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Full Length Article

Simultaneous Removal of Reactive Dyes and Hexavalent Chromium by a Metal Tolerant *Pseudomonas* sp. WS-D/183 Harboring Plant Growth Promoting Traits

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Abstract

Textile industry is a continuous source of colored wastewater. This wastewater frequently used for irrigation purpose in many underdeveloped countries including Pakistan. In this study, we isolated the bacterial strains capable of decolorizing dyes and promote the plant growth. Hence to decolorize the reactive red 120 (RR120), the strain WS-D/183 was optimized following response surface methodology (RSM) based modeling approach. Moreover, strain WS-D/183 was also assessed for plant growth promoting characteristics. Results revealed that the strain WS-D/183 showed a good potential for decolorization of structurally diverse types of azo dyes on reaction with a mixture of heavy metal ions (Cr^{6+}, Cd^{2+}, Zn^{2+}, Pb^{2+}). This strain concurrently removed reactive dyes (100 mg L^{-1}) and reduced Cr(VI). Results showed that each dye was decolorized up to 90% except reactive yellow-2 which was decolorized up to 57.4%. Furthermore, the bacterium reduced Cr(VI) by 41 to 95% along with concurrent decolorization of RR120. This bacterium was also found to carry plant growth promoting traits including inorganic phosphate solubilization (497.6 ± 14.8 μg mL^{-1}) and indole-3-acetic acid production (21.07 ± 0.9 μg mL^{-1}). A phytotoxicity evaluation study indicated that irrigation of mung bean [*Vigna radiata* (L.) Wilczek] with RR120, Cr(VI) and RR120+Cr(VI) contaminated waters treated with the strain WS-D/183 enhanced germination along with plumule and radical length of seedlings. Results suggested that *Pseudomonas* sp. WS-D/183 is a valuable addition to the bioresources, which can be used to devise textile wastewater treatment strategies as well as for integrated bioremediation and plant growth promotion in agricultural soils contaminated with textile wastewaters. © 2020 Friends Science Publishers

Keywords: Textile wastewaters; Azo-dyes decolorization; Cr (VI)-reduction; Multi-metal stress; RSM; PGPB; Phytotoxicity; Mung bean

Introduction

In textile industry, a major proportion of dyes used for dyeing fabrics are azo-dyes. These dyes are preferred over other classes of synthetic dyes due to their low price, ease of application and availability in a variety of colors (Shah *et al*. 2014). However, a high fraction of the applied quantity ranging between 15–50% is wiped out in dyeing and washing processes and forms the colored wastewaters (Pratum *et al*. 2011). Release of colored wastewaters also causes entry of azo dyes in water and soil resources (Tony *et al*. 2009). Such dyes disrupt light penetration into water, which effect photosynthetic rate of hydrophytes (Shah *et al*. 2014). It is reported that azo dyes and their metabolites can cause cancer and add poisonous materials in the environment (Carneiro *et al*. 2010). In addition, entry of such wastewaters in soil resources results into a disturbance in soil microbial communities, soil processes and crop productivity (Arif *et al*. 2016; Rehman *et al*. 2018).

Azo dyes and metal-complexed dyes as well as different metal-based salts are also heavily used as mordant for fixation of dyes in textile industry (Desai *et al*. 2009). Consequently, some metals including cadmium, zinc, chromium and lead can be found in textile wastewater above permissible limits (Tuzen *et al*. 2008; Imtiazuddin *et al*. 2014). Presence of dyes and metal ions are the main reason for toxic effects of textile wastewater. Among heavy metal ions, hexavalent [Cr(VI)] commonly co-exists with

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azo dyes in effluents of leather and textile industries (Tuzen *et al*. 2008). Among 17 chemicals, it poses the greatest risk to humans (Quintelas *et al*. 2008). Hence, it is important to develop remediation approaches for concurrent elimination of dyes and hexavalent chromium from wastewater of leather and textile industry.

Previous studies showed that biotic methods for removal of organic and inorganic pollutants are one of innovative, economic and environmental viable techniques (Imran *et al*. 2015a). In this perspective, researchers have identified several bacteria having potential to degrade the textile dyes (Imran *et al*. 2016; Hussain *et al*. 2017). Similarly it has been reported that microbes have the potential to detoxify Cr(VI) metal (Park *et al*. 2006). Furthermore, some efforts of isolating, identifying and characterizing microbes capable of concurrently degrading azo dyes and detoxifying Cr(VI) have met with success (Hussain *et al*. 2017). Therefore, there is a need to isolate more efficient bioresources which can remove dyes and reduce Cr(VI) and exhibit the tolerance against other co-existing metal ions. In this study, we isolated and characterize the metal tolerant bacterium capable of removing dyes and reduce Cr(VI) in a medium spiked with a mixture of Pb^{2+}, Cd^{2+} and Zn^{2+}.

Materials and Methods

Growth media for bacterial growth

A mineral salt medium (MSM) spiked with four heavy metals was used to isolate metal-tolerant, dye degrading bacterial isolate. The ingredients of MSM used in this study are given in Maqbool *et al.* (2016). The mineral salt medium was spiked with four different heavy metal salts as previously reported in Maqbool *et al*. (2016). However, pH of MSM was maintained at 7.2 using basic and acidic solutions.

Isolation, Screening and molecular identification of isolate WS-D/183

For bacterial isolation, effluent samples were taken from outlets of various textile industries. These industries were located near Sargodha Road, Faisalabad, Pakistan. Serial dilution of collected wastewater samples was performed and about 100 μL of 10^{-3}–10^{-6} dilutions was poured on MSM agar plates. These plates were spiked with selected heavy metals and placed in static incubator at 30°C. The bacterial isolates having variable size, color and shape were purified through streaking thrice using MSM agar plates. To prepare inoculum of each isolate, the cultures of all bacterial isolates were inoculated in Erlenmeyer flasks and incubated at 30°C for 72 h. By using sterile MSM, the OD_{600} of all bacterial culture was adjusted as 0.5. Then, 18 mL of MSM containing mixture of heavy metals and RR120 were added separately along 2 mL of isolates in test tubes and incubated at 30°C. After two days of incubation, removal of initially added dye was determined (Maqbool *et al*. 2016). The isolate WS-D/183 was selected for further characterization based on its potential to decolorize the RR120.

A 16S rRNA gene amplification and sequencing was done for molecular identification of the strain WS-D/183 (Maqbool *et al*. 2016). The similarity of this sequence with already known reference sequences of different bacteria was checked as previously reported by Hussain *et al*. (2013); whereas, phylogenetic tree was made by NJ plot (Perriere and Gouy 1996).

