*Article*

# Optimization of phytoremediation in Cd- contaminated soil by using Taguchi method in *Spinacia oleracea*

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## Abstract
Phytoremediation is an environmental friendly technique to the cleanup of polluted soils, which combines the disciplines of plant, soil and microbiology. In this study, four factors including: cow manure, compost, urea fertilizer and Cd-resistant bacteria with three different levels in soils contaminated with cadmium using 50 mg $kg^{-1}$ cadmium chloride ($CdCl_2.H_2O$) were used to optimize of phytoremediation by *Spinacia oleracea*. Taguchi method has been used for experimental design. Results showed that significant factors in the order of importance were: cow manure, Cd- resistant batteries, urea fertilizer and compost. The optimum conditions for the selected levels were inoculate three types of bacteria (CC3, CC4, CC5), compost = 10 (g $kg^{-1}$), urea fertilizer = 0.05 (g $kg^{-1}$) and cow manure = 40 (g $kg^{-1}$). The performance of` these conditions were estimated at 257.27 (mg $kg^{-1}$). Cow manure is the most contribution to efficiency of phytoremediation in *Spinacia Oleracea*.

**Keywords** optimization; *Spinaceae olerace*a; fertilizer; resistance bacteria; cadmium.

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## 1 Introduction
Contamination of heavy metals in soils may affect soil ecology, quality of agricultural products and water resources, human health problem (Thawornchaisit and Polprasert, 2009; Su et al., 2014). Soil are contaminated by heavy metals due to different sources such as agricultural activates; sewage sludge, fertilizers and pesticide (Sayyed and Sayadi 2011; Sayadi and Rezaei 2014). One of heavy metals is cadmium. The amount of cadmium enter into the soil through utilization of sewage sludge and agricultural activates and also as a result of industrial activities such as dye making, rubber making, production of fertilizer from phosphate rock, automobile fuel and metal melting industry (Moteshare-zadeh et al., 2010; Sayadi et al., 2010). Increasing awareness of the environmental and public health hazard of toxic metals pressurizes society to develop management strategies to remediate or restore the contaminated area (Thawornchaisit and Polprasert, 2009; Sayadi and Torabi, 2009). The high cost of existing remediation technologies led to the search for novel

strategies that have the potential to be low cost and impact (Chhotu et al., 2009). Phytoremediation is a technology that plants and their associated rhizospheric microorganisms are applied to remove, degrade and detoxify of soil, sediments, water resources and even atmosphere pollution (Ying, 2002). In this study, four factors include: cow manure, compost, urea fertilizer and Cd-resistant bacteria (after isolation and identity) in Cd-contaminated soil has been used to optimize of phytoremediation by *Spinacia olerace*a. Taguchi method has been used for experimental design. The Taguchi method is a strong design approach to study a large number of parameters with small number of experiments using fractional factorial design (Khosla et al., 2006). Soil bacteria play an important role in the recovery of nutrients, development of the soil structure, removal of toxicity resulting from soil chemical activities, control of plant's pests and also in stimulation of the plant's growth (Moteshare-zadeh and Savaghebi-Firoozabadi, 2010). Kuffnet et al. (2008) revealed the effects of rhizosphere bacteria on absorption and metal concentration in willows in the contaminated soils from lead mine. *pseudomonas, agromyces, streptomyces, flavobacterium, servatia and janthinobacterium* were identified. Among strains, four strains of *pseudomonas* and *serratia* and two strains of *streptomyces* type have the ability of siderophore production and also three strains of *janthino bacterium* and *serratia* have the ability of auxin production. Tu et al. (2000) reported Fertilizers can affect the soil properties such as pH, CEC and ions in soil. All these effects would result in the changes in the forms of heavy metals.

The objectives of the study were to:

1. Choice of appropriate experimental design.

2. Determination the optimal settings of the design parameters which would maximize uptake cadmium in *Spinacia oleracea.*

3. Improving the performance of phytoremediation process.

**2 Materials and Methods**

This research was done to pot culture at the University of Birjand (Iran). Soil samples were collected from a depth of 0-30 cm from the field of faculty of agriculture university of Birjand. Samples were air dried and passed through 4-mesh sieve and mixed uniformly. Physical and chemical properties soil samples were measured. Results are given in Table 1.

