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Modeling of Reactive Black 5 decolorization in presence of heavy metals by the newly isolated *Pseudomonas aeruginosa* strain Gb30

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Abstract

Aim

The effect of heavy metals presence on the decolorization of Reactive Black 5 by *Pseudomonas aeruginosa* was evaluated.

Methods and results

In the current study, a newly isolated strain identified as *P. aeruginosa* strain Gb 30 was selected for its ability to remove high concentration of Reactive Black 5 and resistance to several heavy metals (Cu^{2+}>Zn^{2+}>Cd^{2+}>Cr^{6+}). Strain Gb30 was used to assess the effect of heavy metals presence on RB5 decolorization. The strain growth exhibited different responses at a fixed concentration of EC_{50} (10 h) for each heavy metal. Addition of Zn^{2+} and Cd^{2+} had no effect on decolorization yield after 24 h incubation, whereas Cr^{6+} and Cu^{2+} ions reduced decolorization up to 17%. In order to understand the relationship between heavy metals contamination and decolorization, experimental data relating the initial decolorization rate of RB5 to the concentrations of single and associated heavy metals were fitted to three different inhibition kinetic models.

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Conclusions

In this study, we showed that *P. aeruginosa* strain Gb30 could be used for dyes removal even at high concentrations of heavy metals. The developed models could provide basic informations that may helps for the best management of the bacteria mediated decolorization process at industrial scale.

Significance and Impact of the Study

This study opens new directions for the management of textile industry wastewaters containing dyes and heavy metals using bioaugmentation by *Pseudomonas aeruginosa* strain Gb30.

Key words: decolorization; heavy metals; bacteria; modeling; textile effluent.

Introduction

For many decades, safe discharge of wastewaters initially loaded with both organic and inorganic wastes are considered as a huge threat causing severe environmental damages (Drumond Chequer et al., 2013; Khan and Malik, 2014; Akhtar et al., 2016). Therefore, the environmental management of dye effluents represents a major goal towards ecosystem protection. In addition, ecofriendly and low cost methods which could address limitations to classic physio-chemical treatments are under massive consideration among the social-economic society (Sarayu and Sandhya, 2012). In this context, several studies have recently been carried out highlighting the efficacy of microbial communities in textile effluents detoxification (Saratale et al., 2011; Phugare et al., 2011; Abioye et al., 2014; Yu et al., 2015;

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Prabha et al., 2016; Uday et al., 2016). Considering that textile effluents are a mixture of pollutants containing dye residues, salts, heavy metals, and many other chemicals used during dyeing, detoxification is a multilevel process involving too complex effluent-microbe-environment interactions (Ali et al., 2009; Rawat et al., 2016). Among these pollutants, dyes and heavy metals represent the major threat of aquatic and soil ecosystems (Maqbool et al., 2016). A number of studies suggested biotechnological approaches to overcome this threat using pure culture or consortia for single removal of dyes or heavy metals (Jadhav et al., 2011). Several bacterial strains such as *Providencia* sp., *P. aeuroginosa, A. junii, Enterobacter* sp., *Citrobacter* sp., *B. laterosporus and Bacillus* sp. were investigated in terms of their ability of dyes biodegradation (Wang et al., 2009a, 2009b; Kurade et al., 2011; Phugare et al., 2011; Anwar et al., 2014; Yu et al., 2015). Most of studies on bacterial dye degradation are lab-scale investigation focusing on strains decolorization ability with least attention towards the complexity of the dye biodegradation process in natural conditions. Thus, contaminated systems normally contain high concentrations of salts and heavy metals affecting the dye degradation process by inhibiting bacterial growth or blocking the enzymes activities (Sharma et al., 2008). Khan and Malik (2014) reported the presence several toxic heavy metals in textile effluents such as Ni, Cu, Cr, Pb, Cd, and Zn at concentrations of 0.124, 0.151, 1.533, 0.199, 0.088 and 2.694 mg l^{-1} respectively. Das et al. (2011), Jaishree et al. (2014) and Sarker et al. (2015) confirmed the presence of Cu, Cd, Cr and Zn at variable levels in textile effluents. Previous findings demonstrated that heavy metals might destruct the active site conformation of proteins, which directly leads to enzymes inhibition. For example, Jadhav et al. (2013) reported that the presence of Zn and Hg ions could strongly slow down the decolorization process of Amaranth by *P. aeruginosa* BCH. In the absence of ecosystem-based studies, scientists are invited to consider the environmental conditions surrounding the decolorization process and so to predict the possible fate and consequences

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of azo dyes-heavy metals co-existence into the environment. Some microorganisms such as bacteria, fungi and yeast are able to develop mechanisms of dyes degradation in presence of high concentrations of heavy metals. *A. versicolor* can efficiently decolorize high amounts of Remazol Blue reactive dye in presence of heavy metals (Cr, Cu and Ni) in molasses growth medium (Taştan et al., 2010). Huang et al. (2015) described the ability of *Lactobacillus paracase* CL1107 to co-eliminate toxic chromium and Acid Black (ATT) azo dye. Anwar et al. (2014) reported that *Acinetobacter junii* strain FA10 used for reactive red 120 decolorization exhibited good tolerance to considerable levels of different heavy metals. *P. aeruginosa* strain ZM130 was highly effective in simultaneously removing Cr^{6+} and Reactive Red 120, Reactive Black 5, Reactive Yellow 2, and Reactive Orange 16 azo dyes from a simulated wastewater even in the presence of three other heavy metals Zn, Pb and Cd (Maqbool et al., 2016). The assessment of the inhibitory effects of heavy metal ions on biodegradation of Congo Red by *Pseudomonas* sp. mutant by Gopinath et al. (2011), confirmed that heavy metals contamination interferes with dye remediation. The critical heavy metals concentrations obtained as per Han–Levenspiel inhibition model for Cr, Zn, and Cu were 0.895, 0.302 and 0.204 g l^{-1} respectively.