Metal tolerance of isolate WS-D/183

The metal tolerance of the bacterium was determined by estimating MIC of selected heavy metals. For this purpose, nutrient agar medium was spiked with cobalt, chromium, zinc, lead, nickel, cadmium salts to develop 0–5000 mg L^{-1} of given heavy metals. The strain WS-D/183 was streaked on each plate and preserved for five days to check the growth of WS-D/183. The minimum concentration of a particular metal that inhibited growth of the strain WS-D/183 was considered as MIC for the strain WS-D/183.

Optimization of RR120 decolorization by isolate WS-D/183

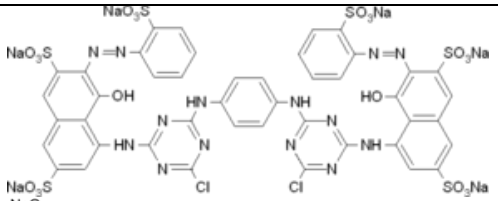
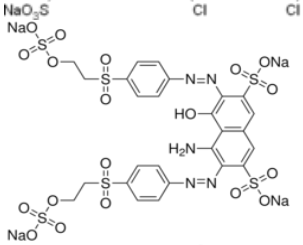
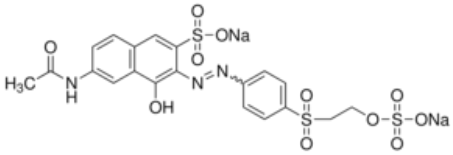
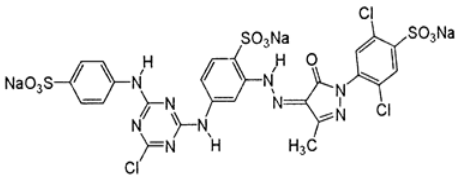
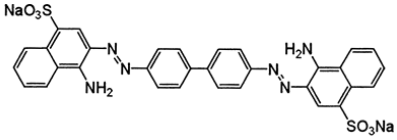
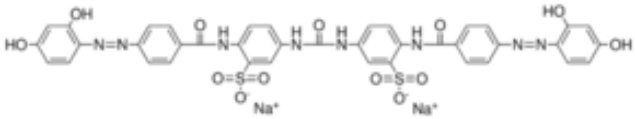
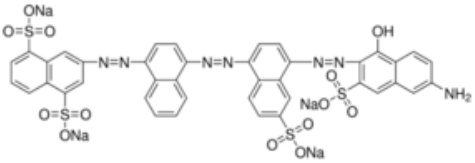
Bio-decolorization of structurally diverse azo dyes: The ability of WS-D/183 for decolorizing different reactive dyes and direct dyes [orange direct (OD), Congo red (CRD) and blue direct (BD)] was assessed in MSM spiked with mixture of metal ions. The general characteristics of these dyes are given in Table 1. The experiment was conducted in glass vials. The cells of the strain WS-D/183 were added to MSM and OD_{600} was adjusted at 0.05. After filter sterilization, each dye was added to maintain 100 mg/L conc. Triplicates of inoculated vials along with un-inoculated control were kept at 30°C. After 24, 48, 72 and 144 h, decolorization was determined as described above.

Optimization of RR120 decolorization by the strain WS-D/183 using RSM: Effect of pH, yeast extract, sodium chloride and metal mixture on removal of RR120 via WS-D/183 was assessed following RSM. Each selected variable was studied at five levels as previously explained in Maqbool *et al*. (2016). Small composite design (SCD) comprising of eight factorial points, eight axial runs and five center points was chosen (Maqbool *et al*. 2016). Decolorization of RR120 determined after 72 h was set as the response variable as explained above. A second order polynomial model was selected to be estimated through observed data by SCD, as given below;

$$E(y|\boldsymbol{x}) = \beta_{\circ} + \sum_{i=1}^{k} \beta_{i} x_{i} + \sum_{i=1}^{k} \beta_{ii} x^{2}{}_{i} + \sum_{i=1}^{k-1} \sum_{j>i}^{k} \beta_{ij} x_{i} x_{j},$$

Where $E(y|x)$ represents expected response given vector $\boldsymbol{x}$ of predictor variables, $\beta_{\circ}$ is a regression constant, β_i is linear regression coefficient, β_{ii} is quadratic regression coefficient and β_{ij} is bilinear regression coefficients.

Table 1: General characteristics of azo dyes

Azo dyes	Molecular weight	Color index number	λ_{max}	Structure
Reactive Red 120	1469.98		535	
Reactive Black 5	991.80	20505	597	
Reactive Orange 16	601.54	17757	494	
Reactive Yellow 2	873.00	18972	404	
Congo red	696.66	22120	497	
Orange Direct	926.81	25430	516	
Blue Direct	965.94	34140	594	

The model adequacy was assessed by lack of fit technique. Lack-of-fit of second-order model indicated that quadratic model lack-of-fit is highly insignificant with low F-value and high p-value. Thus, quadratic polynomial model was more appropriate to describe the relation of response (*i.e.*, decolorization) to the input factors (*i.e.*, pH, NaCl, yeast extract and heavy metal mixture content). The evaluation of variable significance of whole model was checked by R^2 and *F*-test. Moreover, confidence levels were determined to check significance of R^2. Variance inflation factor was also calculated to measure extent of multicollinearity among two or more input variables of polynomial regression model.

Concurrent removal of RR120 and hexavalent chromium using WS-D/183

The strain WS-D/183 was also checked for concurrent elimination of RR120 and Cr(VI) in MSM in the presence of Zn^{2+}, Pb^{2+} and Cd^{2+} mixture. In first step, the bacterium was checked for Cr(VI) removal in MSM taken in glass vials. The supernatants taken over time were analyzed for Cr(VI) content with diphenylcarbazide (DPC) method (Anwar *et al*. 2014). After confirmation of Cr(VI) reduction, it was evaluated for concurrent elimination of RR120 and hexavalent chromium. For this purpose, filter sterilized RR120 was added to MSM spiked with Cr^{+6}, Zn^{2+}, Pb^{2+} and Cd^{2+}. After inoculation, vials along with

un-inoculated controls were placed in an incubator. The supernatant was collected after 0, 30, 60, 90, 120, 150, 180 and 210 h. It was used to measure decolorization and removal of Cr(VI) by using DPC method, whereas, bacterial pellet was used to measure growth of the bacterium. The bacterial pallets were rinsed and suspended in distilled water. OD_{600} was determined using a CO800 Cell Density Meter (Biochrom, England).

