Characterization of Plant Growth Promoting Rhizobacteria Associated with Soybean (Glycin Max L.) Cultivated in Effluent Contaminated Soil.

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ABSTRACT:

Soybean is cultivated widely for the rich source of seed oil and proteins. It is common practice of using such water for irrigation of agricultural lands near the urban area .However, Industrial and Municipal effluents contain the large share of fresh water which is discharged into the rivers and other water bodies. Soil-dwelling microbes play the key role without any side effects on bioremediation of soil quality. Plant Growth Promoting Rhizobacteria (PGPR) were isolated from such soils of soybean rhizosphere. The two isolates have shown good percentage of tolerance to heavy metals and salt. These isolates have also shown plant growth promoting traits like phosphate solubilisation, nitrogen fixation, IAA, siderophore, HCN, and ammonia production. These isolates can be used in the form of consortium for a bioinoculant formulation to augment yield of soybean and to mitigate the adverse impact caused by pollutants.

Keywords: PGPR, effluents, heavy metals, rhizosphere, siderophore assay.

Introduction :

Environmental pollution is an extremely important issue today, affecting every biotic community in multiple ways. In developing as well as underdeveloped countries, industrial effluents are released directly or indirectly into natural water resources, mostly without proper treatment, thus creating a serious threat to the environment. This wastewater composed mainly of phosphorous, nitrogen, organic matter, heavy metals and inorganic salts in addition to suspended and/or dissolved solids and microscopic organisms. The untreated effluent is discharged into stream and water bodies. This damages the normal aquatic life and harmfully affects the quality of ground water table of the locality if allowed to settle into the ground for a prolonged period [14, 18]. These pollutants interfere with physiological activities of plants such as photosynthesis, gaseous exchange, and nutrient absorption and cause overall reduction in plant growth and yield. The lack of enough awareness, economic constraints and poor implementation of laws are the main reasons for the insufficient progress in this issue. It is now realized that there is need to conserve the environment by preventing any further habitat destruction, species extinction and also to restore an undisturbed environment. Due to their toxic effects on plants, animals and human beings, heavy metals and inorganic salts released from different industries are kept under one of the major environmental pollutant category [8].

Bioremediation is one of the well known and effective alternatives for reduction of the toxicity caused by different pollutants like heavy metals, inorganic salts etc. There are the variety of soil dwelling microbes now known to the human being who exhibit this potential to tolerate high concentrations of heavy metals and other pollutants that would normally cause severe toxicity symptoms in higher plants. Isolation and use of microorganisms from such contaminated soils and their use for ameliorating the natural quality of soil from the contaminated site is gaining wide importance today. These bacteria colonize in and around roots of plants are called as rhizobacteria. These are also referred as plant growth promoting rhizobacteria (PGPR or PGPB) [2]. PGPR provide benefits to plant by the variety of direct and indirect mechanisms [20]. They directly

promote plants growth by supplying nutrients (e.g. via the fixation of atmospheric nitrogen (N2), phosphorous (P) solubilisation, segregation of iron (Fe) by siderophores, phytohormone synthesis (e.g. indole-3-acetic acid,),suppression of plant pathogens [3], and by lowering the host's ethylene level due to ACC deaminase activity [11, 16]. Apart from these activites, there are various free-living rhizospheric bacteria that can be applied to heavy metal and salt polluted soils to mitigate lethal effects of heavy metals on the plants. Various strains of *Pseudomonas fluorescence* play a key role in the bioremediation of heavy metals [7, 22]. The *Pseudomonas sp*. also plays the vital role in the utilization of heavy metals. The several mechanisms have been developed by growth promoting rhizobacteriafor their survival under metal stressed environment. These include mobilizing or transforming metals into inactive form to allow the uptake of heavy metal ions [15]. Soybean (*Glycine max* (L.) Merril) is a leguminous plant, occupying large acreages of land worldwide for its rich content of edible oil and proteins. Drastic climatic changes brought irregularities in the monsoon in India. Due to this availability of fresh water is declining in the years for irrigation. Farmers mainly from the urban area are using municipal and industrial waste water for irrigation of soybean fields. This research work was focused on the isolation of PGPR from soybean fields irrigated with such water and evaluation of their heavy metal and salt tolerance and plant growth promoting attributes for boosting the yield of soybean.

