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PRODUCTION AND OPTIMIZATION OF SIDEROPHORE PRODUCING *PSEUDOMONAS* SPECIES ISOLATED FROM TARAI REGION OF UTTARAKHAND

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ABSTRACT

A total thirty five rhizospheric bacteria were isolated from Tarai region of Uttarakhand, India. Among isolated strains, eight isolates shown siderophore production on succinic acid medium and chromo azural S agar medium plate. Three isolates were found to produce more than 60% siderophore units (SU). Amongst them PB19 was found to be the most efficient siderophore producer (78% SU) was identified as *Pseudomonas* spp. Maximum siderophore production was observed at pH 7 and temperature 29˚C. Glucose as carbon source was found to stimulate bacterial growth as well as siderophore production. Lysine was found to be optimum for siderophore production. Maximum siderophore yield was obtained with urea at 0.6gL^{-1} as independent nitrogen source. Iron concentration up to 20 µM was found to be optimum for siderophore production. Shake flask studies revealed that the siderophore production starts after 6 h of growth and reached to maximum productivity of 70.43% SU after 30 hrs. Both types of siderophore produced by selected isolates i.e. wine red color and yellow color formation in supernatant indicated production of hydroxamate type (pyoverdine) and presence of catecholate or phenolate type (pyochelin) siderophore respectively. The present study reveals *Pseudomonas* strain as a promising candidate for crop improvement and protection due to its PGPR activities.

KEY WORDS: **PGPR, Siderophore, CAS, % SU, *Pseudomonas*, Catecholate, Hydroxymate.**

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INTRODUCTION

Rhizosphere is a dynamic environment, which harbours diverse group of microorganisms. Some of the bacteria that directly or indirectly stimulate plant growth have been referred to as Plant Growth Promoting Rhizobacteria (PGPR)[1]. The direct promotion of plant growth involves mechanisms like nitrogen fixation, solubilization of phosphorous and iron from the soil, production of phytohormones like gibberellins, cytokinins and indole-3-acetic acid (IAA) which accelerates root growth[2]. Bacterial Chitinase, Siderophores, HCN etc. produced in the rhizosphere can indirectly support plant growth by suppressing hazardous effects of biotic stresses[3,4] and inhibition of phytopathogens[5]. Siderophores are low molecular weight, non-ribosomal peptides, secreted under low iron stress conditions and capture iron from the environment. Siderophores are also thought to facilitate biocontrol by sequestering iron from pathogens, thus limiting their growth[6,7,8]. Pseudomonas spp. have been employed efficiently as biocontrol agents and presently there are some commercial products available in the market, nevertheless, the applications of purified siderophores, as bacteriostatic or fungi static agents in combination with other antibacterial factors will certainly raise a great interest[9]. Iron is one of the most important microelements used by all microorganisms and necessary for their metabolism. Iron is naturally present in the environment, particularly in soils. However, its bioavailability is relatively low, which is connected with a dramatically decreased solubility of ferric species under physiological pH values owing to their complete hydrolysis. This has resulted in the development of special biologically regulated mechanisms of Fe (III) solubilization, e.g., involving specific natural low-molecular-weight chelating agents (siderophores) which transport iron (III) to the cell surface in the form of a complex, with further Fe (III) release from the latter in the course of its reductive assimilation[10]. Many microorganisms possess high affinity iron uptake system mediated by the action of low molecular weight iron chellators termed as siderophores[11,12]. The maximum use of PGPR creates an alternative way to replace chemical fertilizer, herbicides and other chemical supplements; most of the isolates result in a significant increase in height of plant, shoot and root length and dry matter production of shoot and root of plants. PGPR as a component in integrated management systems in which reduced rates of agrochemicals and cultural control practices are used as biocontrol agents. Selected strains of beneficial PGPR trigger a plant mediated induced systemic resistance (ISR) response that is effective against a broad spectrum of plant pathogens. The present study describes rhizospheric bacterial diversity of Tarai region of Uttarakhand, India. The rhizospheric isolates were evaluated for its potential of siderophore production and the most efficient isolates were optimized for siderophore production.

