Indian Journal of Experimental Biology
Vol. 58, February 2020, pp. 115-121

Heavy metal stress tolerance in *Enterobacter* sp. PR14 is mediated by plasmid

A Sagar[1], R Riyazuddin[1,3], PK Shukla[2], PW Ramteke[2] & RZ Sayyed[4]*

[1]Department of Industrial Microbiology; [2]Department of Biological Sciences, Sam Higginbottom University of Agriculture, Technology and Sciences, Allahabad, Uttar Pradesh-211 007, India
[3]Department of Plant Biology, University of Szeged, 6726 Szeged, Hungary
[4]Department of Microbiology, PSGVP Mandal's, Arts, Science & Commerce College, Shahada, Maharashtra-425 409, India

Received 26 May 2019; *revised 18 September 2019*

Last two decades have witnessed the significant exploitation of many plant growth-promoting rhizobacteria (PGPR) as bioinoculants and biocontrol agents (BCAs). However, PGPR with potential of producing multiple biocontrol traits along with heavy metal stress tolerance and ACC deaminase activity are expected to control phytopathogens and increase tolerance of crop to heavy metal stress, thus helping in bioremediation of heavy metal ions and reducing ethylene level in the root zone. The present work reports the production of multiple biocontrol traits like ammonia (NH_3), hydrogen cyanide (HCN), siderophore (79%), ACC deaminase (0.8 µM/mg/h), chitinase (9.7 U/mL) and tolerance to heavy metal ions (3200 µg/mL) and trace minerals in *Enterobacter* sp. PR14 isolated from the model organic farm of Sam Higginbottom University of Agriculture, Technology and Sciences (SHUATS), Allahabad, India. Elimination of plasmid in the organism resulted in the loss of tolerance of heavy metal ions and trace elements, indicating the role of the plasmid in heavy metal tolerance.

Keywords: PGPR, Biocontrol agents, Bioinoculants, ACC deaminase, Ammonia, Chitinase, HCN, Siderophore, Plasmid curing

Plant Growth Promoting Rhizobacteria (PGPR) based bioinoculants and biocontrol agents (BCAs) have gained significant importance as the best alternatives to traditionally used hazardous agrochemicals[1,2]. Understanding the fundamental mechanisms employed by PGPR should expedite their acceptance as the best suitable and effective adjuncts to agrochemicals.

PGPR control phytopathogens through the production of various metabolites like ammonia (NH_3), hydrogen cyanide (HCN), siderophores[3], ACC deaminase, and chitinase, etc[1,4]. Production of volatile compounds such as NH_3 is yet another indirect mechanism used by PGPR for plant growth promotion and biocontrol of phytopathogens[3,5,6]. Production of HCN is one of the important metabolites produced by PGPR to inhibit phytopathogens[7]. It is produced in Gram-negative bacteria as a secondary metabolite from glycine under the influence of HCN synthase[8]. Among the various volatile compounds, HCN is a well-studied metabolite, it's cyanide ion inhibits most metalloenzymes, especially copper-containing cytochrome c oxidases of phytopathogens[4], and hence HCN producing BCAs have been employed successfully to control phytopathogens.

Chitinase is a chitin-degrading enzyme that hydrolyzes the chitin present in the cell walls of fungal phytopathogens and plays an important role in the biocontrol of fungal pathogens and plant defense mechanisms[9,10].

Siderophores are low molecular weight iron-chelating compounds that chelate the iron and prevent the iron nutrition of phytopathogens[11-14]. Besides biocontrol, siderophore producing PGPR provides iron nutrition to crop plants[14].

The enzyme l-amino cyclopropane I carboxylate (ACC) deaminase helps tolerate drought and salt stress. The enzyme lowers the level of ACC, at this suboptimal level of ACC ethylene production in the plant roots is limited. Reduced level of ethylene increases root length, and larger roots absorb more nutrients, and thus increases crop yield[15,16].

