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Efficient biodegradation and detoxification of Congo red via newly isolated Bacillus strains

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Abstract

Pollution with xenobiotic compounds such as congo red (CR) is a serious problem in the environment. In the present study, an attempt has been made to screen and isolate a potential local bacterial strains from dye contaminated area for decolorization of CR dye as an example of (azo-dye). Out of 52 bacterial isolates demonstrated the ability to grow on CR dye as a sole carbon source, three bacterial isolates showed high dye decolorization percentage ranged from 65.8 to 81.5% after 4 days of incubation (λ max of CR 498 nm). The selected bacterial strains were identified as *Bacillus licheniformis* S2, *Bacillus amyloliquefaciens* S12 and *Bacillus subtilis* S50 based on morphological, molecular and biochemical characteristics. A set of experiments were carried out to optimize the degradation ability of the selected isolates under different physicochemical conditions such as different concentrations of CR, pH range, temperatures and static and shaking incubation. The results revealed that the three *Bacillus* species were able to degrade CR more efficiently at static conditions compared to shaking conditions and the optimum CR dye concentration for the three isolates was 100 μM at 37 ºC and pH 7.0. Spectroscopic techniques were performed to evaluate the isolates degradation abilities and to identify the degradation by-products by UV-Vis and GC-MS, indicated the complete degradation of congo red to other less-toxic compounds after 6 days of incubation. The phytotoxicity test on *Vicia faba* and *Triticum aestivum* seeds revealed that all bacterial degraded dye metabolites had almost negligible effect on both plant germinations compared to untreated dye, which indicating the successful detoxification of CR dye. Therefore, the selected *Bacillus* strains from this study might be useful in the treatment of industrial effluent contaminated with synthetic dyes.

Key words: Biodegradation; Congo Red, *Bacillus*, Decolorization; Synthistic dyes

1. Introduction

Environmental pollution is considered as one of the major health threats to biological life in the modern world. The rapid industrialization leads to uncontrolled release of many chemical pollutants globally, including heavy metals and xenobiotic such as synthetic dyes [1,2]. About one million tons of dyes and pigments are produced every year. The colour of a dye is due to the presence of a chromophore group. Based on the chemical structure of chromophore, there are 20-30 different groups of dyes. Azo, anthraquinone, phthalocyanine, and triarylmethane dyes are the most important groups [3,4]. Azo dyes are the most widely used synthetic colourants in comparison to natural dyes because of their versatile colour range, stable chemical structure, and the ease and cost-effectiveness of synthesis. Congo red (CR), a secondary di-azo dye, is a sodium salt of benzidinediazo-bis-1- naphthylamine-4-sulfonic acid. Congo red is resistant to most of the treatments and its thus found in effluents of many industries [5]. It is also known to cause allergic reactions and to be metabolized to benzidine, a known human carcinogen compound [6,7]. In addition, CR has high solubility in aqueous solutions and high persistent nature once it is discharged into the environment causing great harm to the surroundings due to its strong toxicity and carcinogenic effect [8,9]. Several physico-chemical techniques have been proposed for treatment of dye-polluted effluents. However, these methods are expensive and not always suitable to remove all dyes hence resulting in the production of large amounts of contaminated sludge, which creates a secondary level of soil pollution [10]. Dye biodegradation by microorganisms is a pollution control technology that uses naturally occurring microbes to catalyze the degradation of various toxic chemicals to less harmful forms. It is considered an effective alternative technique to clean up the environment [8]. The prerequisite for the complete mineralization of azo dyes is a combination of reductive and oxidative steps.

Decolourization of azo dyes normally begins with initial results into colorless aromatic amines compounds, then these are further oxidized to simpler nontoxic forms strictly under aerobic conditions [11]. Thus, anaerobic or aerobic processes are important for the complete mineralization of azo dyes [8]. The removal of CR by local bacterial strains is eco-friendly for the remediation of azo dyes pollutants from industrial effluent compared to conventional treatment techniques [8, 10]. Therefore, the aim of our study was focused on exploring the local microbial diversity of textile wastewater and soil and the possibility of using local bacterial isolates for decolourizing a dye-containing solution under different environmental conditions. In addition, postulating the biodegradation pathway of the best selected isolate.