MATERIALS AND METHODS

Collection of soil sample

Rhizospheric soil samples of Kashipur area of district Udham Singh Nagar, a Tarai region of Uttarakhand, were collected and transported to laboratory under sterile conditions.

Isolation of rhizobacteria

Bacteria were isolated from soil by serial dilution technique on nutrient agar medium. Soil suspension was prepared by suspending approximately 1 gm of soil in sterile distilled water and vortexed. The suspension was serially diluted up to 10^{-8} and 100 µl of inoculum was plated in triplicate on nutrient agar and Pikovaskaya agar medium. Plates were incubated at 28˚C for 3 days. Well-isolated colonies were selected, purified and maintained on nutrient agar and Pikovaskaya agar media at 4°C and were used for further studies.

Screening for siderophore production

The isolated rhizospheric bacteria were screened for siderophore production using spectrophotometric method, which was further confirmed by CAS agar method and Universal Chemical Assay (CAS)[13,14].

Production, Detection and Estimation of Siderophore

The isolates were further evaluated for the quantity of siderophore produced. For siderophore production, iron free Succinate medium (SM)[15] consisting of g L^{-1}: K_2HPO_4, 6.0; KH_2PO_4, 3.0; $MgSO_4.7H_2O$, 0.2; $(NH_4)_2SO_4$, 1.0; and Succinic acid 4.0, pH 7.0 was used to inoculate 24 hrs old culture of selected soil isolate at the rate of 1% (v/v) inoculum. It was incubated for 24-48 hrs at 29ºC with constant shaking at 120 rpm. It was centrifuged at 10,000 rpm for 15 min and cell free supernatant was taken for detection and estimation of siderophores. Siderophore produced in culture broth was detected by CAS assay[16].

CAS-shuttle assay

Quantitative estimation of siderophore was done by CAS-shuttle assay[17,18] , in which 0.5 ml of culture supernatant was mixed with 0.5 ml of CAS reagent, and absorbance was measured at 630 nm (Shimadzu, Model-UV1800 ENG240V) against a reference consisting of 0.5 ml of uninoculated broth and 0.5 ml of CAS reagent. Siderophore content in the aliquot was calculated in terms of percent siderophore units (% SU) using the following formula

$$\% \text{ Siderophore Units} = \frac{Ar - As}{Ar} \times 100$$

Where, Ar = absorbance of reference at 630 nm (CAS reagent); As = absorbance of sample at 630 nm.

Morphological and biochemical characterization
The most efficient isolate was further characterized on the basis of its morphological and biochemical characterization. The morphological characteristics of the isolates included were cell shape, size and Gram's nature. The cultural characteristics studied were colony morphology and pigmentation, if any. The purified isolates were further characterized by biochemical tests, Hi 25 ^{TM} (Himedia) test kits for identification of organisms.

Optimization of Cultural Conditions
Siderophore production as a Function of Time
Each selected soil isolate was separately grown in SM (Succinate Medium) by submerged fermentation method with constant shaking of 120 rpm at 29ºC for 48 hrs. Samples were withdrawn after every 6 hrs interval and were estimated for siderophore production.

Effect of pH on siderophore production
The effect of pH at 3.0, 5.0, 7.0, 10.0 and 14.0 on siderophore production was studied on Succinate medium by adjusting the pH before inoculating the strain with 1 N HCl or 1N NaOH and keeping all other conditions constant.

Effect of Sugars and Organic Acids on siderophore production
The effect of different sugars and organic acids on growth and siderophore production was examined. In first set, 100 ml of Succinate medium was externally supplemented separately with 1 g L^{-1} or 0.1% each of glucose, dextrose, sucrose, fructose and mannitol. Second set of SM was individually supplemented with 4 g L^{-1} each of acetic acid and citric acid. Each set was separately inoculated with selected isolates and incubated. Following the 24 hours incubation at 28ºC and 120 rpm, each set was subjected for siderophore quantification.

Influence of amino acids on siderophore production
To observe the effect of amino acids on siderophore production, the Succinate medium was individually supplemented with 1 g L^{-1} or 0.1% of proline, histidine, lysine, arginine, cysteine, and tyrosine. Each set was separately inoculated with selected soil isolate and incubated. Each set was subjected to siderophore quantification after incubation at 28ºC for 14 hrs at120 rpm.

