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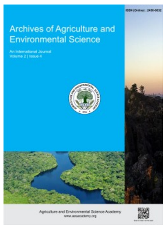

ORIGINAL RESEARCH ARTICLE

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Response surface methodology based optimization of cadmium and lead remediation from aqueous solution by water hyacinth (*Eichhornia crassipes* [Mart.] Solms) and its anatomical study

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ABSTRACT

This experiment was performed to optimize the response surface methodology (RSM) based reduction of cadmium (Cd^{2+}) and lead (Pb^{2+}) from the aqueous solution and to study anatomical effects of Cd^{2+} and Pb^{2+} stress on stomata of water hyacinth (*Eichhornia crassipes* [Mart.] Solms) during phytoremediation. Laboratory experiments were carried out to grow *E. crassipes* plants in six treatments of Cd^{2+} and Pb^{2+} *viz.*, 0 mgL^{-1} (Control), 2 mgL^{-1}, 4 mgL^{-1}, 6 mgL^{-1}, 8 mgL^{-1} and 10 mgL^{-1} in 25 liter capacity glass aquariums. A 2-factor central composite design (CCD) with total 25 experimental runs and the predictor regression model equation was applied to optimize the prime conditions for the Cd^{2+} and Pb^{2+} reduction. Different plant growth attributes *viz.*, translocation factor; kinetic plant growth rate, fresh plant biomass and total chlorophyll content were also found highest up to 4 mgL^{-1} concentration of Cd^{2+} and Pb^{2+}. Structural damage in the stomata of *E. crassipes* was evaluated under microscopic view and found that above 4 mgL^{-1} concentration of Cd^{2+} and Pb^{2+} in the medium, significant structural damage to the stomata of leaves of the *E. crassipes* occurred. The results of this study concluded that *E. crassipes* can remediate Cd^{2+} and Pb^{2+} from the medium more efficiently at 1.22 mgL^{-1} concentration and the developed model can be used to navigate the design space. Furthermore, the different plant growth attributes were also affected above 4 mgL^{-1} concentration of Cd^{2+} and Pb^{2+} in the medium.

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INTRODUCTION

Environmental pollution caused by heavy metals has become a global issue, which extremely affects growth and development of agricultural crops, aquatic plants as well as the native flora. The devastating impacts of heavy metals are reduction in growth and development, photosynthetic rate, chloroplast, photosynthetic pigments and more importantly disturbed plant water relation. Heavy metals in soil and water can also induce the alteration in anatomical parameters of plants (Batool *et al.*, 2015). However, tolerance to these toxic metals is attained and varies among diverse species, and even within populations of a same plant species. Additionally, the study of the effects of toxicants including heavy metals on the growth and development of plants represents not only a theoretically but also practically relevant problem with respect to increasing industrialization (Sayed, 1997; Freitas *et al.*, 2007).

The aquatic macrophytes are believed to eliminate heavy metals from aquatic bodies through bio-accumulation process, where the high amounts of the heavy metals are captured in their body parts and it is likely to be useful for the reduction of the pollutants along with heavy metals from the industrial effluents by an environmental friendly approach (Wei *et al.*, 2014). Hyper-accumulator plants are able to remove and store significant amount of toxic and metallic contaminant (Letachowicz *et al.*, 2006; Kumar and Chopra 2016; Di *et al.*, 2015; Zaranyika and Nyati, 2017). These plants can be transplanted to sites for bio-filtering heavy metals from wastewater. Higher the affinity of the metal for the sorbate plant species, the latter is attraction and is bounded with different mechanisms. The process of bioremediation continues until an equilibrium is established between the amount of toxicant sorbate species and its portion remaining in the solution (Mahmood *et al.*, 2010). Earlier studies of aquatic plants also stated that toxicant acts as an on/off switch for stomata (Baruah *et al.*, 2012; Iida *et al.*, 2016). Hyper-accumulating aquatic plants like water hyacinth (*Eichhornia crassipes*), water caltrop (*Trapa natans*) and water lettuce (*Pistia stratiotes*) which are capable to remove heavy metals from contaminated water bodies commonly known as phytoremediation of heavy metals from wastewaters (Liao and Chang, 2004; Deka and Sarma, 2011; Kumar *et al.*, 2017a; Kumar *et al.*, 2018).

Heavy metals are actively captured by plant roots and then transferred to upper sections of the plants *viz.*, stem, leaves and fruits (Perfus-Barbeoch *et al.*, 2002). Heavy metals such as copper (Cu), zinc (Zn), cobalt (Co), and iron (Fe) are essential in trace amounts in catalyzation of metabolic activities in plants. However, excess of any kind of metal adversely affects plant metabolism and growth rate (Hall, 2002; Chandra and Kang, 2016). Stomata aperture is strongly regulated by divergent exogenous stimuli, such as light, drought stress, pathogens, temperature and others (Acharya and Assmann, 2009). Rapid stomata closure in leaves occurs in response to water deficiency and optimizes water use efficiency, thereby playing crucial roles in drought stress tolerance. Like this, stomata closing may also be affected by a direct interaction of the toxic metal at the guard cell level. Alternatively, increased stomata resistance may also be a consequence of toxic effects in other plant tissues, leading to decreased water accessibility in leaves and finally the stomata regulation (Cai *et al.*, 2017). These characteristics may be used as heavy metal sensitivity markers in aquatic plants. The aquatic plants are well adapted to grow in a wide range of environmental features including pH, electrical conductivity and temperature. Plants being used for the phytoremediation must be capable to tolerate the multiple forms and concentrations of the contaminants present in their environment (Kumar and Chopra, 2016; Kumar *et al.*, 2017a, c). Furthermore, the phytoremediation efficiency is depended upon leaf structure (Hessini *et al.*, 2008) because the leaf traits are often linked to the resource use efficiency of plants (Singh *et al.*, 2012).