In this concern, our study tried to isolate a multi heavy metal resistant strain, *Pseudomonas aeruginosa* Gb30, a model specie for both heavy metal resistance and dye biodegradation, to assess the effect of heavy metals on bacterial growth and finally to provide mathematic models describing the single and the associated inhibitory effects of heavy metals on decolorization process.

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Materials and methods

Chemicals and reagents

Technical grade Reactive Black 5 (RB5) dye was purchased from Sigma-Aldrich (Germany). A stock solution of a final concentration of 10 mol l^{-1} was used during the study. The heavy metals stock solutions used in this study were $CdCl_2$ (0.5 mol l^{-1}), $CuSO_4$ (0.25 mol l^{-1}), $K_2Cr_2O_7$ (1 mol l^{-1}) and $ZnSO_4$ (1 mol l^{-1}). Methanol used for HPLC analysis was of analytical grade.

Bacterium isolation and culture conditions

The bacterial strain was isolated from desert soil collected at the vicinity of Kebeli in the South west of Tunisia (33° 42′ 18″ Nord, 8° 57′ 54″ Est). Soil sample was collected from 1 to 10 cm in depth and stored in a sterile bottle before use. 0.5 g of desert soil sample was suspended in 20 ml of non-selective nutrient broth medium (5 g l^{-1} NaCl, 5 g l^{-1} peptone, 2.5 g l^{-1} yeast extract, 10 g l^{-1} meat extract and 15 g l^{-1} agar, pH was adjusted to 7) and incubated at 45 °C for 18 h at 150 rpm. Serial dilutions of the obtained bacterial culture were plated separately onto nutrient agar plates. After 24 h of incubation, individual colonies were picked and streaked twice on new plates and incubated for 2 days then stored at 4 °C for a short time period.

Strain Gb30 was selected, among isolated bacterial collection, for its color removal ability as well as its heavy metal resistance capability. Study of the heavy metals effects on bacterial growth as well as on decolorization ability of the bacterial strain was carried out in nutrient broth (NB) medium prepared by dissolving 5 g l^{-1} NaCl, 10 g l^{-1} peptone and 5 g l^{-1} yeast extract. pH was adjusted to 8 using standard NaOH and HCl solutions.

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Strain identification

For DNA extraction, the strain was grown overnight at 37°C in a 250 ml flask containing 25 ml of Nutrient Broth medium. Biomass was harvested by centrifugation (11000xg, 10 min). DNA extraction was monitored according to Su et al. (2007) with some modifications. Almost complete 16S rRNA gene was amplified through PCR using the bacterial domain universal primer sets fD1 (5' AGAGTTTGATCCTGGCTCAG 3') and rP2 (5' ACGGCTACCTTGTTACGACTT 3') (Weisburg et al., 1991). 16S rRNA gene was amplified in a 25 µl reaction volume containing: 2.5 µl of *Taq* buffer (10X) (Invitrogen, USA), 1 µl $MgCl_2$ (25 mmol l^{-1}), 1 µl dNTPs (20 mmol l^{-1}), 1 µl (10 µmol l^{-1}) fD1 and rP2 primer set (Invitrogen, USA), 1U *Taq* DNA polymerase (Invitrogen, USA), and about 5 ng of genomic DNA. The thermal cycling program was: initial denaturation at 95 °C for 5 min, followed by 35 cycles at 95 °C for 15 s, 54 °C for 15 s and 72 °C for 90 s, with a final elongation at 72 °C for 7 min. Appropriate positive as well as negative controls were run in parallel for each reaction to ascertain absence of contamination or PCR failure. PCR products were then purified and sequenced directly using a BigDye197 Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and analysed with an ABI-3130xl 48-capillary DNA sequencer (Applied Biosystems). The obtained sequence was compared to the nearest neighbour sequences available in the NCBI using BLASTn program (http://www.ncbi.nlm.nih.gov/BLAST) and strain identity was ascertained when unambiguous high identity was scored. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The obtained 16S rRNA gene sequence was deposited in GenBank database under accession number KY655217.1.