2. Materials and Methods

2.1. Isolation of azo dye degrading bacteria from polluted soil

Polluted soil samples were collected from the dyes waste disposal site of textile factory, harboring dyes, detergents, chemicals, and tanneries, in Shubra El Kheima, Cairo, Egypt. The soil samples were transferred in sterile falcon tubes to the laboratory. Isolation of dye-degrading bacteria was carried out by enrichment technique. Ten grams from each soil sample suspended in 90 ml MY medium contain (g/L): Na_2HPO_4 (2); KH_2PO_4 (1); $(NH_4)_2SO_4$ (2); $MgSO_4 \cdot 7H_2O$ (0.4); NaCl (3); $CaCl_2 \cdot 2H_2O$ (0.1); $FeCl_3 \cdot 6H_2O$ (0.05); yeast extract (0.2); supplemented with 14 µM congo red at a pH of 7. After seven days of incubation at 37 ℃, one ml was inoculated into another fresh medium supplemented with 140 µM congo red for another seven days. Aliquots from the cultures were serially diluted up to 10^{-7} dilutions using sterile saline solutions. The diluted suspensions (10^{-5}, 10^{-6} and 10^{-7}) were spread onto MY agar plates. After incubation at 37 ℃ for 48 h, the bacterial colonies surrounded by clear or bright zone were selected for further purification and evaluation.

2.2. Congo red decolorization assay

To evaluate the selected bacterial isolates for congo red decolorization, Erlenmeyer flasks (250 ml) containing 50 ml of MY broth media with 100 µM CR were inoculated with 1% (v/v) fresh bacterial suspension. The flasks were kept in a static incubator at 37 ℃ for six days. After three days of incubation, aliquots (1 ml) were drawn every 24 h from each bacterial culture. The collected aliquots were centrifuged at 10000 rpm for 10 min then were filtered through a cellulose acetate filter with 0.45 µm pore size. The bacterial filtrates were measured at different wavelengths including 497, 510, 530 and 560 nm using spectrophotometer (Jenway 6305, Staffordshire, UK). The control was medium containing the same concentration of dye without inoculums. Decolourization percentage was recorded by measuring the absorbance of the bacterial supernatant using the following formula: % of Decolourization = $\frac{A0 - At}{A0}$ x 100 where A0; is the absorbance of uninoculated broth and At; is the absorbance of bacterial supernatant at time (t).

2.3. Identification of the best dye-decolorization bacterial isolates

Based on the bacterial decolorization assay, the best bacterial isolates were selected for further characterization. The selected isolates (S2, S12 and S50) initially were examined for colony characteristics then under the microscope for Gram stain, cell size and morphology, and spore formation ability. Then biochemical and physiological tests were carried out with specific requirements for each test as outlined in Bergey's Manual of Determinative Bacteriology [12]. The biochemical tests including, catalase activity test, Oxidase activity test, methyl red and Voges-Proskauer test, indole production test, nitrate reduction test, citrate utilization test, starch hydrolysis test, gelatin hydrolysis, mannitol fermentation test, growth at 10% NaCl and growth at 55 ℃. The identification was confirmed by the amplification of 16S rRNA gene by PCR and sequencing of the produced amplicon as described before [2] and the API 50 CHB Kit specialized for the identification of *Bacillus* and related genera [13]. The sequences of 16S rRNA gene of three isolates were compared to references of 16S rRNA gene sequences of other bacterial isolates retrieved from National Center for Biotechnology Information (NCBI) database. The MEGAX software was used to build the phylogenetic relationship for the chosen bacterial isolates using the maximum likelihood approach and the Kimura 2-parameter model.

2.4. Physicochemical characteristics affecting dye decolorization

In order to obtain maximum decolorization for the selected bacterial isolates, series of different CR concentrations (50, 100, 200, 300, 400, 500 µM) were assayed by the three selected isolates at 37 °C, pH 7 and static condition. The cell-free supernatant of each culture was measured at λ max 498 nm of CR after three days of incubation. In addition, the decolorization of CR was evaluated at various incubation temperatures (30, 37, 40 and 45 °C) or in the same medium adjusted to different values of pH including 5, 7, and 9. The degree of bacterial

decolorization was calculated from triplicate experiments as described above. Furthermore, the effect of agitation (static and shaking incubation) on CR decolorization was tested in two sets of triplicates of 140 μm CR inoculated with 1% (v/v) of each bacterial strain suspension. One set was incubated at 150 rpm while another set was kept under static conditions. After three days of incubation at optimum temperature and pH, the effect on decolorization was compared.

