Original Research Article

In-vitro Evaluation of Chromium Tolerant Plant Growth Promoting Bacteria from Tannery Sludge Sample, Dindugal, Tamil Nadu, India

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A B S T R A C T

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Industrial waste is one of the most essential sources of contamination in the environment. Chromium (Cr) is a toxic heavy metal, a major contaminant in tannery wastes and its accumulation in soil and water is a major environmental concern today. In the present study, an attempt was made and investigated the status of different beneficial microbes particularly plant growth promoting rhizobacteria (PGPR) from tannery sludge samples collected from tannery effluent treatment plant at Dindugal, Tamil Nadu, India. Experiments were conducted and evaluated their chromium heavy metal tolerance abilities and plant growth promoting activities under *in-vitro*. Based on molecular analysis, the PGPRs were identified as *Achromobacter xylosoxidans* (LK391696), *Azotobacter vinelandii* (LK391702) and. The production of IAA was found to be high by *Achromobacter xylosoxidans* (46µg/ml) followed by *Azospirillum lipoferum* (30µg/ml). Phosphate solublization activity was also found to be positive in all these PGPR isolates. Significance of these results revealed that there is a possibility of using these potential PGPRs for bioremediation of chromium contaminated sites and also as good plant growth promoter.

Introduction

Environmental pollution is an extremely important issue today, affecting all of us in one way or the other. In developing as well as underdeveloped countries, the industrial effluents are released directly or indirectly into natural water resources, mostly without proper treatment, thus posing a serious threat to the environment. Heavy metals released from different industries are kept under environment pollutant category due to their toxic effects on plants, animals and human beings (Chidambaram *et al.,* 2009).

The untreated tannery effluent discharged into stream and water reservoirs damages the normal life and if allowed to settle into ground for a prolonged period, harmfully affects the ground water table of the locality (Mondel *et al.,* 2003; Prasad, 2007). They interfere with physiological activities of plants such as photosynthesis, gaseous exchange and nutrient absorption and cause reduction in plant growth, dry matter accumulation and yield.

In Tamil Nadu there are many industries like textile, dyeing, tannery, foundry and metal casting located in various districts and all these industries discharge effluents which cause pollution. Rehabilitation of heavy metal contaminated soils has of late, gained importance to restore the vegetative cover and the industries are giving serious thought to the problem. Economic constraints and lack of sufficient awareness of the importance of the problem are the main reasons for the insufficient progress in this aspect.

There is now a realization of the need for conservation of the environment to prevent any further habitat destruction and species extinction and also to rebuild an undisturbed environment. Bioremediation is well known to be effective in eliminating the toxicity caused by different heavy metals. Many microbes and macro fungi exhibit tolerance to high concentration of heavy metals that would normally cause severe toxicity symptoms in higher plants. The symbiotic association of plant roots with vesicular arbuscular (VA) mycorrhizal and ectomycorrhizal (ECM) fungi (Colpaert and Van Assche, 1992) in metal contaminated soils gives a new dimension to biologically changed bioavailability by exploiting an essentially greater soil volume than roots can do and by volatilization of heavy metals.

Using microorganisms for decontamination of different contaminated sites have received greater interest. Hence, it is essential to isolate and identify heavy metal tolerant potential beneficial microbes from the polluted samples for bioremediation of heavy metal contaminated sites. In the present study, an attempt was made to investigate the status of beneficial bacteria from tannery sludge waste samples collected from tannery effluent treatment plant at Dindugal, Tamil Nadu, India and determine their heavy metal tolerance and plant growth promoting abilities under *in vitro* conditions.

Materials and Methods

Sample collection

The tannery sludge samples were collected from tannery effluent treatment plant located at Dindugal, Tamil Nadu, India, in a sterile polythene bags and brought to the laboratory for further analysis.

Physico-chemical parameters of tannery sludge samples

The tannery sludge samples collected from the study location were analysed for their physico-chemical parameters such as pH, Electrical Conductivity (EC), Bulk density, Organic carbon, available Nitrogen (N) , Phosphorus (P), Pottassium (K) and Calcium, Magnesium, Copper, Zinc, Manganese and heavy metals, were analysed in Soil and Water Testing Lab of IFGTB, Coimbatore, by using standard procedures.