Assessment of PGPR traits of WS-D/183

We studied the indole acetic acid (IAA) and phosphorus solubilization as indices of PGPR traits. Gordon and Weber (1951) method was employed to determine IAA production by the bacterial strain. Briefly, LB broth containing L-tryptophan, IAA precursor, was inoculated with the strain WS-D/183. After 48, 120 and 240 h of incubation, IAA content and pH of the supernatant were determined. For IAA measurement, two drops of H_3PO_3 and 1 mL Salkowski reagent were added to supernatants and kept at room temperature for few minutes. The strength of pink color developed was assessed at 530 nm. A calibration plot was developed using IAA standards and used to determine IAA.

For measuring phosphate solubilization, NBRIP broth medium having tri-calcium phosphate as insoluble phosphorus source was prepared. The broth was inoculated with the culture of the strain WS-D/183 and incubated under static conditions. After 48, 120 and 240 h of incubation, phosphorus content and pH of the filtrate were determined. Aliquots taken after regular intervals were filtered using Whattman No. 1 filter paper. Afterwards, the filtrate was subjected to centrifugation to get clear supernatant. The supernatants were used for measuring phosphate solubilization as previously reported by Maqbool *et al*. (2018).

Phytotoxicity Evaluation of the wastewater treated with WS-D/183

Three MSM flasks containing 500 mg RR120 L^{-1}, 25 mg Cr(VI) L^{-1} and RR120+Cr(VI) were inoculated with the strain WS-D/183 for 72 h in order to obtain treated water for phyto-toxicity evaluation of the strain. The untreated RR120, Cr(VI) and RR120+Cr(VI) along with their respective treated counterparts were used to determine their toxicity on seed germination of mung bean [*Vigna radiata* (L.) Wilczek]. Ten seeds were sown in sand taken in petri plates. The sand plates containing seeds according to the below given eight treatments were watered. Experiment was conducted in triplicate in growth chamber where plants received the light for 16/8h light/dark periods at temperature of 25/30°C and day/night humidity of 70/90%. After 10 days, germination (%), radical length and plumule length was measured.

Treatments: T_0 = No dye, T_1 = RR120 @ 500 mg L^{-1}, T_3 = Cr(VI) @ 25 mg L^{-1}, T_4 = RR120 @ 500 mg L^{-1} + Cr(VI) @ 25 mg L^{-1}, T_5 = No dye + WS-D/183, T_6 = RR120 @ 500 mg L^{-1} + WS-D/183, T_7 = Cr(VI) @ 25 mg L^{-1} + WS-D/183, T_8 = RR120 @ 500 mg L^{-1} + Cr(VI) @ 25 mg L^{-1} + WS-D/183.

Statistical analysis

Analysis of the observed experimental data in RSM was carried out using Design Expert 9 software. Fitness of the model used in this study was evaluated using a number of parameters. Moreover, one way analysis of variance (ANOVA) was used for determination of significance of treatment effects on growth of mung bean. Tukey's HSD test was used for multiple means comparisons for parameters where significant treatment effects were found. Tables and figures contain means and standard errors of means from three replicates. All statistical analyses were performed using Statistix8.1 software.

Results

Molecular identification of the isolate WS-D/183

More than 200 bacteria were isolated and checked for decolorization of RR120 in MSM spiked with a mixture of Cr^{6+}, Pb^{2+}, Cd^{2+} and Zn^{2+}. The decolorization ranged from 1.3±1.1 to 95.6±3.2% for various bacterial isolates. The maximum decolorization of the RR120 was shown by the isolate WS-D/183. Bioinformatic analysis of 16S rRNA gene of the isolate WS-D/183 through BLASTn revealed that it had the highest similarity with several species of genus *Pseudomonas*. Moreover, during phylogenetic analysis the isolate WS-D/183 (GeneBank Accession No. MG547881) was clustered with bacterial spp. belonging to genus *Pseudomonas* (Fig. 1). Moreover, phylogenetic tree revealed that WS-D/183 belongs to genus *Pseudomonas* and designated as *Pseudomonas* sp. WS-D/183 (Fig. 1).

MIC of metal ions for *Pseudomonas* sp. WS-D/183

The MIC of six metals for growth of *Pseudomonas* sp. WS-D/183 is presented in Table 2. The strain WS-D/183 showed variable resistance towards metal ions. The MIC of Co^{2+}, Cr^{6+}, Zn^{2+}, Pb^{2+}, Ni^{2+} and Cd^{2+} for the bacterium were 13.58, 9.62, 30.59, 4.83, 3.41 and 44.48 m*M*, respectively.

Potential of WS-D/183 to decolorize structurally diverse textile dyes

Pseudomonas sp. WS-D/183 showed excellent removal of selected reactive and direct dyes (Table 3). However, it was observed that it showed higher decolorization of reactive than direct dyes. After 24 h treatment, the strain WS-D/183 showed maximum decolorization of RB5 (53.5±1.1%) followed by RR120 (18.3±1.3%) and RY2 (17.7±3.2%) as compared to 14.7% (±1.7), 7.5% (±2.7) and 3.4% (±1.1) decolorization of BD, CRD, OD, respectively. However, after 144 h, more than 96% of the added RR120

Table 2: Metal resistance (MIC) of different heavy metals by WS-D/183

Metals	Source	MIC (m*M*)
Cobalt	$Co(NO_3)_2$	13.58
Chromium	$K_2Cr_2O_7$	9.62
Zinc	$ZnSO_4$	30.59
Lead	$Pb(NO_3)_2$	4.83
Nickel	$NiCl_2.6H_2O$	3.41
Cadmium	$CdCl_2$	44.48

Table 3: Potential of *Pseudomonas* sp. WS-D/183 for removal of reactive and direct dyes

Dyes	Color removal (%)			
	24 h	48 h	72 h	144 h
Reactive Red 120 (RR120)	18.3 ± 1.3	26.3 ± 0.8	65.0 ± 2.1	96.1 ± 2.3
Reactive Orange 16 (RO16)	4.9 ± 2.4	31.4 ± 1.2	49.9 ± 2.7	63.7 ± 2.4
Reactive Black 5 (RB5)	53.5 ± 1.1	82.4 ± 2.2	86.2 ± 1.9	89.2 ± 1.1
Reactive Yellow 2 (RY2)	17.7 ± 3.2	58.4 ± 2.1	60.6 ± 3.4	64.2 ± 2.1
Congo Red direct (CRD)	7.54 ± 2.7	23.7 ± 1.4	43.2 ± 2.1	72.5 ± 1.5
Orange Direct (OD)	3.4 ± 1.1	11.8 ± 2.3	26.7 ± 1.7	34.6 ± 3.2
Blue Direct (BD)	14.7 ± 1.7	33.8 ± 2.6	41.5 ± 1.8	48.2 ± 2.6

