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Full Length Article

Metal-tolerant *Pseudomonas aeruginosa* Strain ZM130 has the Potential for Concurrent Dye Decolorization and Plant Growth Promotion

Zahid Maqbool[1,2], Sabir Hussain[1*], Faisal Mahmood[1], Muhammad Shahid[3], Tanvir Shahzad[1], Tanvir Ahmed[4], Amna Sahar[5], Muhammad Imran[6], Zulfiqar Ahmad[7] and Farhan Hafeez[8]

[1]*Department of Environmental Sciences and Engineering, Government College University, Faisalabad, Pakistan*
[2]*Department of Soil and Environmental Sciences, University of Poonch, Rawalakot, Azad Jammu Kashmir, Pakistan*
[3]*Department of Bioinformatics and Biotechnology, Government College University, Faisalabad, Pakistan*
[4]*Department of Statistics, Government College University, Faisalabad, Pakistan*
[5]*Department of Food Engineering/National Institute of Food Science and Technology, University of Agriculture Faisalabad, Faisalabad, Pakistan*
[6]*Institute of Soil & Environmental Sciences, University of Agriculture, Faisalabad-Pakistan*
[7]*State Key Laboratory of Water Resources and Hydropower Engineering Science, Wuhan University, Wuhan, Hubei 430072, China*
[8]*Department of Environmental Sciences, COMSATS University Islamabad, Abbotabad Campus, Tobe Camp University Road Abbottabad, Pakistan*
[*]For correspondence: sabirghani@gmail.com; Tel: +92 300 77 95 494

Abstract

Dye decolorizing *Pseudomonas aeruginosa* strain ZM130 was characterized for its potential to decolorize reactive yellow 2 (RY2) dye as well as for its characteristics to promote plant growth under stressed conditions in the presence of a multi-metal mixture (Cr^{6+}, Pb^{2+}, Cd^{2+} and Zn^{2+}). The bacterial growth and RY2 decolorization by this strain were significantly ($p \leq 0.05$) increased when the multi-metal-stressed medium was enriched with yeast extract. The individual and interactive impacts of levels of NaCl, pH and concentration of yeast extract as well as the multi-metal mixture were evaluated following response surface methodology (RSM) modeling based on quadratic polynomial equations. Based on RSM data, optimal decolorization of RY2 was predicted at 14.4 g L^{-1} NaCl, 7.9 pH, 5.8 g/L yeast extract and a multi-metal mixture (Cr: 11.65 mg/L; Pb: 23.30 mg/L; Cd: 11.65 mg/L; Zn: 23.30 mg/L). This strain also showed a good potential for production of IAA (indole-3-acetic acid) and inorganic phosphorus (P) solubilization in the presence of multi-metal mixture and RY2. Assessment of plant growth promotion in a pot study indicated that the strain ZM130 had the potential to significantly enhance the growth of maize (*Zea mays*) in normal soil as well as the soil contaminated with multi-metal mixture and RY2. On the basis of its multifarious characteristics, the strain ZM130 is envisaged as a potential bioinoculant to devise the strategies for integrated bioremediation and plant growth promotion in agricultural soils contaminated with textile wastewaters.

Keywords: *Pseudomonas*; Dye decolorization; Response surface methodology (RSM); Plant growth promotion; IAA production; Phosphate solubilization

Introduction

Azo-dyes are extensively used for dyeing different types of textile products. However, their release into the environment in the form of textile wastewaters is a matter of concern for the general public and scientific community. They are undesirable not only because of the aesthetic problems associated with their presence in the environment but also because of their toxic effects on different living organisms (Imran *et al*., 2015b). Eco-toxicological data suggest that several azo-dyes as well as their degradation products are also carcinogenic and mutagenic in nature (Imran *et al*., 2015b). A number of diseases in humans including diarrhea, dermatitis, skin ulceration, haemorrhage and respiratory problems are also reported to be associated with exposure to azo-dyes (Imran *et al*., 2015b). Along with azo-dyes, various metal ions are also a constituent of textile effluents because of being part of metal complexed azo-dyes and due to use of metal-containing chemicals while dyeing the textile products (Imran *et al*., 2015b). A number of studies have indicated the presence of various metal ions in textile wastewaters above their permissible limits (Imran *et al*., 2015b). These metal ions not only have negative impacts on different living organisms including plants, animals, human beings and microorganisms (Mahar *et al*., 2016) but also disturb the growth and activity of the microbes involved in decolorization of the azo-dyes (Imran *et al*., 2015a; Abbas *et al*., 2016). In nutshell, the presence of metal ions in textile

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wastewater serves as a reason to reduce the efficiency of the biological treatment processes focusing on the removal of azo-dyes. Hence, there is a need to devise the strategies for their remediation from the environment.

Recently, use of microbes for remediation of dyes has emerged as a cost effective, economical, environment-friendly and feasible solution, and a number of azo-dyes degrading bacteria have been identified and characterized (Khalid *et al*., 2012; Anwar *et al*., 2014; Imran *et al*., 2015a, b; Najme *et al.*, 2015; Abbas *et al.*, 2016; Maqbool *et al*., 2016; Hussain *et al*., 2017). Many such bacteria decolorize azo-dye effluents and also mineralize the intermediate byproducts of azo-dyes. The reduction and degradation of azo-dye molecules involve different enzymes including azoreductase, laccase, lignin peroxidase and tyrosinases (Imran *et al*., 2015b; Imran *et al*., 2016b; Mahmood *et al*., 2016; Maqbool *et al*., 2016). Moreover, a wide range of studies have also been conducted on describing the potential of the microorganisms either to resist or to detoxify the heavy metal ions (Lakshmipathy *et al*., 2010; Ilias *et al*., 2011; Maqbool *et al.*, 2015). However, nowadays, isolation and characterization of multifunctional microbial strains having the potential to cope with more than one problem simultaneously is a topic of interest for the scientific communities (Zaidi *et al*., 2006; Dwivedi *et al*., 2011; Maqbool *et al*., 2016; Hussain *et al*., 2017). For example, few bacterial strains resistant to NaCl salt and different metal ions were found to have the spotential of decolorizing different azo-dyes (Hussain *et al*., 2013; Imran *et al*., 2015a; Abbas *et al*., 2016). Similarly, the isolation and characterization of microbial strains capable of simultaneously removing the dyes and hexavalent chromium were also reported (Mahmood *et al*., 2013; Maqbool *et al*., 2016; Hussain *et al*., 2017). Despite that multifunctional microbial strains dealing with two or more than two different types of pollutants have been reported, very little is known about the plant growth promoting bacteria having the potential to decolorize the azo-dyes (Mahmood *et al*., 2017). Such isolates might be exploited for plant growth promotion in parallel to function of remediation in the stressed soils often irrigated with textile wastewaters.