2. Materials and Methods

2.1 Collection of Soil Samples

The soil samples from the fields irrigated with effluent was collected in sterilized zip pouches from the nearby industrial area of Nasik MIDC and Municipal Corporation, Maharashtra, India. These samples were brought to the laboratory for further analysis.

2.2 Isolation of PGPR from Soil

Serial dilution and plating techniques as described by Parkinson *et al* (1971) and SubbaRao (2007) was adopted for isolation and enumerating the population of bacteria. For this, approximately one gram of soil sample is mixed in 100 ml sterile saline containing Erlenmeyer's flask. Content is mixed well for 10 minutes on the vortex mixer. After mixing it is allowed to settle, this became 10-2 dilution. One ml of supernatant is pipetted out aseptically from this into 9 ml sterile saline containing test tube and mixed well to get 10-3 dilution. Similar steps are repeated serially up to 10-7 dilution. 0.1 ml suspension from the last dilution is plated on sterile Ashby's Nitrogen free mannito lagar, Pikovskay's agar and Soil extract agar plates respectively. Control was set by using soils irrigated with well water from each region. Plates were incubated at room temperature up to five days.TVC and Colony characters of well-isolated colonies were recorded.

2.4 Determination of Heavy Metal Tolerance of Isolates

The method described by Upadhyay *et al* (2009) was adopted for determination of heavy metal tolerance by isolates. Sterile minimal broth tubes supplemented with various concentrations (0.1mM, 0.5 mM, 1.0 mM, 2.0mM) of Copper (Cu), Nickel (Ni), Mercury (Hg), and Chromium (Cr) were prepared and loop full of culture was inoculated and tubes were incubated at 28±2ºC for 3 days along with tube of nutrient broth without heavy metal as a control. Tolerance is measured by recording turbidity at 600 nm.

2.5 Determination of Salt Tolerance

Sterile nutrient broth tubes containing 0.5, 5, 7.5 and 10% concentration of NaCl, were inoculated separately with 24 hours old loop full culture of each isolate. The negative control is kept without inoculation. Tubes were incubated at room temperature for 3 days. The cell growth was measured at 600 nm.

Evaluation of Plant Growth Promoting Attributes of Heavy metals and Salt tolerant isolates

Isolates that showed maximum tolerance to the higher concentration of heavy metals and NaCl were selected for further analysis of Plant growth promoting abilities.

3.1 Nitrogen Fixation

Freshly grown cultures were inoculated into sterile nitrogen free mineral agar medium**,** 2% Glucose and bromothymol blue indicator. The visual detection of nitrogen fixing activity was observed by the change in the color of the medium after 3 to 7 days of incubation at 300C [21].

3.2 Phosphate Solubilisation

Heavy metals and salt tolerant isolates obtained were tested for phosphate solubilization ability in Pikovskaya‘s agar medium. Freshly prepared suspensions were stab inoculated with the help of sterile toothpicks into sterile Pikovskaya‘s agar plates in triplicates. Plates were incubated at 300C for 6 days. Development of halo zone around the colony was considered as presumptive confirmation of phosphate solubilisation. Halo size was calculated by subtracting colony diameter from the halo zone. Solubilization efficiency (SE) was calculated by the formula as given below: [18].

$$SE = \frac{\text{Diameter of halo zone (solubilisation diameter)}}{\text{Colony diameter (growth diameter)}} \text{ X } 100$$

3.3 Indole Acetic Acid (IAA) Production

The method described by Bent *et al* [4] was adopted to conduct an experiment to determine plant growth hormone production by different PGPR isolates. Sterile tubes containing nutrient broth with 2 mg/ml tryptophan were inoculated with 0.1 ml 24-hour old culture of PGPR isolates. These tubes were incubated for 4 days at room temperature. Incubated culture broth is centrifuged at 10,000 rpm for 30 minutes and the pellet was discarded. In a clean test tube 1ml of the supernatant was taken and to this 2 ml of freshly prepared Salkowski s reagent (50ml 35% HClO4 + 1ml 0.5M FeCl3) was added. Tubes were observed for development of Pink color after incubation for 30 minutes in dark. The absorbance was measured at 530 nm.

3.4 Siderophore Production

Method, as described by Clark & Bavoil(9), was used for the assay for siderophore production by PGPR. Chromeazurole S agar plates were prepared and spot inoculated with test organism and incubated at 30oC for 5 days. Development of yellow-orange halo around the colony was considered as positive for siderophore production.