Values are means of three replicates followed by ± standard error of means (n = 3)

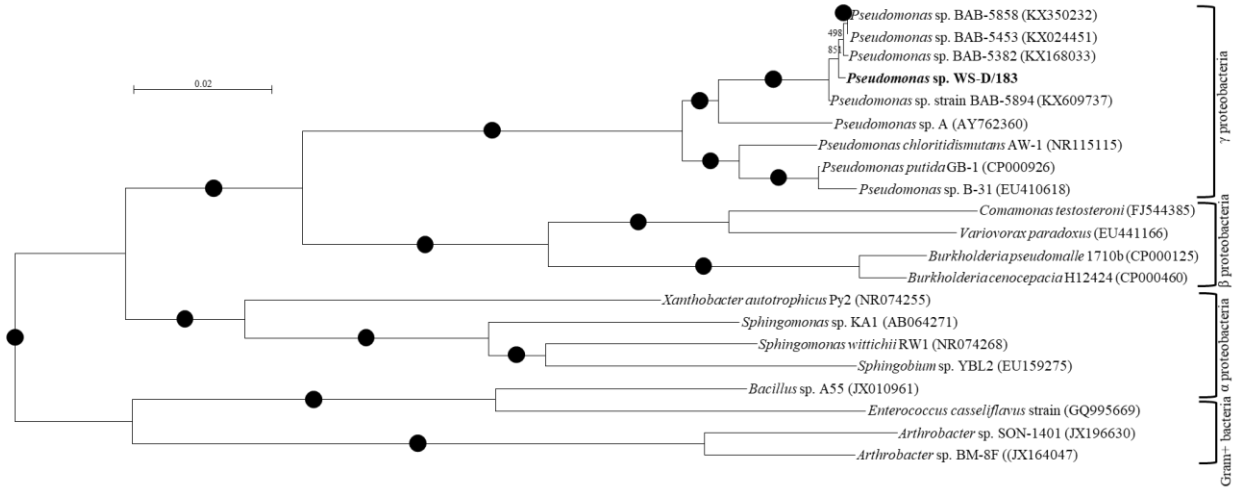


Fig. 1: Phylogenetic tree analysis using multiple alignment of 16S rRNA gene sequence of *Pseudomonas* sp. WS-D/183

was decolorized followed by RB5 (89.2±1.1%), RY2 (64.2±2.1%) and RO16 (63.7±2.4%). The other dyes CRD, BD and OD dyes were decolorized up to 72.5 (±1.5%), 48.2 (±2.6%) and 34.6 (±3.2%) respectively, over the same treatment period.

Optimization of RR120 dye decolorization by *Pseudomonas* sp. WS-D/183 using RSM

Validation and importance of RSM model: Response surface methodology was used to optimize four different variables for biodecolorization of RR120. The quadratic model showed lowest sequential p-value (0.0001). Data for sequential model selection suggested preferring quadratic over two-factor interaction model because it had very low *p*-value (Table 4). Moreover, cubic model terms were found different from each other. The model validation showed a high value of R^2=0.8531 with adjusted-R^2=0.6736. This indicated a big share of variation in response that can be addressed by quadratic polynomial model. Furthermore, "Adeq precision" ratio was found as 9.846. Predicted residual sum of square (PRESS) determines how well the hypothesized model is being estimated by the design. Lower PRESS value showed good performance of the model. Quadratic model showed lower PRESS as compared to other models.

The estimated regression model is presented below which shows the contribution of different second-order model terms for the response.

$$\text{Decolorization} = 21.42 - 8.63x_1 + 13.06x_2 + 15.96x_3 - 1.55x_4 + 2.21x_1^2 + 6.88x_2^2 - 8.79x_3^2 - 0.24x_4^2 - 9.65x_1x_2 - 3.21x_1x_3 + 7.67x_1x_4 + 6.20x_2x_3 - 2.03x_2x_4 + 10.22x_3x_4$$

Salt (x_1) and metal concentration (x_4) are contributing

Table 4: Summary of data statistics regarding model selection

Source	Sequential p-value	Lack of Fit p-value	Adjusted R-Squared	Predicted R-Squared	
Linear	0.0463	0.0017	0.3102	-0.1671	
2FI	0.4105	0.0013	0.3479	-4.5158	
Quadratic	0.0001	0.9802	0.9793	0.9910	Suggested
Cubic	0.9802		0.9742		Aliased

Table 5: Analysis of variance (ANOVA) for RR120 decolorization using quadratic model

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	7802.11	14	557.29	65.30	0.0001	significant
A-Salt	596.51	1	596.51	69.90	0.0004	
B-pH	1364.51	1	1364.51	159.89	< 0.0001	
C-C-Source	2265.36	1	2265.36	265.45	< 0.0001	
D-Metal Conc.	19.10	1	19.10	2.24	0.1949	
AB	372.20	1	372.20	43.61	0.0012	
AC	82.37	1	82.37	9.65	0.0266	
AD	235.24	1	235.24	27.57	0.0033	
BC	307.64	1	307.64	36.05	0.0018	
BD	16.42	1	16.42	1.92	0.2240	
CD	834.97	1	834.97	97.84	0.0002	
A^2	120.69	1	120.69	14.14	0.0131	
B^2	1164.92	1	1164.92	136.50	< 0.0001	
C^2	989.08	1	989.08	115.90	0.0001	
D^2	1.37	1	1.37	0.16	0.7055	
Residual	42.67	5	8.53			
Lack of Fit	0.007411	1	0.007411	0.00069	0.9802	not significant
Pure Error	42.66	4	10.67			
Corrected Total	7844.78	19				

negatively to remove RR120 dye by WS-D/183, though the contribution of metal concentration is comparatively very low. The pH (x_2) and Yeast extract (x_3) are positively contributing for the response, so their quadratic effects and the interaction are also high. It is interesting to note that metal concentration has low linear as well as quadratic effects but this is strongly positively contributing for response when interacts with salt and Yeast extract. Salt and pH also have strong but negative contribution for biodecolorization of RR120 azo dye.