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Heavy metals effects on bacterial growth

To investigate the heavy metals effects on bacterial growth, median effective concentration of each heavy metal after 10 hours' incubation EC_{50} (10 h) was determined. 500 µl of a freshly prepared culture (OD_{600} = 1.0) were added to 20 ml NB medium containing different concentrations of the appropriate heavy metal. A metal-free medium inoculated with the bacterial strain and media supplemented with different heavy metals concentrations without microorganism inoculation were used respectively as positive and negative controls. During the first 10 hours of incubation, 2 ml of inoculated cultures were withdrawn at different time intervals and OD_{600} were measured. Growth inhibition rates at different concentrations of each heavy metal were calculated according to the following equation:

$$I(\%) = (\mu_c - \mu_m) * \frac{100}{\mu_c} \qquad Eq(1)$$

I (%): growth inhibition rate

μ_c: growth rate of the metal free control

μ_m: growth rate of the metal containing culture

The plot relating heavy metal concentration to the bacterial inhibition rate was built using a linear regression method and EC_{50} (10 h) of each heavy metal was determined.

The studied heavy metals concentrations in order to determine EC_{50} (10 h) of each one are summarized in table 1.

Dye removal experiments

Dye decolorization experiments were conducted in NB with addition of 50 mg l^{-1} of Reactive Black 5 at 37 °C under static condition. Color removal was checked by measuring the absorbance of culture supernatant at the dye λ_{max} (597 nm). Decolorization percentage was calculated as follows:

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$$Decolorization\ (\%) = \frac{OD_I - OD_S}{OD_I} * 100 \qquad Eq\ (2)$$

Were:

OD_I: initial dye absorbance at 597 nm

OD_S: decolorized sample absorbance at 597 nm

Dye removal analysis in presence of heavy metals

Cultures supplemented with EC_{50} (10 h) concentrations of Cd^{2+}, Cu^{2+}, Cr^{6+}, Zn^{2+} and mixture of the four selected heavy metals at a concentration of $EC_{50}/8$ were carried out at 37 °C, pH 8 and 50 mmol l^{-1} initial dye concentration under static condition to determine the single and associated heavy metals effects on dye removal. Batch experiments were conducted in 250 ml flasks containing 100 ml NB medium inoculated with 2.5% (v/v) inoculum (OD_{600} = 1.0). Aliquots were withdrawn at different time intervals and were centrifuged at 11000 g for 10 min then the supernatant was analyzed at 597 nm. RB5 degradation in cultures added with EC_{50} (10 h) concentrations of Zn^{2+}, Cd^{2+}, Cu^{2+}, Cr^{6+} and mixture of the four selected heavy metals at a concentration of $EC_{50}/8$ were analyzed using HPLC and UV-Visible spectroscopy to determine the single and associated heavy metals effects on dye removal. RB5 biodegradation products were monitored into a DIONEX UltiMate 3000 (Thermo Scientific) C-18 column at room temperature. The mobile phase was water / methanol (60 / 40 %) with a flow rate of 1 ml min^{-1}. Compounds were detected using an UV/VIS detector at 597 nm. Cultures samples were scanned using a JENWAY 7315 UV-Visible spectrophotometer in the range of 200-800 nm.

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Effect of heavy metals concentrations on decolorization yield

Decolorization kinetics of RB5 in presence of different concentrations of heavy metals were evaluated during 48 h of incubation. Heavy metals concentrations ranged from ($3\times EC_{50}$ to $EC_{50}/2$), ($2.5\times EC_{50}$ to $EC_{50}/4$), ($1.5\times EC_{50}$ to $EC_{50}/8$) and ($1.5\times EC_{50}$ to $EC_{50}/8$) respectively for Zn^{2+}, Cd^{2+}, Cu^{2+} and Cr^{6+}.

Heavy metals inhibition kinetics

To determine the inhibitory parameters of added heavy metals, different kinetic equations were applied (Liu et al., 2008; Wang and Wan, 2009; Wang et al., 2008). These models were used to fit the obtained experimental data in order to study the effect of single and associated heavy metals concentrations on dye decolorization (table 2). Decolorization rate was calculated for the first 24 h by plotting the linear regression of RB5 decolorization yield at different heavy metals concentrations. In order to investigate the associated effect of studied heavy metals, initial decolorization rate of RB5 by the strain was evaluated in presence of serial dillutions of heavy metal mixture containing EC_{50} (10 h) concentration of each heavy metal where 1 Arbitrary Unit (AU) is equivalent to (1.525 + 0.629 + 1.605 + 0.462) mmol l^{-1} respectively of Zn^{2+}, Cd^{2+}, Cu^{2+} and Cr^{6+}. The kinetic parameters estimation of each model was carried out with non-linear regression method using Matlab, R2010a (The MathWorks, Natick, USA) software. The quality of each models' fitting has been tested by calculating the coefficient of determination (R^2), the adjusted coefficient of determination (R^2_{adj}), the root mean squared error (RMSE) and the sum of squared error of prediction (SSE), described in the following equations:

$$R^2 = 1 - \frac{\sum_{i=1}^{n}\left(Y_i - \hat{Y}_i\right)^2}{\sum_{i=1}^{n}(Y_i - \bar{Y}_i)^2} \qquad Eq(6)$$

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$$R^2{}_{adj} = 1 - (1 - R^2) \cdot \frac{n-1}{n-p} \qquad Eq(7)$$

$$RMSE = \sqrt{\frac{\sum_{i=1}^{n}\left(Y_i - \hat{Y}_i\right)^2}{n-p}} \qquad Eq(8)$$

$$SSE = \sum_{i=1}^{n}\left(Y_i - \hat{Y}_i\right)^2 \qquad Eq(9)$$

with Y_i:experimental value; $\hat{Y}_i$: calculated value; $\overline{Y}_i$: arithmetic mean of all experimental values; n: number of experimental values; p: number of model parameters. The best model was chosen from the comparison between the four previously defined statistical criteria.