Due to uprising global water scarcity, wastewaters are often used to irrigate different fodder crops in numerous developing countries (Ahmed *et al*., 2016; Arif *et al*., 2016). However, due to the use of wastewater generated by textile industry for irrigation purpose, a huge amount of dyes as well as metal ions are accumulated in the soils. The azo-dyes in concentrations as high as 456 mg kg^{-1} along with considerable concentrations of heavy metal ions have been reported in some soils near textile units (Imran *et al*., 2015b, c). These azo-dyes in the soils has been reported to adversely affect the crop productivity as well as growth, activity and functioning of the soil microorganisms primarily due to the presence of azo-dyes and metal ions (Topac *et al*., 2009; Imran *et al*., 2015c; Ahmed *et al*., 2016; Arif *et al*., 2016). Hence, there is a need to isolate and characterize the microbial strains which have the potential not only to remove such pollutants from the soils but also to promote plant growth in such stressed soils. In this context, a dye decolorizing bacterium *Pseudomonas aeruginosa* strain ZM130 was characterized only for decolorization of reactive yellow-2 (RY2) in the presence of a mixture of heavy metal ions (Cr^{6+}, Pb^{2+}, Cd^{2+}, Zn^{2+}) under varying cultural conditions using response surface methodology (RSM) but also examined for characteristics of plant growth promotion including IAA production and P solubilization in the presence of RY2 azo-dye and multi-metal mixture in aqueous and soil media.

Materials and Methods

Strain, Culture Media and Chemicals

A previously isolated reactive red 120 decolorizing bacterial strain ZM130 belonging to *Pseudomonas aeruginosa* (Maqbool *et al*., 2016) was used in the present study for its optimization for decolorization of reactive yellow-2 (RY2) dye. The RY2 dye as well as the analytical grades of all the chemicals used in this study were purchased from Sigma-Aldrich. The mineral salt (MS) medium used in this study contained Na_2HPO_4 (1.0 g L^{-1}), KH_2PO_4 (1.0 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (0.5 g L^{-1}), $CaCl_2 \cdot 2H_2O$ (0.1 g L^{-1}), NaCl (1.0 g L^{-1}) and yeast extract (3.0 g L^{-1}) (Anwar *et al*., 2014). This MS medium was added with a multi-metal mixture including Cr (5 mg L^{-1} as $K_2Cr_2O_7$), Cd (5 mg L^{-1} as $CdCl_2 \cdot 2H_2O$), Zn (10 mg L^{-1} as $ZnSO_4 \cdot 7H_2O$) and Pb (10 mg L^{-1} as $Pb(NO_3)_2$] in most of the experiments; any change in composition otherwise mentioned. The IAA production and P solubilization potentials of the strain were estimated in LB (Luria-Bertani) medium and NBRIP (National Botanical Research Institute's Phosphate) medium, respectively.

Characterization of RY2 Decolorizing Capabilities of the Strain ZM130

Impact of carbon co-substrates on RY2 decolorization: Four carbon/energy substrates (yeast extract, glucose, maltose and D-mannitol) were tested for their impact on RY2 decolorization by ZM130 in MS medium spiked with a mixture of four metals (Cr^{+6}, Cd^{2+}, Pb^{2+} and Zn^{2+}) as described in section 2.1. For this purpose, MS broth containing multi-metal mixture was prepared and supplemented with 3 g L^{-1} of each substrate separately along with a control without any co-substrate. For inoculum preparation, the cells of the strain ZM130 grown MS broth were harvested by centrifugation (6,000 rpm for 20 min), washed twice with saline water (0.9% NaCl) and resuspended in MS medium. The bacterial cells were then inoculated in the above media containing different substrates to develop initial bacterial cell density of 0.05 at

600 nm (OD_{600}). Filter sterilized RY2 stock solution was added to make a final concentration as 100 mg L^{-1} and the experiment in triplicates was incubated under static condition (microaerophilic incubation) at 30°C along with un-inoculated controls. After regular time interval up to 40 h, aliquots from each sample were centrifuged (6000 rpm for 10 min) and the supernatants were used to measure the absorbance at 404 nm (λ_{max} for RY2) using a UV-Visible spectrophotometer (Shimadzu UV/Vis). The decolorization was estimated using the following equation:

$$\text{Decolorization (\%)} = (A - B)/A \times 100 \qquad \text{(Eq. 1)}$$

Where, A and B represent the absorbance of un-inoculated control and inoculated sample, respectively. At the end of incubation, 1 mL of the aliquots from each culture centrifuged (6000 rpm for 20 min), the bacterial pallets were washed twice with distilled water and dissolved in 1 mL of distilled water for measuring bacterial cell density (OD_{600}) using a CO800 Cell Density Meter (Biochrom, England).

Based on the results of this study, yeast extract was added as a carbon co-substrate in MS medium in all the subsequent experiments.

Optimization of RY2 Decolorization by the Strain ZM130 Using Response Surface Methodology (RSM)

Impact of four input variables (Level of NaCl concentration, pH and concentration of yeast extract as well as the multi-metal mixture) on decolorization of RY2 dye (100 mg L^{-1}) by the strain ZM130 was estimated by following an RSM based approach. Response surface methodology is an important statistical tool which has already been used for optimization of the process of bacterial decolorization of dyes by studying the interaction among the different experimental variables while studying their impact on the response (Anwar *et al*., 2014; Maqbool *et al*., 2016). In this study, each of the input variable was studied at five different levels following a 21-run small composite design (SCD) comprising of 8 factorial points, 8 axial runs and 5 center points (Table S1). The whole of the experiment was incubated at 30°C under static conditions. After 12 h incubation, RY2 decolorization was estimated as a response variable as described earlier.

A second order polynomial model was chosen to be estimated through observed data by SCD. The second order model for estimated response is given as:

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

Where $E(y|x)$ represents expected response (% decolorization of RY2 by the strain ZM130) given vector $\boldsymbol{x}$ of predictor variables, $\beta_{\circ}$ is regression constant, β_i*'s* are the linear regression coefficients, β_{ii} are the quadratic regression coefficient and β_{ij} are the bilinear regression coefficients.