Response surface methodology (RSM) is a set of mathematical and statistical methods applied for designing, refining, validating and optimizing procedures and experiments (Anderson and Whitcomb, 2005). This technique is used for evaluating the impacts of discrete factor, their comparative significance and the dependency of two or more variables and finding the best conditions for preferred responses or results of an experiment (Wantala *et al.*, 2012). RSM is used to find the optimum operating conditions for the system and to estimate a region that fulfils the operating conditions (Mourabet *et al.*, 2012).

A number of studies have been reported on the toxic effect of different heavy metals on the structure of stomata in various aquatic macrophytes which are being used for phytoremediation purposes. But, the present investigation was a novel study for optimizing response surface methodology (RSM) based reduction of Cd^{2+} and Pb^{2+} from the aqueous solution along with to study anatomical effects of Cd^{2+} and Pb^{2+} toxicity on stomata of water hyacinth (*Eichhornia crassipes* [Mart.] Solms) during phytoremediation.

MATERIALS AND METHODS

Test plant species (*E. crassipes*) for phytoremediation experiments

E. crassipes is a free floating aquatic plant and belongs to the family Pontederiaceae, was used to as test plant for this experiment. *E. crassipes* is a rapid growing aquatic macrophyte and its name Eichhornia was derived from the famous 19^{th} century Prussian politician J.A.F. Eichhorn. *E. crassipes* is an aquatic plant having good feasibility of phytoremediation of wastewaters containing metallic and other kind of chemical pollutants (Kumar *et al.*, 2017a, b). For this experiment, juvenile plants of *E. crassipes* were collected from the adjacent ponds situated at Jamalpur Kalan (29°91'20"N and 78°13'11"E) Haridwar (Uttarakhand), India. The healthy and disease free plants of *E. crassipes* were accurately weighted before and then used for the phytoremediation experiment.

Preparation of Cd^{2+} and Pb^{2+} stock solutions

The stock solutions of cadmium (Cd^{2+}) and lead (Pb^{2+}) of 100 mgL^{-1} concentration were prepared by dissolving solid $CdSO_4 . 2H_2O$ (pure analytical grade, Sigma Aldrich Inc.) and solid $PbSO_4$ (pure analytical grade, Sigma Aldrich Inc.), respectively, into appropriate amount of heavy metal free bore well water (BWW). Further, the stock solutions were diluted to obtain 2 mgL^{-1}, 4 mgL^{-1}, 6 mgL^{-1}, 8 mgL^{-1} and 10 mgL^{-1} concentrations. The stock solutions were standardized accordingly to make sure that the correct concentration was achieved.

Design of phytoremediation experiment

The phytoremediation experiment using *E. crassipes* was conducted in the Multipurpose Experimental Area (MEA) located at Department of Zoology and Environmental Science, Gurukula Kangri Vishwavidyalyaya, Haridwar (Uttarakhand), India (29° 55'13"N and 78°7'23"E). Glass aquariums of 25 liter capacity were used as phytoremediation vessel. Three replicates of each concentrations of Cd^{2+} and Pb^{2+} *viz.*, 0 mgL^{-1} or control (BWW), 2 mgL^{-1} (T_1), 4 mgL^{-1} (T_2), 6 mgL^{-1} (T_3), 8 mgL^{-1} (T_4) and 10 mgL^{-1} (T_5) were made accordingly and used as growing medium of *E. crassipes*. Bore well water (BWW) was used as control/blank (0 mgL^{-1}) which was analyzed for heavy metals before the experiment, and found free of Cd^{2+} and Pb^{2+} (Kumar *et al.*, 2017b). For this experiment the glass aquariums were filled with 20 liter volume of growing medium and set in an order as shown in the Figure 1 and three replicates of each treatment were implemented.

Heavy metals characterization of growing medium

The growing medium was characterized for two heavy metals *viz.*, Cd^{2+} and Pb^{2+}. The concentration of Cd^{2+} and Pb^{2+} were analyzed before, during and after the phytoremediation experiments. The analysis of Cd^{2+} and Pb^{2+} was performed at every 15 days interval (Initial day, 15th day, 30th day, 45th day and 60th day) by following the standard methods and procedures prescribed by the AOAC (2005); APHA (2012) and Chaturvedi and Sankar (2006). Cd^{2+} and Pb^{2+} were analyzed by using an Atomic Absorption Spectroscopy (Model- PerkinElmer, Analyst 800, GenTech Scientific Inc., Arcade, NY).

Determination of growth attributes of *E. crassipes* plants

Total fresh biomass, total chlorophyll content and kinetic plant growth rate of *E. crassipes* plants were determined before and during the phytoremediation experiments at intervals of 0, 15, 30, 45 and 60 days in each of treatment. Fresh weight of *E. crassipes* plants was determined by using a digital balance. Total chlorophyll content (chlorophyll a and b) of *E. crassipes* was analyzed using acetone (80%) extraction method and the single beam absorbance was recorded with help of a UV-Vis spectrophotometer (Agilent, 60 Cary UV-Vis) (Aron, 1949; Kumar *et al.*, 2017a, b). The quantity of chlorophyll a, chlorophyll b and total chlorophyll of *E. crassipes* were calculated using the equation 1, 2 and 3.

Total chlorophyll content: 20.2(A645) + 8.02(A663) (1)

Chlorophyll a: 12.7(A663) – 2.69(A645) (2)

Chlorophyll b: 22.9(A645) – 4.68(A663) (3)

Where, A645 and A663 are the absorbance taken at 645 and 663 nm, respectively.

The kinetic plant growth rate of *E. crassipes* plants was determined by comparing the final weight with the initial weight. The equation 4 was used to calculate the kinetic plant growth rate (Hunt 1978; Kumar *et al.*, 2017a, b).

$$\text{Kinetic growth rate} = \frac{\text{InW}_2 - \text{InW}_1}{(t_2 - t_1)} \quad (4)$$

Where, InW_2 and InW_1 are initial and final fresh biomass of plants at harvest, respectively, and (t_2-t_1) is the time of the experiment in days. The results were represented as increase of biomass per unit mass per day ($gg^{-1}d^{-1}$).

