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Isolation and Characterization of Aluminium and Copper Resistant 'P' Solubilizing Alkalophilic Bacteria

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Aluminium and copper resistant bacteria, isolated from contaminated soil, showed metal tolerance level of 3106 μ*M* and 4398 μ*M*, respectively as evident by four select isolates based on their phosphorus solubilization potential (0.2-6.4 μg mL^{-1}). All four isolates could grow in a *p*H range of 5-11. Three isolates are member of Pseudomonadaceae family, *Pseudomonas* sp. (CD7), *P. pseudomalli* (CG13) and *P. maltophila* (TH18). The isolates, CD7 and CG13, belong to siderovar group as the *Pseudomonas* sp. strain B10, and are able to grow at 42°C. The CG13 is osmotolerant (10% NaCl). These isolates could be better bioinoculant candidate(s) for the contaminated alkalophilic sites.

Keywords: metal resistance, growth promotory microorganisms, phosphorus solubilization, siderotyping, osmotolerant, alkalophile

Introduction

Heavy metals, which are regularly being introduced into the environment from various sources viz. sludge dumping, industrial effluent and mine tailing (Sani *et al,* 2001), are used in industry as catalysts, preservative to prevent microbial growth, insecticides, herbicides and as disinfectants in clinics, hospitals and homes (Misra, 1992). Such metals form an important class of pollutants. However, organisms require metals (as micronutrients) as a cofactor of certain enzymes. Heavy metals, at higher concentration, are toxic to cells and may cause cell death by interacting with nucleic acids and enzymes active site (Ohsumi *et al,* 1998; Hazel & Williams, 1990; Cervantes & Gutierrez-Corana, 1994).

Almost every index microbial metabolic activity (methanogenesis, respiration, motility, organic matter decomposition, etc.) can be adversely affected by elevated concentration of heavy metals. *Azotobacter* sp., when inoculated into heavy metal contaminated soil, inhibits N-fixation (Brieley & Thornton, 1983). Presence of Cu and Cd are found toxic for denitrification process observed in three environmental isolates of *Pseudomonas* sp. (Bollay & Barawarg, 1979). In surface water, Cu^{++} (1.0 mg l^{-1}) is toxic to some fish and aquatic plants (Sawyer *et al,* 1978). Aluminium, in acidic soil, gets precipitated in the form of its phosphate either on root surface or in root walls and hampers root development in many crops because of unavailability of soluble P (Barlett & Riego, 1972). Ash originating from the *poha* industry, where paddy husk is used as a fuel, contains 0.91% Al_2O_3 (Sawarkar & Dikshit, 1990). Cu and Al are very toxic for heart and lung tissues, as the former causes the damage of lung and the latter affects heart.

Like microorganisms, metal pollution has potential to cause sickness and death in plants, animals and humans. Plants are of particular concern because they extract metals from polluted sites and mine waste and, in turn, make the metal available to animals. Thus, plant uptake of heavy metals gets magnified at higher level in the food chain.

Heavy metal resistant microorganisms, which grow not only under contaminated environment but also possess growth promotory properties, are of particular importance for the degraded and/or polluted land-use practices. Roots absorb mineral nutrients as ions in soil water. Phosphorus is one of the macronutrients limiting plant growth. Most of the essential plant nutrients remain in insoluble form in soil (Abd-Alla, 1994; Silver & Misra, 1988). A large portion of inorganic phosphates applied to soil as fertilizer is rapidly immobilized after application and becomes unavailable to plant (Yadav & Dadarwal, 1999).

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Thus, the release of insoluble and fixed form of phosphorus is an important aspect of increasing soil phosphorus availability. Microorganisms, which make insoluble nutrient elements available to the plants, are known as plant growth promotory microorganisms. Organisms under this category have the ability to solubilize insoluble soil phosphates, chelate iron by producing siderophores and protect plants from soil pathogens by producing antibiotic metabolites, thus increasing the plant health and yield.

Microorganisms have developed the mechanism to cope with a variety of toxic metals for their survival. Heavy metal resistant bacteria are prevalent in the environment enriched with such metals. Such bacteria are often found on the plasmids and transposable elements (Silver & Misra, 1988). Genetic determinant for resistance to heavy metals (such as Hg^{2+}, Ag^{+}, AsO_2^{-}, BO_3^{3-}, Cd^{2+}, Co^{2+}, CrO_4^{2-}, Cu^{2+}, Ni^{2+}, etc) are contained by many bacterial strains. This study was undertaken for isolation and characterization of Al and Cu resistant phosphate solubilizing bacteria.