Results

Identification of the dye decolorizing heavy metal resistant bacteria

Strain Gb30 was selected among several bacterial strains due to its ability to decolorize the RB5 and to grow in the presence of different heavy metals. Phylogenetic analysis based on16S rRNA gene showed that strain Gb30 is closely related to members of *Pseudomonas aeruginosa* with a sequence similarity value of 99% (Figure 1).

Heavy metals effects on bacterial growth

The evaluation of heavy metals effect on bacterial growth of *P. aeruginosa* strain Gb30 was performed by the determination of the inhibition percentage of the bacterial growth rate induced by Zn^{2+}, Cr^{6+}, Cu^{2+} and Cd^{2+} addition in the culture medium. The effect of heavy metals on strain Gb30 was different among the four tested metals. Cr^{6+} and Cd^{2+} ions most affected the bacterial growth, which confirms the toxic nature of these tow heavy metals. In

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contrast, the strain presented a great tolerance toward Zn^{2+} and Cu^{2+} ions compared to Cr^{6+} and Cd^{2+} ions. The EC_{50} (10 h) was measured for each metal and was as fellows: 1.525 mmol l^{-1} for Zn^{2+}, 0.629 mmol l^{-1} for Cd^{2+}, 1.605 mmol l^{-1} for Cu^{2+} and 0.462 mmol l^{-1} for Cr^{6+}. Bacterial growth was totally inhibited after 10 hours' incubation at concentrations of 3.05 mmol l^{-1} for Zn^{2+}, 1.258 mmol l^{-1} for Cd^{2+}, 3.21 mmol l^{-1} for Cu^{2+} and 0.924 mmol l^{-1} for Cr^{6+} (Figure S1).

Dye removal analysis in presence of heavy metals

The decolorization rate of RB5 by *P. aeruginosa* was affected by the addition of single metals (Cu^{2+}, Cr^{6+} and Cd^{2+}) except for Zn^{2+} (Figure 2). Zn^{2+} addition in the culture media had a very little or no effect on color removal. Decolorization yield was 79% and 76% respectively in metal free (control) and Zn^{2+} containing cultures after 20 h of incubation while only 67 % dye removal was reached in 20 h for Cd^{2+} containing cultures. Nevertheless, for both cultures, complete color removal was obtained at 38 h. In contrast, significant reduction in RB5 decolorization rate were recorded when similar ranges of concentrations (EC_{50} (10 h)) of Cr^{6+} or Cu^{2+} were added to the culture media. No dye decolorization was recorded in the first 20 h of incubation and decolorization rate did not exceed 17% for both heavy metals (Cr^{6+} and Cu^{2+}) after 48 h. UV-Vis spectra of untreated dye, metal free culture and cultures added with heavy metals confirmed the previously obtained results (Figure 3). These results are consolidated with the HPLC spectra at 597 nm. After 48 h of incubation, total decolorization of RB5 only in presence of Zn^{2+} and Cd^{2+} was reached (Figure S2). However, important peaks were observed in presence of Cr^{6+} and Cu^{2+} suggesting the persistence of the initially added dye or the accumulation of its intermediates.

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Effect of heavy metals concentrations on decolorization yield

Kinetics of RB5 decolorization by strain Gb30 were carried out to investigate the effect of heavy metals concentrations on RB5 removal. The strain decolorization performance was demonstrated as a dose-dependent response upon exposure to studied heavy metals except in case of Zn^{2+}. RB5 decolorizing ability maintained the same level with increasing Zn^{2+} concentration from 0.7 to 4.5 mmol l^{-1} (Figure 4a). In all tested Cd^{2+} concentrations, the strain exhibited high RB5 removal ability and total decolorization was finally achieved (Figure 4b). In case of Cu^{2+} ions, decolorization percentage didn't exceed 17% and total decolorization has never been reached for all concentrations (Figure 4d). The increase in Cr^{6+} concentration in the culture media stopped decolorization process during the first 20 h of incubation. After this lag-time, decolorization processed progressively to reach total decolorization after 48 h of incubation only for the $EC_{50}/4$ and $EC_{50}/8$ concentrations (Figure 4c).