2.5. Biodegradation metabolites analysis

The ability of the bacteria to degrade of CR was investigated initially by UV-Visible spectrophotometer recording of spectrum from wavelength of 200 to 650 nm. Gas Chromatography Mass Spectroscopy (GC-MS) spectra technique was applied to compare the final metabolites result from CR degradation by the three selected strains with its abiotic control and was used for identification of the produced CR metabolites. After six days of incubation, 100 ml of liquid culture was centrifuged at 10000 rpm for 10 min and equal volumes of ethyl acetate was used to extract metabolites from the supernatant. The extracts were evaporated with anhydrous Na_2SO_4 in a rotary evaporator, dried at 40 °C and used for further analysis [14].

The extract obtained from rotary evaporator re-dissolved in methanol (high performance liquid chromatography grade) for GC-MS analysis. The GC-MS analysis of the dye was carried out utilizing a TRACE™ GC Ultra gas chromatograph instrument coupled with a ISQ Single Quadrupole Mass Spectrometer detector (Thermo Scientific, CA, USA) stands National Research Center. The GC-MS instrument was equipped with a TG-5MS column (30 m x 0.25 mm i.d., 0.25 μm film thickness). The analyses were performed with a split ratio of 1:10. The initial temperature was 60 °C for 1 min that rising 3.0 °C per min till 240 °C then injector and detector were held for one min. The CR metabolites and control samples were diluted in hexane (1:10, v/v) and 2 μL of the samples were injected. Mass spectra were recorded at 70 eV of electron ionization (EI), using a m/z spectral range from 40 to 450. Finally, the bacterial degradation products were identified by the comparison of both products' retention time and fragmentation patterns and the library of authentic chemicals mass spectra (Wiley spectral library and NSIT library collection).

2.6. Phytotoxicity assay

The phytotoxicity of CR dye before and after treatment with the selected bacterial species was carried out on two seeds representing monocot; *Triticum aestivum* and dicot; *Vicia faba* plants following the method described before [15]. The seeds were tested at early seedling growth with the CR dye, bacterial treated dye and sterile distilled water used as a control. The cell-free supernatant of each bacterial culture was obtained by centrifuging at 10000 rpm for 15 minutes then filtration by syringe filter (0.2 μM). The untreated dye (10 μM) and dye filtrates (10 ml) were used to irrigate ten seeds placed in sterilized Petri dishes. All samples were incubated at the same environmental conditions, each treatment was performed in three replicates. The percentage of germination was recorded after 7 days. Two controls are included in the experiment. The negative control was irrigated with sterilized distilled water while the positive control was irrigated with the dye buffers without the bacteria.

2.7. Statistical analysis

The percentage of decolorization is presented as the mean ± standard deviation (SD) from at least three replicates. The obtained data are analysed statistically using IBM SPSS (Statistics, 20), to determine the degree of significance using one-way analysis of variance (ANOVA) at a probability level $(P) \leq 0.05$.

3. Results
### 3.1. Screening of dye degrading isolates from contaminated soil samples

The enrichment techniques performed in this study successfully yielded 52 bacterial isolates demonstrated the ability to grow on CR dye as sole carbon source. The bacterial isolates were purified, and only 16 isolates were able to grow at higher concentration of CR (140 μM) then the isolates with similar morphological characteristics were excluded. Based on the clear zone formed after bacterial dye decolorization, six morphologically distinct isolates were chosen (Figure 1).
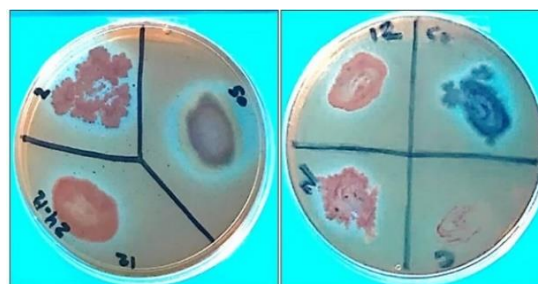
Fig. 1. The efficiency of congo red decolorization by the bacterial isolates S2, S12, S50 and negative control isolate in MY medium supplemented with 140 μM congo red.

