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Siderophoregenic *Acinetobacter calcoaceticus* isolated from wheat rhizosphere with strong PGPR activity

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ABSTRACT

Thirty-two bacterial isolates were obtained from wheat rhizosphere in black cotton soils of North Maharashtra region and subsequently tested for *in-vitro* siderophore production. Wheat isolate SCW1, being a strong siderophore producer, was selected, identified and confirmed as *Acinetobacter calcoaceticus*. The strain produced catechol type of siderophores during exponential phase which was influenced by iron content of medium. Seed bacterization with siderophoregenic *A. calcoaceticus* improved plant growth in pot and field studies. Such PGPR activity was attributed to the ability of strain to solubilise phosphates and produce IAA. Siderophore mediated antagonism was observed against common phytopathogens *viz., Aspergillus flavus*, *A. niger, Colletotrichum capsicum* and *Fusarium oxysporum.*

Keywords: Acinetobacter, Siderophore, PGPR, wheat growth, phytopathogens

INTRODUCTION

Siderophores are low molecular weight bio-molecules secreted by micro-organisms in response to iron starvation for acquisition of iron from insoluble forms by mineralization and sequestration (Lankford, 1973). Although some siderophores are known to chelate other ions, their specificity and avidity for iron is the most consistent feature (Chincholkar *et al*., 2007a). Lost ability to synthesize siderophores lead to loss of ability to synthesize cyanide and thus marred biocontrol abilities of strain proves physiological importance of iron (Voisard *et al.,* 1989). Siderophores produced by rhizosphere inhabitants has been studied well and it has been reported that ability to produce siderophores not only improve rhizosphere colonization of producer strain but also play an important role in iron nutrition of plant (Vansuyt *et al.,* 2007) and antagonism against phytopathogens (Chincholkar *et al*., 2007b). Role of siderophores in induced systemic resistance (ISR) in plants was also well appreciated (De Meyer *et al*., 1999).

Such unequivocal importance of iron in plant growth-promotion and biological control encouraged us to screen new plant growth promoting rhizobacteria (PGPR) on the basis of its ability to produce siderophores to quench iron. Black cotton soil of North Maharashtra region is unique and known as elite soil for crops like cotton, wheat and banana. Long-standing cash crop like banana with its long big leaves and stem avoids exposure of sunlight to soil and maintains high moisture making the soil conductive for growth of microflora. Rotation of crops *viz.* cotton; gram, wheat and groundnut enrich and subsequently cherish the biological nature of the soil which forms an un-explored treasure.

This research has brought a strain in focus which is siderophoregenic and highly efficient plant growth promoter. Although identified as *Acinetobacter calcoaceticus*, which is a known opportunistic human pathogen and there is apprehension to use opportunistic human pathogens of non clinical origin lacking major pathological features like DNase and hemolytic activity as biocontrol agents; recent literature indicated that it could be reliable and relevant (Bevivino *et al*., 1994; Hebbar *et al.,* 1999).

MATERIALS AND METHODS

Materials

All media components were purchased from Hi-Media Laboratories Pvt. Ltd (India), unless otherwise specified in the text. Throughout the experimentation, analytical/ guaranteed reagent (AR/GR) grade chemicals from S. D. Fine chemicals. Pvt. Ltd (India) and ultra-pure water (Millipore, USA) were used. Glassware was cleaned with 6N HCl to remove residual iron and rinsed with pure water followed by drying. All growth media and reagents were handled carefully to avoid iron contamination.

Culture maintenance

Phytopathogens *viz. Aspergillus flavus*, *A. niger, Colletotrichum capsicum* and *F. oxysporum* were maintained on potato–dextrose agar (PDA) and the same

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medium was also used for checking antifungal activities of *Acinetobacter calcoaceticus*. Bacterial isolates obtained during this study were routinely cultivated on nutrient agar and preserved in 30% (v/v) glycerol at –86 °C for long-term.

Isolation from Rhizosphere

The sampling sites were located in Jalgaon district of North Maharashtra (Location, 21.05N; 75.40E; soil, black cotton type; annual rainfall, 700 mm; annual temperature, above 27.5 °C; commercial crops, banana, wheat, cotton and sugarcane). Healthy looking wheat plants in the vicinity of diseased plant were selected for sampling. After removal of plant from soil, root portion was cut and packed in sterile plastic bags. These bags were transported to laboratory under cold conditions for immediate processing. Adhering soil was carefully brushed off and plant roots were washed with sterile buffered saline ten times. These roots were then vigorously shaken with new batch of sterile buffered saline for 5–10 min. After serial dilution, 100 µL aliquots of desired dilution (10^{-4}–10^{-6}) were then platted in triplicate on nutrient agar. Plates were incubated at 28 °C for three days. Well-isolated colonies were selected, purified and maintained as specified above.

