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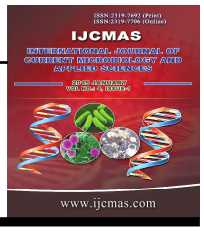

Original Research Article

Estimation of plant growth promoting potential of a nickel accumulating isolate obtained from dhapa industrial wasteland (Kolkata, India) soil on Indian yellow mustard (*Brassica hirta*)

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Keywords

Nickel accumulation; IAA production; Phosphate solubilization; *Brassica hirta* Bioremediation

A B S T R A C T

Plant growth promoting bacteria (PGPB) are known to influence plant growth by various direct or indirect mechanisms. Present study was conducted with an aim to estimate the PGP potential of one nickel tolerant bacterial isolate from Dhapa industrial wasteland, Kolkata, India. Isolate I (Gram negative coccobacilli) was observed to tolerate and accumulate significant amounts of nickel and also have multiple Plant Growth Promoting (PGP) activities like IAA production and phosphate solubilization. Present study also shows that seeds of yellow mustard (*Brassica hirta*) inoculated with the test isolate individually, significantly enhanced root and shoot growth and also protected the plant from the various phytotoxic effects of nickel.

Introduction

Heavy metal pollution of soil is becoming one of the most severe environmental hazards and has received great attention in the last few years because of its negative impact on human health and agriculture. Economically, excessive accumulation of heavy metals is most toxic to crop plants and results in decreased soil microbial activity, soil fertility and yield losses (McGrath *et al.*, 1995). Various lines of data (Guo *et al.;* 1996,Mclaughlin *et al.;* 1999; Sanita *et al.*, 1999; Chen *et al.*, 2003;) indicate that accumulation of heavy metal also threaten human health through the food chain. Although Nickel (Ni^{+2}) in general is known as an essential micronutrient and components of some plant enzymes (Eskew *et al.*,1983; Andreeva *et al.*, 2001) but it is highly phytotoxic when present in excessive concentrations. It has been reported that Ni^{+2} toxicity is correlated with growth inhibition, changes in chloroplast and chlorophyll

concentrations, disturbance of other metabolic and physiological processes especially photosynthesis in plants (Boominathan and Doran, 2002). Industrialization and various anthropogenic activities have been responsible for increased heavy metal release to the environment. Previous studies (Smit *et al.*, 1997) indicated that long term exposure of heavy metals showed decrease in microbial diversity and metabolic processes. Soil bacteria can be found in the rhizosphere and have been considered to promote plant growth directly or indirectly.

Several scientific investigation (Wu *et al.* 2005; Shrivastava and Kumar, 2011; Shrivastava, 2013) reveal that plant growth promoting rhizobacteria (PGPR) actively colonize on and around the plant root surface and increase plant growth and yield. PGPRs promote plant growth by various mechanisms which include: the ability to produce phytohormones (Egamberdiyeva, 2007), asymbiotic N_2 fixation (Mrkovacki and Milic 2001), antagonism towards phytopathogenic microorganisms by production of siderophores (and other chelating agents) that can affect trace metal mobility and availability to the plants (Abou-Shanab *et al.*, 2003; Idris *et al.*,. 2004) the synthesis of antibiotics, enzymes and/or fungicidal compounds (Jeun *et al*,. 2004, Ahmad *et al.*, 2006;) and solubilization of mineral phosphates (and redox changes) and other nutrients (Cattelan *et al.*, 1999). Unfortunately, no such research work has extended on Dhapa industrial wasteland, Kolkata. Therefore we have undertaken this research work on that locality. The present study will help us in obtaining more meaningful and realistic knowledge of isolation and characterization of nickel accumulating and plant growth promoting microbes from industrial wasteland soil and their growth promoting and nickel accumulating effects in yellow mustard (*Brassica hirta*)

Materials and Methods

Sampling

Moist, non clayey soil samples were collected in quasi sterile sampling conditions from Dhapa (N 22° 31' 40.066"/E 88° 24' 42.5628"), West Bengal, India. Samples were collected in sterilized glass sampling bottles and transported to the laboratory (at 25±2°C) within 20 minutes of collection for screening of viable and culturable nickel tolerant bacteria.