3.2. Evaluation of CR degradation abilities in liquid broth

The disappearance of congo red color had been measured at different wavelengths to evaluate the abilities of different isolates to decolorize the congo red color. The results revealed that at all wavelengths the same trend of degradation percentage was obtained (Figure 2). The S50 was the best isolate reached 88.2% after 4 days of incubation at 530 nm followed by isolate S2 (72.3%) and S12 (69.2%) at the same incubation period. Prolong incubation to 5 and 6 days did not improve the decolorization percentage at all tested wavelengths. Therefore, these three bacterial isolates were selected for identification and further analysis.
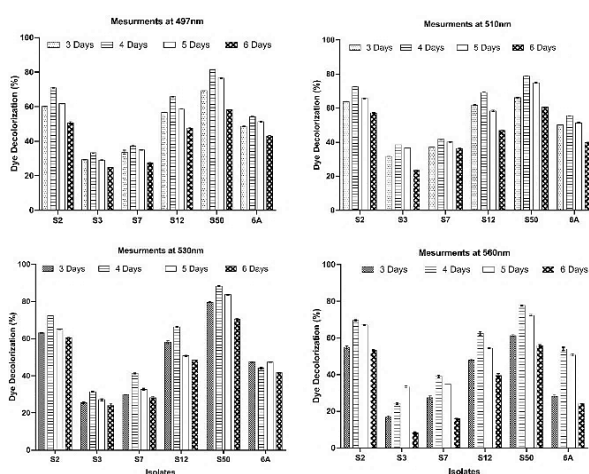
Fig. 2. The decolorization percentage of Congo Red at different wavelength (497, 510, 530 and 560) by the dye-degrading isolates (S2, S3, S7, S12, S50 and 6A). The bars represent the average values of three different replicates ± standard deviation.

## 3.3. Identification of the selected dye-degrading isolates

The examination of isolates colony morphology showed that the three isolates were different in shape and appearance, isolate S2 and S12 showed wrinkled colonies with some slime formation, while S50 showed smooth colonies. The results of microscopic examination revealed that all isolates were motile Gram-positive, rod shaped with sizes (2.9 by 0.75 µm) and were able to form subterminal endospore (Table 1), suggested that the isolates is belonging to the genus *Bacillus*. The biochemical characteristics were used to identify the three dye-degrading isolates to the species level. The isolates characteristics suggest that isolates S2 more related to *B. licheniformis* as it was the only isolate that can grow at 10% NaCl and 55 °C and hydrolyze arginine. The isolate S12 was positive for

citrate utilization and negative for β-galactosidase and isolate S50 was positive for Methyl red. These results were matched with those in Bergey's Manual of Systematic Bacteriology and isolates S12 and S50 were identified as *Bacillus amyloliquefaciens* and *Bacillus subtilis* respectively.

Table 1. Results of biochemical tests on dye-degrading bacterial isolates S2, S12 and S50

Characteristics	Bacterial isolates		
	S2	S12	S50
Gram Reaction	+	+	+
Spore formation	+	+	+
Motility	+	+	+
Anaerobic growth	+	+	+
Catalase	+	+	+
Voges Proskauer	+	+	+
Methyl red	-	-	+
Indole production	-	-	-
Oxidase	+	+	+
Citrate utilization	-	+	-
Hydrolysis of			
Starch	+	+	+
Gelatin	+	+	+
Casein	-	+	+
β-galactosidase	+	-	+
Arginine dihydrolase	+	-	-
Acid from			
Glucose	+	+	+
D-Mannitol	+	+	+
Nitrate to nitrite	+	+	+
Growth at NaCl (6.5%)	+	+	+
Growth at NaCl (10%)	+	-	-
Growth at 45 ℃	+	+	+
Growth at 55 ℃	+	-	-
Identification	*B. licheniformis*	*B. amyloliquefaciens*	*B. subtilis*

Furthermore, the 16S rRNA genes sequence analysis confirmed that the three isolates were belonging to Bacillus genera. The isolates S2, S12 and S50 exhibited a high degree of sequence similarities (99.9–100%) with 16S rRNA gene from other references species of *B. licheniformis*, *B. amyloliquefaciens* and *B. subtilis* in GenBank respectively. The phylogenetic tree of the three isolates with the references related *Bacillus* species were generated using the program MEGAX using the maximum likelihood method by 1000 bootstrap analyses (Figure 3).
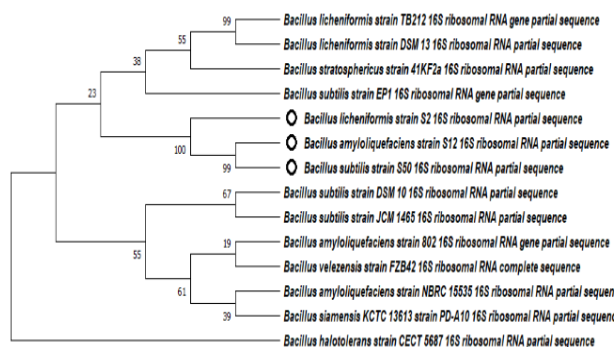
Fig. 3. The evolutionary analysis of the best biologically active bacterial isolates as analyzed by phylogenetic tree constructed by the maximum likelihood method using MEGAX software for the 16S rRNA gene sequences of *Bacillus* sp. S2, S12 and S50. The numbers at nodes represent the percentage values given by 1000 bootstrap samples analysis.