Isolation of PGPRs from sludge sample

Serial dilution and plating techniques as described by Parkinson *et al* (1971) and Subba Rao (2007) was adopted for enumerating the population of bacteria. One gram of soil sample was weighed and suspended in 100 ml sterile water blank aseptically. The flask was vortexed for 1 minute. This constitutes 10^{-2} dilution. 0.1ml from this dilution was aseptically transferred to another water blank with 9 ml of sterile water using sterile pipettes. This is 10^{-3} dilution. Similarly, successive dilutions of the samples till 10^{-6} were prepared by serial dilution method. 1ml of aliquots of 10^{-5} and 10^{-6} was serially transferred to sterile labeled petri dishes using sterile pipettes. 20ml of the respective sterile media were poured

onto the plates. The plates were gently rocked manually for uniform distribution of the diluted sample in the medium. The plates were allowed to solidify and then incubated at 28 ± 2ºC for 3-5 days. After incubation, selective colonies were picked up and transferred to fresh medium plates. For *Azotobacter* sp., Jensen's agar medium was used. Rojo-Congo (RC) agar medium was used for *Azospirillum* sp. isolation. Phosphate Solublising Bacteria (PSB) was isolated in Pikovskaya's agar medium.

*Azotobacte*r colonies were selected based on the appearance of mucoid, transparent, gummy colonies. *Azospirillum* colonies appeared as scarlet pink, round colonies. PSB were identified based on the halo zone formed around the colonies. Population density of PGPR was determined by counting Colony forming units/gm of soil (Subba Rao, 2007 and Rodriguez, 1982). The population density of PGPR organisms was recorded.

Isolation and identification of Cr tolerant isolates

The method described by Upadhyay *et al* (2009) was adopted for identification of chromium (Cr) tolerant isolates. The nutrient agar plates supplemented with various concentration (0mM, 0.5mM, 1.0 mM, 2.0 mM) of Cr were prepared and loop full of culture was streaked and the plates were incubated at 28±2ºC for 5-7 days and the culture inoculated on nutrient agar plate without heavy metal act as control.

The heavy metal tolerance was recorded based upon growth of the isolate and the best isolates were selected and subjected to minimum inhibitory concentration (MIC) assay.

Determination of Minimum Inhibitory Concentration (MIC)

Minimum Inhibitory Concentration (MIC) of different PGPR isolates towards different concentrations of chromium heavy metal was determined by broth dilution method as described by Nieto *et al.* (1989). Chromium (Cr) was added separately to nutrient broth at various concentrations ranging from 0.5mM – 2mM and loop full of bacterial isolates were inoculated and incubated at 30°C for 4 days. Nutrient broth without chromium metal ion was acts as control. The minimum inhibitory concentration (MIC) was determined as the lowest concentration of heavy metal that completely inhibited growth of the bacteria and cell growth was measured using spectrophotometric method at 600nm.

Analysis of plant growth promoting characteristics of Cr tolerant isolates
Determination of Indole Acetic Acid (IAA) production

An experiment was conducted to determine plant growth hormone production by different PGPR isolates by adopting the method described by Bent *et al* (2001). The tubes containing nutrient broth with tryptophan (2mg/ml) was sterilized and inoculated with 1ml of PGPR isolates. Then the tubes were incubated for 7-8 days. After incubation, the culture broth was centrifuged at 10,000 rpm for 30 minutes and the pellet was discarded. 1ml of the supernatant was taken in a clean test tube. To this 2ml of freshly prepared Salkowski's reagent (50ml 35% $HClO_4$ + 1ml 0.5M $FeCl_3$) was added. The tubes were incubated in dark for 30 minutes for the development of pink colored complex. After 30 minutes, the absorbance was measured at 530 nm.