Screening for siderophore production

For inoculum development, a loopful culture of each isolate from nutrient agar slant was separately inoculated in 100 ml of low strength nutrient medium and incubated for 18 h at 28 °C on rotary shaker at 120 rpm. These grown cultures were inoculated (1% v/v inoculum) in sterile succinate medium (Meyer and Abdallah, 1978) separately and incubated on rotary shaker at 28 °C, 120 rpm. Supernatants of 36 h old cultures were tested for siderophore production by using Universal Chemical Assay comprising Chrom Azurol S (CAS) reagent as described by Schwyn and Neilands (1987). All positive cultures were compared for siderophore production ability as described by Payne (1994) where percent decolorization was calculated by using the following formula.

$$Percent \quad decolorization = \frac{Ar - As}{Ar} \quad X100$$

Where,
Ar = Absorbance of reference
As = Absorbance of sample at 630 nm.

Identification of selected strain

Selected strain was subjected to identification as per Bergey's Manual of Systematic Bacteriology (Juni, 1984) and 16S rDNA gene sequencing with eubacteria specific primer set 16F27N (5'-CCAGAGTTTGATCMTGGCTCAG-3') and 16R1525XP (5'-TTCTGCAGTC TAGAAGGAGGTGWTCCAGGC-3') (Pidiyar *et al.,* 2004). In order to check the sensitivity of the isolate towards different antibiotics, disc diffusion was done where culture was grown on Muller Hinton Agar in presence of antibiotic impregnated sterile filter paper discs dispensable with octa-disc dispenser (Hi-Media, Mumbai). After 48 h incubation of plate at 28 °C, either development of zone of inhibition was observed and interpreted as sensitive (S) or resistant (R).

Sideroanalysis

The presence of hydroxamate and catecholate type of iron chelating functional groups on siderophore molecules present in culture supernatant were detected by performing Csaky test (Csaky, 1948) and Arnow test (Arnow, 1937) respectively. Siderophore production by *A. calcoaceticus* as a function of time was studied by growing it in synthetic succinate medium at 28 °C and shaken at 120 rpm. Growth was monitored optically at 610 nm and siderophore production at 510 nm (Arnow, 1937) at an interval of 6 h up to 60 h. To get more siderophores, culture was grown in 12 L reactor (Navin Process systems, Pune) with 7 L succinate medium and incubated for 36 h with air 0.75 VVM, agitation 100 rpm at 28 °C. Biomass was separately harvested by centrifugation at 10, 000 rpm for 10 min at 4 °C. Siderophores in this cell-free supernatant were concentrated on HP-20 (Mitsubishi chemical corporation, Japan) column with a small modification of method described by Budzikiewicz (1993). Briefly, cell free supernatant was acidified to pH-6 with 12 M HCl and passed through HP-20 column at the rate 1-2 bed volumes per hour (40 x 2.5 cm). This was followed by washing consecutively with water, 50% methanol, 75 % methanol (@ 4-8 bed volumes per hour). For recovery of siderophores, column was eluted with 100% methanol (@ 1 bed volume per hour). Methanolic elute was concentrated on rotary evaporator at reduced pressure to obtain yellow-brown colored powder. Spectrum of solution of purified product in methanol was determined on UV-visible spectrophotometer (Nano-drop Technologies, USA). Moreover, pure product was subjected to Fourier Transform Infrared Spectroscopy (Test scan, Shimadzu FTIR 8000 series) in potassium bromide recording from 4000 to 500 per cm.

Phytopathogen inhibition

Phytopathogen inhibition by *A. calcoaceticus* was studied. A 100 µL of siderophore rich bioinoculants was spread on Czapek Dox agar medium amended with/without 50 µM of $FeCl_3$ separately. A 7 mm diameter mycelial disk taken from actively growing phyto-pathogens (grown on Czapek Dox agar) was placed at the centre. In the control supernatant sample was replaced by equal volume of sterile water. Plates were incubated at 28 °C and mycelial diameter was measured for 5 days. Pure siderophore of *A. calcoaceticus* in the range of 50–500 µL/mL was added in C-Dox agar medium separately and tested against phytopathogen.