Influence of Iron on siderophore production
In order to determine the threshold level of iron at which siderophore biosynthesis is repressed in soil isolates under study; the cultures were grown in SM, externally supplemented with 1-100µM of iron ($FeCl_3$). Following the incubation at 29°C and 120 rpm, siderophore content were estimated.

Influence of Nitrogen sources on siderophore production
In this experiment, ammonium sulphate in SM was replaced separately by different concentrations of urea (commercial grade) in the range of 0.2, 0.4, 0.6, 0.8 and 1.0 g/L, and sodium nitrate, soy flour at the rate of 1.0 g/L. Siderophore production in this media was compared with that of SM containing ammonium sulphate.

Characterization of siderophore
Hydroxymate type of siderophore was determined by hydrolyzing 1ml supernatant of overnight grown culture with 1ml of 6N H_2SO_4 in a boiling water bath for 6h or 130°C for 30 min. Further, this hydrolysed sample was buffered by adding 3ml of sodium acetate solution. To this, 0.5ml iodine was added and allowed to react for 3-5 min followed by iodine was destroyed with 1 ml of sodium arsenate solution. Finally 1 ml α- napthylamine solution was added for appearance of colour. Wine red colour formation indicates production of hydroxymate type of siderophore[19], while catecholate type of siderophore was determined by adding 1 ml nitrite-molybdate to 1 ml of supernatant followed by 1 ml NaOH solution. Finally 1ml of 0.5 N HCL was added and allowed to develop colour. Yellow colour formation indicates production of catecholate type siderophore[20].

RESULTS

Total 35 rhizobacteria were isolated from rhizospheric soils of Tarai region and were recognized as PB series 1, 2, 3 and so on. These isolates were further screened for the siderophore production potential individually.

Screening for siderophore production
Siderophore production is one of the important traits of PGPR. In the same manner, the rhizospheric isolates were screened for their siderophore production potential and it was found that 8 isolates out of 35 isolates were positive for the siderophore production and were used for the further study. The positive isolates were PB2, PB7, PB10, PB16, PB19, PB26, PB29, and PB33. This was further confirmed by qualitative CAS test where instant decolourization of CAS reagent from blue to yellow or orange red was observed (Fig 1).

Production, detection and estimation of siderophore
Isolates selected on the basis of siderophore production by CAS test were further analysed by CAS–shuttle assay. The assay revealed that out of eight, three isolates were producing more than 60 % SU under iron starvation conditions in succinic acid medium. Isolate PB2, PB19, PB29 were found to produce 63%, 78% and 70% SU respectively after 48 hours of incubation. Maximum siderophore production was obtained in PB19 that was further characterized and subjected to optimization of siderophore production under *in vitro* conditions.

Morphological and biochemical characterization of most efficient isolate
It was found that the most efficient isolate was PB 19. It was Gram negative rod, arranged singly and was catalase, oxidase positive and showing growth at 5ºC as well as at 41ºC. On the basis of biochemical characterization, isolate PB19 was identified as *Pseudomonas* strain (Table 1).

Table 1
Biochemical characterization of PB19 bacterial isolate evaluated using the Hi^{25} enterobacteriaceae Himedia biochemical test kit

Tests	Result	Tests	Result
β-galactosidase activity	+	Esculin hydrolysis	+
Lysine utilization	+	Arabinose	-
Ornithine utilization	+	Xylose	-
Urease	+	Adonitol	-
Phenylalanine deamination	-	Rhamnose	-
Nitrate reduction	-	Cellobiose	-
H_2S production	-	Melibiose	-
Citrate utilization	V	Saccharose	-
Voges Proskauer's	-	Raffinose	-
Methyl red	-	Trehalose	-
Indole	-	Glucose	-
Malonate utilization	-	Lactose	-
Oxidase production	+		

Where + is positive test, - is negative test and V is 11-89% positive reaction.

Optimization of cultural conditions

Siderophore production as a Function of Time

The bacterial isolates did not exhibit any traces of siderophore synthesis during beginning hours of incubation whereas the bud in the vegetative growth was observed. Siderophore production by isolate PB19 was started after 10 hrs of inoculation. Optimum SU (70.43%) was observed at 30 hours of incubation and started decreasing beyond 42 hours of incubation (Fig 2).