Design Expert 10 was used to analyze the whole of the data. The model adequacy was checked by applying the lack of fit test. The validity of model, the significance of whole model and the individual model terms were checked by coefficient of determination (R^2) and *F*-test through ANOVA (analysis of variance). Moreover, confidence limits were established to check the significance of regression coefficient estimates. The magnitude of multicollinearity was measured by computing the variance inflation factor (VIF) among two or more input variables of polynomial regression model.

Plant Growth Promoting Characteristics of the Strain ZM130

IAA production: In order to estimate IAA production, LB broth (pH 7.2±0.1) was amended with tryptophan (100 μg mL^{-1}) and inoculated with the bacterial culture of the strain ZM130. The samples were tightly sealed and incubated at 30°C under static conditions along with un-inoculated control. After regular intervals, pH of the growth medium was measured; aliquots were centrifuged (6000 rpm for 10 min) and the supernatants were mixed with orthophosphoric acid (two drops) and Salkowski reagent (1 mL). The pink color was allowed to develop by keeping mixture in dark for about 10-15 min and its intensity was measured using UV-Visible spectrophotometer at 530 nm (λ_{max}). The amount of IAA production was measured by following a standard curve made by using standard IAA solutions. Moreover, in another experiment, different concentrations of RY2 (0 to 500 mg L^{-1}) were made in tryptophan (100 μg mL^{-1}) amended inoculated and un-inoculated LB broth media, incubated under similar conditions as above and processed for IAA measurement and RY2 decolorization.

Inorganic Phosphate Solubilization

The potential of the strain ZM130 for inorganic phosphate solubilization was also determined. For this purpose, NBRIP broth media amended with tri-calcium phosphate (TCP) (1000 μg mL^{-1}) was used, inoculated with the strain ZM130 in triplicate and incubated at 30°C under shaking (150 rpm; partially aerobic incubation) for 7 days along with un-inoculated control. After regular intervals, pH of the growth medium was measured and aliquots were drawn, filtered through Whatman filter paper No. 1. The filtrates were centrifuged (10,000 rpm for 20 min) and supernatants (1 mL) were mixed with Barton's reagent (2.5 mL) and final volume was made up to 50 mL with distilled water. The mixture was kept at room temperature for 10-15 min until the yellow color developed whose intensity was measured at 430 nm (λ_{max}) on UV-visible spectrophotometer. The concentration of P solubilized was estimated using a standard curve made by using different concentration of the standard K_2HPO_4 solutions. Moreover, in another experiment, different concentrations of RY2 (0 mg L^{-1} to 500 mg L^{-1}) were made in TCP (1000 μg mL^{-1}) amended

inoculated and un-inoculated NBRIP broth media, incubated under similar conditions as above and processed for P solubilization and RY2 decolorization.

Pot Experiment for Assessment of Plant Growth Promotion by ZM130

The potential of ZM130 for decolorization of RY2 and for promotion of plant growth was also tested in a pot study using maize (*Zea mays*) as a test crop. For this purpose, inoculum of ZM130 was prepared by culturing it in mineral salt medium spiked with 150 mg L^{-1} of RY2 under shaking (150 rpm) at 28°C for 48 h. The cells were harvested by centrifugation (6000 rpm) and washed twice with fresh mineral salt medium. A sandy loam soil (never exposed to textile effluents) was collected from a research field of Ayub Agricultural Research Institute (AARI) Faisalabad. The soil was sterilized by autoclaving and filled in pots (500 g soil/pot). The experiment consisted of four treatments (T1: Control non-contaminated soil, T2: Non-contaminated soil inoculated with ZM130, T3: Soil contaminated with multi-metal mixture and RY2, T4: Soil contaminated with multi-metal mixture and RY2 but inoculated with ZM130) in triplicates. For treatments T3 and T4, the soils in each pot were spiked and homogenized with a mixture of metal ions and RY2 [Cr^{2+} (10 mg kg^{-1}); Pb^{2+} (20 mg kg^{-1}); Cd^{2+} (10 mg kg^{-1}); Zn^{2+} (20 mg kg^{-1}) and RY2 (200 mg kg^{-1})] under aseptic conditions. For treatments T2 and T4, the soils were inoculated by adding the inoculum of ZM130 (10^6 cells g^{-1} of soil). The inoculum was thoroughly mixed with the soils under sterilized conditions. The moisture contents of the soils were maintained at 60% of water holding capacity and then incubated in dark at 28°C for 10 days. Ten surface sterilized seeds of maize were sown in each pot. After germination, only three plants per pot were maintained and allowed to grow for 21 days by following the standard agronomic practices. After harvesting, shoot length, root length, shoot dry weight and root dry weight of all the plants were recorded and statistically analyzed using Least Significance Difference (LSD) test after the analysis of variance (ANOVA) at $p < 0.05$ using the software R (3.4.1). At the end of the experiment, the amount of the remaining RY2 dye in the treatments T3 and T4 was estimated by extracting it by following the procedure described by Imran *et al*. (2015b).

Results

Impact of Different Carbon Co-substrates on RY2 Decolorization

Pseudomonas aeruginosa was found to show only 8.84% (± 1.9) decolorization of RY2 dye in the absence of carbon/energy substrates in the MS broth having multi-metal mixture. However, higher levels of RY2 decolorization were observed in the media added with different types of substrates (Fig. 1A). The strain ZM130 showed 57.31% (± 3.0) decolorization of initially added RY2 (100 mg L^{-1}) in the media amended with yeast extract within 16 h. Whereas, over the same incubation period, only 32.89% (± 2.0), 14.51% (± 2.8) and 3.0% (± 0.7) of the initially added RY2 was decolorized in the media amended with glucose, maltose and D-mannitol, respectively. However, over 40 h incubation, the strain ZM130 exhibited 86.04% (±1.1), 65.86% (± 2.2), 25.53% (± 2.4) and 11.62% (± 1.7) decolorization of RY2 dye in broth supplemented with yeast extract, glucose, maltose and D-Mannitol, respectively. Furthermore, the maximum growth of ZM130 was recorded in the presence of yeast extract as substrate followed by glucose, maltose and D-Mannitol (Fig. 1B). The bacterial density of the strain ZM130 was found positively correlated (R^2 = 0.9883) with the rate of decolorization of RY2 in the presence of different substrates under static conditions (Fig. 1C).