Although many PGPR strains are known to produce one or more biocontrol and plant growth-promoting traits, a bacterium having potentials of producing array of these traits along with stress-tolerant hormone and capable of growing in presence of heavy metal ions can be a dynamic PGPR strain and needs to be explored fully, hence present work

*Correspondence:
E-mail: sayyedrz@gmail.com

was undertaken to isolate PGPR producing multiple biocontrol traits, stress-tolerant enzyme (ACC deaminase) and capable of growing under heavy metal ions stress, in addition, the role of plasmids in heavy metal tolerance by the organism was also undertaken.

Materials and Methods

Bacterial culture

Enterobacter sp. PR 14 was isolated from the model organic farm of SHUATS, Allahabad, India.

Identification of bacterial isolate

Phenotypic characterization

The isolate was identified based on its morphological and biochemical characteristics as per Bergey's manual of determinative bacteriology[17]. For this purpose, *Enterobacter* sp. PR 14 was cultured on pre-sterilized biochemical kits (Hi-Media, Mumbai, India) at 30°C for 24 h and observed for its ability to utilize different carbon sources.

16S rRNA sequencing

The sequencing of 16S rRNA genes of the isolate was carried out as per the method of Sambrook & Russel[18] by using Hi-PurA™ Plant Genomic DNA15. Miniprep purification spin kit (Hi-Media, Mumbai, India). The 16S rRNA sequences were analyzed with gapped BLAST (http://www.ncbi.nlm.nih.gov) search algorithm15. The closely related sequences were aligned by CLUSTAL-W using MEGA version 4.0 software and the evolutionary distances were computed using the neighbor-joining method and the isolate was identified on the basis of similarity of its 16S rRNA genes with that of the gene sequence of known organisms in NCBI database.

Screening and production of biocontrol traits

Screening for production of ammonia (NH_3)

Screening for the production of NH_3 was analyzed by growing the log culture of *Enterobacter* sp. PR14 in 10 mL of peptone water at 30°C for 48-72 h at 120 rpm followed by the addition of 50 µL of Nessler's reagent and observation of the development of colour yellow-brown colour as an indication of ammonia production[19].

Production of hydrogen cyanide (HCN)

For screening of HCN production *Enterobacter* sp. PR14 (5×10^5 cells/mL) was grown at 30°C for 4 days in King's medium amended with glycine (4.4 g/L) and Whatman filter paper No. 1 soaked in a mixture of sodium carbonate (2%) and picric acid (0.5%) solution placed at the top of the plate, and observed for development of yellow to red colour on the filter paper[20].

Screening for production of siderophore

The ability of *Enterobacter* sp. PR14 to produce siderophore was checked by growing it (5×10^5 cells/mL) on chrome azurol sulphonate (CAS) agar plate containing g/L, K_2HPO_4, 3.0; KH_2PO_4, 2.0; $MgSO_47H_2O$, 0.2; NH_4SO_4, 1.0; succinic acid, 4.0; agar, 2.5 and CAS reagent at 30°C for 48-72 h[21] and observed for development of yellow-orange halo around the growth of *Enterobacter* sp. PR14[22].

For siderophore production, *Enterobacter* sp. PR14 (5×10^5 cells/mL) was grown in at 30°C at under shaking (120 rpm) for 48-72 h. After incubation, the broth was centrifuged at 10000 rpm for 20 min[21] and the detection of siderophore from the cell-free supernatant was carried out by CAS test[23]. Quantitative estimation of siderophore was carried out by CAS shuttle assay[23] and the amount of siderophore present in the broth was expressed as % siderophore units (SU)[24].