3.4. The effect of different factors on dye decolorization

To assess the tolerance level and the abilities of the selected bacterial strain on CR decolorization, different concentrations of CR dye were used. The three bacterial strains were found to be highly efficient in decolourizing CR dye in all the tested concentrations. The highest decolorization has been achieved with 100 µM of dye concentration at which *B. amyloliquefaciens* S12 and *B. subtilis* S50 showed maximum decolorization of 81.8 and 81.5 % respectively, while *B. licheniformis* S2 of achieved 76.4% (Figure 4 A, B, C).

The effect of different incubation temperatures was also tested and the results demonstrated that the optimum temperature was 37 °C. A gradual decrease in decolorization activity was observed with increasing in temperatures from 37 to 45 °C. At this optimum temperature *B. licheniformis* S2, *B. amyloliquefaciens* S12 and *B. subtilis* S50 recorded maximum decolorization of 46.4, 56.9 and 53.8 percentage respectively (Figure 4 D, E, F).

The pH has a significant effect on the efficiency of dye decolorization, therefore the CR decolorization was evaluated. The maximum decolorization of CR was observed with all bacterial strains at neutral pH 7, while shifting from neutral pH to alkaline or acidic tends to decrease the decolorization (Figure 5 A, B, C). *B. licheniformis* S2 showed a relatively high decolorization at pH 9 recorded 58.3 compared to 64.6 at pH 7.

In the agitation experiment, it was noticed that the three strains showed a maximum decolorization under static conditions, however under shaking conditions a slight decrease was observed in decolorization percentage (Figure 5 D, E, F).
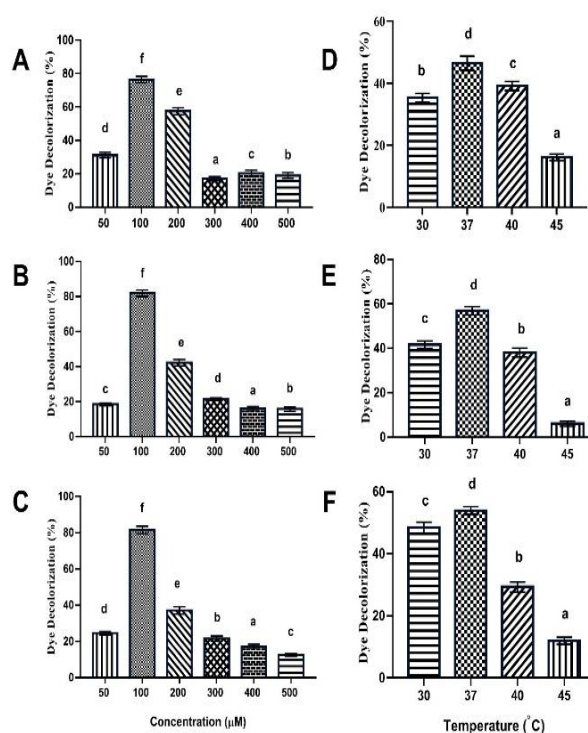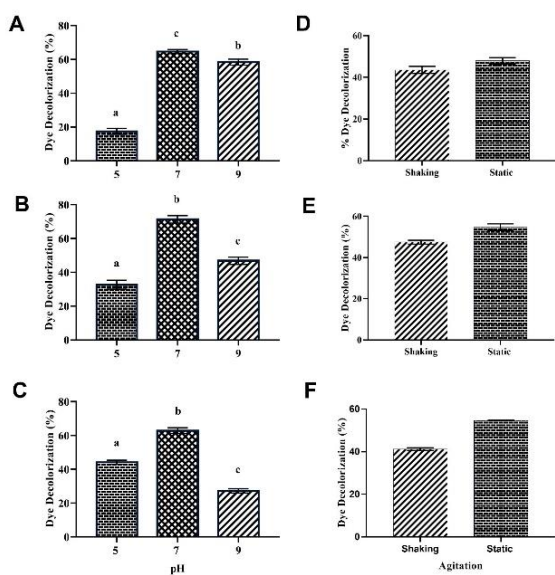

Fig. 4. Effect of different dye concentration and temperature on the decolorization of congo red by the three selected bacterial isolates *B. licheniformis* S2 (A, D), *B. amyloliquefaciens* S12 (B, E) and *B. subtilis* S50 (C, F). The bars represent the percentage of decolorization calculated from three replicate ± standard deviation and the different letters above indicate a significant difference between different factors, while the presence of a common letter are not significantly different ($p < 0.05$)

The effect was more pronounced in *B. subtilis* S50 recorded decolorization of 41.1 compared to 54.6 percentage under static conditions.