Study on Phosphate solubilization efficacy

Chromium (Cr) tolerant PGPR isolates obtained were re-tested by plate assay for phosphate solubilization in Pikovskaya's agar medium. These bacteria were stabbed in triplicate using sterile toothpicks. The halo zone around the colony was presumptive confirmation of phosphate solubilization and was measured after 7 days of incubation at 30ºC. Halo size was calculated by subtracting colony diameter from the total zone of colony and halo zone. Solubilization efficiency (SE) was calculated by the formula as given below:

Solubilization diameter

$$SE = \frac{\text{Solubilization diameter}}{\text{Growth diameter}} \times 100$$

Molecular characterization of PGPR isolates

Chromium tolerant isolates producing highest IAA and showing maximum phosphate solubilization were selected for molecular characterization and identified up to species level.

> * 16S rDNA sequence of the selected PGPR isolates was obtained from SciGenom, Cochin, Kerala, India. This sequence was used to carry out BLAST with the nr database of NCBI gene bank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 4.
> * The sequences of all these isolates with all the required information was submitted to European Molecular Biology Laboratory (EMBL) and accession numbers obtained.

Results and Discussion

Physico-chemical parameters of tannery sludge samples collected from tannery effluent treatment plant.

The results of the physico-chemical parameters are presented in Table 1. The pH was recorded as 7.7 and EC was (9.8 dSm^{-1}). The higher electrical conductivity value of the sample indicates that the discharge of chemicals as cations and anions were higher in the waste effluent water. The higher conductivity alters the chelating properties of water bodies and creates an imbalance of free metal availability for flora and fauna. The presence of organic carbon was higher (2.10%). The available nitrogen was (171.7 kg/ha), phosphorus was (24.26 kg/ha) and available potassium was (475.8 ppm). The presence of heavy metals was ranging from 0.02 to 0.2 ppm. These results were in accordance with the values obtained in a similar study by Faryal *et al* (2007). Logan *et al* (1997) reported that, the analysis of the sludge showed that it contained high amounts of trace elements especially arsenic, chromium, cadmium, lead, mercury and zinc which all have a negative impact on plant growth.

Population density of PGPR in tannery sludge samples

Survey undertaken and collected tannery sludge samples in tannery effluent treatment plant at Dindugal, Tami Nadu, India. Samples were analyzed and data on population density of PGPR (Phosphate Solubilizing Bacteria, *Azotobacter* and *Azospirillum*) was recorded (Table 2). The population density of beneficial micro flora was found very low in tannery sludge waste

samples ranging from 1.0 - 2.5 x 10^6 CFU/g of soil. The findings of the study are in accordance 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 the less population density of beneficial micro flora examined in tannery sludge samples in the present study.

Determination of chromium heavy metal tolerant

Based on colony morphology, six different colonies were selected and isolated pure cultures for screening of chromium tolerance under *in vitro*. An experiment was conducted for determining heavy metal tolerant property of three phosphate solubilizing bacteria (PSB), two *Azotobacter* spp. and one *Azospirillim* spp. isolates by growing them in agar plates with different concentrations of chromium and the data is presented in Table 3. It was observed that the PSB-1 isolate had maximum growth up to 2mM concentration of chromium. The isolate Azoto-1 and Azosp-1 had maximum growth up to 0.5mM concentration. The growth rate of other isolates was less in heavy metal concentrations over control. The findings of the study are in accordance with the observations made by other researchers earlier (Pal *et al*., 2004; Edward Raja *et al*., 2006). The microbial cells in order to combat the metal stressed conditions have developed several resistance mechanisms such as exclusion of metals by permeability barrier, active transport of metal etc. (Nies, 1999).

Based on above results, all these isolates were screened further to confirm their chromium heavy metal tolerance by minimum inhibitory concentration (MIC)

assay and the results are given in Fig. 1. It was recorded that the MIC of maximum tolerance was noticed in PSB-1 isolate (1.245 OD_{600}) followed by Azoto-1 isolate (1.208 OD_{600}) and Azosp-1 isolate (0.168 OD_{600}). The high tolerance to chromium heavy metal may be due to the presence of high contamination of chromium in sludge sample from which the PGPRs were isolated. Aleem *et al* (2003) reported that the soil pollution with heavy metals could lead to the appearance of heavy-metal resistant PGPR in the soil of industrial regions.