Response Surface Methodology (RSM) based Optimization of RY2 Decolorization by *Pseudomonas aeruginosa* Strain ZM130 using

Assessment of fitness and significance of the model: A number of parameters were measured for evaluation of the RSM model used in this study. Sequential model sum of squares [Type I] showed that cubic vs quadratic model was more significant than the other suggested models but the terms in this model are confounded with each other (Table S2). Therefore, a quadratic model was suggested with significant p-value of 0.0039 that also includes interaction terms. Lack-of-fit test (Table S3) also suggests a second order polynomial model. Moreover, the model summary statistics with R^2=0.9511 and adjusted R^2=0.8672 also supported the selection of quadratic model addressing a high proportion of variation in response (decolorization of RY2).

The observed decolorization of RY2 dye showed high variation among different trials. Thus, to control high variation in the observed response we decided to run the experiment in two blocks. Blocks in coded variables are shown below:

Block I				Block II			
Salt	pH	Yeast extract	Metal conc.	Salt	pH	Yeast extract	Metal conc.
-1	-1	1	-1	-2	0	0	0
1	1	1	-1	2	0	0	0
0	0	0	0	0	0	0	-2
1	-1	-1	1	0	2	0	0
1	-1	1	1	0	0	0	2
0	0	0	0	0	-2	0	0
-1	1	1	1	0	0	-2	0
-1	-1	-1	-1	0	0	0	0
0	0	0	0	0	0	2	0
-1	1	-1	1	0	0	0	0
1	1	-1	-1				

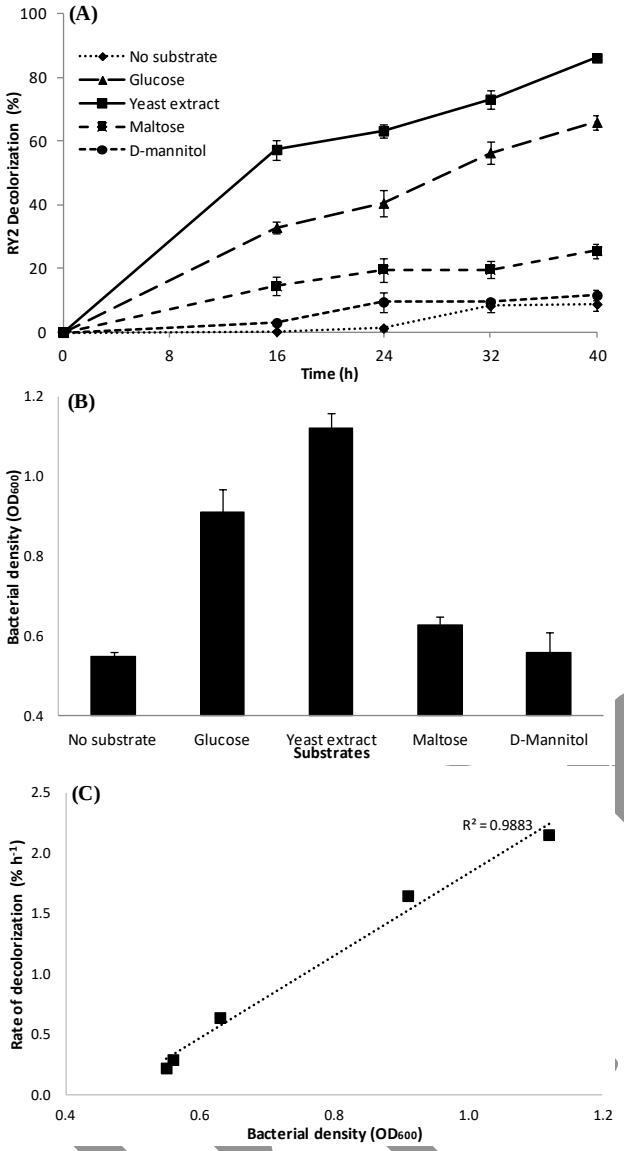

Fig. 1: (A). Decolorization of RY2 by *Pseudomonas aeruginosa* ZM130 in the presence of different carbon/energy substrates, **(B).** Bacterial density (OD_{600}) of *Pseudomonas aeruginosa* ZM130 after 40 h incubation in the presence of different substrates, **(C).** Correlation between the rate of decolorization (% h^{-1}) and the bacterial density (OD_{600}) of *Pseudomonas aeruginosa* ZM130 after 40 h incubation in the presence of different substrates

After blocking, the model was found to be significant (Table S4). Some interaction terms which were highly insignificant for the response were excluded from ANOVA. Based on multiple regression analysis, a second order polynomial model was estimated to explain the relationship between the response variable (decolorization of RY2) and the independent variables under study (Table S5). The equation is given below:

$$y = 68.51 - 17.61x_1 + 8.42x_2 + 14.01x_3 + 6.65x_4 - 9.70x_1^2 - 8.36x_2^2 - 11.77x_3^2 - 3.57x_4^2 - 5.08x_1x_3 + 8.20x_1x_4 + 8.98x_2x_3 - 20.50x_2x_4 + 5.86x_3x_4 + 26.09x_1x_2x_4$$

Contribution of each independent variable was explained by second order polynomial equation. Only x_1 (salt) showed negative linear contribution to response with high intensity (-17.61) and x_3 (concentration of yeast extract) exhibited strong positive contribution in response (decolorization RY2 dye by the strain ZM130) followed x_2 (pH) and x_4 (metal content). All the quadratic terms had negative impact on decolorization (response). x_1x_4 and x_2x_3 had positive and almost equal contribution in response but x_2x_4 showed strong negative contribution in response. The significance of each model term can also be observed in ANOVA table (Table S4). Moreover, coefficient of determination (R^2) and adjusted R^2 also ensure the reliability of model. These values suggested that 86.72% of the variation in the response can be explained through its relation with the independent variable used in this model.

The diagnostic plots for graphical representation of the analysis have been shown in Fig. 2. The perturbation plot here shows that response (decolorization of RY2 by *Pseudomonas aeruginosa* strain ZM130) is more sensitive to variations in salt content (factor A) and concentration of yeast extract (factor C) as compared to other factors *i.e.*, B (pH) and D (heavy metal mixture concentration) (Fig. 2A). The normal probability plot shows that the residuals seem to cluster around the straight line which indicated that residuals follow the normal distribution (Fig. 2B). The graph of predicted versus actual response values was a good fit indicating the high potential of the estimated model to predict the response values (Fig. 2C). The Box-Cox plot was also constructed between power transformation variable lambda and the natural log of residuals, to select a suitable power transformation of response values if needed (Fig. 2D). However, in this experiment, there was no need of transformation of the observed values of response for model fitting because suggested transformation is not far away from the current value of lambda, which is 1. The Cook's distances are plotted in (plot No.) to indicate the influence of different design points on the least squares multiple regression analysis (Fig. 2E). Plot shows that four axial runs have maximum influence on the regression estimates which include two axial points when salt is at minimum or maximum while other treatment factors are in the middle levels, and the two axial points when pH is at lowest or highest level while other factors are in the middle. Leverage statistic shows how far away lie the independent observed values of response from the other values (Fig. 2F).