Heavy metals inhibition kinetics

The presence of high concentration of heavy metals in the effluent could slow down the biodegradation of dyes and rather stop it. Modeling of heavy metals effect on dyes removal is crucial to optimize the decolorization process. In the present study, experimental data were fitted by models (1), (2) and (3) mentioned in table 2. The inhibition parameters, the coefficient of determination (R^2), the adjusted coefficient of determination (R^2_{adj}), the root mean squared error (RMSE) and the sum of squares error (SSE) obtained for each model are summarized in table 3. The model having the best fitting is the one having the highest R^2 and R^2_{adj} values and the lowest RMSE and SSE values. Data obtained for each model showed that model (2) greatly correlates with experimental findings for Zn^{2+} (a), Cr^{6+} (c) and Cu^{2+} (d) while Cd^{2+} (b) data were closely fitted to model (1). According to the obtained models, heavy metals inhibition levels on RB5 degradation by *P. aeruginosa* Gb30 could be ranked as

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fellows Cr^{6+}>Cu^{2+}>Cd^{2+}>Zn^{2+}. Non-linear curves describing the different models are shown in Figure 5. The obtained results showed that the strain presented high tolerance to Zn^{2+} and the initial decolorization rate slightly decreased by the addition of 6 mmol l^{-1} of Zn^{2+} ions. This result confirms the hypothesis that this metal have a very weak effect on RB5 decolorization yield. The critical concentration wich totally inhibits the decolorization process in presence of Cd^{2+} ions was estimated by the first model to be 2.491 mmol l^{-1} thing which confers to the strain its performance in dye removal even under high heavy metals concentrations. The RB5 decolorization trend was totally different in Cu^{2+} and Cr^{6+}presence, initial decolorization rate sharply decreased in presence of 1 mmol l^{-1} of the metal in the culture media.

To investigate the effect of associated heavy metals (Zn^{2+}, Cd^{2+}, Cu^{2+} and Cr^{6+}) on RB5 decolorization, the initial decolorization rate variation was investigated in different concentrations of heavy metals mixture. The fitting of experimental data to the three previously described models revealed that the second model was the best to describe the strain decolorization behavior. According to Figure 6, the strain has lost more than 50% of its initial decolorization ability at 0.075 AU of mixture concentration.

Discussion

In the present study, *P. aeruginosa* Gb30 was used as a model strain for the assessment of heavy metals effect on RB5 decolorization. Indeed, the strain exhibited high resistance to heavy metals as well as a great potential in dye decolorization. The performed experiments assessing bacterial growth in presence of heavy metals showed that *P. aeruginosa* strain Gb30 was progressively affected by Cr^{6+}, Cd^{2+}, Zn^{2+} then Cu^{2+}. In previous findings, growth of *P. aeruginosa* strains ZM-130 and ARSKS20 used in dyes removal in presence of heavy metals were progressively affected by chromium > zinc > cadmium and chromium > copper,

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respectively (Soni et al., 2014; Maqbool et al., 2016). In contrast, RB5 decolorization experiments showed high tolerance to Zn^{2+} and Cd^{2+} ions but not for Cr^{6+} and Cu^{2+}. These results suggest that heavy metals addition in culture media may maintain, enhance or affect enzymatic pathways involved in the decolorization process depending on the metal nature (Jadhav et al., 2011). In case of Zn^{2+}, decolorization of RB5 processed normally suggesting that Zn^{2+} ions didn't affected neither growth nor decolorization. Decolorization rate slightly decreased with Cd^{2+} ions addition but total decolorization was finally reached. Cr^{6+} and Cu^{2+} ions sharply affected decolorization with a lag time reaching more than 20 h. The difference in response of the same strain to different metals stress can be explained by the fact that several parameters interfere in dye-heavy metal co-removal such as metals bioaccumulation and/or biosorption capacity of the strain, eventual interactions dye-heavy metal, competition between pollutants for intracellular binding and efficacy of the dye removing enzymatic system. Heavy metals accumulation into the cell may disturb enzymes expression by binding with groups in enzymes molecules and thus disturbing their functions (Gopinath et al., 2011).

The previously obtained results are confirmed by HPLC and UV-Vis spectra, representing the differences in the strain response to each metal. HPLC and UV-Vis spectra of RB5 biodegradation metabolites in presence of Zn^{2+} and Cd^{2+} confirmed the total degradation of the initially used dye and the appearance of new peaks corresponding to the newly formed products. Spectra corresponding to cultures containing Cr^{6+} and Cu^{2+} proved the persistence of most of the initially used dye (Figure S2).

In order to investigate the effect of heavy metal concentration on decolorization efficiency of strain Gb30, several heavy metals concentrations of Zn^{2+}, Cd^{2+}, Cu^{2+} and Cr^{6+} were tested. The lag-time induced by the presence of Cr^{6+} in the media can be explained by the possible

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acclimatization strategy adopted by the strain to cope with the stressful conditions of the media, either by decreasing the metal concentration by bioaccumulation and/or biosorption into the cell (Hansda et al., 2016) or by reducing Cr^{6+} into Cr^{3+} ions which are relatively harmless by producing chromium reductase enzyme (Gopinath et al., 2011). Similarly, inhibitory effects on RB5 decolorization by *Pseudomonas putida* K1 and *Serratia proteamaculans* SL14 were observed when chromium concentration exceeded 2 mg l^{-1} suggesting that, in this condition, RB5 served as an electron donor for chromate reduction. (Mahmood et al., 2013). Although Cu^{2+} ions were the most tolerated by the strain, they were the most harmful for RB5 decolorization process. Soni et al. (2014) reported that Cu^{2+} ions affect the cell transcription mechanism. Many researchers considered copper as one of the most toxic metals. Gopinath et al. (2011) reported that the time required for decolorization of Congo red by *Pseudomonas* sp. in presence of 0.15 g l^{-1} of Cu^{2+} was increased from 27 to 87 hours. Cibacron black W 55 decolorization by *Halomonas* sp. strain was 17% at 0.1 mmol l^{-1} $CuSO_4$ while completely inhibited at 0.5 mmol l^{-1} (Pourbabaee et al., 2011).