3.5. Biodegradation metabolites analysis

To confirm the biodegradation of CR, initially the UV-Visible spectral of three bacterial culture supernatant was analysed from wavelength of 200 to 650 nm. The results revealed obvious decrease of the of CR's major peak in the region at λ max at 498 nm compared to uninoculated control (Figure 6).

The GC-MS was performed at the end of decolorization experiments for ensuring the biodegradation of CR in the culture of the three selected strains. GC-MS analysis showed that there were compounds with lower molecular mass compared to the spectrum of uninoculated YM media supplemented with CR dye such as hexane, benzene, 1,3-bis(1,1-dimethylethyl) and Phenol, 2,4-bis(1,1-dimethylethyl) in the culture supernatants of *B. licheniformis* S2, and *B. subtilis* S50 but no aromatic compounds were detected in supernatant of *B. amyloliquefaciens* S12 (Table 2).

Fig. 5. The percentage decolorization of congo red at different pH and agitation conditions by the selected bacterial isolates *B. licheniformis* S2 (A, D), *B. amyloliquefaciens* S12 (B, E) and *B. subtilis* S50 (C, F). The percentage of decolorization was calculated from three replicates and the different letters above the bars indicate a significant difference between different factors, while the presence of a common letter are not significantly different ($p < 0.05$).
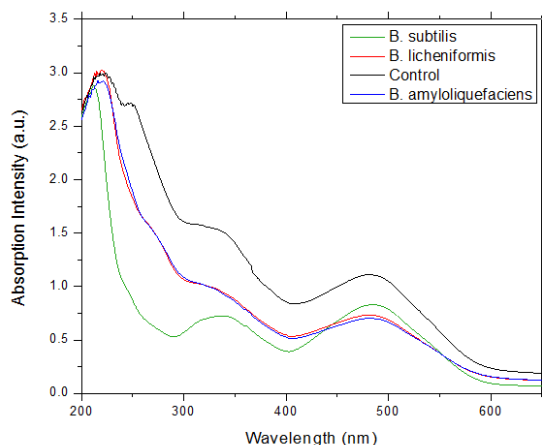
Fig. 6. Representative UV-Visible scan of cell-free supernatant of uninoculated culture supplemented with 100 µM CR (Control), and cell-free supernatant of *B. licheniformis* S2, *B. amyloliquefaciens* S12 and *B. subtilis* S50.

On the basis of biodegradation metabolites listed in Table (2) a pathway was proposed for the biodegradation of CR involved symmetric cleavage of the azo-bond from the removal of azo linkage and breakdown of the complex molecule (Figure 7). The further reactions including degraded intermediates

into low molecular weight fragments and then converted them into CO_2 and water.

Table 2. The CR's detected metabolites by GC-MS during the biodegradation by the selected bacterial isolates after 6 days of incubation.

| Retention time (Rt.)	M.Wt.	Area (%)	Compounds	Isolate(s)
1.13	86.18	15.58	Hexane	S2
1.13	88.11	19.79	2-Butanone, 4-hydroxy	S12
2.79	142.28	1.02	2,6-Dimethyldecane	S2
3.84	144.21	1.16	2,4-Dimethylhexanedioic acid	S2
4.80, 4.77	190	9.35, 4.47	Benzene, 1,3-bis(1,1-dimethylethyl)	S2, S50
5.02, 5.00	212	2.99, 2.68	Dodecane, 2,6,10-trimethyl	S2, S50
9.90, 9.89	206	1.37, 0.87	Phenol, 2,4-bis(1,1-dimethylethyl)-	S2, S50
14.58, 14.57	212.41	1.37, 1.28	Tetradecane, 2-methyl	S2, S50
13.62	282	2.66, 0.31, 2.22	Hexadecane, 2,6,11,15-tetramethyl	S2, S12, S50
18.09, 17.92, 18.08	282	0.75, 0.29, 0.90	Hexadecane, 2,6,10,14-tetramethyl	S2, S12, S50

3.6. Phytotoxicity assay

To confirm the complete degradation of CR dye, phytotoxicity experiments were performed and results demonstrated that the percentage of seeds germination was significantly affected by the crude CR dye compared to control in both tested plants. The treatment of seed with the CR dye treated with all *Bacillus* species significantly increased the germination percentage of both plants. The best germination percentage was recorded for CR dye treated with *B. licheniformis* on *Triticum aestivum* and *Vicia faba* seeds (Table 3).