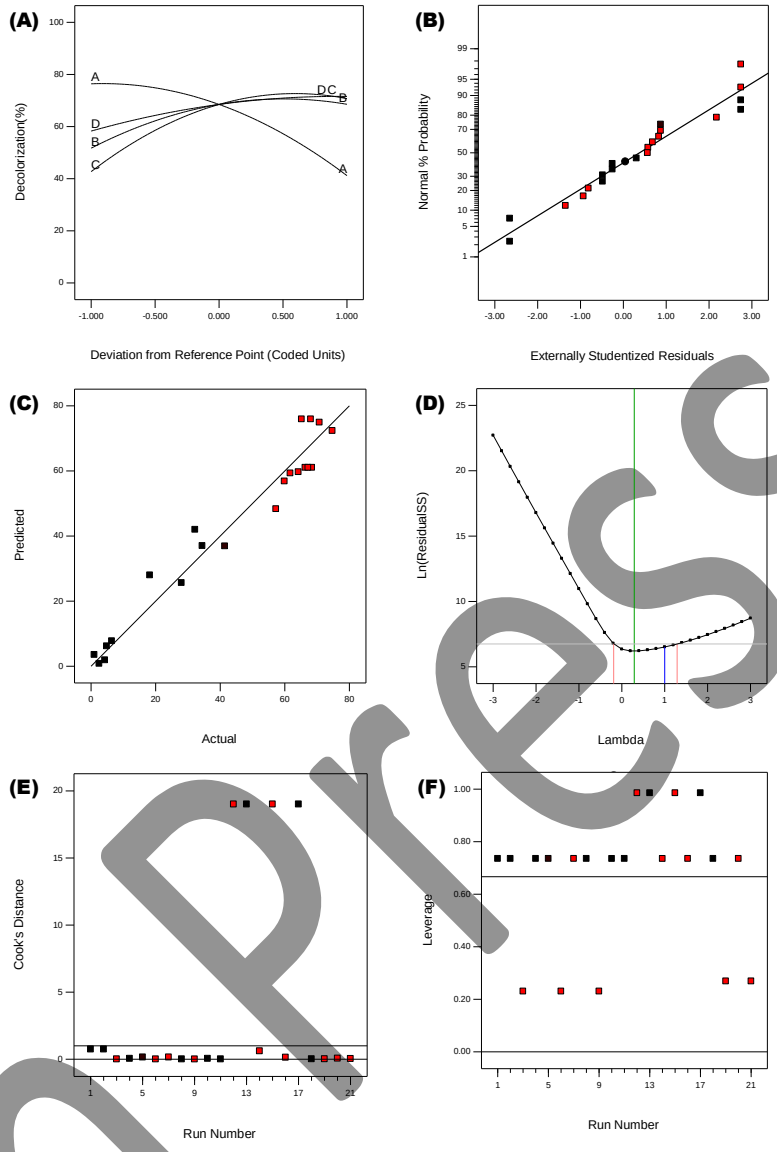

Fig. 2: Diagnostic Plots for model validation including normal plot of residuals, **(A)** Perturbation Plot, **(B)** Normal Plot of Residuals, **(C)** Predicted vs. Actual plot, **(D)** Box-Cox Plot, **(E)** Cook's Distance Plot and **(F)** Leverage vs. Run Plot

Impact of Modeled Parameters on RY2 Decolorization

The response plots (3D) showed how the decolorization changes with a variation in two interacting factors, while keeping the remaining two factors at central position (Fig. 3). Interactive impact of the levels of yeast extract (substrate) and the NaCl (salt) content on RY2 decolorization by ZM130 has been presented in Fig. 3A. It was noticed that RY2 decolorization was maximum when the level of salt content was low and the level of yeast extract was higher, while keeping the other two factors at central position. It is noteworthy that decolorization of RY2 was significantly improved when the concentration of yeast extract at the lowest salt content was increased, while such significant increase in RY2 decolorization was not observed in the media having high salt content. However, increase in salt content was observed to have a negative impact on RY2 decolorization even when yeast extract was present at a higher concentration. Hence, the maximum RY2 decolorization was observed in the cultures containing salt content at lower levels and concentration of yeast extract at highest level. The interactive impact of the level of multi-metal mixture content and the concentration of NaCl salt content on RY2 decolorization by ZM130 has been presented in Fig. 3B. It is interesting to note that an increase in the level of salt content had a negative impact on RY2 decolorization in the presence of lower as well as higher concentrations of the multi-metal mixtures.

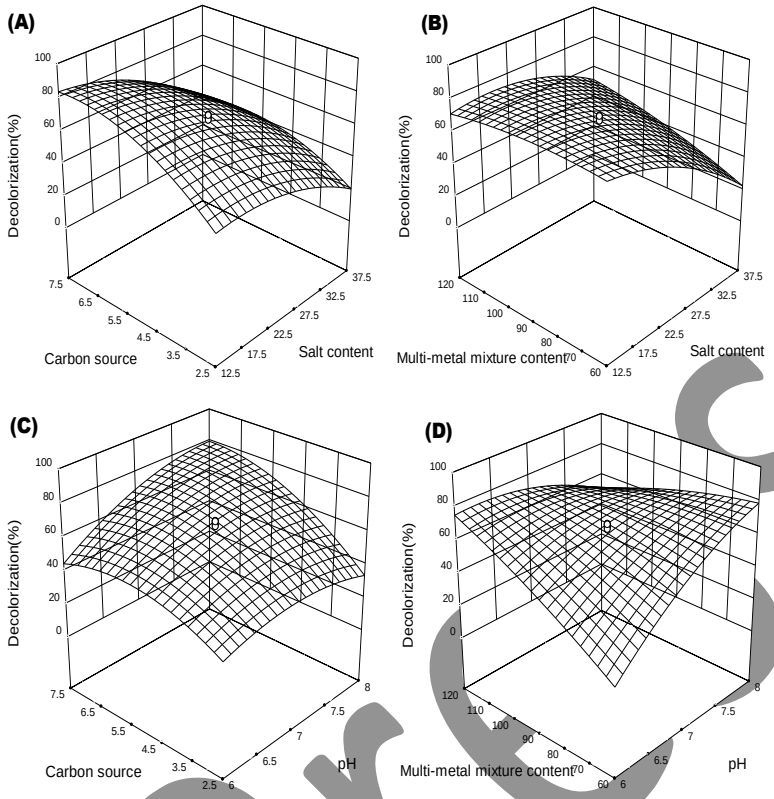

Fig. 3: 3-D Response plots for different factors exhibiting interactive effects of independent variables on decolorization (%) of RY2 by *Pseudomonas aeruginosa* ZM130. **(A)** concentration of co-substrate (yeast extract) and salt content, **(B)** concentration of multi metal mixture and salt content, **(C)** concentration of co-substrate (yeast extract) and pH, **(D)** concentration of multi metal mixture and pH