Translocation factor of Cd^{2+} and Pb^{2+} in roots and leaves of *E. crassipes*

Translocation factor (T_f) is important attribute for screening hyper accumulator's plants suitable for phytoextraction of heavy metals. Metals that are accumulated by plants and largely stored in the roots of the plants are indicated by T_f values. Greater the T_f value more is the s translocation of heavy metal in the aerial parts of the plant (Mellem *et al.*, 2009). This is the ratio which represents the ability of a plant to translocate metals from its roots to its aerial parts (Mellem *et al.*, 2012). T_f of *E. crassipes* for Cd^{2+} and Pb^{2+} was calculated using the equation 5.

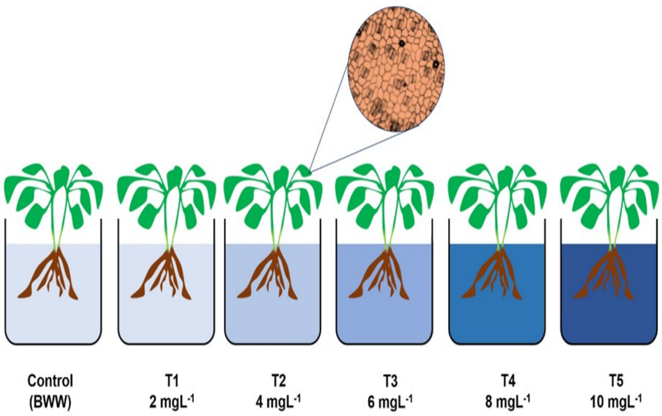

Figure 1. *Experimental setup for phytoremediation using E. crassipes.*

$$\text{Translocation factor } (T_f) = \frac{C_a}{C_r} \quad (5)$$

Where, C_a and C_r are the concentration of metal in aerial parts and roots of *E. crassipes*, respectively.

Optimization of Cd^{2+} and Pb^{2+} reduction using response surface methodology

The reduction of Cd^{2+} and Pb^{2+} from the growing medium was optimized by response surface methodology (RSM). There are three steps, essentially used to optimize any RSM model *viz.*, statistical designing the experiment, determining the coefficient values of mathematical model and performing response prediction and validity of the model (Mondal *et al.*, 2013). A 2-factor Central-Composite Design (CCD) was applied to evaluate the effect of the selected parameters on the reduction of Cd^{2+} and Pb^{2+} from aqueous solutions by *E. crassipes*. A total 25 experimental runs were designed (Table 1) and performed to evaluate the reduction of Cd^{2+} and Pb^{2+} separately. Two factors *viz.*, X_1: Cd^{2+} and Pb^{2+} treatment concentration (2, 4, 6, 8 and 10 mgL^{-1}) and X_2: Experimental times (0, 15, 30, 45 and 60 day) were selected as the independent variables. While, Y_1 and Y_2 for percent (%) reduction of Cd^{2+} and Pb^{2+} from the growing medium were taken as dependent variables to study the response of independent variables, respectively.

The selected variables were coded according to the equation 6 given below.

$$x_i = \frac{X_i - X_0}{\Delta X_i} \quad (6)$$

Where, x_i is the coded value of an independent variable, X_i is the real value of an independent variable, X_0 is the real value of an independent variable at the center point and ΔX_i is the step change value.

The percent reduction (Y_i) of Cd^{2+} and Pb^{2+} was calculated according to the equation 7 (Zheng and Wang, 2010).

$$Y\ (\%) = \frac{C_0 - C_t}{C_0} \times 100 \quad (7)$$

Where, *Y* is the reduction efficiency (%), and C_0 and C_t are the initial and residual (after t days) concentrations of Cd^{2+} and Pb^{2+} in the aqueous solution (mgL^{-1}). The experimental results were analyzed using Design Expert Version 11.0 (Stat Ease) software package and polynomial regression model was used as per equation 8 (Salehi *et al.*, 2017).

$$Y\ (\%) = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij}\, X_i X_j \qquad (8)$$

Where, Y is the response; X_i and X_j are the independent variables; β_0 is an established coefficient and β_i $\beta_{i,j}$ and βii are the regression coefficients, respectively.

Preparation of slides and microscopic analysis

Fresh leaves of *E. crassipes* were used to prepare slides for microscopic observations. Fresh leaf samples from each aquarium were collected at intervals of every 15 days (Initial day, 15th day, 30th day, 45th day and 60th day) at 12:00 pm noon (IST/ +5:30GTM). Sharp and new blades were used to dissect the leaves carefully, while the sections were cut with a dissecting scope by placing blade on its axis i.e. perpendicular to the angle of view. The prepared slides were stained with Safranine-O reagent solution (10%) according to standard method described by Hultine and Marshall (2001). For assessing the relative changes in stomata structure and density, plant leaf peel was expanded on the slides, dried and rewetted. Images were captured with help of a compound microscope fitted with digital camera (Model- Olympus, CH20i). Stomata counting were performed under the microscope at resolution of 40X i.e. number of total stomata in per view (50) and the number of damaged stomata. Stomata were counted in four microscopic views in each slide and the calculated mean value was used as average stomata damage count. The degree of stomata damage was calculated with the equation 9 given below:

$$\%\ \text{Stomata Damage}\ (S_D) = \frac{(DS \times 100)}{(TS)} \qquad (9)$$

Where, DS is the number of damaged stomata and TS is the total number of stomata per view.

Statistical analysis

The results of the present study were the mean of three replicates. The experimental results were analyzed with Design Expert Version 11.0 (Stat Ease). A single way Analysis of Variance (ANOVA) test was applied to the obtained data with the help of MS Excel 2013 and the graphs were plotted with the help of OriginLab Pro Version 9 software.