4. Discussion

Soil and water contamination by azo-dyes effluent from industrial activities is one of the major environmental concern due to the negative impact on the biological ecosystem as well as human health [4]. The bacterial based biological treatment of azo-dyes contamination, especially textile dyeing waste, has gained scientists' attention as an effective and economic substitute to conventional physiochemical techniques such as activated carbon adsorption, electrochemical oxidation and membrane separation [4,8]. However, the crucial factor affecting the biological treatment is the selection of dye-decolorizing microorganism that has advantages over

others. Therefore, in this study the soil microbial diversity was explored to select an effective bacterial isolate able to decolorize a solution containing a CR dye in aerobic conditions.
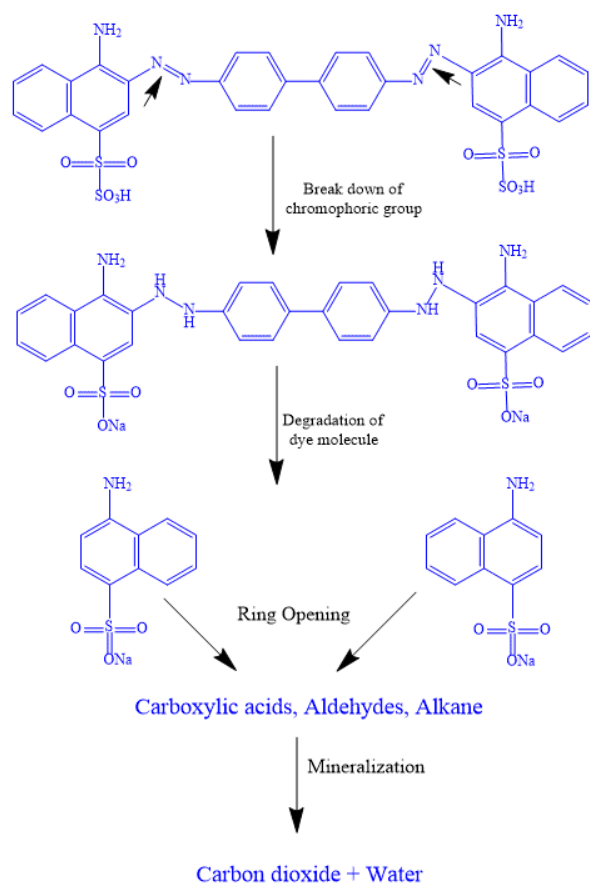
Fig. 7. The proposed pathways for the degradation of CR by the selected bacterial strains

Table 3. The effect of CR dye treated with the selected Bacillus species on *Triricum aestivum* and *Vicia faba* seeds germination.

Treatment	Germination (%)	
	Triticum aestivum	*Vicia faba*
Control (sterile water)	100 ± 0.00 [a]	100 ± 0.00 [a]
Untreated CR dye	26.67 ± 5.77 [c]	20.00 ± 10.00 [d]
Treated with *B. licheniformis* S2	80.00 ± 10.00 [b]	76.67 ± 5.77 [b]
Treated with *B. amyloliquefaciens* S12	63.33 ± 5.77 [b]	50.00 ± 10.00 [c]
Treated with *B. subtilis* S50	76.67 ± 11.55 [b]	56.67 ± 5.77 [c]

The enrichment technique for isolation of CR degrading bacteria was effective to obtain 52 bacterial

isolates able to utilize CR as a carbon source. A similar method was applied in some studies to isolate potential bacterial species in the biodegradation of different pollutant compounds such as crude oil [16], pesticides [17] and synthetic dyes [18]. The decolorization capability was evaluated among each single isolate by increasing the initial concentration in the culture media to select highly resistant isolates that can effectively utilize CR. In this regard, many studies reported the toxicity of CR to bacterial cells especially at high concentrations due to the imbalance between bacterial biomass and dye, obstruction of active sites of oxidizing and reducing enzymes by dye molecules with different structures [19,20]. Furthermore, the high concentration of azo dye in the media may increase the accumulation of toxic aromatic amines as degradation metabolites that retard the bacterial growth of some bacterial species [21].