Evaluation of PGPR activity of *A. calcoaceticus*

PGPR activity was evaluated in triplicate using wheat seeds (variety Lokvan). Seeds were surface sterilized with 0.1% $HgCl_2$ solution and washed three times with sterile distilled water, subsequently soaked for 1 h in siderophore rich bio-inoculants (10^8 cfu per mL) containing 0.5% CMC. Aseptic conditions were maintained throughout the process. After drying on surface sterilized polyethylene sheet under clean air, bacterized seeds were sown (8 seeds/pot) in pots containing black cotton soil (pH 7.3). Untreated seeds served as control. Pots were watered after every 48 h. Observations like rate of germination, root and shoot length, dry weight of roots and shoots were recorded after 28 days of sowing.

Large-scale field trial was carried out at Vadgaon, Dist Jalgaon in winter (November-February, 2005-2006) with different treatments of PGPR strains *viz. A. calcoaceticus* SCW1, *Pseudomonas putida* DFC31, *P. aeruginosa* ID 4365 (PAID), and P-suraksha (*Pseudomonas* sp.), *Azotobacter* sp. and PSB (Phosphate solublizing bacteria) a commercial formulation of International Panacea, Delhi were used to observe the influence on growth of wheat (variety: Lokvan Mahyco). Bacterized seeds as mentioned above were used for field trials on 5 acres of land. Data on shoot height, root length, chlorophyll in leaves after 50 days, grain yield after 120 days were recorded.

Detection of IAA synthesis and phosphate solubilization

Indole acetic acid (IAA) production was detected by a method described by Sharaf and Farrag (2004). Briefly, bacterial strain was grown for seven days in tryptophan (500 µg/mL) supplemented nutrient medium at 28 °C. A 2-mL aliquot of the supernatant was acidified with 2 drops of ortho-phosphoric acid and mixed with 4 mL of Salkowski's reagent (50 mL, 35% perchloric acid + 1 mL 0.5% $FeCl_3$) and allowed to stand at room temperature for 20 min for development of pink color. Phosphate solubilization by *A. calcoaceticus* was studied by growing the organism on Pikovskaya's agar (1948). After 72 h plate was observed for zone of clearance around bacterial growth.

RESULTS AND DISCUSSION

PGPRs are commonly used as inoculants for improving the growth and yield of agricultural crops, however screening for the selection of effective PGPR strains needs to be very critical. This study focuses on the screening for an effective PGPR strain(s) on the basis of their potential to produce siderophore *in-vitro* because of crucial role of iron in plant nutrition and plant growth promoting activity at large. Siderophoregenic strains have ability to mediate plant growth promotion directly as well as indirectly. Iron-siderophore complex is accepted by plants to quench iron thirst in soil, a direct mechanism, (Sharma and Johri, 2003; Vansuyt *et al.,* 2007) moreover chelation of soluble iron by microbial siderophores leads to growth inhibition of phytopathogen (Bano and Musarrat, 2003).

While performing isolation, only healthy plants in the vicinity of stunted plants were selected. During isolation of bacteria from these plants, loosely associated microflora was discarded as best PGPR strains are known to colonize root surface more intimately as shown by Glandorf *et al.* (1994). Moreover, fields having record of consistent high yield of cash crops were selected where probability of isolating better PGPR was high. Altogether thirty-two isolates were obtained from different rhizospheres. All isolates were screened for siderophore production as per Universal Chemical Assay given by Schwyn and Neilands (1987) and with its extrapolation, its comparison was done according to method given by Payne (1994). Out of thirty two strains, eight strains were able to grow in iron deficient synthetic medium and produced siderophores among which SCW1 showed maximum decolorization of CAS reagent (Figure 1). Instant conversion from blue to golden yellow color after reaction of cell free extract with CAS reagent confirmed production of siderophores with 58% de-colorization as compared to reference containing an equal amount of un-inoculated SM. After thrice repetition of this experiment with same outcome, strain SCW1 was selected for further studies. This strain, forming smooth, pale yellow colony on nutrient agar, was identified as *Acinetobacter calcoaceticus* as per Bergey's Manual of Systematic Bacteriology (Table 1) and the identity was confirmed by 16S rDNA gene sequencing method.

