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Research Article

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HYDROXAMATE AND CATECHOLATE SIDEROPHORE SYNTHESIZING ARSENIC RESISTANT PGPR ISOLATED FROM WEST BENGAL

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Abstract

Arsenic has emerged a global environmental problem due to its occurrence in groundwater of sedimentary aquifers. Diverse industrial applications and agriculture practices of arsenic compounds have aggravated the metal contamination problem. Siderophores are specific ferric iron chelator under iron-starved conditions. Two strains WB (*Lysinibacillus* sp) and PG (*Dietzia maris)* showed a high degree of resistance to arsenic upto 1000ppm. Both strains were exhibited battery of plant growth promoting traits including enhanced production of plant growth hormone indole acetic acid (IAA), phosphorus solubilization and siderophore production. Strain WB was found potential IAA producer in the presence of arsenic 1000ppm concentration (67µgl^{-1}). The rate of P-solubilization and siderophore production were obtained maximum in strain PG with 90.0µgml^{-1} and 81% siderophore units respectively, in arsenic stressed medium. On Infra red screening isolates PG and WB acclaimed hydroxamate and catecholate nature of siderophores respectively. Our results evidenced that the strong pressure in the rhizosphere selected strains able to improve plant fitness by triggering different growth promotory attributes to improve performance upon biotic and abiotic challenges.

Keywords: Arsenic, Siderophoregenesis, Plant growth promoting rhizobacteria (PGPR)

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Introduction

Arsenic contaminated ground water is most serious problem worldwide. North India and Bangladesh are badly contaminated, in northern districts of Bangladesh almost every house has a person suffering from arsenicosis (Mohan et al. 2006). Arsenic is a silent killer found in two major toxic forms, arsenate and arsenite. Arsenate acts as a phosphate analog due to structural similarity with phosphate. In addition, arsenite disrupts sulfhydryl groups of proteins and interferes with enzymatic functions (Jackson et al. 2003; Srivastava et al. 2010).

When sources of water pollution are enumerated, agriculture is listed as a major contributor. One of the major factors determining uptake and toxicity to plants is the form of arsenic (Banejad et al. 2011). Crop research center (Pantnagar) is agriculturally practiced land, its environment has been stressed by various metals, as the consequence of enormous fertilizer and pesticide application.

Due to its immense requirement, the strains were selected for the present study to know its interaction with the siderophore producing plant growth promoting bacterial strains in metal contaminated soil. In India, 90,000 MT of technical grade pesticides are used annually to control pests and plant diseases (Chauhan et al. 2006).

Microorganisms elaborate a variety of biogenic chelating agents, for instance siderophores, because of limited availability of iron which is due to rapid oxidation of Fe^{+2} to Fe^{+3} state. These siderophores solubilize ferric iron in the environment and transport it into the cell (Banejad et al. 2011) and determine the growth of microorganisms under competitive conditions when availability is a limiting factor(Nagoba and Vedpathak 2011). Microbial siderophores and derivatives are classified as hydroxamates, catecholates, carboxylates and mixed type, based on chemical nature of their coordination sites. Bacterial hydroxamates are composed of hydroxylated and acylated alkylamines. Hydroxamic acid-based siderophores also have applications in medicine for the treatment of thalassemia patients and as potential anti-cancer agents. In addition catecholates, which are produced only by bacteria, composed of catecholate and hydroxy groups (Nagoba and Vedpathak 2011). Arsenic detoxification has been reported in number of bacterial species such as *Escherichia coli, Bacillus* spp., *Staphylococcus aureus, Staphylococcus xylosus, Chromobacterium violaceum, and Pseudomonas* sp (Srivastava et al. 2010; Freibach et al. 2004; Patel et al. 2004). This study was executed on metal induced siderophore production by two native plant growth promoting strains WB and PG, and their chemical characterization using Infra red spectra. Siderophore production by these plant growth promoting bacteria would greatly influence the efficacy of arsenic decontamination in affected areas, plant growth promotion and disease control also.

Materials and Methods

Procurement and revival of test strains

The test strains WB(*Lysinibacillus* sp) and PG(*Dietzia maris*) were isolated from waste fed canal near Cooch Behar district (West), and cultured on Nutrient agar media by incubation at 28ºC for 24h. Isolates were tested for their resistance or sensitivity to As metal using $Na_2HAsO_4.7H_2O$ salt respectively at different concentrations ranging from 0, 500 and 1000 ppm on Nutrient agar plates after incubation at 28ºC for 48 hours. Selection was based on their arsenic tolerance capability.