Production of ACC deaminase

For detection of ACC deaminase activity of *Enterobacter* sp. PR14, it was grown on minimal medium (MM) containing g/L, KH_2PO_4, 2.0; K_2HPO_4, 0.5; $MgSO_4$, 0.2 and glucose, 0.2; ACC, 0.3 or $(NH_4)_2SO_4$, 0.19 at 30°C for 24-72 h and observed for the appearance of bacterial growth[25]. ACC deaminase activity was measured by growing the isolate in MM containing 5 mM of $(NH_4)_2SO_4$ as a substitute of ACC at 30°C at 200 rpm for 48-72 h to induce the production of ACC deaminase. Following the centrifugation of broth, ACC deaminase activity was estimated by measuring the production of α-ketobutyrate generated by the cleavage of ACC[26]. The amount of α-ketobutyrate produced was measured from the calibration curve prepared with α-ketobutyrate (0.1 and 1.0 µM). The ACC deaminase activity was expressed as the amount of α-keto-butyrate produced per mg of protein per h[26].

Screening for production of chitinase

Screening for chitinase production was carried by growing *Enterobacter* sp. PR14 (5×10^5 cells/mL) on MM containing colloidal chitin, 0.5%; Na_2HPO_4, 0.2%; KH_2PO_4,0.1%; NaCl, 0.05%; NH_4Cl, 0.1%; $MgSO_4.7H_2O$, 0.05%; $CaCl_{27}H_2O$, 0.05%; yeast extract, 0.05% at 30°C for 4-6 days, and observed for zone of chitin hydrolysis around the colony[27].

Chitinase activity was determined by incubating 1 mL of culture supernatant with 1 mL of 1% colloidal chitin in 0.05 M phosphate buffer, (pH 7.0) at 30°C for 1 h[28]. After centrifugation of the reaction mixture, the amount of N-acetyl-d-glucosamine (GlcNAc) released in the supernatant was determined as per the method of Miller[29] using GlcNAc as a standard (100-1000 µM). One unit of chitinase activity was defined as the amount of enzyme that liberated 1 µMo f GlcNAc per h under the conditions of the study. Protein estimation was carried out as per Lowery *et al.*[30].

Tolerance to heavy metals and trace elements

The ability of the isolate to tolerate heavy metals and trace elements was detected by agar diffusion method[23]. For this purpose each nutrient agar (NA) was separately amended with 0.6 to 3200 µg/mL concentration of various soluble heavy metals like Cr, Pb, As, Ag, Au, Hg and trace elements like Al, Zn, Mo, Mn, Cu, Ni and separately grown with log culture of isolate at 30°C for 48 h. Plates were observed for the presence or absence of growth and degree of growth was taken as a degree of tolerance or resistivity to heavy metal ions.

Plasmid curing

A log culture of *Enterobacter* sp. PR14 was grown in Luria-Bertani (LB) medium containing 100 µg/mL of acridine orange (mutagenic agent) solution at 30°C for 24 h followed by dilution (1:100) with fresh LB containing sub-lethal concentrations of markers, such as penicillin (10 µg/mL), tetracycline (5 µg/mL) and chloramphenicol (10 µg/mL), and grown at 30°C for 30-45 min under shaking at 100 rpm to ensure expression of antibiotic resistance genes followed by addition of penicillin G (104 U/mL) and shaking for 1.5 h, surviving cells were harvested by centrifugation (10000 rpm for 10 min), suspended to the same volume in saline, and plated on non-selective LB agar for 24 h at 30°C, the colonies were then replica plated onto LB agar containing specific antibiotic (penicillin) at inhibitory concentrations and re-incubated for 24 h at 30°C. Other antibiotic markers (tetracycline and chloramphenicol) were also tested to make sure the loss of plasmid DNA and that a mutation had not occurred[31].

Tolerance to heavy metals and trace elements after plasmid curing

The cured cells were grown on the NA agar sseparately supplemented with various metal ions by agar diffusion method as mentioned above[22]. Each plate was incubated at 30°C for 48 h and observed for the ability of *Enterobacter* sp. PR 14 to tolerate and grow in the presence of heavy metal ions. The presence or absence of growth was taken as an indication of plasmid-mediated resistance or sensitivity due to loss of the plasmid.