Modeling of the heavy mtals effect on RB5 decolorization was used as a mathematical tool describing the decolorization behavior of the strain in presence of single and mixed heavy metals. The application of the three described models provided basic informations that may help for the best management of the bacteria mediated decolorization process at industrial scale. A simple comparaison of the calculated critical concentration of each heavy metal, confirms the hypothesis that decolorization was increasingly affected by $Zn^{2+} > Cd^{2+} > Cr^{6+} > Cu^{2+}$. Our finding are in contrast with the results previously described by Gopinath et al. (2011) which reported that Congo red decolorization by mutant *Pseudomonas* sp. was increasingly damaged by $Cu^{2+} > Zn^{2+} > Cr^{6+}$ ions addition in the media and the critical heavy metals concentration were respectively 204, 302 and 895 mg l^{-1}.

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Most reported data were interested in the study of the single effect of heavy metals on decolorization but in the present study, we tried to assess the effect of heavy metals co-existence on RB5 decolorization. It is clear that the strain decolorization ability was relatively damaged by the accumulation of high heavy metals concentrations in the same media. Hussain et al. (2013) demonstrated that *Pseudomonas* sp. isolate RA20 was able to completely decolorize RB5 in the presence of a multi-metal mixture containing Cr (2 mg l^{-1}), Cd (10 mg l^{-1}), Cu (10 mg l^{-1}), Zn (20 mg l^{-1}), Ni (20 mg l^{-1}) and Pb (20 mg l^{-1}) in a longer period of time compared to the control.

In the present work, heavy metals addition affected differently RB5 decolorization, thing which needs a genomic and proteomic level studies to understand the mechanisms involved in the dye-heavy metal bioremediation process. Modeling of the single heavy metal as well as the mixture effect on decolorization yield can improve the textile wastewater treatments performance. In order to minimize the time required for dye removal, genetic engineering investigation might be proposed to enhance the metal tolerance and/or removal by the strain. Strain Gb30 seems to be a profitable matrix for such investigations.

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Conflict of interest

The authors declared no conflict of interest

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Supporting Information legends

Figure S1. Effect of heavy metals concentrations on the growth rate of *P. aeruginosa* Gb30 during first 10 h incubation; • Zn^{2+}; ▪ Cd^{2+} ◆ Cu^{2+}; ▲ Cr^{6+}

Figure S2. HPLC analysis of untreated RB5 and biodegradation products in presence of heavy metals; a: untreated dye; b: Zn^{2+}; c: Cd^{2+}; d: Cu2+; e: Cr^{6+}

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Table 1: Heavy metal concentrations used for EC_{50} (10 h) determination

Metal	Source	Concentrations (mmol l^{-1})
Cadmium (Cd^{2+})	$CdCl_2$	0.1, 0.25, 0.5, 0.75, 1
Copper (Cu^{2+})	$CuSO_4$	0.5, 0.75, 1, 2, 3
Chromium (Cr^{6+})	$K_2Cr_2O_7$	0.1, 0.25, 0.5, 0.75, 1
Zinc (Zn^{2+})	$ZnSO_4$	0.5, 0.75, 1, 2, 3, 5

Table 2: Models used to evaluate the inhibitory effect of heavy metals on decolorization of RB5 by *P. aeruginosa* Gb30.

Model	Equation		References
(1)	$r = r_{max}\left(1 - \frac{C}{C_{crit}}\right)^m$	$Eq\ (3)$	(Wang and Wan, 2009)
(2)	$r = \frac{r_{max}}{1 + (C/K_c)^m}$	$Eq\ (4)$	(Wang et al., 2008)
(3)	$r = \frac{r_{max}\, K_C}{K_C + C}$	$Eq\ (5)$	(Liu et al., 2008)

Where r: initial decolorization rate (mmol l^{-1} h); r_{max}: maximum decolorization rate (mmol l^{-1} h); C: heavy metal ion concentration (mmol l^{-1}); C_{crit}: critical heavy metal ion concentration (mmol l^{-1}); K_c: metal inhibition constant (mmol l^{-1}) and m: empirical constant.

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Table 3: Inhibition constants and statistic parameters derived from the fitting of the RB5 decolorization experimental data with models (1), (2) and (3) for Zn^{2+}, Cd^{2+}, Cu^{2+} and Cr^{6+} ions and the mixture of all of them ($p \leq 0.05$).

