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Bioaccumulation of cadmium by *Enterobacter* sp. and enhancement of rice seedling growth under cadmium stress

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ABSTRACT

Bacteria-mediated plant growth promotion and bioremediation of heavy metal containing soil is a widely accepted eco-friendly method. The present study is aimed to screen out cadmium resistant bacterial strain from metal contaminated rice rhizosphere and evaluate its effects on the growth of rice seedlings under cadmium stress. Among four different isolates (designated as S1, S2, S3 and S5), the S2 isolate was screened on the basis of different PGP traits and multi heavy metal resistance (minimum inhibitory concentration for cadmium, lead and arsenic were 3500, 2500 and 1050 µg/ml respectively). The selected S2 strain has ability to produce ACC deaminase (236.11 ng α-keto-butyrate/mg protein/h), IAA (726 µg/ml), solubilize phosphate (73.56 ppm) and fix nitrogen (4.4 µg of nitrogen fixed/h/mg protein). The selected strain was identified as *Enterobacter* sp. on the basis of phenotypic characterization, MALDI-TOF MS analysis of ribosomal proteins, FAME analysis and 16 S rDNA sequence homology. The high cadmium removal efficiency (> 95%) of this strain from the growth medium was measured by Atomic Absorption Spectrophotometer and it was due to intracellular cadmium accumulation evidenced by SEM-EDX-TEM-EDX study. SEM analysis also revealed no distortion of surface morphology of this strain even grown in the presence of high cadmium concentration (3000 µg/ml). Inoculation of this strain with rice seedlings significantly enhanced various morphological, biochemical characters of seedling growth compared with un-inoculated seedlings under Cd stress. The strain also exhibited alleviation of cadmium-induced oxidative stress, reduction of stress ethylene and decreased the accumulation of cadmium in seedlings as well that conferred cadmium tolerance to the plant. Thus the S2 strain could be considered as a potent heavy metal resistant PGPR applicable in heavy metal contaminated agricultural soil for bioremediation and plant growth promotion as well.

Main finding: A cadmium resistant plant growth promoting *Enterobacter* sp. was isolated that accumulated cadmium evidenced by SEM-TEM-EDX study. It reduced Cd uptake and enhanced growth in rice seedlings.

1. Introduction

Population explosion and industrialization are responsible for contamination of soil, groundwater, surface water, sediments and air with heavy metals (Nriagu and Pacyna, 1988). Cadmium (Cd) is highly toxic heavy metal in biological systems (Begg et al., 2015). Anthropogenic release of Cd due to industrialization predominantly via non-ferrous ore processing, combustion of fossil fuels and manufacturing of Cd-containing products pollute both terrestrial and aquatic ecosystems (Joseph, 2009; Begg et al., 2015). The world production of Cd has increased alarmingly by > 1000 fold from the beginning of the twentieth century to about 20,000 t per years (Nriagu and Pacyna, 1988). For its non-degradable character, it can easily enter into the food chain (Begg et al., 2015). It is estimated that average human ingests 30 µg Cd per day through their normal foodstuff (Joseph, 2009). Intracellular Cd induces oxidative stress resulting severe damages to several organs such as liver, lungs, kidneys, testes, pancreas, bones and placenta (Cuypers et al., 2010). Moreover, Cd is considered as a potential carcinogen by the International Agency for Research on Cancer (IARC) (Joseph, 2009).

Cd is one of the most toxic pollutants and needs special attention to control it particularly in agricultural fields. The elevated concentration of Cd in the soil affects soil fertility, disordered physiology and metabolism of the plant that showed a limitation in plant growth, symbiosis,

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crop production and yield (Wani et al., 2007). Also, it has deleterious effects on photosynthetic process, mineral elements uptake and transport resulting massive agricultural loss (Tran and Popova, 2013). Thus it is a high time to alleviate Cd as well as other heavy metals' toxicity mainly from agricultural fields. Several heavy metals alleviating practices including phytoremediation through hyper-accumulators have been implemented but the best result can be scored by bio-augmentation assisted phytoremediation (Agnello et al., 2015). Nevertheless, heavy metal resistant plant growth promoting rhizobacteria (PGPR) can be exploited to enhance plant growth promotion as well as bioremediation (Wan et al., 2012). Many heavy metal resistant PGPR can ameliorate plant growth through several PGP traits, viz., facilitating resource acquisition (nitrogen fixation, phosphate solubilization, and iron sequestration), ACC deaminase activity, IAA production, HCN production etc. (Glick, 2012).

Bacterial response to both excess and low concentration of metal ions mainly controlled through metalloregulatory protein which is a metal sensing regulatory transcription factor (Chandrangsu et al., 2017). High Cd^{2+} markedly dysregulates metal homeostasis (both Zn^{2+} and Mn^{2+}) in *Streptococcus pneumoniae* causing severe damage to the bacterial cells (Begg et al., 2015). Again elevated Cd can cease microbial population through inhibiting many essential processes such as cell division, transcription, translation or by their membrane disruption, DNA damage and protein denaturation (Khan et al., 2009). In spite of all these odds, few can perform as contaminant degraders (Dary et al., 2010), contaminant transformers (i.e., the transformation of toxic metals into its less toxic forms) or contaminant accumulators (Chen et al., 2016). Considering their metal alleviating as well as plant growth promoting ability, PGPR are now used globally by many research workers for the betterment of crop productivity to meet the present demand particularly for contaminated agricultural lands near an industrial area. Many investigations regarding Cd-tolerant PGPR have been reported since last decade under the genus *Variovorax, Rhodococcus, Flavobacterium* (Belimov et al., 2005)*, Alcaligenes, Mycobacterium* (Dell'Amico et al., 2008)*, Ochrobactrum* (Pandey et al., 2010)*, Bacillus* (Ahmad et al., 2014, 2016)*, Micrococcus* (Prapagdee et al., 2013)*, Chryseobacterium* (Moreira et al., 2014), *Stenotrophomonas* (Ahmad et al., 2014), *Ralstonia* (Moreira et al., 2014; Prapagdee and Khonsue, 2015), *Serratia* (Wan et al., 2012; Ahmad et al., 2014)*, Pseudomonas* (Dell'Amico et al., 2008; Ganesan, 2008; Chen et al., 2014; Kamran et al., 2015)*, Arthrobacter* (Prapagdee and Khonsue, 2015)*, Leifsonia* (Ahmad et al., 2016; Chen et al., 2016), *Enterobacter* (Chen et al., 2016; Płociniczak et al., 2013; Ahmad et al., 2016), *Klebsiella* (Prapagdee et al., 2013; Ahmad et al., 2014, 2016; Pramanik et al., 2017)*,* etc.

