C with maximum activity attained at 37°C. Further increase in temperature resulted in maginal reduction in decolorization activity. Decline in decolorization activity at higher temperature can be attributed to the loss of cell viability or to the denaturation of the azoreductase enzyme (Cetin and Donmez, 2006).

Effect of agitation: It was observed that under static conditions, the decolorization of all dyes tested was higher than under agitation. Hence, static conditions were preferred to investigate bacterial dye decolorization in further experiments (Fig. 5a, b). It was found out that under agitation conditions, presence of oxygen deprives the azoreductase from obtaining electrons needed for cleavage of azo dyes under static conditions and these electrons are available to azoreductase from NADH to decolorize azo dyes (Khalid *et al*., 2008).

Effect of incubation time: In a trial to determine the best incubation period for dyes decolorization, the decolorization activity was measured after 24, 48, 72, 96 and 120 h, the best decolorization results were recorded at 72 h in all cases and the increase in decolorization activity after 72 h was not affective.

UV-visible spectra: Decolorization of the dye solution by bacteria could be due to adsorption to microbial cells or to biodegradation. In adsorption, examination of the absorption spectrum would reveal that all peaks decreased approximately in proportion to each other. If dye removal is attributed to biodegradation, either the major visible light absorbance peak would completely disappear or a new peak would appear.

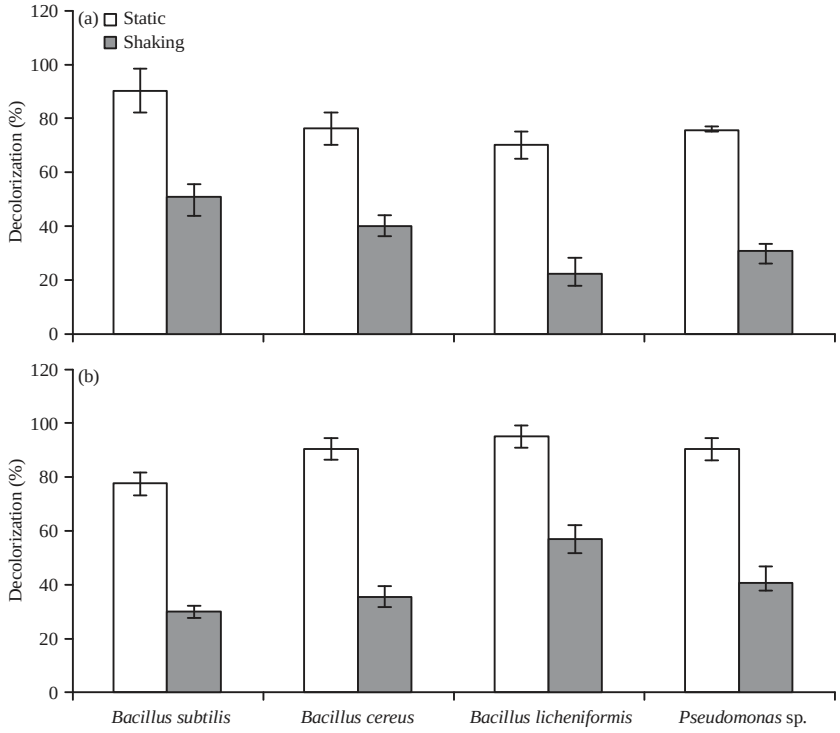

Fig. 5(a-b): Decolorization of (a) Black B and (b) Congo red at static and shaking conditions by *Bacillus subtilis*, *Bacillus cereus*, *Bacillus licheniformis* and *Pseudomonas* sp., values shown are the mean of three independent experiments, error bars represent the standard deviation

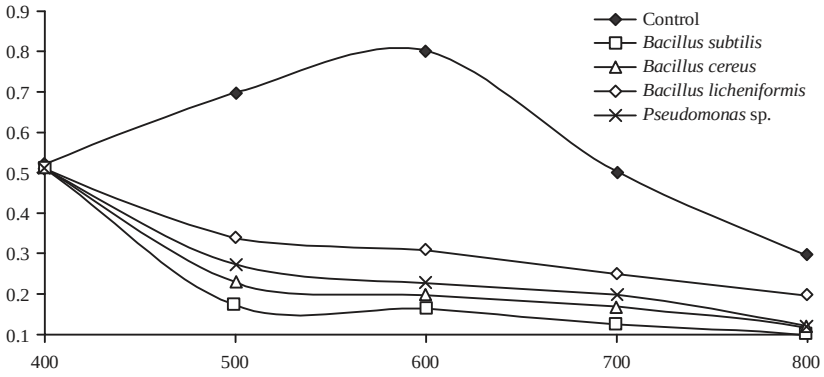

Fig. 6: UV-Visible spectral analysis of black B before and after treatment with bacterial strains