Effect of pH on siderophore production

pH plays an important role in the solubility of iron and thereby its availability to the growing organism in the medium. Optimum siderophore yield of 81.95% was estimated at pH 7.0. With increasing pH (towards alkalinity), siderophore production was found decreasing (Fig 3).

Effect of Sugars and Organic Acids on siderophore production

Among the various sugars tested, glucose was found to have stimulatory effect on PB19 with SU 68.05% (Fig 4). All the sugars adversely affected the siderophore production. Among organic acids, citric acid was suitable for maximum siderophore production with SU 41.33% (Fig 5).

Effect of Amino acids on siderophore production

All the tested amino acids positively affected the siderophore production. 83.99% SU production was observed in lysine utilization, 80.36% SU in histidine, and 79.05% SU in cysteine (Fig 6).

Effect of Iron on siderophore production

Among the different iron concentration tested, the maximum siderophore production was observed at 20 µM (87.90% SU). Siderophore production was decreasing with increase in iron concentration (Fig 7).

Effect of nitrogen sources on siderophore production

Out of various nitrogen sources tested at different concentration, optimum siderophore yield of 88.61% siderophore units by PB19 was obtained in SM supplemented with urea at 0.6gL^{-1}. Urea was proved to be the best utilizable nitrogen source; it showed maximum siderophore units within a short period of incubation. However Soy flour did not favour siderophore production because of the fact that these nitrogen sources are rich in iron content (Fig 8).