Isolation of Nickel tolerant bacteria

The samples were agitated under shaking conditions (110 rpm) for 20 minutes and allowed to settle for the solid and large suspended particles to precipitate. The bacterial isolates were screened by dilution plating of the filtrate (0.2 ml) in Nutrient Agar (NA) media plates (sterile, 90 mm diameter) (gms / L): Peptic digest of animal tissue 10, Meat extract 10, Sodium chloride 5, agar 15; final pH 7.2±0.2 at 25°C) containing varied concentrations of Ni^{+2} (mM : 0.5, 1-10) as analytical grade salts of

$NiCl_2$ from their sterilized stocks (100mM).

Plates were incubated at 35±2°C for 24 hours. Nickel tolerant bacterial colonies that developed at highest concentration of Ni^{+2} supplemented media were selected as Ni tolerant isolates for further experimental study. The isolates were maintained as axenic cultures by several periodic subculturing onto Ni^{+2} supplemented Nutrient Agar slants.

Assay for phosphate solubilization

Phospahte solubilization assay was performed as per Singh et al (1994) with slight modifications. Pikovaskya agar (gms / L: Yeast extract 0.5, Dextrose 10, Calcium phosphate 5, Ammonium sulphate 0.5, Potassium chloride 0.2, Magnesium sulphate 0.1, Manganese sulphate 0.0001, Ferrous sulphate 0.0001, agar 15; pH 7.2±0.2 at 25°C) plates were streak inoculated with a loopful of overnight (18-20 h.) culture (cell density approx. 10^7-10^9 cells/ml assessed turbidometrically at 600 nm) of the bacterial isolates (grown at 35±2°C, 110 rpm in Nutrient broth).

Presence of clear zone around the bacterial colonies after 120 hours at 35±2°C indicated positive response to phosphate solubilization. Phosphate Solubilization Index (PSI) is the ratio of total diameter .i.e. clearance zone including bacterial growth and the colony diameter.

PSI = [Colony diameter + Halozone diameter] / Colony diameter

Assay for detection of Indole acetic acid (IAA) production

IAA quantification was performed following the method of Glickmann et al (1994) with little modifications. Bacterial strains were grown for 72 hours at 35±2°C, 110 rpm on NB with or without sterilized (under 0.21 micron filter) tryptophan (trp) (500 µg /ml) and then the bacterial cells were removed from the culture medium by centrifugation (10,000 rpm, 25°C, and 10 minutes).

One ml of the supernatant was mixed vigorously with 4 ml of Salkowski's reagent (150 ml conc. H_2SO_4, 250 ml H_2O, 7.5 ml 0.5 M $FeCl_3$. $6H_2O$), and the absorbance was measured at 535 nm with UV-Visible double beam spectrophotometer (Jasco V-600). The results were done in triplicate and IAA content were measured from standard curve of IAA absorbance performed elsewhere. This assay along with phosphate solubilization was set to be limiting factor screening protocol for further tests to be done.

Heavy metal tolerance assay

Minimum inhibitory concentration (MIC) for the test metal viz. Ni^{+2} were determined using standard tube dilution techniques using Luria broth (gm/L; Tryptone 10,yeast extract 5, NaCl 10 ; pH 7.2±0.2 at 25°C) supplemented with different concentrations of Ni^{+2} respectively. Overnight (18-20 hours) bacterial inoculations (parameters as stated before) were used as inoculant. Growth was recorded after 24 hours of incubation at 35±2°C (at 110 rpm for broth cultures). The lowest concentration of metal that completely inhibited microbial growth was considered as the MIC. Development of growth indicated by turbidity in the broth medium was observed and accordingly the MIC was determined.

Cell mediated nickel removal assay

0.25 ml of overnight grown (18-20 h) cell suspensions were inoculated to 75 ml of TMMG (Tris Minimal medium supplemented with glucose [TMMG], (gm/L): Tris base: 6.05, glycerol -2-phosphate: 0.67, $(NH_4)_2SO_4$: 0.96, KCl: 0.62, $MgSO_4$: 0.063, $FeSO_4$: 0.0003, glycerol: 0.6; 0.8% glucose; pH was adjusted to 7.00 with 2 M HCl) medium in 250 ml conical flasks having 2 mM of Ni and incubated on a shaking incubator(110 rpm) at 35±2°C.