However, an increase in the level of multi-metal mixture concentration had almost no impact on RY2 decolorization at lower salt content and resulted into a clear increase in RY2 decolorization at higher salt content. The optimal value of decolorization being in the presence of lowest salt content also indicates the significant negative impact of the level of salt concentration on RY2 decolorization by ZM130. Fig. 3C clearly presents that an increase in pH as well as the concentration of yeast extract content were positively interacting with each other since the maximum RY2 decolorization by ZM130 was observed when both pH and yeast extract were at their highest levels. The interactive impact of the level of multi-metal mixture concentration and pH on RY2 decolorization by the strain ZM130 has been presented in Fig. 3D. It was interesting to note that an increase in RY2 decolorization by ZM130 was observed when pH was increased in the presence of lower level of multi-metal mixture concentration as well as when the level of the multi-metal mixture concentration was increased at lower pH. However, a decrease in the RY2 decolorization was observed when the level of multi-metal mixture concentration was increased at alkaline pH values. In the same way, a decrease in the RY2 decolorization was observed when the pH was increased in the presence of higher level of multi-metal mixture concentration. The optimal values of NaCl salt concentration, the level of yeast extract and pH and concentration of multi-metal mixture for maximum RY2 decolorization predicted from the model were observed to be 14.4 g L^{-1}, 5.8 g L^{-1} and pH 7.9, respectively, with an optimal level of the multi-metal mixture concentration (Pb: 23.30 mg L^{-1}; Cr: 11.65 mg L^{-1}; Cd: 11.65 mg L^{-1}; Zn: 23.30 mg L^{-1}).

Evaluation of Plant Growth Promoting Characteristics of ZM130

In this study, the strain ZM130 was found to produce IAA (Fig. 4A). About 22 μg mL^{-1} of IAA was produced by the strain ZM130 in 96 h incubation under static conditions. Moreover, the pH of the medium was found to gradually decrease from 7.5 to 4.9 during the incubation period. However, it was interesting to note that IAA producing activity of *Pseudomonas aeruginosa* ZM130 was significantly decreased when the medium was added with RY2 at different concentrations (Fig. 4B). As compared to control without dye, approximately 34.2% and 43% decrease in IAA production by the strain ZM130 was recorded when the medium was added with 250 μg mL^{-1} and 500 μg mL^{-1} of RY2 dye, respectively.

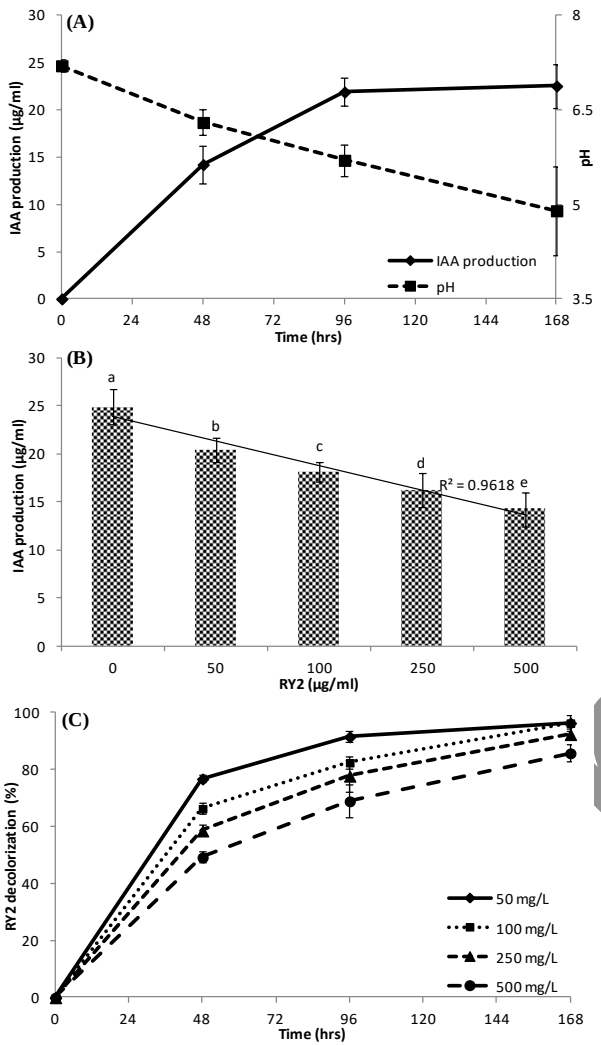

Fig. 4: Potential of *Pseudomonas aeruginosa* ZM130 for Indole acetic acid (IAA) production in liquid culture. Error bars indicate standard error (n = 3). **(A).** The primary Y-axis indicates IAA production by the strain *Pseudomonas aeruginosa* ZM130 and the secondary Y-axis indicates change in pH of Luria-Bertani broth amended with tryptophan, **(B).** The figure indicating RY-2 azo-dye concentrations dependent inhibition of IAA activity, **(C).** Decolorization of RY2 by the strain *Pseudomonas aeruginosa* ZM130

The level of IAA production was found to be negatively correlated (R^2=0.9618) with the level of RY2 present in the medium. However, over the incubation period, the added RY2 was also observed to be decolorized by ZM130 (Fig. 4C).

The qualitative analysis (clear halo-zone on Pikovskaya's agar) indicated for the potential of the strain ZM130 for inorganic phosphate solubilization (data not shown). Hence, the strain ZM130 was inoculated in liquid medium amended with tri-calcium phosphate (1000 μg mL^{-1}) for quantitative assay for inorganic phosphate solubilization. According to the results, the strain ZM130 showed considerable potential for phosphate solubilization (Fig. 5A).