Metal	Model	r_{max}	C_{crit} or K_c	m	R^2 (%)	R^2_{adj} (%)	RMSE	SSE
Zn^{2+}	Model 1	2.013	50	1.193	87.76	79.60	0.041	0.005
	Model 2	2.033	50	0.849	91.46	85.77	0.034	0.004
	Model 3	2.017	37.37	-	88.96	86.20	0.034	0.005
Cd^{2+}	Model 1	1.991	2.491	0.882	97.62	95.24	0.080	0.013
	Model 2	1.996	1.481	1.298	96.18	92.37	0.102	0.021
	Model 3	2.050	1.611	-	95.16	93.55	0.094	0.026
Cu^{2+}	Model 1	2.071	487.9	2166	97.85	96.78	0.135	0.073
	Model 2	2.055	0.189	2.675	98.87	98.30	0.098	0.039
	Model 3	2.069	0.107	-	96.07	95.29	0.164	0.134
Cr^{6+}	Model 1	2.051	1.762	25	98.49	97.73	0.111	0.050
	Model 2	2.056	0.048	1.629	99.31	98.96	0.075	0.023
	Model 3	2.065	0.033	-	98.18	97.81	0.109	0.060
Mixture	Model 1	2.496	0.1329	0.9667	0.9924	0.9873	17.76	946.4
	Model 2	2.078	0.06574	4.112	0.9977	0.9962	9.686	281.4
	Model 3	5.252	0.01586	-	0.9077	0.8847	53.59	1.15E+04

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Figures legends

Figure 1 16S rRNA sequences based phylogenetic tree showing the evolutionary relationships between newly isolated strain Gb30 and its closest relatives

Figure 2 Decolorization kinetics of RB5 in presence of EC_{50} (10 h) concentration of single and associated heavy metals; : control; : Zn^{2+}; : Cd^{2+}; :Cu^{2+};: Cr^{6+}; : heavy metals mixture (EC_{50}/8)

Figure 3 UV-Vis spectra of untreated dye, metal free culture and cultures added with Zn^{2+}, Cd^{2+}, Cu^{2+} and Cr^{6+} after 48 h of incubation at an initial metal concentration = EC_{50} (10 h); : untreated dye; : control; : Zn^{2+}; : Cd^{2+}; :Cu^{2+};: Cr^{6+};

Figure 4 RB5 decolorization kinetics in presence of different concentrations of Zn^{2+} (a): : control; : 3*EC_{50}; : 2.5*EC_{50}; :2*EC_{50};: EC_{50};: EC_{50}/2, Cd^{2+} (b): : control; : 2*EC_{50}; : EC_{50}; :EC_{50}/2;: EC_{50}/4; Cu^{2+} (d):): : control; : 1.5*EC_{50}; : EC_{50}; : EC_{50}/2;: EC_{50}/4;: EC_{50}/8,and Cr^{6+} (d): : control; : 1.5*EC_{50}; : EC_{50}; : EC_{50}/2;: EC_{50}/4;: EC_{50}/8

Figure 5 Heavy metals inhibition kinetics adjusted with models (1), (2) and (3); (a): Zn^{2+} ions; (b): Cd^{2+} ions; (c): Cr^{6+} ions; (d): Cu^{2+} ions; -: Model (1); -.- : Model (2) ; -- : Model (3)

Figure 6 Heavy metals mixture inhibition kinetic adjusted with model (2)

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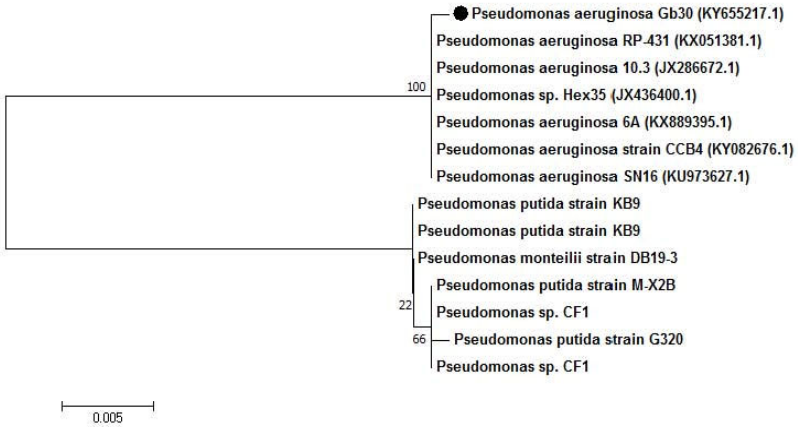

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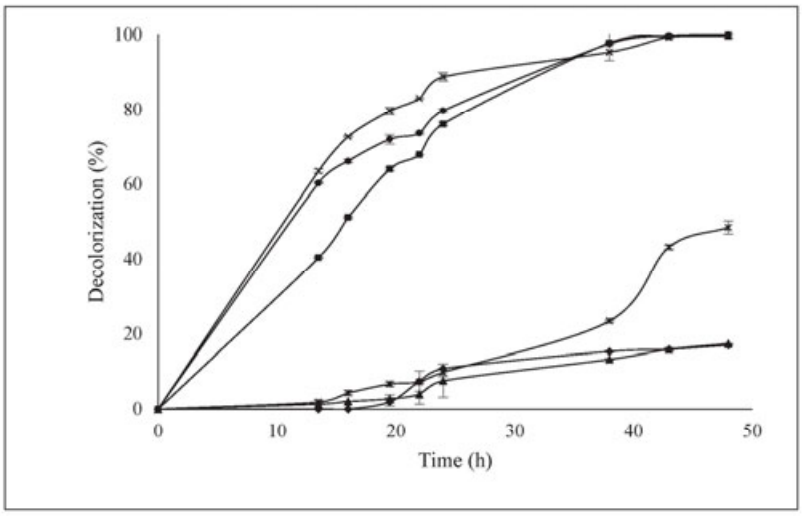