RESULTS AND DISCUSSION

Effect of Cd^{2+} and Pb^{2+} treatment on growth attributes of *E. crassipes*

The results showed that after 60 days of phytoremediation experiment the total fresh biomass of *E. crassipes* plants increased up to 4 mgL^{-1} and then started decreasing above this concentration of Cd^{2+} and Pb^{2+}. Figure 2 represents the total fresh biomass in Cd^{2+} and Pb^{2+} treatments at different concentrations. The highest fresh biomass was observed in the T_2 treatment (4 mgL^{-1}) for both Cd^{2+} and Pb^{2+} *viz*., 206.75 gm/kg and 193.25 gm/kg, respectively. Fresh plant biomass was found higher than in control treatment i.e. 155.43 gm/kg which indicated that $CdSO_4.2H_2O$ and solid $PbSO_4$ salts enriched the growing medium and boosted the plant weight below T_2 treatment. Kumar *et al.* (2017a, b) also reported the increased fresh biomass of *E. crassipes* and *Pistia stratiotes* plants grown in sugar and paper mill effluents in which, appropriate concentration of Cd, Pb, Al, Cu and Fe were present. They also stated that higher the concentration of heavy metals in the effluents acts as toxicants for *E. crassipes* plants.

Kinetic plant growth rate and total chlorophyll content of *E. crassipes* during phytoremediation experiments were noted at different concentration of Cd^{2+} and Pb^{2+} treatments as shown in Figures 3, 4. Increasing kinetic plant growth rate 2.211 gg^{-1}d^{-1}, 2.261 gg^{-1}d^{-1} for Cd^{2+}; 2.101 gg^{-1}d^{-1}, 2.182 gg^{-1}d^{-1} for Pb^{2+}; and total chlorophyll content 2.260±0.10 mg/gfwt for Cd^{2+}; 2.070±0.10 mg/gfwt for Pb^{2+} were observed *viz*., highest at T_1 and T_2 treatment (2 mgL^{-1} and 4 mgL^{-1}) due to the conformity of the efficient uptake of these metals and to achieve the maximum growth of plant as earlier reported by (Sooknah and Wilkie, 2014; Kumar *et al.*, 2016) while, the concentration above 4 mgL^{-1} plant growth progressively declined after 30-60 days due to metal induced toxicity in the plant which might be due to the inhibition of chlorophyll processes and biosynthesis (Mukherjee and Kumar, 2005). The reduction in the total chlorophyll content is associated with the higher concentrations of toxic heavy metals treated to the aquatic plant as earlier reported by researchers for water lettuce (De *et al.*, 1985), Cd and Hg treatment by *Hydrilla verticillata* and *Lemna minor* (Chatterjee and Nag, 1991), Pb treated by the *Salvina natans* (Sen and Bhattacharyya, 1993), Pb and Cr treatment by *Ipomea aquatica* (Alam and Chatterjee, 1994) and Zn, Cu, Cd and Cr treatment of wastewater using water hyacinth and water lettuce (Kouamé *et al.*, 2016). Therefore, the results indicated that the plant growth attributes viz., total fresh biomass, total chlorophyll content and kinetic plant growth rate of *E. crassipes* were strongly affected by the different concentrations of Cd^{2+} and Pb^{2+}. The optimum concentration of Cd^{2+} and Pb^{2+} was between 2-4 mgL^{-1} where, total fresh biomass, total chlorophyll content and kinetic plant growth rate was radially increased.

Translocation factor of Cd^{2+} and Pb^{2+} in leaves and roots of *E. crassipes*

The transport of Cd^{2+} and Pb^{2+} from aqueous solution to the roots and again to the leaves of plant of *E. crassipes* potency is due to necessity and accumulation power, which is controlled by the several physiological and biochemical processes (Kumar *et al.*, 2017a). Figure 5 presented the translocation factor of Cd^{2+} and Pb^{2+} in roots to leaves of *E. crassipes* after 60 days of phytoremediation experiments. T_f was observed highest at 2 mgL^{-1} concentration for both Cd^{2+} and Pb^{2+} treatments i.e. 4.56 and 2.29 correspondingly at 30 day. Cd^{2+} was found to have high T_f value than of Pb^{2+}, which means that *E. crassipes* translocate Cd^{2+} in more quantity in comparison of Pb^{2+} at 2 ppm concentration (T_f- Cd^{2+} >T_f- Pb^{2+}). Also, the capability of a plant to translocate metals from the roots to the shoots is estimated by using the translocation factor. Yoon *et al.* (2006) reported that greater the T_f factor values <1 higher they are capable to absorb heavy metals from their environment. Thus, the higher T_f value (4.56 and 2.29) which was observed at T_1 treatment of both Cd^{2+} and Pb^{2+} at 30th days, indicated that maximum uptake of these metals occurs at 2 mgL^{-1} concentration by *E. crassipes*.

Optimization analysis for Cd^{2+} and Pb^{2+} reduction

One of the main objectives of this study was to find out the optimum concentration of Cd^{2+} and Pb^{2+} that could be efficiently reduced by *E. crassipes* from the aqueous solution during the phytoremediation. Table 1 shows that 96% of Cd^{2+} and 94% of Pb^{2+} was reduced from the aqueous solution at run number 5. As the concentration of metal was increased the percent reduction (Y%) was decreased substantially. Determining the prime value for controller variables (factors i.e. independent variables) is one of the main aims of RSM that can take full advantage of a response over a certain area of importance (Khuri and Mukhopadhyay, 2010; Darajeh *et al.*, 2016). The maximum model sum of squares suggested by the Design Expert software was a Two-Factor Interaction (2FI) model for percentage reduction of Cd^{2+} and Pb^{2+}. The ANOVA for Cd^{2+} and Pb^{2+} reduction is presented in Table 2. The 2FI ANOVA models for Cd^{+2} and Pb^{+2} reduction had Xi and X_2 as significant factors with Prob > F of 0.0001. High percentage removal of Cd^{2+} and Pb^{2+} was observed at lower metal concentrations. However, the percent reduction of Cd^{2+} and Pb^{2+} was decreased as the concentration of metal treatment was enhanced in the medium.