The ANOVA is presented in Table 5. Highly significant model with a low p-value again qualifies the second-order model. The significance of R^2 was calculated by estimating confidence limits; all R^2 were found in range of confidence limits. High R^2 = 0.8531 and adjusted R^2=0.6736 confirmed the model reliability.

Fig. 2 shows 3D plots for graphic appreciation of analysis. Fig. 2 (a–c) show normal probability plot constructed for externally studentized residuals and normal % age probabilities, a plot constructed for predicted versus externally studentized residuals and a plot for run number (design point) against externally studentized residuals, respectively. These are diagnostic plots used to assess the leverage of each design point. We can observe that one design point is exerting undue leverage for the response which may be regarded as an outlier. The analysis of data in the presence of an outlier may be misleading. Therefore, for the further analysis we ignore that outlier. From Fi. 2(d) we can see that actual data is lying along predicted line, which means that it is a good fit of quadratic model. Fig. 2(e) presents Box-Cox plot drawn for lambda (power variable) vs the natural log of sum of squares of residuals. The lambda value corresponding to the lowest residual value is the natural choice. The perturbation plot depicted the impact of an input variable on the response when it is varied from a reference point, which is usually the central level. Clearly, the response is highly insensitive to varying levels of metal concentration. Cook's distances are plotted in Fig. 2(g) showing the random effect changes if a point is deleted. All cook's distances lie within the acceptable range. Fig. 2(h) shows the plot of leverage values against runs. All leverage values lie in the interval of 0 and 1. Difference in fits (DFFITS) measures the amount of influence the ith observation has on the predicted response. All DFFITS values lie in the acceptable interval (-2, 2) (Fig. 2(i)). Another measure which is closely related to DFFITS is known as DFBETAS, which measures changes in the model related to each regression coefficient (Fig. 2j). Most values lie along the straight line representing 0.

Separate and interactive effects of input variables on output variable

Individual, quadratic and interactive effects of selected four parameters on RR120 decolorization by WS-D/183 are shown in Table 5. Effect of salt concentration, pH and yeast extract content was significant but metal mixture concentration did not show a significant linear contribution in RR120 decolorization. Similarly, quadratic effects of salt concentration, pH and yeast extract content are also significant (p<0.05) but the quadratic effect of metal mixture concentration is not significant (p>0.05). The salt

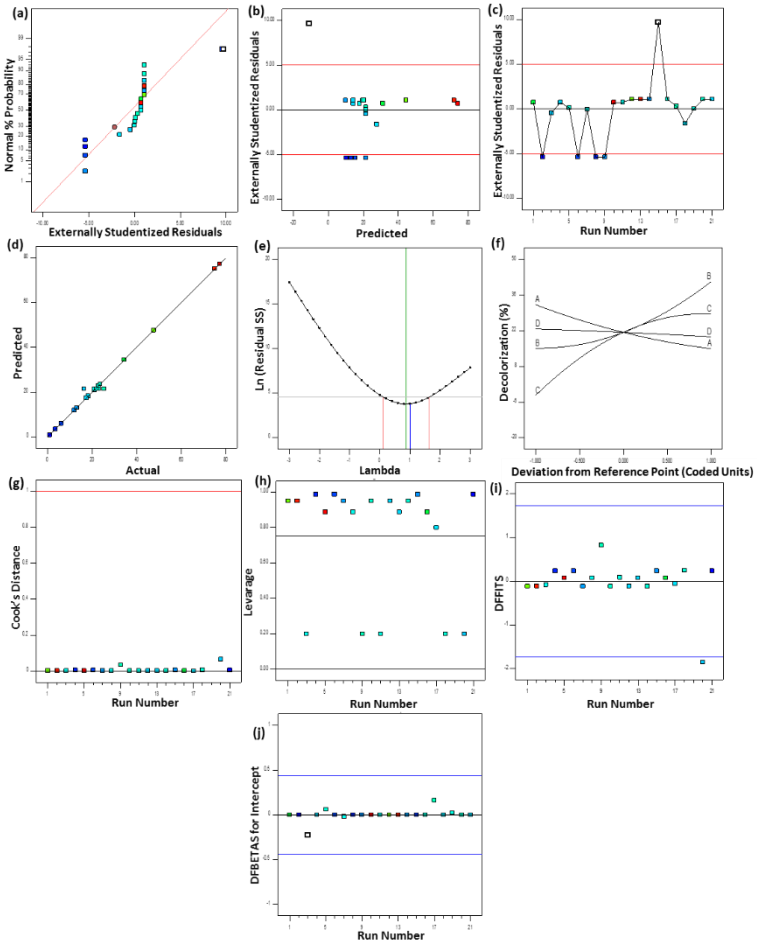


Fig. 2: Diagnostic plots for model validation

concentration and pH, salt concentration and metal mixture concentration, salt concentration and Yeast extract content, pH and Yeast extract content, and Yeast extract content and metal mixture concentration showed significant (p<0.05) interactive effects on RR120 decolorization by WS-D/183.

A 3-D response plots showed interactive effects of four input variables including pH, NaCl, yeast extract and metal mixture content on decolorization of RR120 by strain WS-D/183 (Fig. 3). The selected salt concentrations did not significantly affect decolorization of RR120 at low pH (Fig. 3a). However, at higher pH, increasing the salt concentration significantly (p<0.05) decreased RR120 decolorization. Results also indicated that increased concentration of yeast extract resulted in an increase in RR120 decolorization in the presence of lower as well as higher salt concentration levels (Fig. 3b). However, the effect of yeast extract concentration was more pronounced at lower salt concentration. Fig. 3(c) shows the interactive effect of salt and multi-metal mixture concentration on decolorization of RR120 and explains that RR120 decolorization was comparatively less sensitive to changing levels of salt and metal concentration. The interactive effect of pH and yeast extract content significantly increased the decolorization with increasing values of both variables with the maximum decolorization achieved at highest values of both of these variables (Fig. 3d). Fig. 3(e) shows that decolorization is insensitive to changing levels of multi-metal concentration in contrast to pH levels which increases decolorization as its level increases. It was also observed that higher concentration of yeast extract was found to have a positive impact on RR120 decolorization in the presence of lower as well as higher concentrations of the multi-metal mixture while keeping the pH and salt concentration at their middle level (Fig. 3f). Moreover, it was interesting to notice that higher content of multi-metal mixture was found to decrease decolorization of RR120 at lower concentration of yeast extract. According to the model, the optimized values of pH, salt, yeast extract concentration and multi-metal mixture content to achieve the highest were predicted to be 8.0, 12.5 g L^{-1} and 7.5 g L^{-1} and a multi-metal mixture (Cr: 20 ; Pb: 40; Cd: 20 and Zn: 40 mg L^{-1}) respectively.