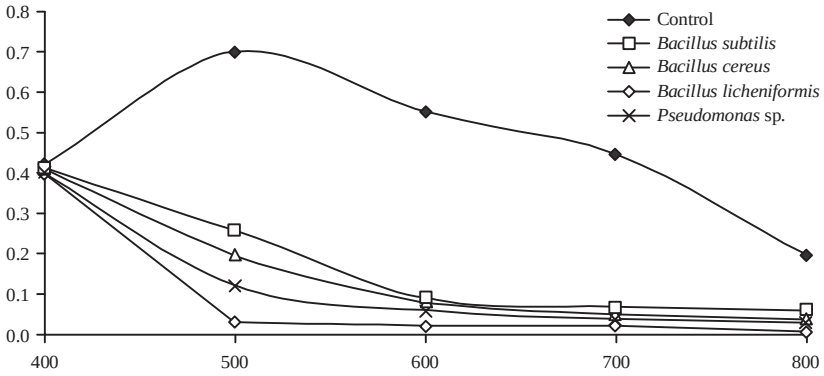

Fig. 7: UV-Visible spectral analysis of congo red before and after treatment with bacterial strains

Moreover, dye adsorption would result in cell mats which are deeply colored because of adsorbed dyes, whereas those retaining their original colors are accompanied by the occurrence of biodegradation.

In the present study, all bacterial isolates pellets retained its original color and did not become deeply colored because of adsorbed dyes. This indicate that, color removal was due to degradation not to adsorption (Chen *et al*., 1999). The UV-vis spectra from 400-800 nm corresponding to initial and final samples of black B and congo red are shown in Fig. 6 and 7. The initial dye solution showed high peak at the wavelength of 597 and 490 nm for black B and congo red, respectively. The decolorized dyes showed disappearance of peaks with all isolates, which confirmed that the decolorization is due to dye degradation. Further characterization of these bacterial strains and their enzymes are necessary for intense field application study.

Toxicity assays: Detoxification of the azo dyes by microbial cultures was attributed to the conversion of azo-nitrogen to non-toxic metabolites. The initial step of biodegradation of azo dyes is a reductive cleavage of the azo group, this reaction is catalyzed by a variety of biological systems and leads to the accumulation of aromatic amines. Aromatic amines generated by the reductive cleavage of the azo dyes are potentially toxic, mutagenic and carcinogenic (Chen *et al*., 2007). Interest is now focused on the bacteria which can perform high rate of discoloration and provide detoxification of aromatic products under aerobic environment. In the present study, no zone of inhibition observed surrounding the wells containing decolourized dye water, indicated that the biodegraded or decoulourized products were non-toxic to beneficial soil bacteria. Resulted illustrated in Table 1 showed good germination rate as well as significant growth of plumule and radical for *Vicia faba* seeds observed in degraded metabolites as compared to the dye sample. In raw dyes, less germination (16.67-32.22%), radical (2.36-3.22 cm) and plumule growth (1.65-2.50 cm) was observed. After bacterial treatment of dyes, the maximum germination (65.67-70.90%) radical (7.98-8.55 cm) and plumule growth (5.80-6.36 cm) were observed. The phytotoxicity tests indicated that degraded metabolites had almost a

Table 1: Phytotoxicity studies of black B and congo red and their metabolites formed after biodegradation by bacterial strains on germination and seedlings of *Vicia faba*

		Black B					Congo red				
Parameters	Distilled water	Raw dye	*Bacillus subtilis*	*Bacillus cereus*	*Bacillus licheniformis*	*Pseudomonas* sp.	Raw dye	*Bacillus subtilis*	*Bacillus cereus*	*Bacillus licheniformis*	*Pseudomonas* sp.
Germination (%)	100.00±0.00	16.67±3.11	60.32±2.25	54.45±1.28	50.20±2.36	46.55±3.18	32.22±2.21	62.03±1.98	56.32±1.46	70.90±2.45	65.67±3.00
Radical (cm)	10.77±0.20	2.36±0.60	6.60±0.01	4.26±0.54	3.90±0.22	4.34±0.34	3.22±0.12	5.19±0.32	6.77±0.50	8.55±0.21	7.98±0.36
Plumule (cm)	7.40±0.13	1.65±0.15	5.80±0.14	3.11±0.58	3.02±0.14	2.70±0.05	2.50±0.34	3.70±0.10	3.47±0.11	6.36±0.21	4.16±0.34

negligible effect on the plant seeds as compared to that of dye, which is indicative of the less toxic nature of the metabolites. In the same line, Jadhav *et al*. (2010) observed that the *Pseudomonas aeruginosa* strain BCH was able to detoxify the dye, direct orange 39 effectively which was tested with *Triticum aestivum* and *Phaseolus mungo*.

CONCLUSION

This study recommended the application of the these bacterial strains in the decolorization of the azo dyes in the industrial effluents under all nutritional and environmental conditions in Egypt.

SIGNIFICANCE STATEMENTS

- Azo dyes are the most widely used synthetic colorants in industry and they are regarded as pollutants
- *Bacillus subtilis*, *Bacillus cereus*, *Bacillus licheniformis* and *Pseudomonas* sp., have shown significant biodegradation of azo dyes
- The toxicity test concluded that the degradation products were less toxic compared to wild dyes and the phytotoxicity study showed good germination rate of *Vicia faba* seeds
- This study recommended the application of the these bacterial strains in the decolorization of the azo dyes in the industrial effluents under all nutritional and environmental conditions in Egypt

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