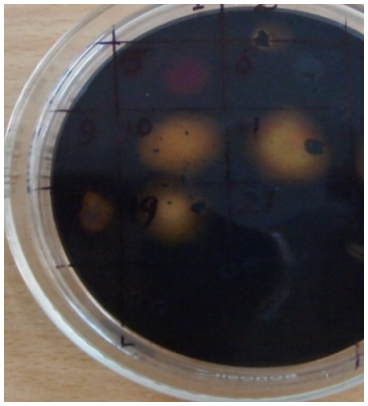

Figure 1
CAS Assay

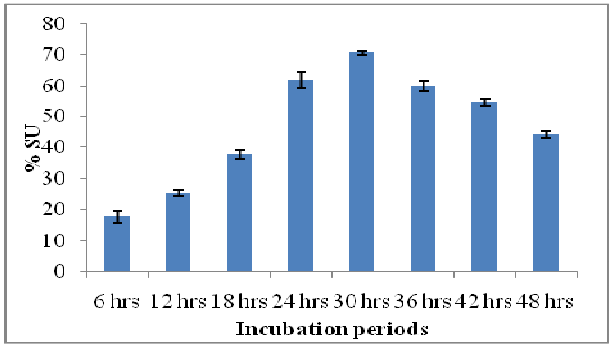

Figure 2
Effect of incubation period

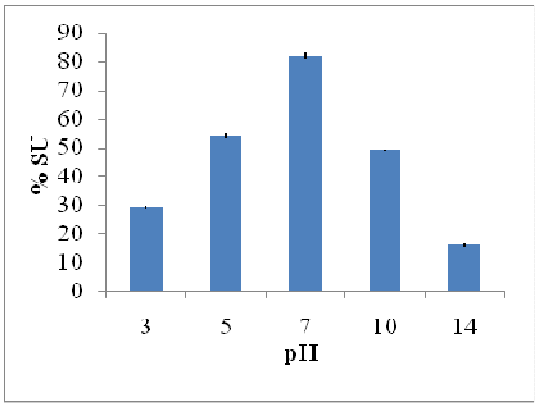

Figure 3
Effect of pH

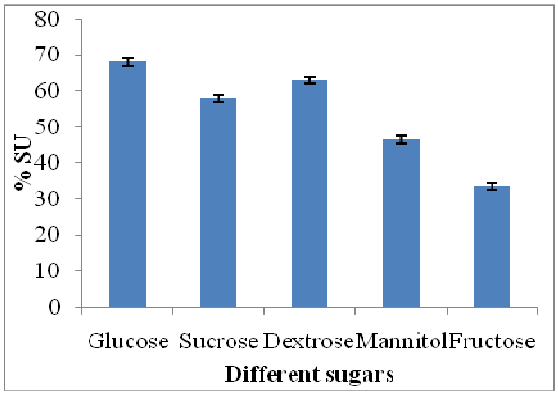

Figure 4
Effect of sugars

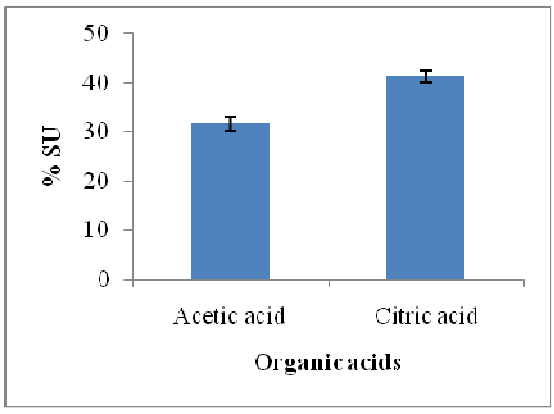

Figure 5
Effect of Organic acids

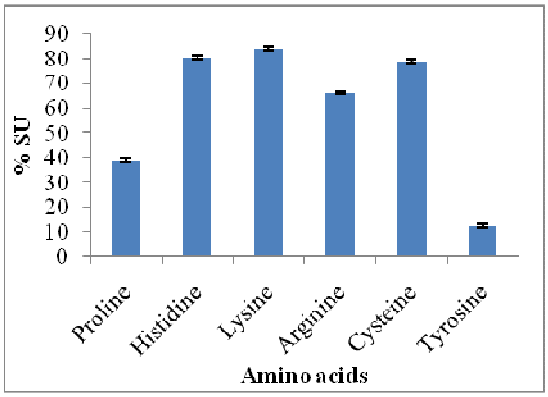

Figure 6
Effect of Amino acids

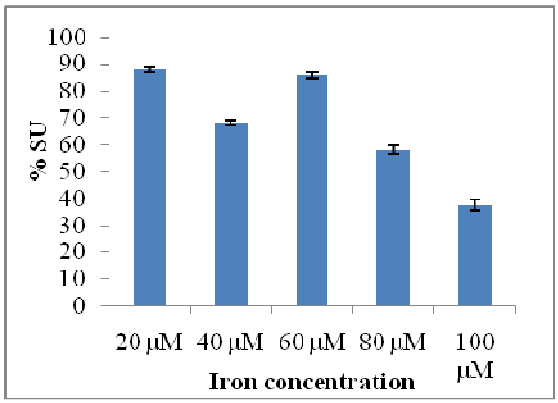

Figure7
Effect of Iron concentration

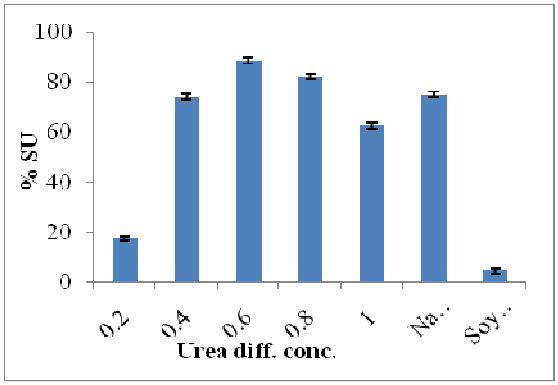

Figure 8
Effect of nitrogen sources

Characterization of siderophores

Isolate PB19 has showed both types of siderophore production i.e. hydroxamate type and catecholate or phenolate type (pyochelin) confirmed by wine red colour and yellow colour formation respectively in supernatant. The maximum siderophore production was detected in succinate medium as compare to other media and continues during the log phase in parallel with growth (Fig 9). This is due to pyoverdine, in which the 3-amino moiety of the chromophore is substituted with various groups derived from succinate, malate and α- ketoglutarate.