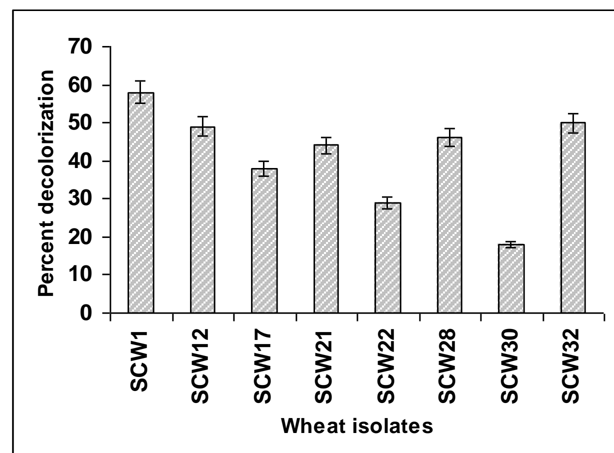

Figure 1: Percent de-colorization of CAS reagent by siderophore containing cell-free supernatant of various wheat isolates

Antibiotic sensitivity/resistance assay of isolate revealed that this strain is sensitive to amikacin, ampicillin, chloramphenicol, ciprofloxacin, colistin, gentamicin, netillin, norfloxacin, tobramycin, piperacillin, where as resistant to carbenicillin, ceftazidime and cephoxitin. Higher sensitivity of strain to clinical antibiotics is consistent with the fact that this is a rhizosphere isolate unlike multi-drug resistant

clinical isolates described in literature. Culture supernatant of *A. calcoaceticus* showed that Arnow's test gave a strong positive reaction, indicating the presence of the catecholate group on siderophores. However in Csaky's test the intensity of reaction was weak, indicating the presence of hydroxamate group on siderophores in the supernatant.

Table1: Biochemical properties of SCW1 (wheat isolate)

Biochemical test	**Result**
Gram Character	Gram negative
Shape	Short rods
Motility	Non-motile
<u>Enzyme production</u>	
Catalase	Positive
Urease	Negative
Oxidase	
Gelatinase	
<u>Pigment production</u>	
King's B medium	No pigmentation
King 's A medium	
Nitrate reduction	Negative
<u>H_2S production</u>	
Blood hemolysis	Negative
DNase	
<u>Carbon source utilization</u>	
Glucose	
Ribose	
Xylose	
Arabinose	
Citrate	Positive
Succinate	
Fumarate	
Pyroglutamate	
Lactate	
Adonitol	
Lactose	
Sorbitol	Negative
Mannitol	
Other PGPR Properties	Positive

Initiation of siderophore production in first quarter of exponential growth phase was indicated by the time function of siderophore production in succinate as well as lactate medium. Synthesis continues till the end of stationary phase and its catabolism initiates with initiation of death phase (Figure 2). *A. calcoaceticus* produced optimum siderophore at 36 h of incubation period.

Growth of cells was observed maximum at 24 h and 36 h in lactate and succinate medium respectively. However, growth and siderophore production occurred earlier in lactate medium indicating low generation time in lactate containing medium. Siderophore production was found to be inversely proportional to iron concentration (Figure 3) whereas growth was directly proportional to iron concentration. Biosynthesis of siderophores in this strain is suppressed completely at 40 µM although major suppression occurred at 20 µM which emphasized the need of iron and its availability to cells.

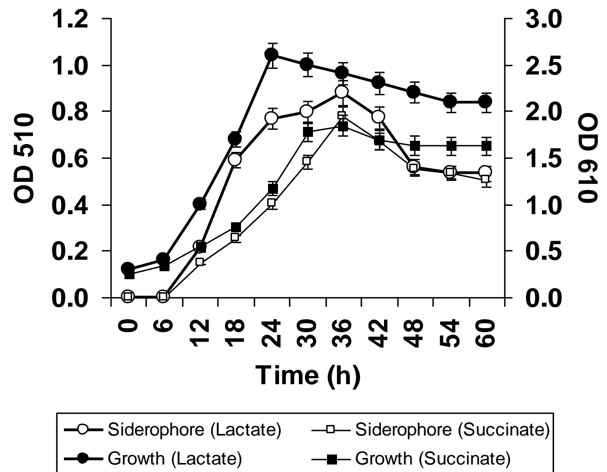

Figure 2: Comparison of *A. calcoaceticus* cell growth (610 nm) and siderophore synthesis (510 nm) in succinate and lactate containing medium as function of time