Simultaneous removal of reactive dyes and Cr(VI) using WS-D/183

The bacterium did simultaneous removal of all four reactive

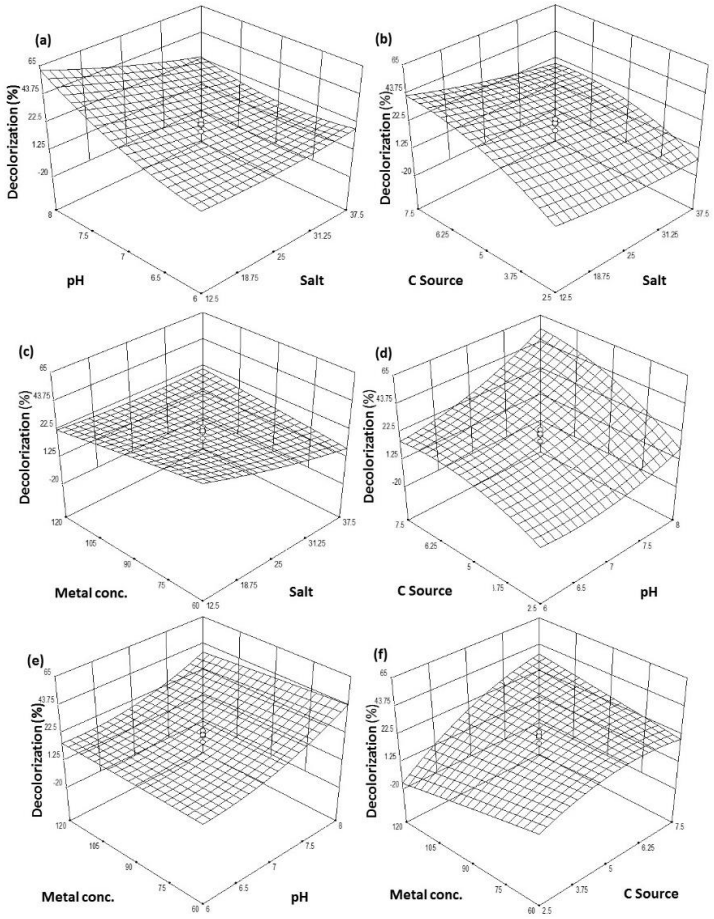


Fig. 3: 3-D Response plots for different factors exhibiting interactive effects of independent variables on decolorization (%) of RR120 by *Pseudomonas* sp. WS-D/183

dyes and Cr(VI) in a medium spiked with Zn^{+2}, Cd^{+2} and Pb^{+2} mixture (4a – 4d). It was observed that during the first 60 h, rate of Cr(VI) reduction by the strain WS-D/183 was faster than decolorization of reactive dyes. About 62.1% (±2.1), 79.8% (±1.7), 26.2% (±1.1) and 43.5% (±2.5) of 25 mg Cr(VI) L^{-1} was removed within the first 60 h in the presence of RR120, RY2, RO16 and RB5, respectively. In parallel to Cr(VI) removal, RR120, RY2, RO16 and RB5 were decolorized up to 21.4% (±0.9), 1.5% (±0.6), 2.2% (±1.1) and 4.6% (±1.3), respectively during the same incubation period. However, at the end of experiment, decolorization of different dyes reached up to 58–90%, whereas Cr(VI) removed up to 42–95%. Also, the growth of strain WS-D/183 was monitored and found to be increased during simultaneous removal of textile dyes and Cr(VI) (Fig. 4e). Furthermore, the Cr(VI) removal and bacterium growth were significantly correlated to each other (Fig. 4f).

Plant growth promoting traits of the strain WS-D/183

Table 6 shows that the strain WS-D/183 has considerable potential of IAA production in LB broth amended with tryptophan. IAA content measured after 48 h was relatively low (2.23 ± 0.2), however it increased by 8.46 fold after 240 h. Moreover, a gradual decrease in pH of medium (6.9–5.3) was also recorded during incubation period (0–240 h).

Clear halo-zones were found around the culture of strain WS-D/183 grown on Pikovskaya's agar plates due to solubilization of TCP in NBRIP medium (data not shown). Based on qualitative analysis, the strain WS-D/183 was inoculated in liquid medium containing tri-calcium phosphate (1000 μg mL^{-1}). Results revealed that the strain WS-D/183 showed good potential for phosphate solubilization (Table 6). It was observed that 222 μg mL^{-1} (±8.5) soluble phosphate was released within 48 h of incubation under shaking conditions. However, at the end of the experiment (240 h), 497.6 μg mL^{-1} (±14.8) soluble phosphate was released by the strain WS-D/183 in the liquid medium under shaking condition at 30°C. Moreover, gradual decrease in pH (6.2 to 4.2) of the growth medium was also recorded (Table 6).

Phytotoxicity evaluation

Data regarding the impacts of untreated and treated wastewater containing RR120 azo-dye as well as Cr(VI)

Table 6: Potential of *Pseudomonas* sp. WS-D/183 for IAA production, Phosphate solubilization and change in pH of media

Time	Indole acetic acid production (µg mL^{-1})	pH	Phosphate solubilization (µg mL^{-1})	pH
48	2.23 ± 0.2	6.9 ± 0.2	222.0 ± 8.5	6.2 ± 0.3
120	6.16 ± 0.4	5.9 ± 0.3	442.1 ± 9.0	5.1 ± 0.3
240	21.07 ± 0.9	5.3 ± 0.1	497.6 ± 14.8	4.2 ± 0.2

Values are means of three replicates followed by ± standard error of means (n = 3) $p<0.05$

Table 7: Evaluation of phytotoxicity of the treated and untreated reactive red-120 (RR120) and hexavalent chromium [Cr(VI)] on Mungbean germination