IAA production and Phosphate solubilization efficacy

Another experiment was conducted to determine the efficacy of IAA production and phosphate solubilization by chromium tolerant isolates of different PGPRs isolated from tannery sludge samples and the results are presented in Fig. 2 and Fig. 3. It was found that there is a variation in quantity of IAA production and phosphate solubilisation among the isolates. IAA production was maximum in PSB-1(46.0µg/ml), followed by Azosp-1 (30.0µg/ml) and Azoto-1 (29.0µg/ml). Maximum phosphate solubilisation efficacy was found in isolate PSB-1 (90%), followed by Azosp-1 (40%) and Azoto-1 (25%). Many PGPRs are tolerant to heavy metals and play an important role in mobilization of heavy metals and also play an important role for enhancing plant growth (Gadd, 1990).

Molecular identification of efficient isolates of PGPR

A total of 6 PGPR isolates were obtained from tannery sludge samples. Of these, 3 bacterial isolates were selected based on their heavy metal tolerance, IAA production and phosphate solubilization efficacy. These bacterial isolates were characterized at

molecular level and identified as *Achromobacter xylosoxidans*, *Azotobacter vinelandii* and *Azospirillum lipoferum.* The 16s rDNA sequences of these isolates were deposited in the EMBL Nucleotide Sequence Database (LK391696, LK391702 and LK391703) accession numbers.

Many earlier studies revealed that the PGPR had plant growth hormone production and phosphate solubilization efficacy. Jha *et al* (2009) isolated and identified *Achromobacter xylosoxidans* as diazotrophic bacteria which showed appreciable level of nitrogenase activity, IAA production and P solubilization ability. The present investigation is the first report which clearly indicates that the bacterial isolate, *Achromobacter xylosoxidans* is isolated from tannery sludge waste samples and also it showed maximum chromium heavy metal tolerance up to 2mM concentrations under *in vitro* condition. Piperidou *et al* (2000) reported that *Azotobacter vinelandii* had gained much importance in bioremediation and mineral solubilization (Sashidhar and Podile, 2009) and nitrogen-fixation (Ravikumar *et al.,* 2004). Raut *et al* (2008) isolated *Azospirillum lipoferum* and they determined its heavy metal tolerance and IAA production abilities and proved it as good biofertilizer.

Table.1 Physico-chemical parameters of tannery sludge samples collected from effluent treatment plant, Dindugal, Tamil Nadu

S.No.	Physico-chemical parameters	Tannery sludge sample
1	pH	7.7
2	Electrical Conductivity (dSm^{-1})	9.8
3	Bulk density (gm/cc)	0.7
4	Organic carbon (%)	2.10
5	Available Nitrogen (kg/ha)	171.7
6	Available Phosphorus(kg ha-1)	24.26
7	Available Potassium (ppm)	475.8
8	Calcium (meq/100g)	4.2
9	Magnesium (ppm)	0.20
10	Arsenic (ppm)	0.2
11	Cadmium(ppm)	0.1
12	Copper(ppm)	0.05
13	Lead(ppm)	0.2
14	Mercury(ppm)	0.02
15	Zinc(ppm)	0.5
16	Chromium(ppm)	0.2

Table.2 Population density of PGPR isolated from tannery sludge samples

| Sl. No.	Media used for beneficial bacteria isolation	Cfu/g soil[*]
1.	Pikovskaya's agar	2.5×10^6
2.	Jenson's medium	2×10^6
3.	Rojo- congo agar	1×10^6

[*]**Mean of 5 replications**

Table.3 Chromium tolerance of tannery bacterial isolates in agar plate method

Sl. No.	PGPR Isolates	Chromium heavy metal concentrations				
		0mM	0.1mM	0.5mM	1mM	2mM
1.	PSB 1	+++	+++	+++	+++	+++
2.	PSB 2	+++	+	-	-	-
3.	PSB 3	+++	+	-	-	-
4.	Azoto 1	+++	+++	+++	++	++
5.	Azoto 2	+++	++	-	-	-
6.	Azosp 1	+++	++	+	+	+