3.5 Ammonia Production

The ammonia production was detected by Cappuccino and Sherman (1992) method using Nessler’s reagent. Freshly grown cultures were inoculated into 10 ml peptone water in each tube and incubated for 48 h at 30◦C. Isolates were tested for the production of ammonia by adding Nessler’ s reagent (0.5 ml) to each tube. Development of brown to yellow color was a positive test for ammonia production.

Hydrogen Cyanide Production

Sterile nutrient agar medium containing 4.4 g per liter of glycine is streaked with freshly grown cultures of isolate. A Whatman filter paper No.1 soaked in 0.5% picric acid solution (in 2% sodium carbonate) was placed inside the lid of a plate. Plates were sealed with para film and incubated at 30°C for 72hrs. Development of light brown to dark brown color was considered positive for HCN production [6].

4. Results and Discussion

4.1 Physicochemical Analysis of waste water

In Table.1 physico-chemical parameters of wastewater are presented. The pH was recorded as alkaline [8, 7] and electrical conductivity value as 9.8 dSm-1. Higher electrical conductivity value is an indication of heavy discharge of chemicals in the form of cations and anions in the effluents. This alters chelating properties of water bodies. Due to this, there is an imbalance of free metal availability for flora and fauna. Heavy metals were present from 0.25 to 7 ppm amount. Similar values were obtained in the study conducted by Faryl *et al*. (2007). The sludge that contained high amounts of trace elements especially arsenic, chromium, cadmium, lead, mercury and zinc which all have a negative impact on plant growth [12]. The amount of organic carbon was 2.10% which is higher than prescribed limit. The concentration of other elements was i) available nitrogen 171.7 kg/ha, ii) phosphorus 24.26 kg/ha, and iii) available potassium was (445.8 ppm).

Table 1: Physicochemical analysis of waste water collected from Nasik Municipal corporation's effluent treatment plant.Sr no.	**Parameter**	**Values**	**Maximum permissible limit ***
1	pH	8.7	**6-9**
2	Electrical Conductivity (dSm^{-1})**	9.8	**1000**
3	Biological Oxygen Demand$(BOD)_3$,27^0C	355	**100**
4	Chemical Oxygen Demand (COD)	300	**270**
5	Total Suspended Solids (TSS)	150	**100**
	Available Nitrogen (kg/ha)	171.7	**NS***
6	Available Phosphorus(kg ha^{-1})	24.26	**NS***
7	Available K (ppm)	445.8	**200**
7	Chlorides (ppm)	1400	**900**
8	Zinc (ppm)	5	**1**
9	Iron (ppm)	7	**10**
10	Copper (ppm)	6	**5**
11	Trivalent Chromium (ppm)	9	**4**
12	Nickel (ppm)	2	**NS***
13	Arsenic (ppm)	1	**NS***
14	Cyanide (ppm)	0.5	**NS***
15	Lead (ppm)	3	**NS***
16	Mercury (ppm)	3	**NS***

*****Source: Ministry of Environment, Forest and Climate Change, New Delhi, Official Gazette of India. Notification 1st January 2016,

* **NS** = Not specified

**** dS/m** = deciSiemen/metre in SI Units (equivalent to 1 mmho/cm)

4.2 Population Density in soil irrigated with effluent water

After analysis of population density of PGPRs isolated from the soil irrigated with water of effluent treatment plants from different locations of Nasik Municipal Corporation area which is shown in the Table2. It is observed that number of beneficial microflora was drastically decreased from 35 to 15 x 106 CFU/ gm of soil

sample as compared with control. This study corresponds with the observations made by Revathi *et al* (2011). They reported that the heavy metal contaminants like Hg, Pb, Zn, As, Cd, Cr, Na, K, Cu, etc. destroy bacteria and other beneficial microorganisms in the polluted soil. This may be the reason for diminishing the TVC of beneficial microflora examined in effluent water samples.