The methods for bacterial isolation and identity of resistant microorganisms to heavy metals have been reported previous in (Jahanbakhshi et al., 2011). Isolation and purification of native resistant bacteria from soil samples were done before the performance of pot culture experiments (Table 2). Cd- contaminated soil samples with concentration of 50 mg $kg^{-1}$ Cd were simulated. Cadmium solution was prepared from $CdCl_2.H_2O$. Four other parameters including: compost, cow manure, urea and Cd-resistant bacteria in three levels were also formulated. Characteristic of compost and cow manure are shown in Table 3.

The plant species *Spinacia oleracea* was used to remove or reduce concentrating cadmium (Jahanbakhshi, 2011). Pots were made of polyethylene material and weighed about 250 g with diameter of 14.2 cm and height of 13 cm. In each pot 1000g of sieved soil were added. The pots were placed in a laboratory environment with lighting of 14 hours during the mean day and night temperatures of 25°C and 18°C. Soil moisture was maintained by weighing the pots and replenishing the water loss daily. Seedlings were fifteen plants in each pot and grown for about four weeks. Taguchi method has been used for experimental design.

After planting period, shoot samples were harvested and washed with distilled water and placed in the special packets and dried in oven at 70°C for 48 hours. The dry weight was recorded. The plant sample was grinded with pestle and mortar. Sample was prepared for Cd determination by wet digestion method where nitric acid (65%) and percholoric acid (70%) were used to chop off the organic component of the sample (Ebrahimpour and Mushrifah, 2008). The extracts were determined for Cd using model Shimadzu 6300 AA atomic absorption spectrophotometer (Japan). The optimization of the observed values was determined by analysis of variance (ANOVA) which was based on the Taguchi method.

**Table 1** Characteristic physical and chemical of used soil (sandy-loam).

| Clay (%) | Sand (%) | Silt (%) | FC (%) | OM (%) | EC (dS $m^{-1}$) | pH | Total N (%) | Available P (mg $kg^{-1}$) | Available K (mg $kg^{-1}$) | Cd (mg $kg^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|
| 12 | 56 | 32 | 15 | 0/4 | 2.21 | 7.5 | 0/04 | 10 | 250 | 0/02 |

FC: Field Capacity, OM: Organic matter, EC: Electrical conductivity.

**Table 2** Characteristic of activate Cd- Cr resistant bacteries (Jahanbakhshi et al, 2011).

| Number bactery | Morphology | Gram reacts | Status colony | Size colony | Color colony |
|---|---|---|---|---|---|
| $CC_1$ | Coco basil | Negative | Soft | Point | White |
| $CC_2$ | Cocsi | Negative | Soft | Point | White |
| $CC_3$ | Cocsi | Negative | Soft | Point | White |
| $CC_4$ | Cocsi | Negative | Soft | Point | White |
| $CC_5$ | Coco basil | Positive | Soft | Point | White |

**Table 3** Characteristic of compost and cow manure.

| Type of component | pH | Electrical conductivity extract ECe | Available K | Available P | Total N | Cd |
|---|---|---|---|---|---|---|
| | - | dS $m^{-1}$ | mg $kg^{-1}$ | mg $kg^{-1}$ | % | mg $kg^{-1}$ |
| compost | 7.7 | 5.85 | 8100 | 6300 | 2.1 | - |
| cow manure | 7.8 | 3.45 | 7500 | 2600 | 3.02 | - |

Taguchi replaces the full factorial experiment with a lean, less expensive, faster, partial factorial experiment. Taguchi's design for the partial factorial experiment is based on specially developed Orthogonal Arrays (Christopher and Tpwle, 2001). The Taguchi method having a orthogonal array table to affect the design process (Gerjan et al., 2000). OA (Orthogonal array) is a matrix of numbers arranged in rows and columns. Each row represents the level of factors in each run and each column represents a specific level for a factor that can be changed for each run (Khosla et al., 2006).