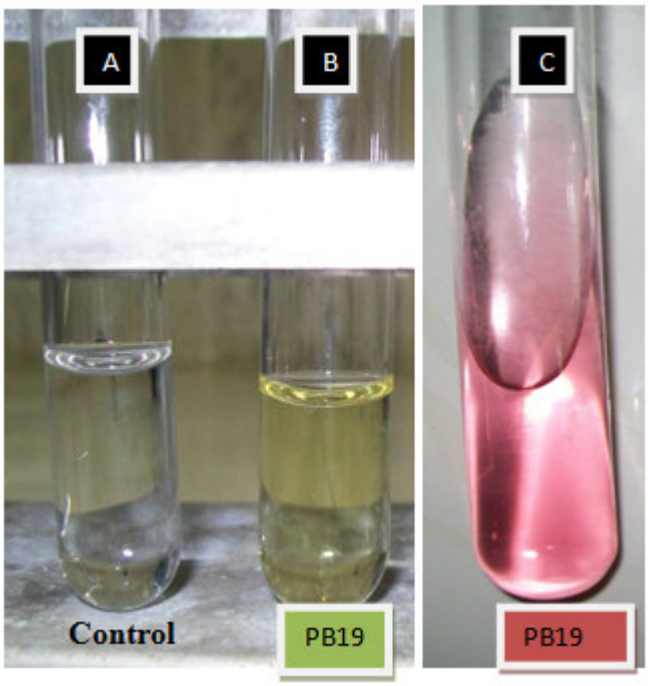

Figure 9

Tube A: Control Tube
Tube B: Yellow color showing Siderophore Production (Catchol type)
Tube C: Wine red color showing Siderophore Production (Hydroxymate type)

DISCUSSION

The rhizospheric bacterial isolates were isolated from the different areas of kashipur, Tarai region. They were identified on the basis of their microscopic, morphological and biochemical characteristics. Microscopic characteristics of the isolates showed that the isolates were Gram negative and confirmed as *Pseudomonas* spp. Siderophore production by *Pseudomonas* isolates were confirmed by color change from blue to orange in CAS plate assay. The color change from blue to orange resulting from siderophore is due to removal of Fe from the dye[21]. Siderophores production reached a maximal value with 150µ/ml Fe^{3+} siderophore production was maximum at this iron concentration. The succinate medium was found a suitable medium for high sidrophore production to achieving optimal iron concentration. Same result was observed by Raaska,[22] who examined siderophore detection in *Pseudomonas* spp growing cultures. Maximum siderophore production was 87.84% units. Meyer and Abdallah[15] had previously shown that the amount of pigment released per unit of cell mass was inversely related to the concentration of the factor limiting growth. Siderophores are low-molecular weight iron-binding compounds which are secreted under limited iron stress and found that iron concentration was inversely proportional to the siderophore, produced by the isolates[23]. At pH (7.0), maximum siderophore yield (81.95%) was obtained. Siderophore are very sensitive to high pH. This may be due to the fact that pH helps in excess solubilisation of ion, which increases the iron content of the medium[16,24]. Among the various sugars tested, glucose was found to have stimulatory effect (68.05% SU) On the contrary; all the sugars adversely affected the siderophorogenesis. All tested amino acids positively affected siderophore production. However, lysine resulted in the production of maximum siderophore units i.e. (83.99% SU) for selected isolate. The amino acid lysine resulted in the maximum siderophore units followed by histidine and cysteine. Among organic acids, citric acid was found suitable for optimum siderophorogenesis for isolate. Out of various nitrogen sources tested, optimum siderophore yield of 88.61% siderophore units by selected isolate was obtained in SM supplemented with urea at 0.6gL^{-1}. Both type of siderophores have been produced by the isolates i.e. hydroxamate type and catecholate or phenolate type confirmed on the basis of colour formation in supernatant. Most of the developing countries do not have the industrial development, which helps to subsidize high input agriculture in developed countries. So scientists are exploring new ways of meeting the nutritional needs of crop plant aiming at high productivity. Soil microorganisms like bacteria and fungi have a particular important role in exploration of these new approaches. One of the beneficial activities of the organism is the production of siderophores. So, siderophore producing organisms will be making the soil fertile and they also have antifungal activity against phytopathogens[25]. In order to satisfy their need to iron, microorganisms start to excrete large amounts of specific Fe3+ scavenging molecules (siderophores), when cells are grown under iron deficiency[26]. The Fe (III) siderophore complex is then transported into bacterial cell via cognate-specific receptor to enzymatic reduction[27,28]. Pyoverdine (PVD), the fluorescent siderophore produced by the rRNA group I species of genus *Pseudomonas*, constitutes a large family of iron chelators[29]. Microorganisms are able to siderophores production can protect themselves by toxic metals binding (Al, Pb, Cd,)[30,31]. Although essential metals