Table2: Population Density of soils obtained from four different area

Sr no	Medium	TVC /0.1ml *
1	Pikovskay's	$15X10^{6}$
2	Ashby's Nitrogen free Mannitol Agar	$17X\ 10^{6}$
3	Soil Extract Agar	$35\ X10^{6}$
4	Control	$105X10^{6}$

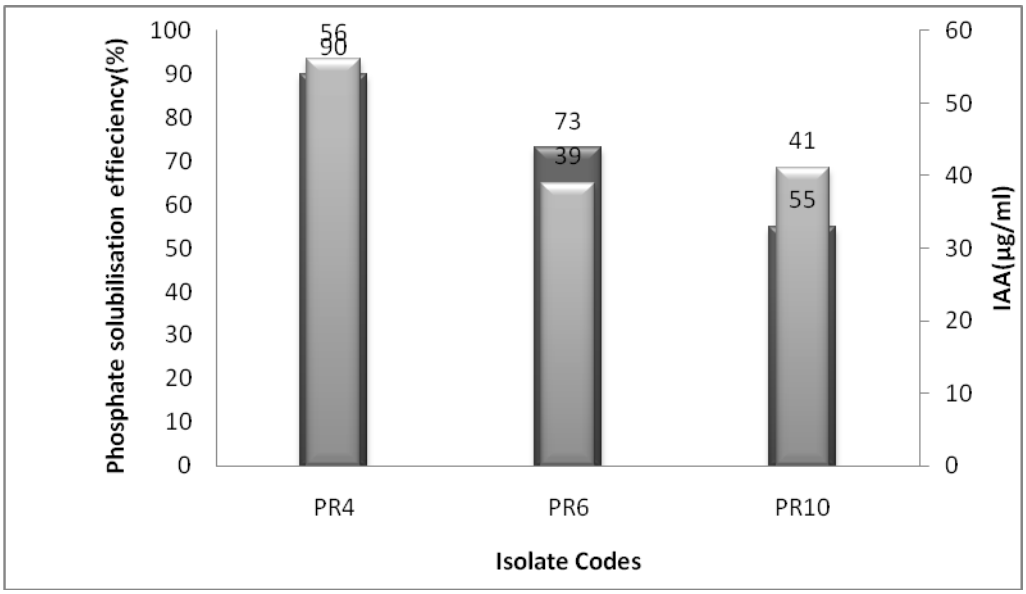
Table 3: Multiple PGPR traits of heavy metal and salt tolerant strains

Isolate Code	Nitrogen fixation	Phosphate Solubilization	IAA production	Siderophore production	Ammonia Production	HCN production
PR4	+	+	+	+	+	+
PR10	+	+	+	+	+	+

Table 4: Characterization of selected strains

Tests / Isolate codes	Gram character	Shape	Motility	Catalase	Oxidase	Amylase	Gelatinase
PR4	Positive	Rods	-	-	-	+	-
PR10	Positive	Rods	-	+	-	-	+

Fig 3: Graph of phosphate solubilization efficiency and Indole Acetic acid production by the heavy metal and salt tolerant isolates



4.3 Heavy Metal Tolerance

Growth response of isolates in different concentrations of Cu, Ni, Hg, and Cr is recorded in figure 1. Two isolates (PR4, and PR10) out of ten have shown maximum growth in the form of turbidity in the tube containing the heavy metal concentration of 2 mM. Less growth was observed for other isolates in heavy metal concentrations. To overcome metal stressed conditions resistant microbes have developed several mechanisms such as active transport of metals, exclusion of metals by permeability barrier etc. [15]. Soil Pollution with heavy metals could lead to the appearance of heavy-metal resistant PGPR in the soil of industrial regions [1].

4.3 Salt Tolerance

After the screening of all bacterial isolates for growth in different salt concentrations; three isolates (PR4, PR6, and PR10) grown luxuriantly in 7.5% NaCl concentration. These were selected for further evaluations. It has been reported earlier that bacteria isolated from saline soil are more likely to withstand high saline conditions in drought affected area [24, 25, 13] and if these bacteria also possess plant growth promoting traits, they would be ideal for use in sustainable agriculture [10].

5. Conclusion

The present study was aimed to isolate PGPR from the rhizosphere of the soybean field which is irrigated with industrial and municipal waste water and evaluate their plant growth promotion activities. The PGPR isolates have exhibited concurrent tolerance to heavy metals and salts in addition to good production of IAA, siderophore, HCN, ammonia, nitrogen fixation and phosphate solubilization. Hence these attributes of PGPR can be used to improve the yield of the soybean crop taken in such soils.

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