It was recorded that 71.5 μg mL^{-1} soluble phosphate was released in liquid medium by the strain ZM130 after 168 h incubation under shaking conditions. Moreover, gradual decrease in pH from 7.0 to 4.3 of the growth medium was also noticed. However, inhibition assay for phosphate solubilization in the presence of different concentrations of RY2 showed a respective significant decrease in P solubilization by ZM130 (Fig. 5B). As compared to control without dye, approximately 29.4% to 61.2% decrease in P solubilization by ZM130 was observed when the medium was added with 50 μg mL^{-1} to 500 μg mL^{-1} of RY2 dye. Moreover, % decrease in P solubilization by the strain ZM130 was found to be positively correlated (R^2 = 0.9396) with the increase in concentration of RY2 in the medium. Interestingly, over the same incubation period, the RY2 added in the medium was also found to be decolorized (Fig. 5C).

The plant growth promoting potential of ZM130 was also assessed in soil through a pot study. The results presented in Table 1 clearly indicate that shoot length (cm), shoot dry weight (g) and root dry weight (g) were significantly increased when the non-contaminated soil was inoculated with the strain ZM130. Root length (cm) was also found to increase in response to inoculation however, this increase was statistically non-significant as compared with the control. Spiking of the soil with multi-metal mixture and RY2 resulted into a significant decrease in all growth parameters except the root length which was also decreased but statistically non-significantly. In contaminated soil, all the four parameters (root length, shoot length, root dry weight & shoot dry weight) were significantly increased in the soil inoculated with ZM130 as compared with their respective un-inoculated controls. The amount of the RY2 dye extracted from T3 and T4 at the end of the experiment indicated a significant decrease (>80%) in the remaining RY2 in T4 as compared to T3.

Discussion

In order to cope with the prevailing water scarcity, wastewaters including the textile effluents are commonly used to irrigate agricultural fields in several developing countries including Pakistan. Irrigation with textile effluents not only pollutes the soils but also reduces plant growth/yields. This study was focused on characterization of metal tolerant dye-decolorizing strain ZM130 which also harbors plant growth-promoting traits. The results indicate that ZM130 requires carbon/energy substrate for faster decolorization of RY2. Among the substrate compared, yeast extract was found as a better substrate compared to others.

Table 1: Effect of ZM130 inoculation on growth parameters of *Zea mays* in normal and contaminated soils

Treatments	Shoot Length (cm)	Shoot Dry Weight (g)	Root Length (cm)	Root Dry Weight (g)
Control soil (T1)	36.3 b	0.507 b	15.2 ab	0.596 b
Control soil + ZM130 (T2)	39.9 a	0.639 a	16.1 a	0.694 a
¶Contaminated soil (T3)	32.7 c	0.442 c	14.4 b	0.463 c
¶Contaminated soil + ZM130 (T4)	38.2 ab	0.553 b	15.6 a	0.621 b
LSD** values	3.17	0.06	1.05	0.07

¶Soil spiked with a mixture of metal ions [Cr^{2+} (10 mg kg^{-1}); Pb^{2+} (20 mg kg^{-1}); Cd^{2+} (10 mg kg^{-1}); Zn^{2+} (20 mg kg^{-1})] and RY2 (200 mg kg^{-1})
** Least Significant Difference

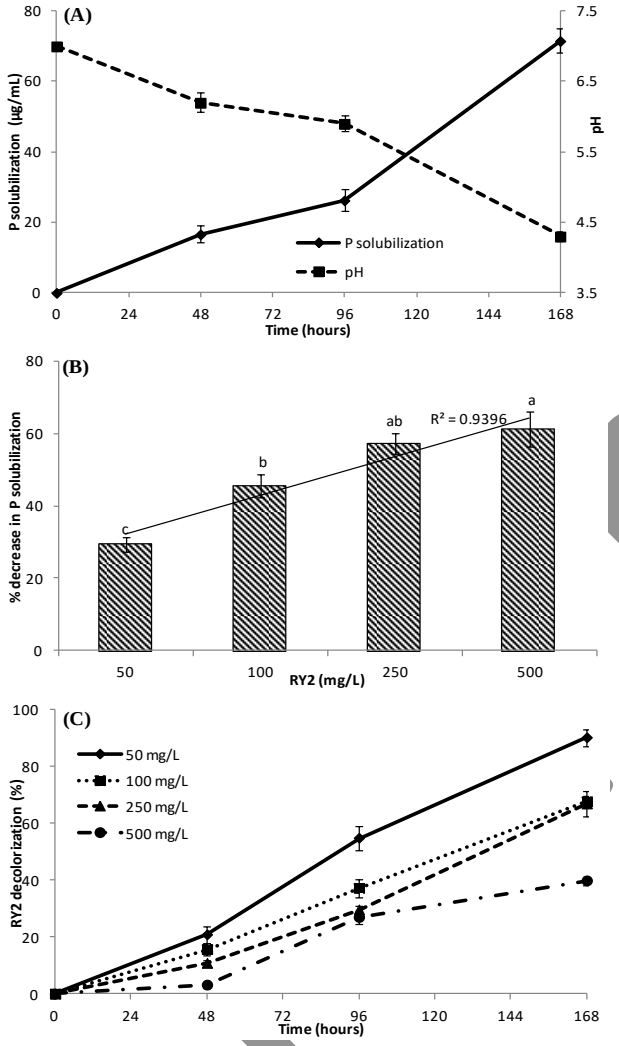

Fig. 5: Potential of *Pseudomonas aeruginosa* ZM130 for P solubilization in liquid culture. Error bars indicate standard error (n = 3). **(A).** Phosphate solubilizing activity by the strain *Pseudomonas aeruginosa* ZM130 as a function of time. The primary Y-axis indicates the amount of soluble phosphate determined from the absorbance data using the calibration curve with KH_2PO_4 at 430 nm. The secondary Y-axis indicates the change in pH of the NBRIP broth medium during growth at different time points. **(B).** The figure indicating RY-2 azo-dye concentrations dependent inhibition of P solubilization, **(C).** Decolorization of RY2 by the strain *Pseudomonas aeruginosa* ZM130