Statistical analysis

Data obtained from each experiment were statistically analyzed using mean, analysis of variance, and Tukey's multiple comparison tests. Values of $P \leq 0.05$ were taken as statistically significant[32].

Results

Identification of bacterial isolate

Phenotypic characterization

In the preliminary characterization according to Bergey's manual of determinative bacteriology, all the morphological and biochemical characteristics of isolate closely resembled the characteristic features of *Enterobacter* sp. Thus, the isolate was preliminarily identified as *Enterobacter* sp.

16S rRNA sequencing

The comparison of BLAST search of 16S rRNA gene sequences of the isolate with 16S rRNA gene sequences of NCBI GenBank database and phylogenetic analysis revealed 100% similarity and evolutionary relatedness of the isolate with *Enterobacter* sp. (Fig. 1). Thus, it was identified as *Enterobacter* sp. The gene sequence of the isolate was submitted to NCBI GenBank under the name *Enterobacter* sp. PR14 with accession number KP226570.

Production of biocontrol traits

Production of NH_3

Addition of Nessler's reagent in peptone water broth inoculated with *Enterobacter* sp. PR14 resulted

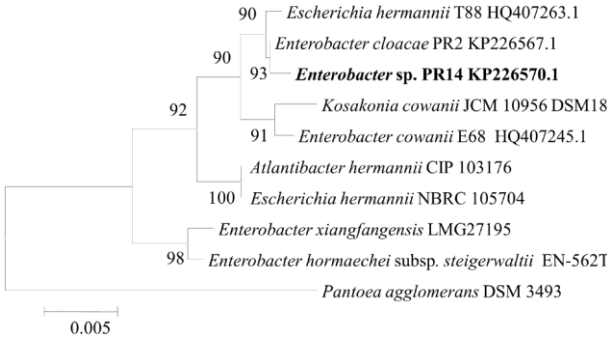

Fig 1 — Phylogenetic analysis of *Enterobacter* sp. PR14 based on 16s rRNA gene sequence drawn using the neighbor-joining method (MEGA 5.0 software) with evolutionary distances computed using Kimura's two-parameter method showing the relationship of PHB depolymerase producing bacteria with the validly published sequences of related genera.

in the formation of yellow colour indicating the production of the copious amount of ammonia by *Enterobacter* sp. PR14.

Production of HCN

Growth of *Enterobacter* sp. PR14 on modified King's B agar plate resulted in a change in the colour of HCN paper from yellow to brown. This indicated the production of HCN by the isolate.

Production of siderophore

After 30 h growth of the organism on CAS agar, the colour of CAS agar changed from blue to orange, while the colour of un-inoculated CAS agar (reference) remained unchanged. This indicated the ability of *Enterobacter* sp. PR14 to excrete siderophore. The addition of cell-free supernatant to CAS solution also changed the blue colour of CAS to orange colour while the colour of un-inoculated SM (reference) remained unchanged. CAS agar test indicated the siderophore producing ability of the isolate and CAS test confirmed the result of CAS agar. The amount of siderophore produced during 30 h submerged growth of *Enterobacter* sp. PR14 in SM was 79% SU.

Production of ACC deaminase

Growth of isolate on MM containing ammonium sulfate indicated its ability to secrete ACC deaminase and utilize ammonium sulfate as a substitute of ACC. After 48 h growth, the isolate produced ACC deaminase activity of 0.8 μM α-ketobutyrate mg/h.

Production of chitinase

Good growth of *Enterobacter* sp. PR14 was observed on MM containing colloidal chitin as the only source of carbon; which reflected the ability of the isolate to excrete chitinase that hydrolyzed the chitin present in the medium. The submerged growth of the organism in MM yielded an optimum chitinase activity of 9.7 U/mL with 657 μg/mL protein content.