Treatments	Removal (%)	Germination (%)	Plumule length (cm)	Radical length (cm)
No dye	---	83.3 ± 5.8 a	9.8 ± 0.4 a	5.63 ± 0.49 abc
RR120*	---	60.0 ± 5.0 cd	5.3 ± 0.7 de	3.53 ± 0.40 d
Cr(VI)**	---	53.3 ± 2.9 d	5.6 ± 0.8 d	3.30 ± 0.53 d
RR120* + Cr(VI)**	---	36.7 ± 2.9 e	4.4 ± 0.6 e	2.80 ± 0.66 d
No dye + WS-D/183	---	86.7 ± 7.6 a	9.6 ± 0.9 ab	5.97 ± 0.40 a
RR120* + WS-D/183	92.1 ± 2.5	71.7 ± 5.8 b	10.3 ± 0.5 a	5.83 ± 0.90 ab
Cr(VI)** + WS-D/183	81.4 ± 3.1	70.0 ± 5.0 b	8.6 ± 0.7 bc	4.97 ± 0.32 c
RR120* + Cr(VI)** + WS-D/183	> 80	68.3 ± 10.4 bc	8.4 ± 1.1 c	5.13 ± 0.38 bc
LSD		8.6	1.04	0.77

* RR120 added @ 500 mg L^{-1}, ** Cr(VI) added @ 25 mg L^{-1}

Values are means of three replicates followed by ± standard error of means (n = 3). The means sharing different letters in a column differ significantly at $p<0.05$ from each other

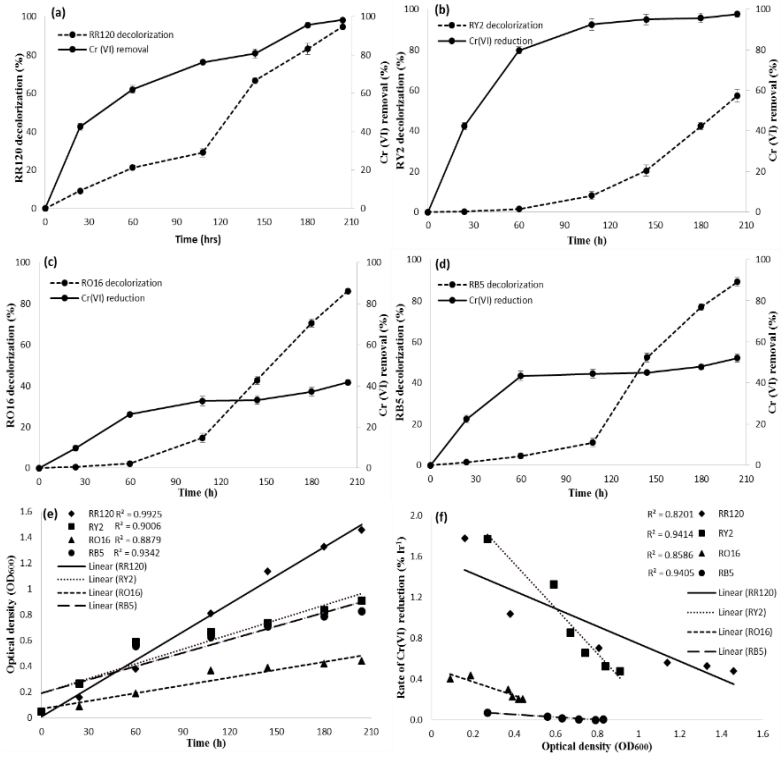


Fig. 4: Simultaneous removal of Cr(VI) and reactive dyes by *Pseudomonas* sp. WS-D/183 in MS broth medium containing three other heavy metals (Zn, Cd, Pb). (a) Simultaneous removal of Cr(VI) and reactive red-120. (b) Simultaneous removal of Cr(VI) and reactive yellow-2. (c) Simultaneous removal of Cr(VI) and reactive orange-16. (d) Simultaneous removal of Cr(VI) and reactive black-5. (e) Correlation between growth and time of incubation for removal of Cr(VI) and reactive dyes by the strain *Pseudomonas* sp. WS-D/183. (f) Correlation between growth and rate of Cr(VI) reduction by the strain *Pseudomonas* sp. WS-D/183

residues on germination, radical length and plumule length of mung bean showed that about 60.0% (± 5.0), 53.3% (±2.9) and 36.7% (±2.9) seeds of mung bean germinated when they were irrigated with untreated RR120 solution, Cr(VI) solution and RR120+Cr(VI) solution, respectively (Table 7). However, germination of mung bean seeds increased significantly when they were irrigated with treated RR120 solution, Cr(VI) solution and RR120+Cr(VI) solutions with 71.7% (± 5.8), 70.0% (± 5.0) and 68.3% (± 10.6) germination, respectively. Similarly, the plumule length was significantly reduced when mung bean was irrigated with RR120 solution (5.3 ± 0.7 cm), Cr(VI) solution

(5.6 ± 0.8 cm) and RR120+Cr(VI) solution (4.4 ± 0.6 cm) as compared to that of water (9.8 ± 0.4 cm). However, plumule length of mung bean significantly increased when irrigated with treated RR120 solution, Cr(VI) solution and RR120+Cr(VI) solution as compared to their respective untreated solutions (Table 7). Similarly application of untreated RR120 solution, Cr(VI) solution and RR120+Cr(VI) solution significantly decreased radical length. However, application of these solutions after bacterial treatment significantly increased radical length compared to respective untreated solutions (Table 7).

Discussion

Among more than 200 bacterial isolates tested in this study, the isolate WS-D/183 showed maximum decolorization of RR120. Based on analyses of its 16S rRNA gene, the isolate designated as *Pseudomonas* sp. WS-D/183 (Fig. 1). Previous studies showed that a few bacterial strains of genus *Pseudomonas* can degrade azo-dyes (Hussain *et al*. 2013; Maqbool *et al*. 2016). It was observed that isolate WS-D/183 has this ability even in the presence of multiple heavy metals ion, which is more commonly encountered in real world.

The strain WS-D/183 showed variable capacity to decolorize the reactive and direct dyes. This might be due to their structure or the varying extent of adaptation of the bacterium to dye environment or differences in electron affinity (Chen *et al*. 2011; Imran *et al*. 2015a). Varying decolorization of structurally different dyes have been reported (Imran *et al*. 2014; Najme *et al*. 2015; Abbas *et al*. 2016). Moreover, higher and quicker decolorization of reactive dyes compared to direct dyes suggested that the strain WS-D/183 is more specialized in degrading reactive dyes (Imran *et al*. 2015a).