Materials and Methods

Microorganisms, Medium and Cultural Condition

Heavy metal resistant bacteria were isolated from two different sites (soil affected by paper and pulp mill effluent and insecticide treated soil of crop research centre at Pantnagar, India). Isolation was done on King's B agar medium (protease peptone, 20; $MgSO_4.7H_2O$, 0.4; K_2HPO_4, 1.0; and agar, I5 g; glycerol, 8.0 and distilled water 1000.0 ml; *p*H 7.0±0.2) supplemented with four concentrations of Al (as $AlCl_3$) (207, 1035, 2071 and 3106 μ*M*) and Cu (as $CuCl_2$) (293, 1466, 2932 and 4398 μ*M*) at 28°C.

Screening and Characterization

All the isolates recovered were characterized morphologically, by catalase and oxidase test. Screening was based on phosphate solubilizing potential of isolates. 'P' solubilization was checked on Pikovskaya agar medium (yeast extract, 0.5; dextrose, 10.0; calcium phosphate, 5.0; ammonium sulphate, 0.5; potassium chloride, 0.2; $MgSO_4$, 0.1; and agar 15 g; $MnSO_4$ and $FeSO_4$, 1 mg each; distilled water, 1000 ml, *p*H 7.0±0.2). Phosphorus quantification for select isolates was checked in NBRIP broth (Nautiyal, 1999), where tri-calcium phosphate (5%) was used for 7 days.

Siderophore Production Test

Siderophore secretion was checked by CAS (chrome-azurol 'S') assay (Schwan & Neilands, 1987), followed by quantification (Meyer & Abd-Allah, 1978). Absorbance was taken at 400 nm using extinction coefficient and the amount of siderophore was calculated. Siderotyping was done at Universite Louis-Pasteur, France (Munsch *et al*, 2000).

Antibiotic Sensitivity Test

It was checked against six antibiotics—Streptomycin, Gentamicin, Chloramphenicol, Ampicillin, Tetracycline and Bacitracin—using discs on the nutrient agar plates after spread plating of each culture. Further, four selected isolates were characterized at Microbial Type Culture Collection, IMTECH, Chandigarh, India.

Results and Discussion

Bacterial Strains

Of the 36 isolates recovered at different levels of metal concentration, 21 were from effluent affected soil and 15 from insecticide treated soil.

Toxicity to metals at different concentrations were checked both on solid and liquid media. Results of solid medium substantiate liquid medium growth profile. The availability of metal compound is higher in liquid than in solid medium. Thus, a particular concentration becomes more toxic for organism while grown in liquid broth. Therefore, the tolerance level for a metal was greater in solid medium than in liquid medium.

Biochemical Characteristics

Most of the isolates were Gram -ve short rods. About 50% isolates showed positive zone for 'P' solubilization on Pikovskaya agar plate. Eight isolates were strong positive, four from each soil (Table 1). Two isolates (CD7 and CGI3) with maximum 'P' solubilizing potential together with two isolates (TD8 and TH18) with minimum potential were chosen for further experimentation (Table 2). The antibiotic sensitivity profile (Table 3) revealed that all the four isolates were sensitive for Bacitracin and Ampicillin but resistant against Gentamicin, Chloramphenicol, Tetracycline and Streptomycin.

Table 1—Qualitative 'P' solubilization potential of recovered isolates

Metal	$AlCl_3$			$CuCl_2$		
Clearance Zone	+	++	–	+	++	–
No. of Isolates	9	4	4	2	4	14

++: Strong positive; +: Moderate; - :Negative

Table 2—'P' solubilization potential and heavy metal tolerance of select isolates

S No	Isolates	'P' solubilized ($\mu g\ ml^{-1}$)[a]	$AlCl_3$ 3106 μM	$CuCl_2$ 4398 μM	$CuCl_2$ 2932 μM
1	CD7	6.4	+	–	–
2	TD8	0.4	+	–	–
3.	CG13	6.0	-	–	+
4.	TH18	0.2	-	+	+

–: No growth; + : Positive growth; [a]Each reading is a mean of three replicates

Table 3—Antibiotic sensitivity profiling of select isolates

	Sm	Am	Cm	Bc	Gm	Tc
CD7	0.4	-	0.2	-	0.9	1.0
TD8	0.7	-	1.3	-	1.1	1.1
CG13	0.7	-	0.5	-	1.3	0.8
TH18	0.8	-	1.4	-	1.25	1.3