Tolerance to heavy metals and trace elements

Enterobacter sp. PR14 grew well in the presence of a variety of heavy metals and trace elements. While in the case of heavy metal ions the isolate could tolerate high concentration (400 μg/mL) of Pb (Fig. 2), whereas other heavy metals viz. Au and Hg inhibited the growth of the isolate. Among the trace elements, the highest threshold level (3200 μg/mL) was observed with Al followed by Zn and Mo while minimum (50 μg/mL) tolerance was observed with Cu (Fig. 2).

Discussion

Production of volatile compounds such as ammonia is yet another indirect mechanism followed by PGPR for plant growth promotion and phytopathogens suppression[3]. Joseph *et al.*[33] have also reported ammonia production in PGPR associated with chickpea. Dutta & Thakur[34] have reported ammonia production in various species of *Enterobacter* isolated from the tea rhizosphere. The production of volatile compounds has been reported in several species of PGPR. Production of ammonia helps in plant growth promotion and biocontrol or BCA[11]. The inhibition of fungal pathogens' growth by volatiles produced by bacteria or fungi is well reported[10].

In HCN test, change in the colour of filter paper from yellow to brown-red is due to the secretion of HCN that reacts with picric acid solution amended on the filter paper[11,35]. Shaikh *et al.*[36] have also reported a similar colour change during HCN production in *Pseudomonas* sp. isolated from the banana rhizosphere. HCN is one of the several volatile compounds produced by a wide variety of biocontrol strains of *P. aeruginosa* and various other PGPR[11]. Production of HCN in the rhizosphere has been reported as one of the major metabolites of PGPR to inhibit the growth of phytopathogens and pests[3,4]. Rijavek & Lapanje[35] have reported HCN producing *Pseudomonas* sp. CHAO as a BCA against fungal plant pathogens and found that HCN is involved in metal tolerance and phosphate nutrition of PGPR through phosphate solubilization.

In siderophore test, change in the colour of CAS agar and CAS solution is due to the chelation of iron from HDTMA of CAS by siderophore present in the broth[22]. The maximum production of siderophore in

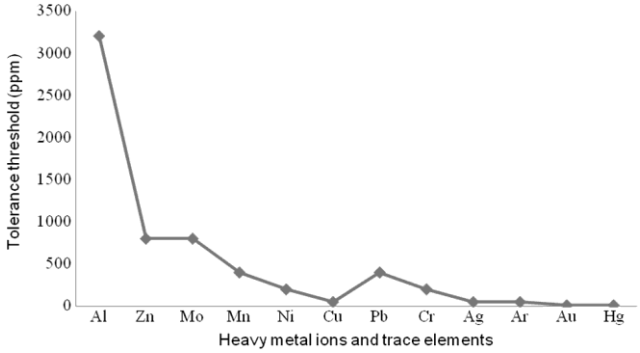

Fig 2. — The threshold level of tolerance of various heavy metal ions and trace elements in *Enterobacter* sp. PR14 as determined by agar diffusion method on each nutrient agar separately amended with various soluble heavy metals and trace elements at the concentration ranging from 0.6 to 3200 μg/mL.

SM is attributed to the iron-free nature of the medium. Siderophore production as a means of iron solubilization, prevention of iron nutrition of host and other metal ion chelators have been reported in a wide variety of PGPR including *Enterobacter* sp.[34,36]. Siderophore excreted in the rhizosphere region of the plant provide iron nutrition to the plant[29] serve as the first defense against root invading parasites[12] and helps in removing toxic metals from polluted soil[29].

Production of ACC deaminase is due to the presence of ACC in minimal medium, it is one of the important mechanisms of stress tolerance, and many PGPR are known to alleviate the effects of stress through excretion of ACC deaminase[1]. ACC exuded by the plant is degraded by the action of ACC deaminase secreted by PGPR present on/in plant roots or in the rhizosphere[37]. Kruasuwan & Thamchaipenet[38] have reported the ACC deaminase activity of 0.1 μM α-ketobutyrate mg/h in endophytic *Enterobacter* sp. EN-21 and have observed the bacterium to be effective in salt stress tolerance in sugarcane. Gupta & Pandey[39] have also reported ACC deaminase activity of 1.5 μM α-ketobutyrate mg/h in various strains of PGPR and found these strains effective in alleviating salt stress in French bean.