Cells were harvested at 24, 48, 72 and 96 h of incubation by centrifugation (6000 g, 10 min, and 4 °C). To analyze the metal content, the medium supernatants were acid digested with nitric acid and perchloric acid

(5:3) and the volume was adjusted to a known amount. Nickel content in the supernatants was determined using an atomic absorption spectrophotometer (Perkin Elmer Analyst 400, USA). Sets where cells grew without Ni^{+2} were considered as control. The same protocol was applied to the cell biomass to corroborate the results.

Plant growth promotion and chlorophyll content assay

In situ plant growth promotion studies were done with the isolate I. Mustard seeds were surface sterilized by incubating with 1:1 (v/v) solution of 30% H_20_2 and sterile distilled water for 30 minutes under aseptic conditions followed by repeated wash with sterile distilled water. The seeds were then imbibed either with sterilized water or with bacterial suspension for one hour and then sown in solarized clay pots (16.5 cm x 10.0 cm x 12 cm) containing sterilized soil. Nickel content of the soil used for plant growth promotion studies was measured at SGS enterprise (Kolkata) using standard USEPA 3052 protocol. Suitable control measures were taken.

Seedlings were irrigated every other day either with sterilized water (control) or with 100 ml of 250 µM Ni. The choice of such lowered Ni concentration was chosen to avoid severe effects on test plants. The seedlings were harvested after 10 days and measurement of growth parameters was done. The chlorophyll content was determined from leaf slices weighing one gram by extraction from leaf tissue in 85% acetone following the method of Arnon (1949). Absorbance of the extract was obtained at 663 nm and 645 nm respectively. Total chlorophyll content (mg /g fresh weight) = $[(13\times95 \times A_{665} - 6\times88 \times A_{649}) + (24\times96 \times A_{649} - 7\times32 \times A_{665}) \times V]/ (1000 \times W)$, with V being the volume of the extract and W being the fresh weight (g) of leaf tissue.

Estimation of Nickel in plants

Microorganisms were removed prior to the estimation of Ni^{+2} in the root samples by vigorous washing with 0.01 M EDTA and sterilized water to avoid interference of Ni^{+2} accumulated by rhizoplane bacteria. The washed root samples or shoot samples were then dried at 105°C and were digested in a mixture of conc. HNO_3 and $HClO_4$ (4:1, v/v) (Chen *et al*., 2003). The Ni^{+2} content in the digest were determined by atomic absorption spectrometer (AAS).

Result and Discussion

After screening, the nickel resistant bacterial isolate (Isolate 1, Fig. 1 A & B) (from industrial wasteland, containing 73.56 mg/kg residual nickel concentration), showed nickel resistance up to [8±0.04] mM concentration in LB broth. The result was corroborated statistically by several experiments.

Nickel removal assay (Fig. 4) indicates that the percentage of nickel removed from medium supernatant increased significantly with time. The amount of nickel present in the bacterial pellet, accumulating up to 56.94% respectively after a period of 96 hours, was also in corroboration with depletion from supernatant. It indicated that there was no nickel quenching by medium particles. Therefore Isolate 1 was established to be a nickel bioaccumulator.

Assays for phosphate solubilization (Fig.2 A, B & C) revealed that that the isolate has the property of producing phosphatase exoenzyme [PSI = 2.23±0.89].Studies also indicate that the isolate (Fig. 3 A & B) also secrete IAA (of up to 21.43±0.67 µg ml^{-1}at

96 hrs. under tryptophan fed medium) which enhance plant growth.

The comparative root and shoot length of pot experiments for the selected isolate without and with nickel stress respectively in sterilized soil containing residual nickel load of 15.12 mg/kg. Study indicates (in Table 1 and Fig.5) a significant difference between the heights of yellow mustard plants (*Brassica hirta*) between the inoculated and negative control pots. Seedlings from inoculated seeds have significantly greater root increase [77.3%] and shoot increase [27.7%] compared to control, in absence of nickel stress. In presence of nickel stress shoot length of isolate I inoculated plant were increased by 23.2% and for the same root length was also found to increase by 40.7% . Heights of control plants and the plants from nickel containing pots showed a nickel caused decrease of root and shoot by 30.3% and 5.55% respectively. Under Nickel stress, the phytotoxic effects like spots in leaves, yellowing, curling and blackening of leaves were less prominent in inoculated plants than uninoculated ones. The data in table 1 reveal that nickel containing negative control pots show significant chlorophyll loss in plants. On the contrary, inoculated seed containing pots show less chlorophyll loss