have important biological role, at high levels they can damage cell membranes, alter enzyme specificity, disrupt cellular functions, damage the DNA structure[32,33,34] and can reduce crop yields and soil fertility[35].

CONCLUSION

PGPR is increasingly used for crop improvement and protection. In the same context, present study was focused on isolation and characterization of bacterial isolates from Tarai region of Uttarakhand, India. Siderophore production was considered for the present study and the most efficient isolate was characterized and evaluated for optimum conditions for maximum siderophore production. The results are promising for design of potentially active siderophore producing PB19 strain based formulation which would be beneficial for crops improvement and crop protection. The potential of this strain could be investigated in detail and field application shall be studied for its biocontrol potential.

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CONFLICT OF INTEREST

Conflict of Interest declared none.

REFERENCES

1. Bloemberg GV and Lugtenberg BJJ. Molecular basis of plant growth promotion and biocontrol by rhizobacteria. Curr. Opin. Plant Biol, 4: 343-350, (2011).
2. Bhattacharya PN and Jha DK. Plant growth promoting rhizobacteria (PGPR): emergence in agriculture. World J Microbiol Biotechnol, 28 (1327): 1350, (2012).
3. Aeron A, Kumar S, Pandey P and Maheshwari DK. Emerging role of plant growth promoting rhizobacteria in agrobiology. In: Bacteria in agrobiology: crop ecosystems. Berlin Heidelberg: Springer, pp. 1–36, (2011).
4. Ribeiro CM and Cardoso EJBN. Isolation, selection and characterization of root-associated growth promoting bacteria in Brazil Pine (*Araucaria angustifolia*). Microbiol Res, 167 (2): 69–78, (2012).
5. Bashan Y and Holguin G. A proposal for the division of ‘‘plant growth promoting rhizobacteria’’ into two classifications: biocontrol-PGPB and PGPB. Soil Biol Biochem, 30: 1225–1228, (1998).
6. Linget C, Azadi P, MacLeod JK, Dell A and Abdallah MA. Bacterial siderophores: The structures of the pyoverdins of *Pseudomonas fluorescens* ATCC 13525. Tetrahedron Lett, 33 (2): 1737-1740, (1992).
7. Chiriani L, Tobacchioni S and Bevivino A. Interactions between rhizosphere microor-ganisms under iron limitation. Arch. Microbiol. 160 (7): 68-73, (1993).
8. Champomier-Veges, Mch A Stintzi and J. M. Mayer. Acquisition of iron by the non-siderophore producing *Pseudomonas fragi*, Microbiology. 142 (5): 1191-1199, (1996).
9. Dubuis C, Keel C and Haas D. Dialogues of root colonizing biocontrol pseudomonads. Eur J Plant Pathol, 119: 311– 328, (2007).
10. Kamnev AA, Kuzmann E, Perfiliev YD, Vértes A, Ristic′ D, Popovic′ S. and Musi S. Composite ferric oxyhydroxide containing phases formed in neutral aqueous solutions of tryptophan and indole-3-acetic acid. Journal of Radio analytical and Nuclear Chemistry, Vol. 246 (1): 123.129, (2000).
11. Lankford E. Bacterial assimilation of iron. CRC Critical Reviews in Microbiology, 2: 273-311, (1973).
12. Neilands JB. Development of Iron chelators for clinical Use, In: Martell, A.E., Anderson. W.J. and Badman, D.G.North (Eds). Elsevier 13 (3). Holland, Amsterdam (1981).
13. Jalal MAF, Love SK and van der Helm D. Siderophore mediated iron (III) uptake in Gliocladium virens. Properties of cis-fusari- nine, trans-fusarinine, dimerum acid and their ferric complexes. J. Inorg. Biochem. 28, 417-430, (1986).
14. Schwyn B and Neilands JB. Universal Chemical Assay for the detection and deter- mination of siderophores. Anal. Biochem. 140, 47-56, (1986).
15. Meyer JM and Abdallah MA. The florescent pigment *Pseudomonas fluorescens* Microbial 2: 217-230, (1978).
16. Schwyn R and Neilands JB. Universal chemical assay for detection and determination of siderophores. Anal Biochem 160: 47-56, (1987).
17. Payne SM. Detection, isolation and characterization of siderophores. Methods Enzymol, 235: 329-342, (1994).
18. Tank N and Saraf M. Salinity-resistant plant growth promoting rhizobacteria ameliorates sodium chloride stress on tomato plants. J Plant Interact, 5 (1): 5158, (2010).
19. Csaky T. On the estimation of bound hydroxylamine in biological material. Acta Chem Scand 2: 240-254, (1948).
20. Arnow LE. Colorimetric determination of the components of 3, 4-dihydroxyphenylalanine tyrosine mixtures. J Biol Chem, 118: 531- 537, (1937).
21. Wilhelmina MH, Potter AJ, Jennings MP, Jordi R, Hauser AR and Mc Ewan AG. J. Clinical Micriobiology. 42, 2806-2809, (2004).
22. Raaska L, Viikari L and T Mattila- Sandholm. Detection of siderophores in growing cultures of *Pseudomonas* spp. J IInd Microbiol 11: 181–186, (1993).