The individual and interactive effect of different factors on RR120 decolorization by the strain WS-D/183 was examined following RSM based modelling. RSM has already been employed as a tool for optimizing different cultural and incubation conditions for decolorization purpose (Anwar *et al*. 2014; Maqbool *et al*. 2016). The results showed that strain WS-D/183 modified the decolorization rate for RR120. However, no significant effect of multi-metal concentration was observed on bacterial decolorization of RR120 (Fig. 3c–f). It was interesting to note that the individual and quadratic effect of concentration of multi-metal mixture was not significant. However, the interactive effects of metal concentration with yeast extract and salt showed significant decolorization of RR120 (Fig. 3c and d). Such contrasting effects of the concentration on RR120 decolorization can be related to variation in electron withdrawing capacity of metals under variable pH values (Maqbool *et al*. 2016). The optimum value of pH given by RSM was recorded 8.0 for decolorization of RR120. According to previous reports, neutral to slightly alkaline pH is favorable to decolorize the azo dyes (Imran *et al*. 2014; Najme *et al*. 2015). The reason behind this fact might be the negative effects of acidic or highly alkaline pH on growth of bacterium and activities of dye degrading enzymes (Johansson *et al*. 2011).

Results also revealed that higher contents of salt had negative effect on decolorization of RR120 by the bacterium. The optimum value of NaCl contents given by the model was recorded as 12.5 g L^{-1} (Fig. 3). It has been reported that loss of cell integrity, plasmolysis of microbial cells or damage to the enzymatic system of microbial strains involved in decolorization might be the possible reasons for lower decolorization at higher salt contents (Moutaouakkil *et al*. 2003; Gopinath *et al*. 2011). It was also found that increasing the concentration of yeast extract substantially accelerated the decolorization of RR120, and 7.5 g L^{-1} of yeast extract was found optimum. This impact of yeast extract might be attributed to three different roles reportedly performed by yeast extract during the decolorization of dyes. It serves not only as a bacterial growth stimulator by providing carbon and nitrogen but also a source of redox equivalents and a redox mediator (Ong *et al*. 2012; Imran *et al*. 2016). It must be noted that the effect of all the parameters on RR120 was optimized in the presence of multi-metal mixture which corresponds with the situation prevailing in raw textile wastewaters.

The strain WS-D/183 was found highly potent for concurrent removal of reactive dyes and Cr(VI) from simulated wastewater in Cd^{2+}, Pb^{2+} and Zn^{2+} mixture. Recently researchers are focusing on isolation, identification and characterization of bacteria able to degraded dyes and tolerate salts and heavy metals (Anwar *et al*. 2014; Imran *et al*. 2015b). However, few bacteria are reported which are capable of concurrently degrading dyes and reducing Cr(VI) (Anwar *et al*. 2014; Maqbool *et al*. 2016). This study exhibited slow removal of reactive dyes at the start of incubation by WS-D/183. This inhibition at initial stages can be related to the toxic effects of Cr(VI) to the bacterium and preferably reduction of Cr(VI) instead of reactive dyes (Mahmood *et al*. 2013). Though, higher rate of dye removal seen at final stages could be due to either the decrease in toxicity of Cr(VI) or acclimatization of the strain WS-D/183 to Cr(VI) and dyes (Maqbool *et al*. 2016). Moreover, it is observed that various azo-dyes were removed at variable proportions (Table 3) which suggest the possible variation and electron affinity and dye structure. Previously, Anwar *et al*. (2014) observed > 80% removal of Cr(VI) with substantial removal of dyes. But, this study is unique, as concurrent decolorization of dyes and Cr(VI) was studied in the occurrence of Cd^{2+}, Pb^{2+} and Zn^{2+} mixture. Cr(VI) decrease in medium could be due to its biotransformation to Cr(III) by accepting electrons (Wani *et al*. 2007; Maqbool *et al*. 2015) or biosorption of Cr(VI) on the cell surfaces of bacterial strain (Oyetibu *et al*. 2013).

The strain WS-D/183 showed high potential of IAA production and exhibited a time dependent increase in IAA

production. Similarly, it was capable of tri-calcium phosphate solubilization. These results suggest that this strain is capable of promoting plant growth in the presence of multi-metal mixture (Cd^{2+}, $Pb^{2+}Zn^{+2,}$ Cr^{+6}) and dyes (Table 6). Moreover, pH of the medium decreased during IAA production and P solubilization. It might be attributed to the production of low-molecular-weight organic acids (Maliha *et al*. 2004; Ahemad and Khan 2010; Dwivedi *et al*. 2011). Literature showed a number of bacterial strains being used for IAA production and P solubilization (Baig *et al*. 2014; Shahid *et al*. 2015; Akram *et al*. 2016). Researchers also found several bacterial strains having potential to remove dyes and reduce Cr(VI) under metal stress are very few. Recently, Mahmood *et al*. (2017) reported a *Bacillus* sp. carrying PGPR traits and dye decolorization potential.

The RR120 and/or Cr (VI) contaminated water after treatment by the bacterium was tested for toxicity evaluation for plants. Mung bean seeds grown with bacterium treated wastewater showed increased germination, plumule and radical length of mung bean in comparison to seeds grown with untreated wastewater (Table 7). This reduction in phytotoxicity was also supported by the fact that, after inoculation and incubation with strain WS-D/183, >80% of the initially added RR120 and/or Cr(VI) was removed. This indicates that inoculation as well as incubation of the RR120 and Cr(VI) loaded solutions with the strain WS-D/183 might have resulted into their transformation into relatively less toxic products. Our result is in line with previous findings where plants grew better in the azo-dye spiked water after treatment with azo dye decolorizing bacteria (Saratale *et al*. 2009; Najme *et al*. 2015; Mahmood *et al*. 2017). Reduction in phytotoxicity of the decolorized dyes might be attributed to the production of some oxidative enzymes including laccase and tyrosinase during the decolorization process which play their role in degradation of toxic aromatic amines produced during reductive cleavage of azo-dyes into relatively non-toxic/less toxic by-products (Saratale *et al*. 2009). In order to confirm this hypothesis, there is a need to study the enzymology of the decolorization process by the strain WS-D/183.

Conclusion

Findings of this study reported that the strain *Pseudomonas* sp. WS-D/183 can remove dyes and Cr(VI) simultaneously even in existence of other metal ions, which makes it a valuable biotic source for wastewater treatment. Moreover, the distinctive ability of WS-D/183 for IAA production and P solubilization suggested its possible use as a bio-inoculant for increased crop production even in metal stressed conditions. However, there is a need to examine the potential of *Pseudomonas* sp. WS-D/183 for plant growth-promotion in dye contaminated soil using a test crop. Future studies should focused on processes responsible for decolorization of RR120 by the strain WS-D/183 by identifying the genes and enzymes involved in decolorization of azo-dyes using proteomic or metagenomic approaches as well as by characterizing the metabolites using advanced analytical techniques.

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