The present study establishes that the isolate I has the potential for heavy metal accumulation and plant growth promotion. Present investigations lead us to think on molecular mechanisms for using this isolate as biofertilizer for promoting plant growth (especially crop plants) as well as a bioremediator in nickel affected areas.

Table.1 Comparative of plant growth parameters in the pot experiments (± 1 S.E.) (Data for each treatment regime are the mean of 10 observations with SE in parenthesis; CD (*P <0.05) extracted from ANOVA

Parameters	Pot experiment with isolate-I as inoculum					
	Uninoculated Nickel blank	**Inoculated Nickel blank**	**Uninoculated Nickel stressed**	**Inoculated Nickel stressed**	**MS**	**CD (at 5%)**
Shoot length (cm)	33.218 (±0.169)	48.527 (±0.419)	23.130 (±0.598)	37.110 (±0.745)	6.527	2.205
Root length (cm)	17.560 (±0.227)	22.718 (±0.664)	6.990 (±0.152)	15.470 (±0.290)	2.380	1.380
Wet weight (gm)	2.160 (±0.063)	2.740 (±0.092)	1.360 (±0.017)	2.108 (±0.059)	0.056	0.211
Dry weight (gm)	0.460 (±0.018)	0.510 (±0.013)	0.260 (±0.019)	0.390 (±0.012)	0.004	0.042
Chlorophyll content (mg gm^{-1})	2.280 (±0.089)	2.770 (±0.027)	1.480 (±0.036)	2.220 (± 0.033)	0.027	0.147

Fig.1 A: Nickel tolerance profile of Isolate1 in LB in ascending order from right to left; **B:** Comparative of Nickel tolerance in Isolate 1 with other isolates found (±1 S.E.)

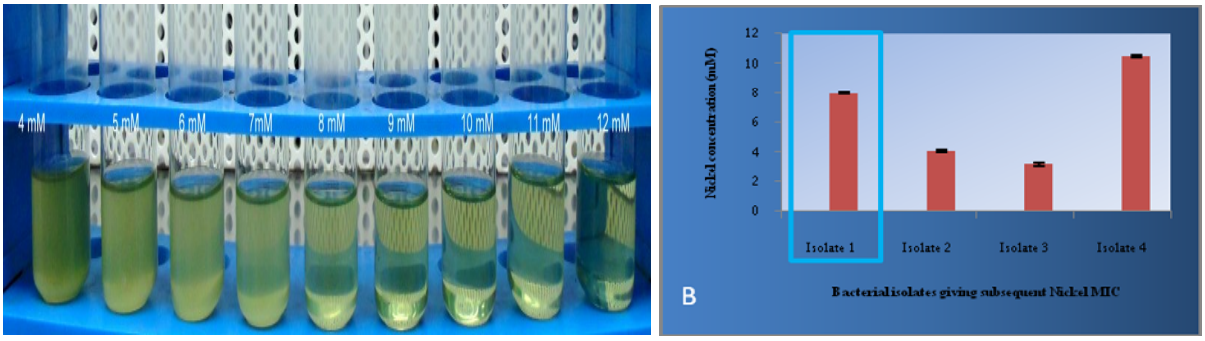


Fig.2 Initial screening profile of the isolate in Pikovskaya agar, **A:** isolate I, **B:** uninoculated control, **C:** comparative PSI values of all the isolates giving positive result for Phosphate solubilizing assay (±1 S.E.)