23. Budzikiewicz H. Secondary metabolites from fluorecent pseudomonads. FEMS Microbiol, Rev. 104, 209-228, (1993).
24. Olsen RA, Clark RB and Bennet JH. The enhancement of soil fertility by plant roots. AM Sci, 69: 378-384, (1981).
25. Girish PK, Shrikant SB, Sunil AM and Manish ND. Exploring the potential of *Pseudomonas* sps as phosphate solublizer plant growth promoter, Biocontrol agent and pesticide degrader. Asian.j.Exp.Biol.Sci.Spl. 40-41, (2010).
26. Braun V and Braun M. Iron transport and signalling in *Escherichia coli*. FEBS Lett., 529: 78-85, (2002).
27. Meyer JM. Pyoverdins: Pigments, siderohores and potential Taxonomic markers of fluorescent Pseudomonas species. Arch. Microbiol. 174: 135- 142, (2000).
28. Cornelis P and Matthijs S. Diversity of siderophore-mediated iron uptake systems in fluorescent pseudomonads: not only pyoverdines. Environ. Microbiol 4: 767-798, (2002).
29. Wahyudi AT, Indri R and Giyanto. Screening of *Pseudomonas* sp. Isolated from rhizosphere of soybean plant growth promoter and biocontrol agent. Am. J. Agric. Biol. Sci., 6: 134-141, (2011).
30. Mureseanu M, Renard G, Galarneau A and Lerner DA. A demonstration model for a selective and recyclable uptake of metals from water: Fe (III) ions complexation and release by a supported natural fluorescent chelator. Talanta, 60: 515-522, (2003).
31. Olmo AC, Caramelo and San Jose C. Fluorescent complex of pyoverdin with aluminum. J. Inorg. Biochem. 97: 384-387, (2003).
32. Bruins MR, Kapil S and Oehme FW. Microbial resistance to metals in the environment. Ecotoxicol. Environ. Safety, 45: 198-207, (2000).
33. Canovas D, Cases I and Lorenzo VD. Heavy metal tolerance and metal homeostasis in *Pseudomonas putida* as revealed by complete genome analysis. Environ. Microbiol. 5: 1242-1256, (2003).
34. Teitzel GM, Geddie A, De Long SK, Kirisits MJ, Whiteley M, and Parsek MR. Survival and growth in the presence of elevated copper: transcriptional profiling of copper-stressed *Pseudomonas aeruginosa*. J. Bacteriol. 188: 7242-7256, (2006).
35. Stuczynski TI, McCarty GW and Siebielec G. Response of soil microbiological activities to Cadmium, Lead and Zinc salt amendments. J. Environ. Qual., 32: 1346-1355, (2003).