## 3 Results

For our experiments, we considered four parameters. These four main factors for convenience are represented by the letters A~D (Table 4). The levels of the parameters are listed in Table 5. For these experiments, $L_9(3^4)$ OA was selected, which represents 9 experiments with four three-level factors. The selected OA is represented in Table 6.

**Table 4** Main factors to optimization of phytoremediation.

| Factor | Description |
|---|---|
| A | Compost |
| B | Resistant Bacteria |
| C | Urea Fertilizer |
| D | Cow Manure |

**Table 5** Factors and levels.

| **Factor** | **Level** | | |
|---|---|---|---|
| | **1** | **2** | **3** |
| **A** | 10 (gr $kg^{-1}$) | 20 (gr $kg^{-1}$) | 40 (gr $kg^{-1}$) |
| **B** | - | $CC_1$, $CC_2$ | $CC_3$, $CC_4$, $CC_5$ |
| **C** | 0.05 (gr $kg^{-1}$) | 0.1 (gr $kg^{-1}$) | 0.15 (gr $kg^{-1}$) |
| **D** | 10 (gr $kg^{-1}$) | 20 (gr $kg^{-1}$) | 40 (gr $kg^{-1}$) |

**Table 6** Matrix experiments with $L_9$ OA.

| **Experiment** | **Factors and Levels** | | | | **Response of shoots** |
|---|---|---|---|---|---|
| | **A** | **B** | **C** | **D** | **Concentration of Cd ( mg $kg^{-1}$)** |
| 1 | 1 | 1 | 1 | 1 | 197.96 |
| 2 | 1 | 2 | 2 | 2 | 173.50 |
| 3 | 1 | 3 | 3 | 3 | 222.79 |
| 4 | 2 | 1 | 2 | 3 | 183.98 |
| 5 | 2 | 2 | 3 | 1 | 169.28 |
| 6 | 2 | 3 | 1 | 2 | 205.51 |
| 7 | 3 | 1 | 3 | 2 | 130.26 |
| 8 | 3 | 2 | 1 | 3 | 222.34 |
| 9 | 3 | 3 | 2 | 1 | 196.29 |

The data produced is analyzed for identifying optimum parameters. The response of each experiment is shown (Table 7). The average response of each factor is calculated at each level. Response table is used for recording the processed data and presents the calculations of the effects from the orthogonally designed experiments.

**Table 7** Average response of each factor at each level (for Concentration of Cd (mg $kg^{-1}$)).

| Factor | Level 1 | Level 2 | Level 3 |
|---|---|---|---|
| A | 198.08 | 186.26 | 182.96 |
| B | 170.73 | 188.37 | 208.20 |
| C | 208.60 | 184.59 | 174.11 |
| D | 187.84 | 169.75 | 209.70 |

The optimum condition is defined by studying the main effects of each of the factors. The levels of the factors are expected to produce the best results can be predicted. From the response table or the response graph, the optimum level of each factor can be predicted as the level that has the highest value of response. Thus, the optimal configuration for the phytoremediation parameters was identified A (1) B (3) C (1) D (3). The corresponding parameter values are listed in Table 8.

**Table 8** Predicted best strategy parameters.

| Factor (Level) | Value( mg $kg^{-1}$) |
|---|---|
| **A(1)** | 594.25 |
| **B(3)** | 624.59 |
| **C(1)** | 625.81 |
| **D(3)** | 629.11 |

The response graph is the graphical representation for the data presented in the response table to quickly identify the effects of different parameters (Taguchi et al., 2005). The response graphs corresponding to the different factors are represented in Fig 1.