IAA(Indole-3-acetic acid) production

Exponentially grown cultures of strain of WB and PG were inoculated in 5ml Nutrient agar media and incubated at 28ºC for 48 h. The broth was taken to centrifugation at 10,000 rpm for 15 min at 4ºC. One ml of *O*-phosphoric acid was added to 2 ml of bacterial supernatant and allowed to stand for 1 h to develop the pink color. Quantification of IAA was estimated at OD 530 nm using standard IAA graph(Bric et al. 1991).

Phosphate solubilization

Pikovaskya (PKV) agar plates were spot inoculated with sterile filter paper discs soaked in overnight grown test cultures WB and PG. After incubation at 28ºC for 5 days, formation of a clear zone around the spot was recorded, this is indicated by a clear zone as compared to the media which is translucent. Quantitative estimation of phosphate solubilization was carried out according to Nautiyal (1999).

Siderophore production

The standard succinate medium was used(Nair et al. 2007) to initiate the growth of test strains for the siderophore production. Colour change in the succinic acid medium was an indicator of siderophore production by test strains. The clear medium turned to fluorescent green after 24 h of incubation and to brown colour after 48 h of incubation. This indicates possible production of siderophore, which is confirmed by CAS assay. The Chrome Azurol S (CAS) assay was used to detect siderophores produced by isolates(Neilands 1989).

Characterization of siderophores

Hydroxamate and catechol functionality of siderophore produced, which are typical functional groups that bind iron(May et al. 2001), was determined by the Csaky test(Csaky 1948) and the Arnow reaction respectively. Optical density was read at 420-450 and 500 nm for hydroxamate and catecholate functional group respectively through UV-VIS-NIR spectrophotometer(Arnow 1937). Siderophores were extracted and purified using acetate extraction method, thereafter Purified siderophore samples were lyophilized and calibrated with thin films on KBr plates, subjected to Fourier transform IR (FT-IR) spectrum. Spectra were recorded in 4000–450 cm-^{1} ranges.

Results

Two most promising strains having plant growth promoting ability were found to be resistant up 1000ppm arsenic and assigned to strain WB (*Lysinibacillus* sp) and PG (*Dietzia maris*) respectively. Exposure of heavy metals gives an advantage of survival to indigenous microorganisms in the polluted environment. Toxic effect of metals led the bacteria to develop a variety of resistance mechanisms to counteract heavy metals. One way to relieve from heavy metal stress in plants might involve the application of siderophore compounds secreted by rhizosphere microorganisms. This approach could be attributed to the fact that siderophore overproducing test strains were isolated from a soil containing high levels of metals have adapted to such environments. Actinomycetes possess various biological activities and have the potential to be developed as efficient plant growth promoter. In addition *Bacillus* species have been successfully reported from industrial landfills, metalliferous soils and nickel contaminated soil. Since *Bacillus and Actinomycetes* were the most abundant, developed a variety of resistance mechanisms to counteract heavy metal stresses. Furthermore besides their metal resistance effect, they are well documented growth promotant.

Plant growth promoting traits

Increasing the arsenic concentration in the medium from 0 to 1000 ppm brought about no significant decline in auxin synthesis. The amount of auxin, produced was 50.3μgl^{-1} for WB and 48.2μgl^{-1} for PG at 500ppm arsenic. The maximum auxin activity 67.0μgl^{-1} was recorded for WB at 1000 ppm concentration of arsenic (Figure 1). The isolates differed in their ability to solubilize phosphate substrates. The average TCP (tri calcium phosphate) solubilization by these isolates ranged from 55 to 60.20μgml^{-1} for WB and PG at 500ppm arsenic respectively. The maximum solubilization 98μgml^{-1} was observed with the isolate PG in 1000 ppm arsenic supplemented medium (Figure 1).