Higher concentration of Cd^{+2} and Pb^{+2} in the aqueous solution was believed to aid as more toxic growing medium of *E. crassipes*. Adequate precision value of 37.48 and 36.83 for Cd^{2+} and Pb^{2+} reduction indicated an adequate signal which implies that this model can be used to navigate the design space. The resulting regression models equations for Cd^{2+} and Pb^{2+} reduction are given in equation 10 and 11, respectively.

$$Y_{Cd}(\%) = 32.486 - 31.19X_i + 34.502X_j - 31.83X_iX_j \quad (10)$$

$$Y_{Pb}(\%) = 29.819 - 29.555X_i + 31.232X_j - 31.14X_iX_j \quad (11)$$

The equations 10 and 11 can be used to make predictions about the reduction of Cd^{2+} and Pb^{2+} for given levels of selected factor. Figure 6 presents the 3D surface plot for Cd^{2+} and Pb^{2+} reduction from aqueous solution with respect to two independent variables viz., X_1: concentration and X_2: experimental time. Mojiri *et al.* (2017) applied RSM-CCD in optimizing the independent factors in biosorption of Cr(IV) by plant powder, including contact time (24–72 hour) and initial concentration of metal (20–80 mgL^{-1}), and their responses. They reported the prime removal efficacy was 92.3 in react time (48.9 hour) at 50.9 mgL^{-1} initial concentration of Cr(IV). The optimization levels given in Table 3 and Figure 7 showed that at 1.22 mgL^{-1} concentration and 54.95 days the model gave maximum percent reduction both of Cd^{2+} and Pb^{2+} from the aqueous solution. Therefore, RSM-CCD model was best fitted to predict and optimize the Cd^{2+} and Pb^{2+} reduction from the aqueous solution.

Effect of Cd^{2+} and Pb^{2+} stress on stomata of *E. crassipes*

Microscopic analysis of slides leaves of *E. crassipes*, stained with Safranine-O showed that Cd^{2+} and Pb^{2+} toxicity in growing medium subsequently brought cellular damages. The stomata were shielded by darker (blackish) region which were unable to open and contribute in the process of transpiration. The degree of stomata destruction was recorded significantly increasing with increase in the heavy metal concentration. However, the effect of Pb^{2+} toxicity was noted higher in comparison to Cd^{2+} (Figures 8, 9 and Table 4). A slight destruction of stomata was observed in control at 45 and 60 days due to aging of the plant. The stomata opening and closing was maximally inhibited at T_5 or 10 mgL^{-1} concentration in both Cd^{2+} and Pb^{2+} due to extensive accumulation and toxicity of heavy metals in the leaves cells of the *E. crassipes*. Stomata are very important structure of the plants they regulate the gaseous exchange, photosynthesis and transpiration rate and ultimately affect the growth of the plants (Cai *et al.*, 2017). The damage in stomata may lead the adverse effects on plant physiology and anatomy which produces stress and consequently affected the remediation efficiency of the *E. crassipes*. Chandra and Kang (2016) also reported the effects of heavy metal stress on photosynthesis, transpiration rate, and chlorophyll content in poplar hybrids. Generally, phytoremediation process is concerned with the metabolism of a plant to uptake and degrades the pollutants. Water content in plants play crucial role in mechanism of photosynthesis and transpiration, and are directly connected with the phytoremediation efficiency of a plant (Sayed, 1997; Sarwar, 2010). The water transport mechanism of plant is affected if the cell membrane permeation is blocked partially or completely by such kind of stress increasing agents, which tend to decrease the phytoremediation rate. Furthermore, the addition of $CdSO_4.2H_2O$ and solid $PbSO_4$ salts increased the pH, EC and TDS after hydrolyzing in the water. The bore well water played significant role in providing the nutritional constituents in the medium like minerals instead of desalted distilled water which cannot support the growth of plants. The optimum value of applicable Cd^{2+} and Pb^{2+} metal ions was found T_2 or 4 mgL^{-1} where the degree of stomata destruction was below 10% and nearby to the damage threshold with maximum metal remediation from the aqueous solution. Due to the strong affinity of *E. crassipes* towards bioaccumulation for heavy metals, they can achieve the process of phytoremediation within a tolerable limit, but beyond this limit the heavy metals acts as a cell deteriorating agent and starts to decrease the phytoremediation potential. Consequently, the results showed that the higher contents (more than T_2 treatment or 4 mgL^{-1}) of Cd^{2+} and Pb^{2+} in the growing medium were found toxic to the stomata of *E. crassipes*.

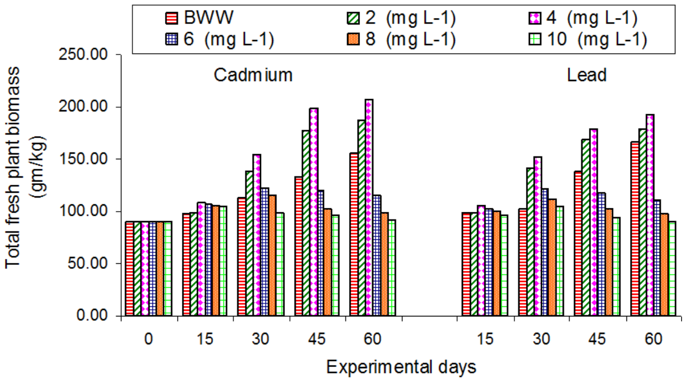

Figure 2. *Total fresh biomass of E. crassipes plants in* Cd^{2+} *and* Pb^{2+} *treatments at different days (BWW: Control).*

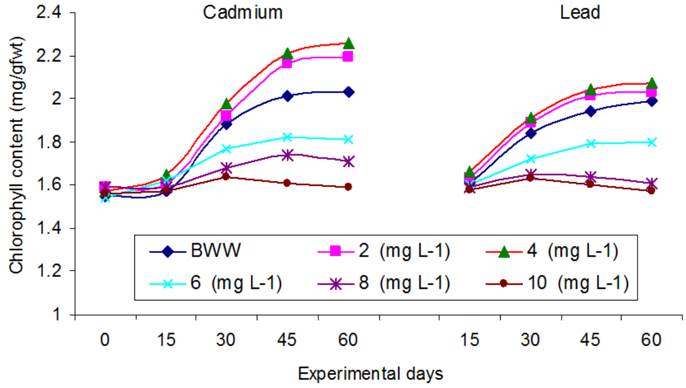

Figure 3. *Total chlorophyll content in plants of E. crassipes plants in* Cd^{2+} *and* Pb^{2+} *treatments at different days.*