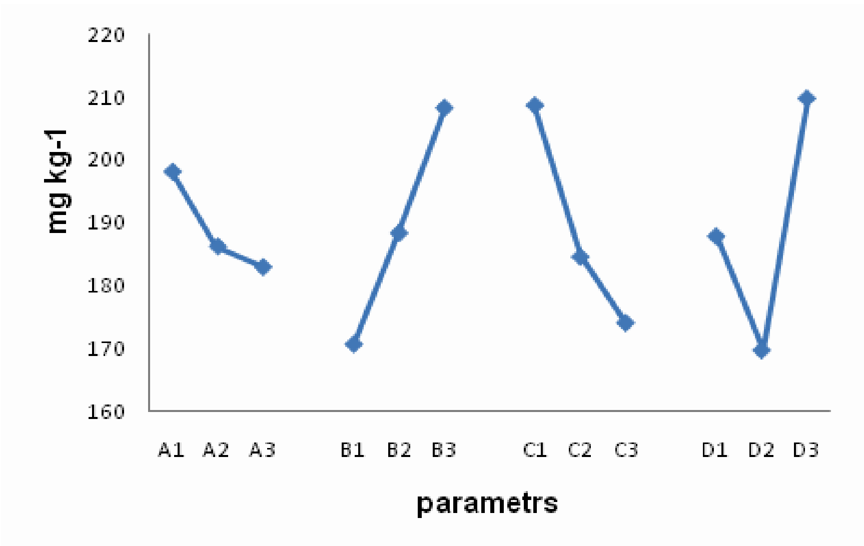


**Fig. 1** Response graphs.

Since the partial experiment is only a sample of the full experiment, the analysis of the partial experiment must include analysis of the confidence that can be placed in the results. A standard technique called Analysis of Variance (ANOVA) is used to provide a measure of confidence. (Christopher and Tpwle, 2001). Table 9 lists the ANOVA for each operating parameter of Optimization of phytoremediation in *Spinacia oleracea* .

**Table 9** ANOVA table.

| Factor | (DOF) | Sum of Squares | Variance | Variance Ratio | Pure sum of Squares | Percent Contribution |
|---|---|---|---|---|---|---|
| | f | S | V | F | S' | P |
| **A** | 2 | 379.33 | 189.66 | 37932 | 379.32 | 5.6 |
| **B** | 2 | 217.63 | 1053.81 | 210762 | 2107.62 | 31.15 |
| **C** | 2 | 1876.25 | 938.12 | 187624 | 1876.24 | 27.75 |
| **D** | 2 | 2400.72 | 1200.36 | 240072 | 2400.71 | 35.5 |
| **e** | 18 | .09 | .005 | 1 | .13 | .001 |
| **T** | 26 | 6764.02 | ….. | …... | ….. | 100 |

DOF: Degree of freedom; e: error; T: Total of all results.

One of the columns of the ANOVA table is percent contribution, which reflects the portion of the total variation observed in an experiment to each significant parameter. It signifies that the parameters with substantial percent contributions are the most important for reducing variation. The contributions of different of phytoremediation in *Spinacia oleracea* parameters are also represented in Fig 2. Cow manure parameter D (3) is the most significant factor and compost the least impact amongst the factors considered for Optimization of phytoremediation in *Spinacia oleracea*. The optimum value obtained was 257.27.

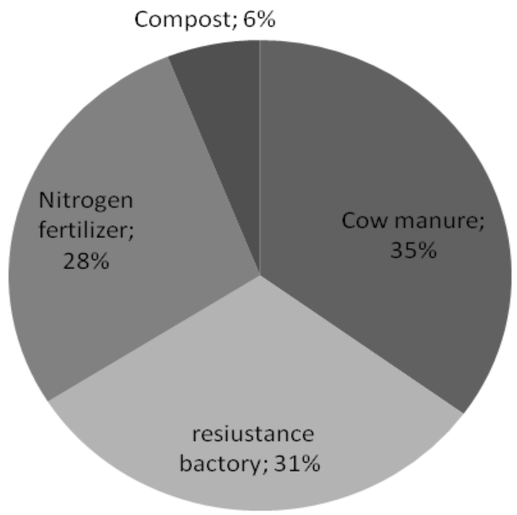


**Fig. 2** Percent contribution of each parameter.

## 4 Discussion

A number of factors affect Cd bioavailability in soil, including soil pH, organic matter presence of other ions, root exudates, types and cultivars of crop plants, and plant age. These factors influence the solubility of Cd compounds and the release of Cd into the soil solution or affect the ability of plants to take up Cd from soil. Some have positive effects while others have negative effects on Cd bioavailability (Sarwar et al., 2010). Results of the analysis showed that the intended soil have suitable physical and chemical properties for greenhouse culture.