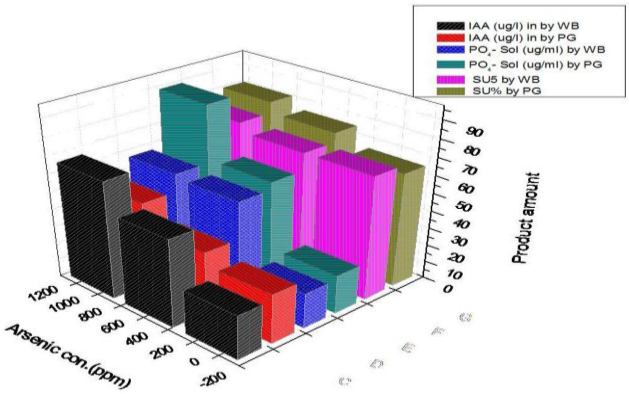

Fig.1. PPGP traits presented by test isolates in arsenic amended treatments

Screening for siderophores

The isolates were found positive for siderophore production with arsenic (500 and 1000 ppm) supplemented standard succinic acid medium. Qualitatively, siderophore was detected by universal CAS (Chrom Azurol S) assay. A significant increase in siderophore synthesis, with the elevated arsenic concentration in medium (succinic acid medium) was recorded during the siderophoregenesis in concerned isolates. Siderophore synthesis was significantly higher for isolate PG with 81.0%SU (siderophore unit) while 75.0%SU was obtained for isolate WB (Figure 1).

Characterization of siderophores

Preliminary biochemical characterization of siderophore confirmed the presence of hydroxamate and catecholate ligends. Strain WB represented a close-up spectral region containing the hydroxamate stretching (Table 1). The infra red frequencies for primary aliphatic group, C=O Streching, C-OH Streching, C=N Streching, OH Group and As-O streching in the spectrum of adsorbed by RK3 that have been assigned to efficient hydroxamate siderophore synthesizer, collectively confirmed the fractions of hydroxamate ligends. (Fig. 2A). The IR spectrum adsorbed by PG suggested additional surface interactions with bacterial catecholate ligends (Table 2) with the stretching frequencies of –C=N, >C=O, >C=C– of benzene nucleus, >C–O– stretching of COOH atom, Ar-O- stretching of phenolic OH atom and m substitute (partial), (Fig. 2B).

Discussion

The plant growth promoting isolates PG and WB exhibited a higher metal tolerance when cultivated under increasing arsenic levels (>1000 ppm) in the growth medium. This high tolerance to heavy metals could be attributed to the fact that the bacteria were isolated from a soil containing high levels of metals have adapted to such environments (Freitas et al. 2004).

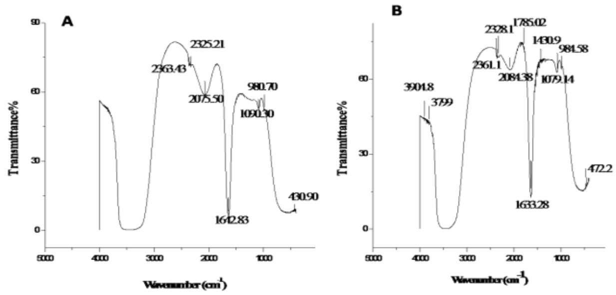

Fig.2. Infra red spectra of siderophores produced by *Pseudomonas aeruginosa* strain WB and PG and arsenic added to the resultant mixture A: Hydroxamate legend, B: Catecholate legend

Table 1. Functional group hydroxamate legend determined in isolate WB based on FT-IR

Bonding	Wavenumber cm^{-1}	Reference
Prim.Aliphatic.group	1090.80	Patel et al. 2009, Sun & Doner 1996
C=O Stretching	1638.45	Patel et al. 2009
C-OH Stretching	1642.83	Patel et al. 2009, Sun & Doner 1996
C=N Stretching	2325.21	Patel et al. 2009
OH Group	3560	Patel et al. 2009
As-O stretching	980.70	Paul&Liu. 1952, Frost et al. 2003, Nair et al. 2007

Table 2. Functional group catecholate legend determined in isolate PG based on FT-IR

Bonding	Wavenumber cm^{-1}	Reference
–C=N	NR	Patel et al. 2009
>C=O	1646.28	Jayesh et al. 2010
>C=C– of benzene Nucleus	1430.90	Jayesh et al. 2010, Freibach and Chen 2004
>C–O– stretching of COOH atom	1785.02	Jayesh et al. 2010, Sun & Doner 1996
Ar-O- stretching of phenolic OH atom	1087.78	Patel et al. 2009
m substitute (partial)	985.58	Patel et al. 2009

Thus, tolerance to heavy metals gives an advantage of survival in the polluted environment. The isolates exhibited a battery of PGP characteristics. A significant production of plant growth hormone IAA, was reported 67.0μgl^{-1} for PG. Isolate PG represented reasonably, P-solubilization with 90μgml^{-1} and excellent amount of siderophore (81.0%SU) was synthesized by WB upto 1000ppm of arsenic metal stress.