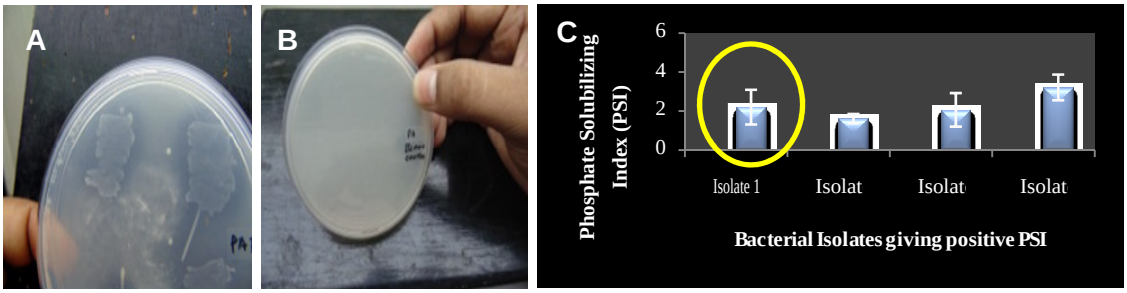


Fig 3 A: IAA production profile of isolate I after 24 hrs; **B:** Comparative IAA production during time lapse assay. Red bar denotes trp supplemented IAA production whereas blue bar denotes trp non supplemented IAA production (± 1 S.E.)

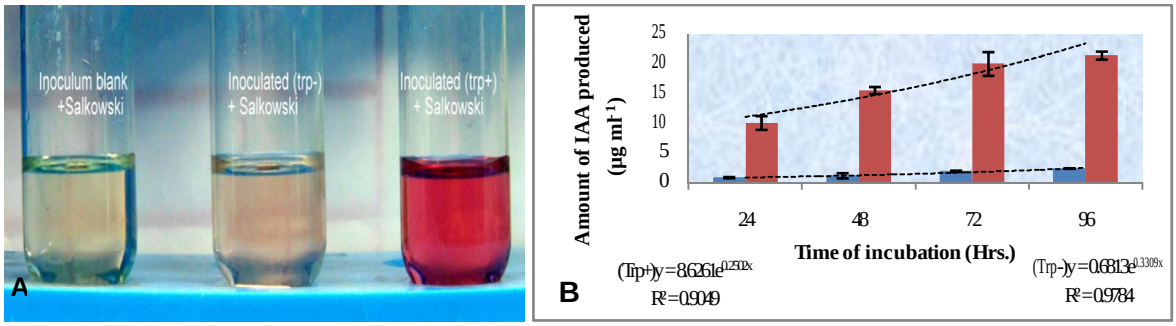


Fig.4 Nickel removal time lapse assay of isolate-I. Blue line indicates nickel content in supernatant while red line indicates nickel content in pellet.

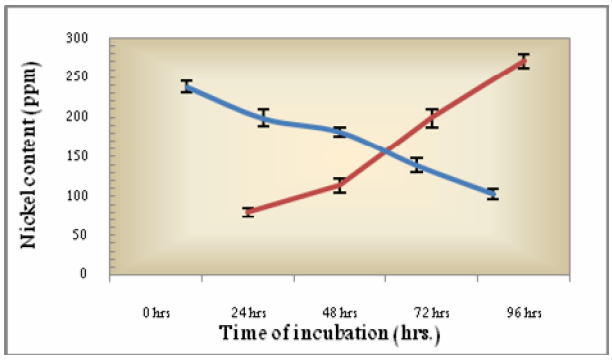
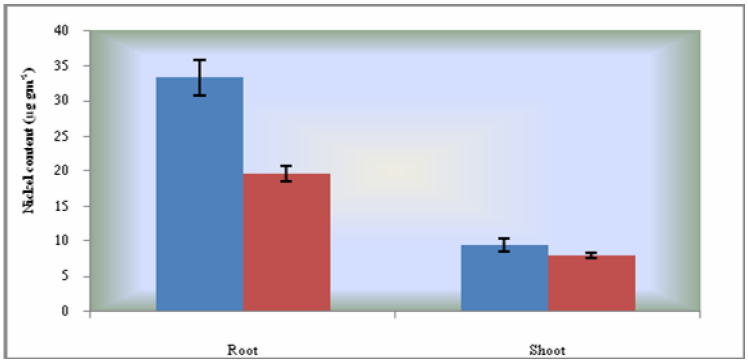


Fig 5 Comparative of plant nickel content in the pot experiments. Red bar indicates nickel content in root and shoot of pot experiments with inocula while blue bar indicates nickel content in root and shoots of uninoculated control



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