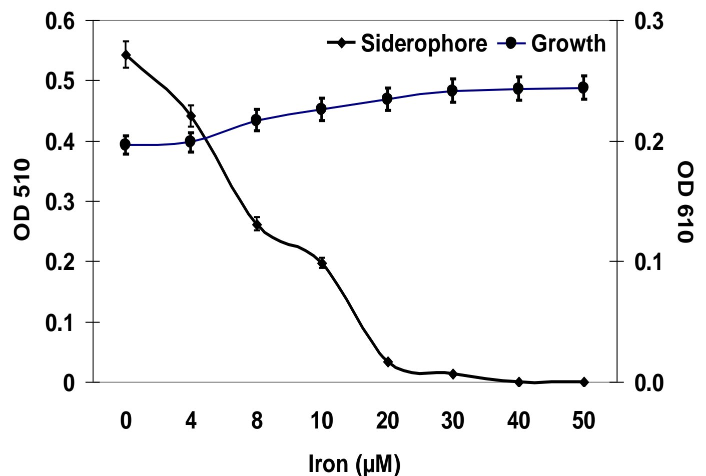

Figure 3: Influence of iron concentration on *A. calcoaceticus* cell growth (610 nm) and siderophore production (510 nm)

As described under materials and methods, catechol type of siderophore was isolated from supernatant of *A. calcoaceticus* and purified (60 mg/L) by using HP-20. Purified siderophore of *A. calcoaceticus* showed positive CAS test, Csaky's and Arnow's test confirming that it contains both of hydroxamate and catechol group.

In UV- visible spectrum, two peaks were observed; major peak at 250 nm while the minor peak at 317 nm (Figure 4) for sample in methanol which showed an absorption spectrum typical of catecholate compounds. These observations are in agreement with literature (Yamamoto *et al.,* 1994).

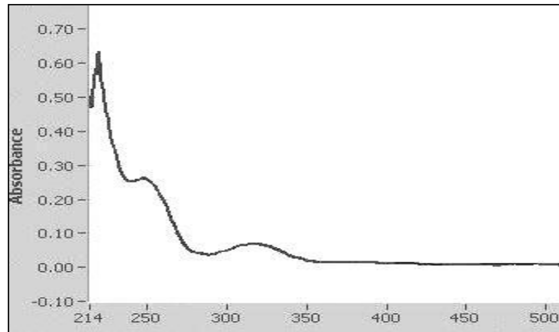

Figure 4: UV-Visible spectra of purified siderophore of *A. calcoaceticus* showing broad peak at 317 nm and narrow peak at 250 nm

Table 2: Bioassay of *Acinetobacter* sp. against various phytopathogens

Phytopathogen	Inhibition (%)	
	Absence of iron	Presence of iron
Aspergillus niger	42.00	24.00
A. flavus	28.57	14.29
Fusarium oxysporum	24.42	06.98
Colletotrichum capsicum	35.29	17.65

Values are averages from triplicates

Table 3: Influence of siderophore producing strain on wheat growth after 28 days

Treatment	Germ. (%)	Shoot		Root	
		Length (cm)	Dry weight (mg)	Length (cm)	Dry weight (mg)
Control	62.5	19.89 (2.58)	107 (4.04)	10.69 (2.51)	60.00 (1.52)
Test	87.7	20.43 (2.55)	133 (2.00)	15.51 (2.50)	67.66 (2.08)
% increase	40.32	2.71	24.29	45.08	12.76

Note: Values in parenthesis indicate standard deviation
Germ.; germination

FTIR spectrum of the pure siderophore of *A. calcoaceticus* showed that peak at 3462 cm^{-1} suggested the presence of phenolic Ar-OH group. Appearance of peaks at 1712 cm^{-1} and 1741 cm^{-1} indicated –C=N stretching frequency of imidazole and oxazoline respectively. The band at 1770 cm^{-1} was due to –C=O of N- hydroxyl amide group. While the peak at 1035 cm^{-1} and 1060 cm^{-1} revealed the presence of C–O–C for ether linkage of oxazoline. The over all UV-Visible and IR spectra data suggested the similarity between the structure of catecholate type of siderophore *i.e.* acinetobactin (Yamamoto *et al.,* 1994) and the siderophore sample of *A. calcoaceticus*.

As the present strain was isolated from rhizosphere of healthy wheat plant, antagonistic and PGPR potential of strain was anticipated. Antifungal activity of siderophore rich supernatant with and without iron in Czapeck-Dox medium, are presented in Table 2. Higher potency of supernatant as inhibitory in absence of iron than in presence of iron may be attributed to presence of siderophores.

