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Gousia Qadir
PhD Microbiology,
Department of Basic Sciences and Humanities, Sheri Kashmir University of Agricultural Sciences and Technology Kashmir, Jammu and Kashmir, India

Prof MY Zargar
Director Research,
Sheri Kashmir University of Agricultural Sciences and Technology Kashmir, Jammu and Kashmir, India

Dr. ZA Baba
PhD soil science,
Department of Soil Sciences Sheri Kashmir University of Agricultural Sciences and Technology Kashmir, Jammu and Kashmir, India

Shakeel Ah Mir
Prof, PhD Agri statistics,
Department of Statistics Sheri Kashmir University of Agricultural Sciences and Technology Kashmir, Jammu and Kashmir, India

GH Najar
Prof, PhD soil science,
Department of soilSciences , Sheri Kashmir University of Agricultural Sciences and Technology Kashmir, Jammu and Kashmir, India

Dr. Mushtaq Ah Malik
PhD biochemistry,
Sheri Kashmir University of Agricultural Sciences and Technology Kashmir, Jammu and Kashmir, India

Correspondence
Gousia Qadir
PhD Microbiology,
Department of Basic Sciences and Humanities , Sheri Kashmir University of Agricultural Sciences and Technology Kashmir, Jammu and Kashmir, India

Isolation and characterization of *Pseudomonas* from oil spill soil under temperate climatic conditions

Gousia Qadir, MY Zargar, Dr. ZA Baba, Shakeel Ah Mir, GH Najar and Dr. Mushtaq Ah Malik

Abstract
The present study was carried out for isolation and characterization of *Pseudomonas* from oil spill soil and 10 isolates were isolated from different locations employing different morphological and biochemical attributes and on the basis of phosphate solubilisation and chitinase production three *Pseudomonas* isolates were employed in microbial consortium and used as effective microbe for treatment of STPs and different parameters were studied like N,P, K,C heavy metals, BOD and COD and it was found there was a decrease from original values after addition of *Pseudomonas* with other microbes.

Keywords: *Pseudomonas*, phosphate solubilisation, chitinase, Solubilisation index

Introduction
Phosphorus (P) is the second imperative key element after nitrogen as a mineral nutrient in terms of quantitative plant requirement. An adequate supply of P is obligatory for proper execution of various metabolic activities of plants. Identification of a potent phosphate solubilizing microorganism capable of transforming insoluble P into soluble and plant accessible forms is considered as the best eco-friendly option for providing inexpensive P to plants The use of phosphate solubilizing bacteria to mobilize large reserves of insoluble P in soils instead of phosphate fertilizers is an ecologically safe and economically reasonable alternative to assure a sustainable agriculture.
The genus *Pseudomonas* is a heterogeneous and environmentally significant group in the rhizosphere of plants, related to bioremediation process. A variety of technologies are currently available to treat the soil and water contaminated with hazardous materials, including excavation and containment in secured landfills, extraction, stabilization and solidification of vapor, soil washing, oil washing, solvent extraction, thermal desorption, vitrification and incineration. In addition to the physical and chemical alternatives, there are biological methods (Bioremediation), The genus *Pseudomonas* is the most heterogeneous and environmentally significant known bacterial group and includes mobile gram-negative aerobic rods, extended in all nature and characterized by its high metabolic versatility given aeruginosa and *Chromobacterium violaceum* also secrete antibiotics (Lippin *et al*. 2008; Taurian *et al*. 2010) and provide protection to plants against soi lborne pathogens (biocontrol) (Khan *et al*. 2002; Vassilev *et al*. 2006; Singh *et al.* 2010).Other physiological traits of PSM involve the release of cyanide, a secondary metabolite which is ecologically important (Wani *et al*. 2007) and gives a selective advantage to the producing strainsy a complex enzymatic system. There are numerous PS bacteria that possess the ability to synthesize a key enzyme, 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Glick *et al*. 2007), which hydrolyses ACC [the immediate precursor of plant hormone ethylene (C2H4)] to NH3 and α-ketobutyrate and thus mitigate the inhibitory effects of C2H4. Some of the compounds synthesized by PS. species of *Pseudomonas* continue to be the preferred organisms of study due to the ease with which they are cultured and the large quantities of pigment produced. *Pseudomonas* species are versatile in respect to phenazine pigment production. Four species of *Pseudomonas*, *P. chlororaphis*, *P. iodinum*, *P. aureofaciens*, and *P. aeruginosa*, have exhibited the ability to synthesize fourteen of the twenty-one naturally occurring phenazines. Half of these have been found to be produced by strains of *P. aeruginosa*.

Table 1: Biochemical Tests of *Pseudomonas*

Biochemical test	Results	Biochemical Tests	Results
Gram Staining	Gram (-) Rods	Urease	(-)
catalase	(-)	lactose	(-)
indole	(-)	Gelatinase	(+)
Methyl red	(+)	Nitrate reduction	(+)
citrate	(+)	Growth at 37 ^{0}C	(+)
VP	(+)	Motility	(+) motile
oxidase	(+)	Glucose	(+)

Materials and Methods: Soil samples were collected from several oil spilled locations and Soils were collected randomly 15-20 cm beneath the surface using sterile spatula and were placed in sterile screw capped vials. The soil samples were placed in sterilized plastic bags during transfer from site to the laboratory. Then serial dilution of soil was done in distilled water and different dilutions of sample were used on appropriate media by spread plate technique. The plates were incubated at 25^0 C for 2-3 days and then were observed for the colony appearance. Isolates exhibiting distinct colonial morphologies were isolated by repeated sub culturing into basal salt medium and solidified basal salt medium until purified strains were obtained. Identification was performed by gram staining and other colony