In general the elevated levels of heavy metals in soil interfere with uptake of nutrients such as phosphorus and lead to plant growth retardation(Zaidi et al. 2006). This deficiency can be compensated by the phosphate-solubilizing ability of strains(Halstead 1969). Further, the plant growth hormone IAA produced by PGPB strain promotes root growth by directly stimulating cell elongation or cell division(Glick et al. 1998). A low level of IAA produced by rhizosphere bacteria promotes primary root elongation whereas a high level of IAA stimulates lateral and adventitious root formation(Xie 1996). The metal resistant bacteria belonging to different genera such as *Pseudomonas*, *Mycobacterium*, *Agrobacterium* and *Arthrobacter* were found to have plant growth-promoting features that can potentially promote plants growth and reduce stress symptoms in plants(Dell'Amico et al. 2005).

In addition, for siderophore production, isolates were grown on standard succinic acid medium.

Succinate was used as a carbon source by PG and WB and increased siderophore yield. Review of previous reports also favored the results obtained, that chromogenic nature of succinate found suitable for better siderophoregenesis.

The finding also indicated that isolates PG and WB, exposed to arsenic stress, and has synthesized a great amount of siderophore with 75% and 81% SU respectively. In fact under arsenic stress cells might be forced to synthesized siderophore to combate stress. Dalip et al.(2003), reported the production of maximum 88.8% and 88.6% siderophore units for *Pseudomonas fluorescence* and *Pseudomonas putida* respectively.

On infra red screening isolates PG and WB acclaimed hydroxamate and catecholate nature of siderophores respectively. The infra red frequencies adsorbed by strain PG and WB suggested additional arsenic interactions with bacterial hydroxamate and catecholate ligands. For the characterstic hydroxamate legend, a hydroxyl stretch, C=N stretch, C–OH stretch and C=O stretching were observed. The similar bendings were previously reported by Patel et al.(2009), in hydroxamate synthesizer probiotic *Bacillus* strain. The fundamental vibrations for NH_2 bending for hydoxalamine were recorded earlier(Paul and Leu 1952).

Infra red spectroscopy showed the presence of aromatic hydroxyl group, >C=O, >C=C–, >C–O stretching of COOH group, aromatic-O (Ar-O) stretching of phenolic OH group and m disubstituted position of the groups in molecules, collectively explained to catecholate type siderophore 2,3-dihydroxy-benzoic acid. These findings are consistent with the findings of Jayesh et al.(2011), reported a catecholate-type siderophore produced by probiotic *Bacillus* spp. Various other reports indicated 2,3 Dihydroxy benzoic acid (2,3-DHBA) and its derivatives from the *Bacillus* sources have been widely reported(Lee et al. 2007). The overall UV-Visible and IR spectra data agreed fairly well with the similarity between the structure of catecholate type of siderophore dihydroxy benzoic acid of *Bacillus* spp. and siderophore sample of *Pseudomonas* sp. This approach can be executed in removing many toxic metals off the soil which poses a serious health threat. The result indicates that PGPR strains PG and WB triggers the plant defensive metabolism by toxic metal chelation via siderophore synthesis.

Conclusion

The recovery of metals by microbial products is one advanced technique, that have many potential applications in both bioremediation and iron chelation therapies. The interaction of heavy metals such as arsenic with siderophores produced by *Lysinibacillus* and *Dietzia maris* warrants future investigation that may help, predict the cycling and fate of heavy metals in contaminated soils. From the above findings, it can be concluded that different type of siderophores produced from isolates WB and PG can be used for remediation of arsenic affected soil. The large-scale application of siderophores to agriculture crops as bioinoculant would be effective as it would substantially reduce the metal contamination which often pollutes the environment.

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