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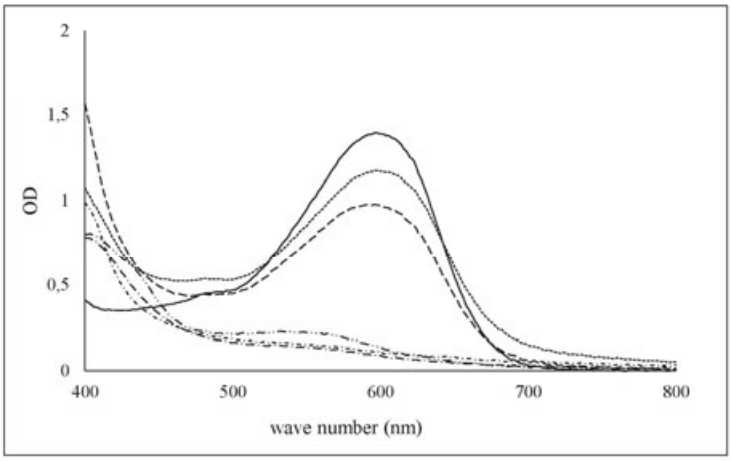

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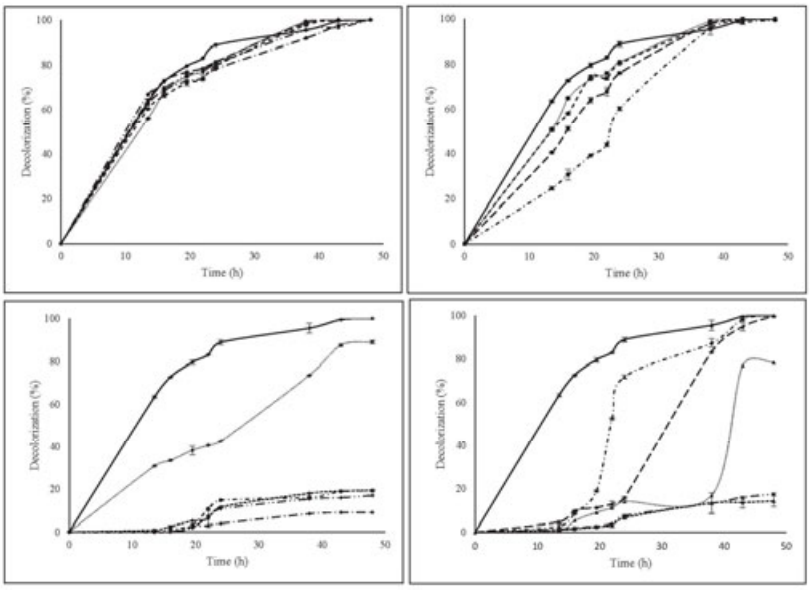

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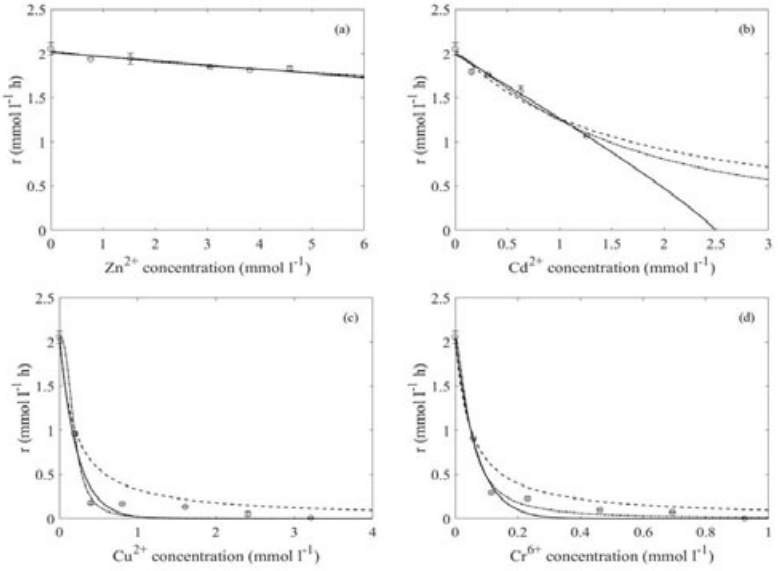

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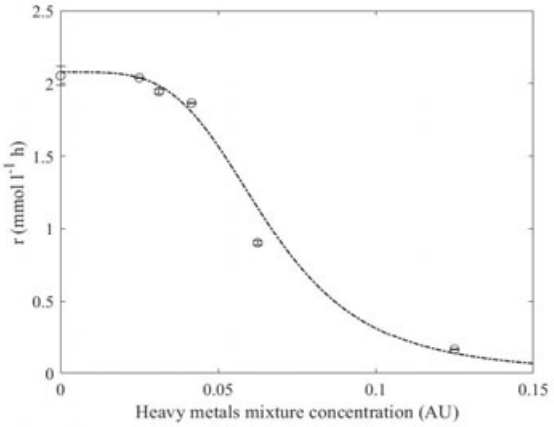

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