Legend: +++ - maximum growth, ++- moderate growth, +- less growth, - - no growth

Figure.1 Represent the Cr tolerance of different bacterial isolates against various concentrations of chromium
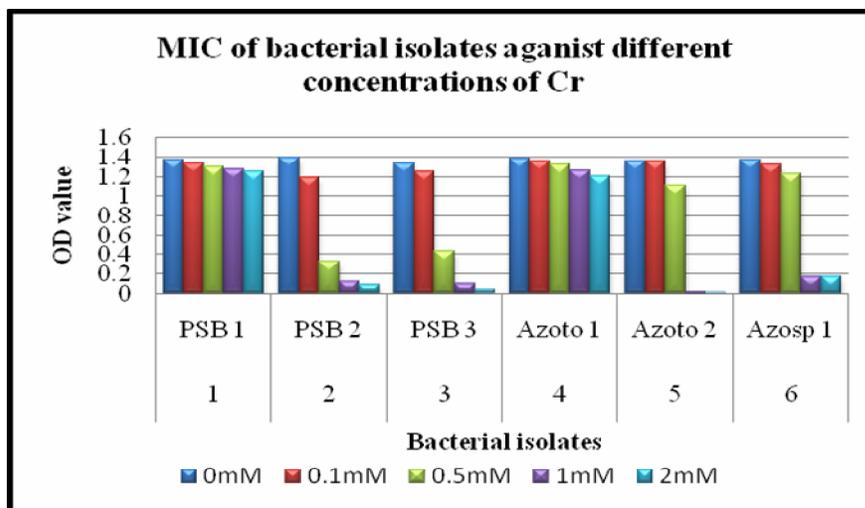
Figure.2 Screening of IAA production by chromium tolerant isolates of PGPRs from tannery sludge samples
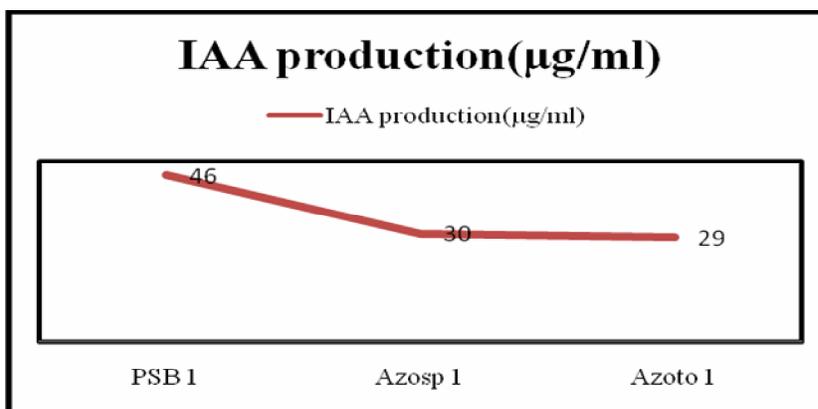
Figure.3 Phosphate solubilizing efficacy of chromium tolerant isolate screened
from tannery sludge samples
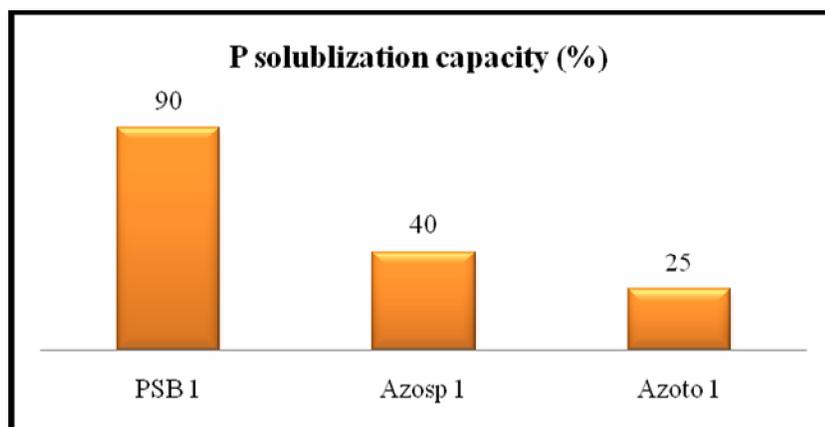
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