The aim of the present study is to isolate potent Cd resistant plant growth promoting rhizobacterial strain from a heavy metal contaminated rice field and identification of this strain by modern approaches of bacterial taxonomy. The study reveals the removal and bioaccumulation efficiency of Cd by the selected strain for effective bioremediation. Attempts are also made to detect the influence of this strain under Cd stress in greenhouse condition particularly morpho-biochemical changes including reduction of Cd and stress ethylene in rice seedlings.

2. Materials and methods

2.1. Site characterization and soil profile

Rhizospheric soil samples were collected from a rice agricultural field near Steel Plant, Durgapur, West Bengal, India (23°33′N, 87°15′E) on 09.10.2014 for the isolation of high Cd resistant PGPR. Different physicochemical characters of the soil were measured (Hseu et al., 2002) particularly emphasized on their Cd, As and Pb contents by using Atomic Absorption Spectrophotometer (GBC avanta, Australia).

2.2. Isolation of Cd resistant PGPR

After collection of soil sample, it was stored at normal refrigerator as soon as possible, removing stones and any organic matter. The isolation procedure was carried out by serial dilution up to 10^{-6} and 10^{-7} (i.e., one gram of soil sample was dissolved in 10 ml of sterile water and then serially diluted) and plated on Davis Mingioli (DM) medium devoid of glucose as carbon source supplemented with ACC (500 μmol) as sole carbon source to isolate stress alleviating ACC deaminase containing bacterial strains. The medium was also supplemented with 1000 μg/ml Cd and the plates were incubated for 72 h at 30 °C. Colonies of varied morphology were picked up and transferred to DM plates for purification through dilution streak method.

2.3. Minimum inhibitory concentration (MIC) determination

The screened four bacterial isolates were allowed to grow under different heavy metal stress. The MIC of respective heavy metals/metalloid ($CdCl_2$, $Pb(NO_3)_2$ and $NaAsO_2$) was determined by the gradual increase of the concentrations both in DM plates and broth until the isolates failed to grow after 7 days of incubation (Pramanik et al., 2016).

2.4. PGP traits of the selected isolates

Further screening was carried out by qualitative analysis of PGP traits, such as nitrogen fixation (isolates were allowed to grow in a nitrogen-free Ashby's medium), IAA production (Bric et al., 1991), phosphate solubilization (Pikovskaya, 1948) and HCN production (Castric, 1975). Quantitative estimation of four important PGP traits of those primarily selected four isolates was done following respective methods.

The bacterial isolates were grown in DM liquid medium separately supplemented with 5 mM ACC (Sigma-Aldrich Co., USA) at 32 ± 1 °C in a rotary incubator shaker (at 120 rpm). After overnight growth, it was centrifuged at 10,000 rpm for 10 min and the pellet was washed with normal saline. The washed pellet was further processed and the ACC deaminase activity was measured by estimating the amount of α-ketobutyrate liberated from the degradation of ACC (Shrivastava and Kumar, 2013). The protein was estimated as stated in Bradford method (Bradford, 1976). Nitrogenase activity was estimated following Acetylene Reduction Assay using Gas Chromatography (VARIAN CP3800) fitted with flame ionization detector (Kaushal and Kaushal, 2015). It was estimated in terms of μg of N_2 fixed/h/mg protein. Protein was estimated by Bradford method (Bradford, 1976).

Quantitative estimation of IAA produced by selected bacterial isolates was done by following the method of Glickmann and Dessaux (1995). Bacterial cultures were grown separately in DM broth supplemented with 0.5% L-tryptophan at 32 ± 1 °C and agitated at 120 rpm in a rotary incubator shaker. After 36 h, the cultured suspensions were centrifuged at 10000 rpm for 10 min and the supernatant (2 ml) was mixed with 2 ml freshly prepared Salkowski reagent (left for 20 min in dark). The development of pink colour indicated IAA production and it was measured in OD at 530 nm by using Spectrophotometer. The OD values were interpolated with a standard curve to estimate the IAA concentration per ml basis.

To determine the phosphate solubilization ability, the bacterial isolates were grown in Pikovskaya's broth and incubated in a rotary incubator shaker (120 rpm) at 32 ± 1 °C for 6 days. After that, the bacterial cultures were centrifuged at 10,000 rpm for 15 min. The supernatant (500 μl) was mixed with the same volume of 10% w/v trichloroacetic acid, 4 ml reagent (3 M H_2SO_4, 2.5% w/v ammonium molybdate, 10% w/v ascorbic acid and distilled water at a ratio 1:1:1:2) in a test tube and incubated the mixture at 28 °C. The reaction mixture developed blue colour and OD was taken at 820 nm. The amount of phosphate solubilized was determined by interpolating the OD values