The screening test demonstrated that three isolates can decolorize CR effectively with short lag phase (three days) and maximum decolorization was recorded after only four days which is in line with observations from several studies performed on bacterial consortium as well as bacterial pure culture [9,18]. All bacterial isolates sediments were not deeply colored which indicated that colour removal was more likely due to degradation but not absorption of CR [22].

Interestingly, the three selected isolates belonged to genus *Bacillus* and were identified as *Bacillus licheniformis*, *Bacillus amyloliquefaciens* and *Bacillus subtilis* for the isolates S2, S12 and S50 respectively. The species in genus *Bacillus* are well known for their high abilities for degradation of a variety of xenobiotic contaminants including different types of Azo-dyes [11,23].

The improvement of useful biodegradation of dye requires application of a potential bacterial strain and its use under the best conditions to achieve the degradation capacity. Therefore, different concentrations of CR were tested and all bacterial strains were able to decolorize the dye at a wide range (50 – 500 µM) of concentrations but 100 µM (dye 71.4 mg/L) was the most suitable concentration. In this regard, the negative impact of high dye concentration on the bacterial growth and resistance to decolorization by microorganisms was previously reported [20,22]. Many studies reported optimum degradation of different bacterial strains at this concentration of CR [9,21].

The ability of the bacterial strain *A. baumannii* YNWH 226 to degrade CR at concentration 100 mg/L at 37°C, pH 7.0 into non-toxic metabolites was reported [24]. In the present study, the dye decolorization activity of the selected *Bacillus* species were maximum at pH 7.0, which may be related to the transport of dye molecules across the cell membrane. Shifting from this pH to lower values increased the H^+ ions that may compete

with dye cations, causing a decrease in colour removal efficiency, Furthermore, at higher pH, the biomass surface acquired a negative charge that may enhance the dye cations through electrostatic force of attraction [5,25]. Similarly, the optimum decolorization temperature was 37 °C and any further increase in temperature resulted in reduction in decolorization activity. The decline in decolorization activity at higher temperatures can be attributed to the loss of cell viability and/or the denaturation of the enzymes responsible for degradation such as azoreductase enzyme [26,27].

The decolorization efficiency of CR was decreased in selected bacterial isolates suggesting that facultative anaerobic condition was favorable, which could be due to the involvement of azoreductase for the decolorization of CR. It was reported that reductive cleavage of azo-bonds (–N=N–) by azoreductase enzyme is inhibited by the presence of oxygen because of the competition in the oxidation of NADH with the electron receptor (oxygen and/or azo-groups) [19,28]. Hence, in this study static conditions were adopted to investigate the bacterial decolorization. Similar finding was reported previously in many studies [27,29].

The UV-visible analysis revealed that the decolorized dye solution showed decrease of the major peak in the visible regions with all *Bacillus* isolates. Similar observation has been reported as an indicator of decolorization in several studies [14,30]. Detoxification of the azo-dyes by microbial cultures was attributed to the conversion of azo-nitrogen to other non-toxic compounds. Generally, the first step of biodegradation of azo-dyes is a reductive cleavage of the azo-group by bacterial enzymes, leading to the accumulation of aromatic amines. These compounds are potentially toxic, mutagenic and carcinogenic [20,24].

GC-MS analysis of the CR dye metabolites showed the presence of derivatives of naphtalene and tolidine with clear evidence of ring cleavage. The complete absence of the aromatic amine compounds such as 4,4′-diaminobiphenyl as well as 3,4-diamino-1-naphthalenesulfonic acid in all tested bacterial supernatants indicated the complete degradation of CR for the bacterial requirements of carbon and energy [11]. These results are in line with the results obtained from the phytotoxicity experiment which demonstrated that significant increase in the percentage of germination for *Vicia faba* and *Triticum aestivum* seeds as compared to the untreated CR dye. The increase in the germination percentage of both tested plants could be attributed to the less toxic nature of the dye residues and produced metabolites. In this regards, similar observations were recorded for the microbial biodegradation of anthraquinone dyes and

azo dyes that produces a safe metabolic products for the plants [15,31].

5. Conclusions

In conclusion the prospective *Bacillus* strains isolated in this study might be potential bacteria in the bioreactor for the treatment and detoxification of industrial effluent contaminated with synthetic dyes.

6. Conflicts of interest

There are no conflicts to declare.

7. Formatting of funding sources

No external funding

8. References

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