The higher rate of bacterial mediated decolorization of azo-dyes in the media added with yeast extract has also been observed by others (Baeta *et al*., 2012; Najme *et al*., 2015; Abbas *et al*., 2016; Imran *et al*., 2016a). Moreover, in the presence of yeast extract, the growth of ZM130 was also higher and significantly (R^2: 0.988) correlated with RY2 decolorization as compared to in the presence of other co-substrates. It indicates that this higher growth of the strain ZM130 might have resulted in generation of more reducing equivalents (NADH, NADPH) essentially required by azoreductase, a key enzyme involved in cleaving azo-bonds during decolorization process (Ong *et al*., 2012; Imran *et al*., 2016a). Relatively higher growth due to yeast extract might be due to the fact that it provides both carbon and nitrogen for microbial growth, whereas, other substrates (glucose, maltose and D-mannitol) contain only carbon for bacterial growth. Moreover, yeast extract also stimulates the activity of azo-dye degrading enzymes resulting into accelerated decolorization (Baeta *et al*., 2012; Imran *et al*., 2016a). For instance, Imran *et al*. (2016a) found that yeast extract accelerated azoreductase activity in a dyes decolorizing strain *Shewanella* ssp. strain IFN4 and that this increase in azoreductase activity was linked with the riboflavin component of yeast extract which served as redox mediator.

The RSM model based approach indicated that all four factors had significant effect on decolorization of RY2. Increase in pH and yeast extract content in medium showed positive impacts on decolorization of RY2, whereas, increase in salt and multi-metal mixture concentration caused significant reduction in decolorization activity of the strain ZM130. However, it was noteworthy that at higher pH and yeast extract concentration, a decrease in negative impacts of the levels of salt concentration and multi-metal mixture concentration on RY2 decolorization was observed. Moreover, it is also noteworthy that RY2 decolorization was increased when the level of multi-metal mixture concentration was increased at lower pH and decreased when the level of multi-metal mixture concentration was increased at higher pH. These contrasting impacts of multi-metal mixture concentration at different pH values might be due to the variations in electron withdrawing power of metal ions at different pH values (Maqbool *et al*., 2016). According to RSM model used in this study, RY2 decolorization by *Pseudomonas aeruginosa* strain

ZM130 was predicted to be optimal at pH value of 7.9. Neutral to slightly alkaline pH has already been reported as an optimal pH range during the decolorization of various azo-dyes by different bacterial strains (Hussain *et al*., 2013; Anwar *et al*., 2014; Najme *et al*. 2015; Maqbool *et al*., 2016). A decline in decolorization at lower or higher pH values might be due to the negative impacts of pH either on growth of the microbes (Rousk *et al*., 2009; Hussain *et al*., 2013) or on enzymatic system involved in decolorization of dyes (Johansson *et al*., 2011). In the present study, it is also noteworthy that almost an un-affected decolorization of RY2 was recorded in the presence of lower as well as higher levels of the concentration of multi-metal mixture. This finding indicates that the strain ZM130 has considerable potential to tolerate the occurrence of the subject multi-metal ions mixture. The ability of the strain ZM130 to tolerate heavy metal ions is also evident from relatively higher MIC of different metal ions for this strain as already reported by Maqbool *et al*. (2016). In this study, higher salt contents were also found to show significant negative effects on decolorization of RY2 and optimum value of salt contents was found to be 14.4 g L^{-1}. The reason behind decreased decolorization at relatively higher concentrations of salt may be associated to the plasmolysis of microbial cells, loss of cell activity or the disturbance of enzymatic system involved in decolorization process (Tan *et al*., 2009; Gopinath *et al*., 2011). This is also in accordance with the findings of a number of previous studies reporting the impact of salt on decolorization of azo-dyes by bacterial strains (Abbas *et al*., 2016; Imran *et al*., 2015a; Najme *et al*., 2015). However, distinguished part of this study is that it explains the impact of salt on decolorization of dye in the presence of multi-metal mixture which is according to the situation prevailing in real textile wastewater.

Results also depicted that ZM130 exhibited potential of IAA production and P solubilization which are two important plant growth promoting traits often observed in bacterial strains. Previously, IAA production and P solubilization potentials have been characterized in several bacterial strains isolated from diverse environments (Khalid *et al*., 2004; Shahid *et al*., 2015). However, the distinguishing point in this study is that it reports IAA production and P solubilization by ZM130, which also harbors other multifarious potentials including dye decolorization, and resistance to salts and different metal ions. The decrease in pH during P solubilization and IAA production by ZM130 might have resulted from a release of low-molecular-weight organic acids in the culture medium (Zaidi *et al*., 2006; Dwivedi *et al*., 2011). We also found that IAA production and P solubilization by ZM130 was significantly decreased when the culture medium was added with RY2. This decrease might be due to the toxic impacts of RY2 dye on the strain ZM130. It is also interesting to note that although IAA production and P solubilization by ZM130 were significantly decreased due to an increase in RY2 concentration in the medium, however, this bacterium did not lose the activity. Interestingly, ZM130 was also found to decolorize RY2 in parallel to IAA production and P solubilization which might be the reason for relatively lesser inhibitory effects at higher RY2 concentrations.

Assessment of potential of the strain ZM130 for plant growth promotion in non-contaminated and contaminated soils indicated that root and shoot growth of maize were significantly enhanced when the soils were inoculated with the strain ZM130. It indicates for the potential of ZM130 to promote plant growth even when the RY2 as well as multi-metal mixture are present as a stress. This finding is a new potential addition in a number studies reporting for the role of plant growth promoting and metal tolerant bacteria in improving the growth of the agricultural crops in the soils under stress due to different types of contaminants including cadmium and lead (Ahmad *et al*., 2014, 2016). The concentration of RY2 in the contaminated soil inoculated with the strain ZM130 was significantly decreased as compared to un-inoculated soil. This reduction in RY2 concentration can be attributed to the presence of ZM130 which had shown a good potential for decolorization of RY2 in broth cultures. Hence, it can be inferred that the strain ZM130 not only promoted growth of plant in contaminated soil but also played its role in reducing the dye contamination in soil as well as liquid media using its dyes decoloring potential.

Conclusions and Perspectives

On the basis of the potentials of ZM130 reported in this study, this strain might serve as a potential bioresource which can be exploited as a super bio-inoculant having environmental and agricultural significance. The distinctive ability of the metal and salt tolerant *Pseudomonas aeruginosa* ZM130 for contemporaneous dye decolorization and plant growth promotion in soil and aqueous media might serve as a tool not only to improve health of the textile wastewater contaminated soils by remediating the dyes but also to sustain and enhance the growth and yield of crops in the soils under stress due to textile wastewaters. However, there is a need to investigate the processes involved in decolorization of RY2 by the strain ZM130 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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