The present study found that Application cow manure led to cadmium concentration increase in shoot of *Spinacia oleracea*. There are several possible explanations for this result. Gregson and Alloway (1984) reported, application of cow manure, the soil pH was increased slightly which may have caused the formation of soluble Cd-organic complexes which can increase metal solubility. In 1994, Ramos et al. reported Cd may form weak complexes with OM and can be easily removed. According to the report of Lamas and Singh (2001), the provision of soluble organic compounds complexes causes Cd solubility. High concentrations of dissolved organic compounds increase metal uptake to root surface (McBride, 1995). Finally, the high biomass productivity in the plants grown in cow manure soil possibly resulted the increase of Cd accumulation in shoot and total Cd uptake (Lamas and Singh, 2001). Narwal and Singh showed significant increase in Cd concentration in wheat when pig manure was the OM source.

With regard to results provided in fig2 Application of inoculants ($CC_3$, $CC_4$ and $CC_5$ have the positive effects of absorbing and translocating Cd to the shoots of *Spinacia oleracea*. Thereby application of these inoculants, better results will be achieved in phytoextraction. According to Yan- de et al. (2007), multiple metal resistance (MMR) have more effects in bacteria than the resistance to one metal, thereby it is possible that in treatments with application of these inoculants, better results will be achieved in phytoremedation.

Native and resistant bacteria, by their effective growth promoting abilities, especially providing necessary iron for the plants by production of microbial siderophores, influencing phytopathogens, could bring out the drought and salinity resistance and also producing indole acetic acid (IAA), and stimulating root, caused enhancement in translocation of metal from soil to the plant and as a result increased the efficiency of phytoremediation (Moteshare-zadeh and Savaghebi-Firoozabadi, 2010). Growth of the plant's roots could increase as a result of production of auxin (IAA) by plant growth promoting rhizobacteria. Also, existence of ACC deaminase enzyme with decreasing of stress ethylene stimulates the plant's growth, so assists in less toxicity of heavy metals in plant (Yan-de et al., 2007).

In this research, Application urea fertilizers led to cadmium concentration increase in shoot of *Spinacia olerace*a. Application of some fertilizers, such as $NH4^+$ fertilizers including urea, ammonium sulfate and monoammonium phosphate (MAP), can enhance Cd availability by lowering pH. Rhizosphere acidification occurs as a result of $NH4^+$ nutrition due to the release of protons ($H^+$) by root cells or nitrification of $NH_4^+$, and this induced acidification can promote mobilization of a localized metal in neutral to alkaline soil contaminated with a particular heavy metal like Cd. The type of N fertilizers applied will determine whether there would be a decrease or an increase in Cd uptake with its application. Compared to $NO^{3-}$ fertilizers, $NH_4^+$containing fertilizers could result in enhanced Cd uptake due to decrease in soil pH (Sarwar et al., 2010).
The addition of organic matter to soil, especially in the form of compost, results in increased mineralization of urea and also micronutrients (Dick and McCoy, 1993). The compost treatment causes the development of microbial populations and influence the metal distribution, during organic matter mineralization or the metal solubilization by decreasing of pH, metal adsorption by the microbial biomass\ metal complexion with the freshly formed humic substances (Hsu and Lo, 2001; Zorpas et al., 2003)

Utilization of compost in soil improves physical properties which can helps to phytoremediation (Zheljazkov and Warman, 2004) due to increased biomass by growing plants, suggesting that more metal can be taken up from the contaminated soils (Tang et al., 2003).

## 5 Conclusions

The optimal configuration for the phytoremediation parameters was identified A (1) B (3) C (1) D (3). Cow manure factor is the most significant factor and compost the least impact amongst the factors considered for Optimization of phytoremediation in *Spinacia oleracea.*

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