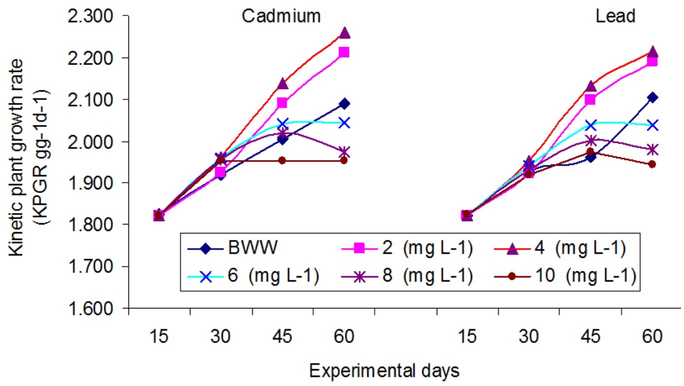

Figure 4. *Kinetic plant growth rate of E. crassipes plants in* Cd^{2+} *and* Pb^{2+} *treatments at different days.*

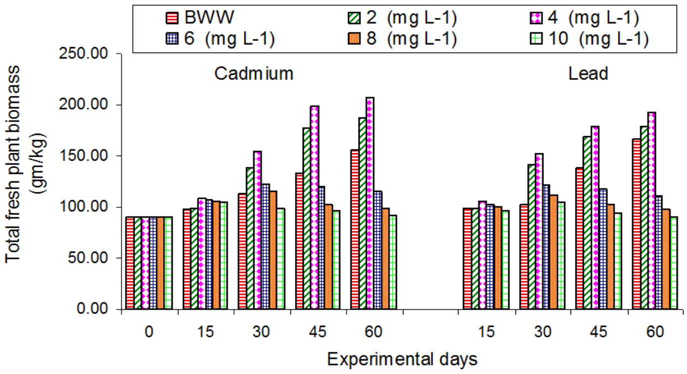

Figure 5. *Translocation factor of E. crassipes plants in* Cd^{+2} *and* Pb^{+2} *treatments at different days.*