Phosphate solubilisation: The principal mechanism for mineral phosphate solubilization of *Pseudomonas* is its production of organic acids and acid phosphatases which play a major role in the mineralization of organic phosphorous. Although several phosphate solubilizing bacteria occur in soil, usually their numbers are not high enough to compete with other bacteria commonly established in the rhizosphere. Thus, the amount of P liberated by them is generally not sufficient for a substantial increase in plant growth. It is generally accepted that the major mechanism of mineral phosphate solubilization is the action of organic acids synthesized by soil microorganisms Production of organic acids results in acidification of the microbial cell and its surroundings. Consequently, Pi may be released from a mineral phosphate by proton substitution for Ca21 the production of organic acids by phosphate solubilizing bacteria has been well documented. Among them, gluconic acid seems to be the most frequent agent of mineral phosphate solubilization. It is reported as the principal organic acid produced by phosphate solubilizing bacteria such as *Pseudomonas* sp

In presents study all the 10 *Pseudomonas* isolates were initially qualitatively screened for TCP solubilization on solid media employing Pikovskaya's medium (Pikovskaya, 1948) amended with tri-calcium phosphate with following composition (g L^{-1}): Yeast extract-0.5; Dextrose 10.0; $Ca_3(PO_4)_2$ 5.0; $(NH_4)_2SO_4$ 0.5; $MgSO_4$ 0.1; KCl 0.2; $MnSO_4$ and $FeSO_4$ traces; Agar-agar 20.0; pH-7.2. Petridishes containing medium were spot-inoculated with 10 µL of overnight grown bacterial cultures and incubated at 28°C.Formation of clear halo zone around the colonies after 3 days of incubation indicates phosphate solubilizing ability. The solubilization zone size was measured after 7 and 14 days of inoculation. SI was calculated. Test bacteria were inoculated into 100 ml PVK's broth and incubated on a rotary shaker at 30 °C for 7 days. Controls consisted of UN inoculated culture medium. After centrifugation at 13,000 rpm for 20 min, concentrations of soluble P released in the media were detected by the colorimetric method as described by Ames (Ames, 1966). The rate of P solubilization was estimated by subtracting the P concentration of un inoculated control from the final concentration of soluble P obtained in the inoculated media. The final pH of media was measured with a pH meter with a glass electrode.

Table 2: Phosphate Solubilization activity of isolated bacterial strains

Isolate	P-released (mg/ml)	Solubilization Index	Solubilization zone
SP1	101.510	1.837	7.057
SP2	96.503	2.303	5.117
SP3	92.333	2.403	6.057
SP4	88.543	2.103	4.090
SP5	105.833	2.203	7.110
SP6	107.443	1.960	8.037
SP7	65.320	1.807	4.220
SP8	47.307	1.187	3.630
SP9	55.823	1.500	2.147
SP10	62.203	1.277	3.570
C.D.	0.204	0.119	0.079
SE(m)	0.069	0.040	0.027
SE(d)	0.097	0.057	0.038
C.V.	0.144	3.728	0.902

Chitinase Assay

The chitinase activity of isolates was estimated as per Ressing *et al* (1995) and for prepreation of colloidal chitin the method of Berger and Reynolds (1958) was adopted.For colloidal chitin prepration, the powdered chitin (10g) was digested over night with concentrated hydrochloric acid (500ml) at 4^{0}C. The contents were centrifuged 10000 rpm for 20 minutes and supernatant removed by filtration. The collected contents were washed three times to remove all acid traces till a pH of 4 was achieved. The pH was adjusted by 2 N NaOH and 1 N HCL. The collected contents were dried in oven at 45^0 C for 20 hours and later added to minimal media (0.3% w/v). All the selected cultures were grown in LB broth.The log phase growing cells (72h old) of each culture 15µl were spotted on already prepared minimal media and plates were incubated at 30^{0}C for 7 days, then iodine was added to these plates. Development of halo zone around the colony after the addition of iodine was considered positive for the production of chitinase enzyme. The halo zone CZ (the clear visible zone around the bacterial colonies indicating chitin hydrolysis. The colony diameter of the isolates (CS) was measured with measuring tape. Then the ratio of both the parameters was calculated (CZ: CS).

Table 3: Chitinase activity shown by isolated strains

Isolate	Chitinase activity (units /ml)	** CS:CZ
SP1	22.18	3.67
SP2	11.05	1.63
SP3	15.33	2.85
SP4	9.41	2.09
SP5	33.34	4.08
SP6	25.12	3.06
SP7	13.82	3.11
SP8	12.73	3.05
SP9	11.21	1.63
SP10	14.69	3.45
Overall Mean	16.88	2.86
CD($p \leq 0.05$)	0.07	0.17
** C: Z depicts ratio of colony size to the zone of clearance		

Results

Qualitative assay of phosphate solubilization Out of 10 bacteria isolated from different oil spilled locations All

Showed clear zones on PVK solid medium supplemented with Ca3 (PO4)2 analysis and however, it was seen that based on the diameter of the solubilizing halos on PVK medium (SI) and other characteristic tests. Three isolates namely SP1, SP5, SP 6and P, were selected for P solubilization the highest SI value was recorded for the isolate SP 3 in plates containing CaHPO4 as sole P source. Similarly Chitinase test was performed for all the 10 isolates of *Pseudomonas* an SP5 showed the maximum activity.

Conclusion

It was concluded that *Pseudomonas* is a potent biodegradation agent and can be used as a efficient bioinoculant for agricultural purposes due its various traits

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