Table 4: Field trial on Lokvan Mahyco of wheat

Treatment	50 days of growth			At harvest	
	Shoot Length (cm)	Root Length (cm)	Chlorophyll content (µg/gm)	Spiklets (gm/100)	Grain Yield Q/H
Control	66.33	9.23	14.82	256.37	26.70
P-suraksha$	73.00	11.24	24.09	287.19	32.55
PSB$	74.00	10.94	15.03	268.23	30.44
DFC31	74.33	13.06	19.97	319.07	34.45
PAID	76.67	10.83	22.15	277.63	33.72
AZO$	74.67	10.04	16.00	269.18	31.45
SCW1	77.67	14.25	26.83	334.00	40.25
F	3.49	44.58	14.01	256.13	2.92
P-value*	0.025	2.48×10^{-08}	3.33×10^{-05}	1.73×10^{-13}	0.046

Each value represents the mean of three replicates; *The difference in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference as P value is < 0.05 (F_{crit} 2.84)
DFC31 = P. putida; SCW1 = A. calcoaceticus; Azo$ (Azotobacter); P-suraksha$ (PGPR) and PSB$ (Phosphate solubilizing bacteria); PAID = P. aeruginosa ID 4365 (marine origin, NMU repository)
$ Commercial formulation of International Panacea, Delhi

Purified siderophore of *Acinetobacter calcoaceticus* at 500 μg/mL concentration inhibited growth of *Aspergillus niger* up to 30.00%, *A. flavus* up to 10.71%, *Colletotrichum capsicum* up to 21.57%, and *Fusarium oxysporum* up to 15.12% inhibition was observed. These results suggest that both siderophore rich supernatant as well as pure siderophore has the inhibitory potential against phytopathogenic fungi.

Influence of this strain on wheat growth (Table 3) showed 25.2% increase in the rate of germination, 45.08% and 12.76% in the root length and dry weight, respectively. Subsequently, 2.71% and 24.29% increase in the shoot length and dry weight respectively were observed over control. Results indicate that *Acinetobacter calcoaceticus* has growth promotion as well as phytopathogen suppression activities.

In field application, the treatments effect was found significant in respect of shoot length, root length, chlorophyll content in leaves, weight of spiklets and grain yield during field experimentation. Seeds treated with SCW1 followed by DFC31 and PAID supported for maximum growth promotion of plants as depicted in Table 4. The grain yield was highest with *A. calcoaceticus* (50.74%) followed by *P. putida* (29.02%) and *P. aeruginosa* (26.29%) over check without seed treatment. Commercial formulation *viz.* P-suraksha, PSB and AZO also significantly increased yield of grain by 21.91%, 14.0% and 17.79% respectively.

Use of siderophoregenic *A. calcoaceticus* improved crop growth and yield compared to other PGPR traits *viz.* P solubilization and N fixation under natural conditions indicates that siderophore production is an efficient parameter for preliminary selection of promising PGPR. Although siderophores are well known for contribution in plant growth promotion and biocontrol activity, other properties of strain also contribute to these feature positively. Considering this fact, further investigation in this direction revealed that like most other PGPRs, *A. calcoaceticus* has ability to synthesize IAA from tryptophan and solubilize tri-calcium phosphate. Previously, Huddedar et al., (2002) have described plasmid pUPI126 mediated indole 3 acetic acid (IAA) production in *Acinetobacter* strains from rhizosphere of wheat. Although, siderophore was dominant secondary metabolites in test organism, it was also capable of producing other metabolites such as IAA, phosphate solublizing organic acid in meager quantity.

Strains of *Acinetobacter calcoaceticus* have been previously isolated from rhizosphere (Sturz *et al*., 2001; Lappin *et al*., 1985). It is also observed that this strain lack of DNase and hemolytic activity (Table 1). This indicates that strain under study is distinct and different from siderophore producing clinical isolates of *Acinetobacter sp.* (Yamamoto *et al.,* 1994). These results are promising for the development of siderophoregenic plant growth promoting Acinetobacter based bio-fertilizer and bio-fungicide. To our knowledge this is first report on *A. calcoaceticus* as an efficient PGPR. The large-scale application of *A. calcoaceticus* to wheat plants as bioinoculant would be attractive as it would substantially reduce the use of chemical fertilizers and pesticides, which often pollute the environment.

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