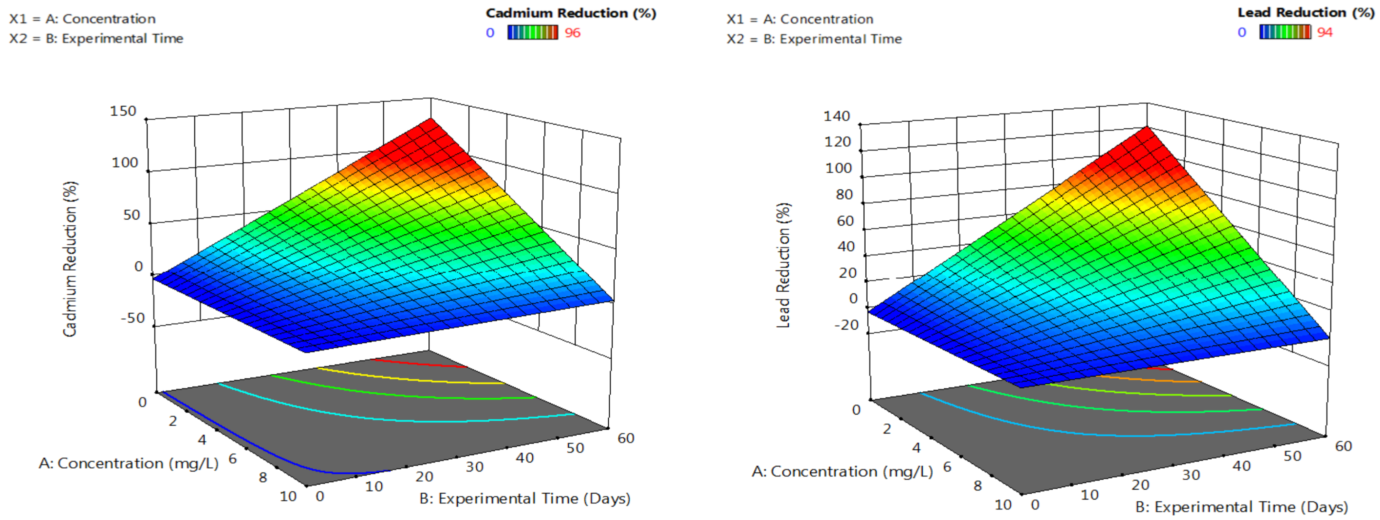

Figure 6. *3D surface Plot for* Cd^{2+} *and* Pb^{2+} *reduction.*

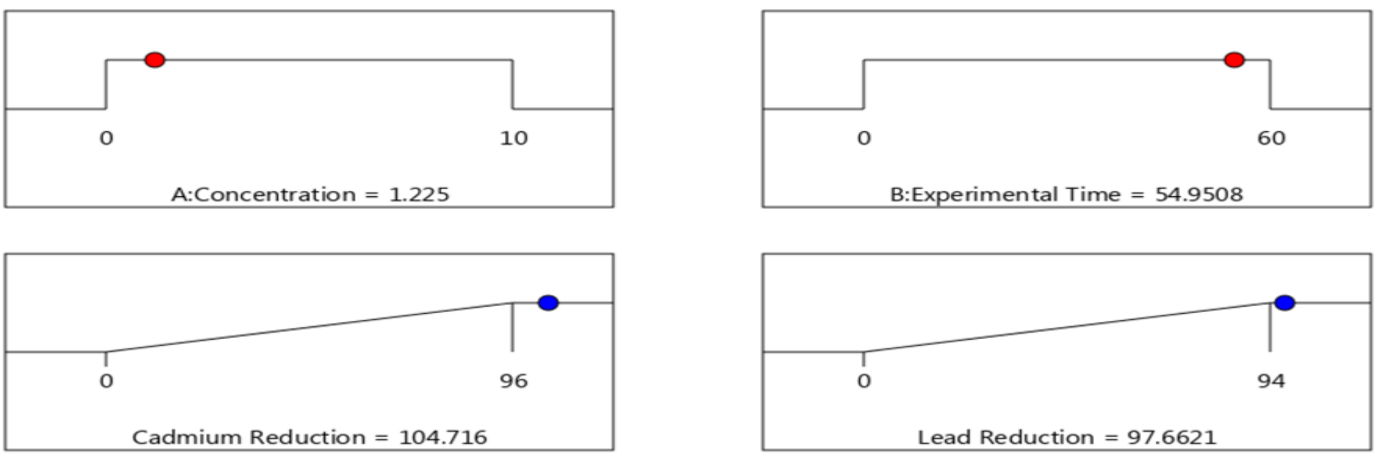

Figure 7. *Optimization of* Cd^{2+} *and* Pb^{2+} *reduction.*

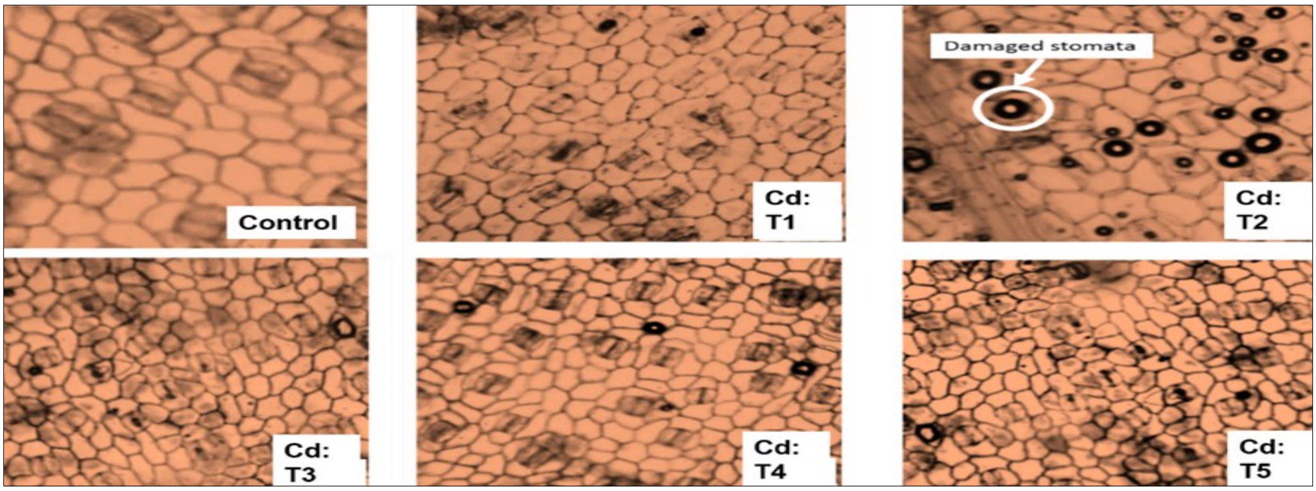

Figure 8. *Microscopic view of damaged leaf stomata of E. crassipes at day 60 due to Cd^{2+} toxicity (resolution: 40X).*

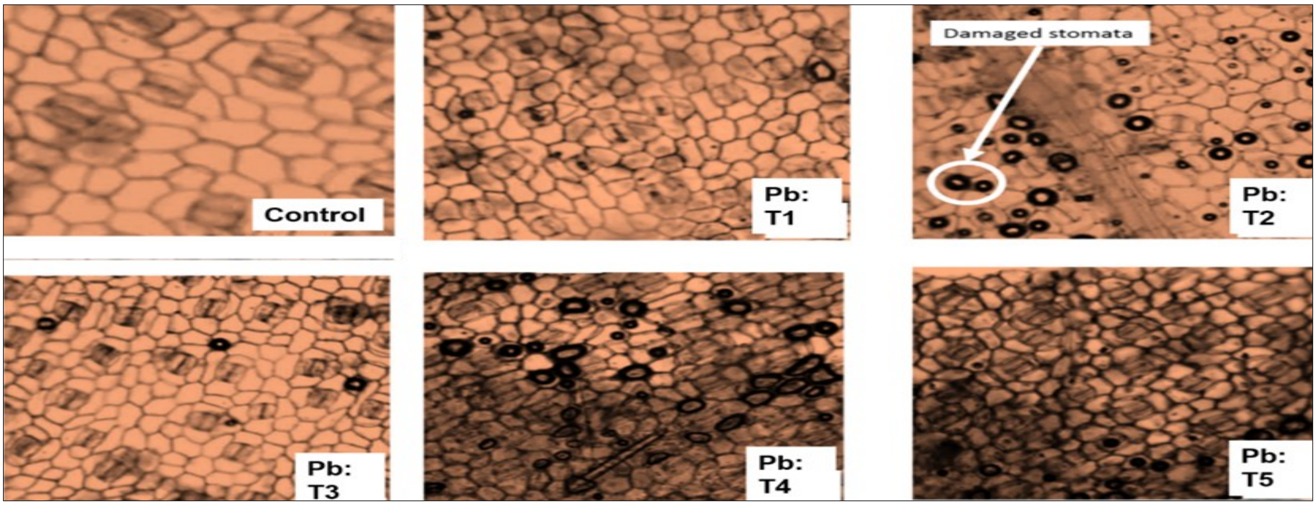

Figure 9. *Microscopic view of damaged leaf stomata of E. crassipes at day 60 due to Pb^{2+} toxicity (resolution: 40X).*

Table 1. RSM design for the phytoremediation experiments.