- : Sensitive; 0.2-1.5: Zone size in cm

Table 4—Outstanding physiological growth characteristics of select isolates

Characteristics		CD7	TD8	CG13	TH18
*p*H	High	**11**	**11**	**11**	**11**
	Low	5	5	5	5
Temp. (°C)	High	**42**	37	**42**	37
	Low	24	24	24	24
NaCl (%)		5	5	**10**	5
Anaerobic growth		–	+	–	–
Oxidation/ Fermentation (O/F)		O	F	O	O
Gelatin liquefaction		+	+	+	+
Arginine dihydrolase		+	+	+	d

–: No growth; +: Positive growth; d: 85-90% isolates are positive

Alkaline and/or acidic soil makes mineral unavailable to plants. Extreme fluctuations, resulting from increasing commercialization and industrialization, have destroyed balance of soil from neutrality. As a result, ions become unavailable to soil and cause deficiency or toxicity of micronutrients, viz. Fe, Mn, Zn and Cu. Under alkaline condition, Fe is rendered unavailable to plant by leaching and is held in the lower portion of the soil. The isolated organisms have the ability to grow well at alkaline *p*H (5.5-6.2 best for most crops). Further, damage to the vegetation and dying of forests have long been associated with acid rain. The aluminium, being more cationic (Al^{+++}) in nature, is one of the major components of acid rain pollution. The H^+ ions present in acid rain are absorbed by soil while, at the same time, other positive ions in the form of cations (K^+, Al^{+++}, Mn^{++}, NH_4^+) are washed out, making soil acidic. Contributions of plant growth promotory metal tolerant pseudomonads have longer been investigated. Cd (180 μM), Ni (420 μM) and Cr (370 μM) resistant mutants of *Pseudomonas* sp. NBRI 4014 have been developed and significant growth promotion was observed in soybean (*Glycine max* PK564) by pot experimentation (Gupta *et al*, 2001).

Growth profile of four isolates shows their alkalophilic nature (*p*H, 5-11). However, CGI3 showed osmotolerant (up to 10% NaCI) characteristic also. The upper temperature limit for the growth of organisms CD7 and THI8 was 37°C, and that for CG13 and TD8 was 42°C (Table 4). Based on physiological and biochemical properties, these isolates were identified as *Pseudomonas* sp. (CD7), *Bacillus circulans* (TD8), *P. peudomalli* (CGI3) and *P. maltophila* (THI8), respectively.

Quick and unambiguous characterization methods of fluorescent pseudomonads are available as "siderotyping" (Meyer *et al,* 2002), which are mainly based on the comparison of isoelectrofocusing (IEF) patterns of pyoverdines and on the characterization of their respective iron transport capacities. Siderotyping showed that isolates CD7 and CG13 are positive for siderophore production, whereas isolate TD8 was negative when tested by the CAS assay (Schwan & Neilands, 1987). IEF showed that strains CD7 and CGl3 produce the same type of pyoverdine with an IEF profile identical to the one of the pyoverdines of *Pseudomonas* sp. B10 (three isoforms with *p*H of 7.5, 5.2 and 4.6, respectively). Moreover, the two strains incorporated iron chelated to pyoverdine (B10) at an equal efficiency as their own pyoverdines. It confirmed that both the isolates belong to the same siderovar as strain *Pseudomonas* sp. B10 (pyoverdine B10) peptide chain sequence: L-Lys-D-*threo*-β-OH-Asp-L-Ala-D-*allo*- THR-L-Ala-D^{δ}-N-OH-Orn (Teintze *et al,* 1981). The result of siderotyping is thus in agreement with the physiological characterization of the isolates.

Growth promotory potential of isolate CD7 has already been reported at 2071 μM and 3106 μM aluminium concentration, respectively (Parmeela *et al,* 2002). Isolate CG13 having several properties together with metal tolerance can better be exploited for copper contaminated sites. However, CD7 as evident by siderotyping has similarity with the first reported rhizobacteria *Pseudomonas* sp. strain B10 (Kloepper *et al*, 1980), which has been confirmed in this work under growth promotory experimentation. Therefore, aluminium tolerance, alkalophilicity, osmophilicity and 'P' solubilizing potential of this strain make it an ideal candidate as bioinoculant.

All the isolates studied are metal tolerant (2900-4390 μ*M*), 'P' solubilizing, alkalophilic and/or osmotolerant that can grow at above mesophilic range (42°C). Further, studies regarding their biosorption and molecular characterization are underway.

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