Run	Factor 1 (A: X_1)	Factor 2 (B: X_2)	Response 1 (Y_1)	Response 2 (Y_2)
	Concentration	Experimental Time	Cadmium Reduction	Lead Reduction
	mgL^{-1}	Days	%	%
1	2	0	0	0
2	2	15	19	16
3	2	30	64.5	55
4	2	45	76.5	74.5
5	2	60	96	94
6	4	0	0	0
7	4	15	10.8	13.5
8	4	30	37.8	32.5
9	4	45	76.3	70
10	4	60	91	83
11	6	0	0	0
12	6	15	6	11.5
13	6	30	18.3	18
14	6	45	38.3	26.7
15	6	60	46.8	35.2
16	8	0	0	0
17	8	15	4.9	5.5
18	8	30	8.3	8.8
19	8	45	15	15
20	8	60	23.5	19.4
21	10	0	0	0
22	10	15	1.8	0.7
23	10	30	5.8	3.6
24	10	45	8	6.7
25	10	60	9.6	8.1

Table 2. ANOVA for 2FI model of Cd^{2+} and Pb^{2+} reduction during phytoremediation.

Source	Cadmium (Cd^{2+})						Lead (Pb^{2+})					
	Sum of Squares	***df***	**Mean Square**	**F-value**	**p-value**		**Sum of Squares**	***df***	**Mean Square**	**F-value**	**p-value**	
Model	21730.56	3	7243.52	141.74	< 0.0001	Significant	18681.78	3	6227.26	135.61	< 0.0001	Significant
A-Concentration	7782.53	1	7782.53	152.29	< 0.0001		6987.98	1	6987.98	152.17	< 0.0001	
B-Experimental Time	13226.53	1	13226.53	258.82	< 0.0001		10838.20	1	10838.20	236.02	< 0.0001	
AB	4052.60	1	4052.60	79.30	< 0.0001		3878.80	1	3878.80	84.47	< 0.0001	
Residual	1073.19	21	51.10				964.34	21	45.92			
Cor Total	22803.74	24					19646.12	24				
Std. Dev.	7.15		R^2	0.9529			Std. Dev.	6.78		R^2	0.9509	
Mean	26.25		Adjusted R^2	0.9462			Mean	23.91		Adjusted R^2	0.9439	
C.V. %	27.24		Predicted R^2	0.9336			C.V. %	28.34		Predicted R^2	0.9324	
			Adeq Precision	37.4893						Adeq Precision	36.8303	

Table 3. Response conditions at optimum value for Cd^{2+} and Pb^{2+} reduction during phytoremediation.

Factor (X)		Responses (Y) at 95% confident level	
X_1: Concentration (mgL^{-1})	**X_1: Experimental Time (Days)**	**Y_1 Cadmium reduction (%)**	**Y_2 Lead reduction (%)**
1.225	54.95	104.71	97.66

Table 4. Damaged stomata count of *E. crassipes* in growing medium (Cd^{2+} and Pb^{2+}) during phytoremediation experiment.

Heavy metals	Treatments	Damaged stomata count*					% S_D after 60 days
		Initial day	15 days	30 days	45 days	60 days	
Control	BWW	0	0	0	2	3	6
Cd^{2+}	2 mgL^{-1}	0.00	0.00	3.00	4.00	6.50	13
	4 mgL^{-1}	0.00	2.00	4.00	7.95	9.26	18.52
	6 mgL^{-1}	0.00	5.64	7.90	9.72	11.19	22.38
	8 mgL^{-1}	0.00	7.61	7.34	6.80	12.12	24.24
	10 mgL^{-1}	0.00	9.82	9.42	9.20	15.04	30.08
Pb^{2+}	2 mgL^{-1}	0.00	0.00	4.00	5.00	7.20	14.4
	4 mgL^{-1}	0.00	3.10	4.80	8.20	10.20	20.4
	6 mgL^{-1}	0.00	5.80	8.20	10.30	12.50	25
	8 mgL^{-1}	0.00	8.20	8.70	11.40	15.60	31.2
	10 mgL^{-1}	0.00	10.20	12.50	13.60	16.70	33.4

BWW: Bore well water.* Values are mean of stomata count in four microscopic views, % S_D: Percent Stomata damage.

Conclusion

The results of this experiment concluded that RSM-CCD model was best fitted to predict and optimize the Cd^{2+} and Pb^{2+} reduction from the aqueous solution. The optimum concentration for Cd^{2+} and Pb^{2+} phytoremediation using *E. crassipes* was found 1.22 mgL^{-1} in 54 days experiment in which maximum amount of Cd^{2+} and Pb^{2+} was reduced from the aqueous solution. Furthermore, the translocation factor kinetic plant growth rate, fresh plant biomass and total chlorophyll contents were also found positively correlated with the Cd^{2+} and Pb^{2+} concentration up to 4 mgL^{-1} and after it negatively. Moreover, the concentrations <4 mgL^{-1} of Cd^{2+} and Pb^{2+} produced structural damage to the stomata in the leaves of *E. crassipes*. The number of damaged stomata of *E. crassipes* was also increased with the increase in the contents of Cd^{2+} and Pb^{2+} in the growing medium. The higher contents (T_5 or 10 mgL^{-1}) of Cd^{2+} and Pb^{2+} in the growing medium were found very toxic to the stomata of *E. crassipes*. Among both the metals, Pb^{2+} produced more damage to stomata of *E. crassipes* in comparison of Cd^{2+}. This study suggested that the anatomical and ultra-structural characteristics may be used as a part of the studies on the modifications caused by the potentially toxic metals and other plant pollutants and using *E. crassipes* for phytoremediation purposes by enabling to control heavy metals levels within its tolerable limits using the proposed RSM-CCD. Further research is required to study the effects of heavy metals toxicity on anatomical, physiological and biochemical processes like water balance, gaseous exchange, transpiration rate and photosynthetic rate of *E. crassipes* which likely affects the phytoremediation potential of *E. crassipes*.

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