Excretion of chitinase in minimal media containing colloidal chitin is in response to chitin that induces the production of chitinase. The yield of chitinase produced by *Enterobacter* sp. PR 14 is higher than the earlier reports. Shaikh *et al.*[40] have reported 1.5 U/mL of chitinase in *S. maltophilia* after 48 h of submerged growth in MM. Higher yields of chitinase can be attributed to more induction of chitinase synthesis in the presence of colloidal chitin this reflects the metabolic potential of the isolate. Woo & Park[41] have demonstrated chitinase production in response to chitin or chitosan induction in *Bacillus* sp. Dahiya *et al.*[42] have reported antifungal chitinase from *Enterobacter* sp. NSG 4. Chitinase producing PGPR are known to lyse fungal cell wall through degradation of chitin polymer of the fungal cell wall and hence are of crucial importance in biocontrol of fungal phytopathogens[6,7].

Growth of *Enterobacter* sp. PR14 in presence of heavy load of metal ions is attributed to its ability to produce siderophore and ACC deaminase. Siderophore has been reported to chelate a variety of metal ions[22,43]. Higher tolerance towards aluminum may be due to the ability of an organism to accumulate aluminum. While lower tolerance to copper may be due to the bactericidal effect of copper. Behnsen & Raffatellu[12] have reported that siderophore binds to iron as well as non-iron metal ions such as Cu and Zn and is responsible for making the PGPR strain resistant to the toxicity of Cu and overcome the limitations of Zn. Sayyed *et al.*[43] have also reported the ability of *Enterobacter* sp. RZS5 to grow at elevated levels of different metal ions. Rijavec & Lapanje[35] have reported metal tolerance in HCN producing *Pseudomonas* CHAO.

Rhizobacteria are known to produce a variety of mechanisms to tolerate various types of stresses, ACC deaminase is one such enzyme[44]. Besides this, the other possible mechanisms of stress tolerance include the presence of metal uptake systems and the production of bio-surfactants and exopolysaccharides, etc.[29]. Good growth in the presence of elevated levels of heavy metal ions has been reported in *Enterobacter* sp. PR14. Sayyed *et al.*[14,43] have reported pronounced growth and production of various metabolites in *Enterobacter* sp. RZS5 in the presence of higher levels of various metal ions.

Absence of DNA bands in cured cells of *Enterobacter* sp. PR14 indicated the complete loss of plasmid DNA. The total CFU after curing was zero confirming the absence of viable bacteria either in the initial population or after curing[45]. The absence of growth of cells devoid of plasmid indicated the loss of the ability of an organism to resist the toxic effects of heavy metal ions, thus confirming the heavy metal resistivity as a plasmid-borne character[46].

Conclusion

Although a large number of PGPR strains are known to secrete an array of plant-growth-promoting and biocontrol traits, only those PGPR which could produce a variety of these traits along with stress-tolerant hormone and are able to grow at higher levels of various heavy metal ions can be regarded as dynamic PGPR. We report production of various biocontrol traits and stress tolerating enzyme; ACC deaminase in heavy metal resistant *Enterobacter* sp. PR 14 further the heavy metal tolerance of the isolate was confirmed as a plasmid-mediated character. The results of this study demonstrated the potential of *Enterobacter* sp. PR14 to produce multiple biocontrol traits, such as NH_3, HCN, chitinase, siderophore, and ACC deaminase, besides its tolerance to higher levels of various heavy metal ions, makes it a potential BCA, a good candidate for stress tolerance and best

tool for bioremediation of heavy metal contaminated soil, and thus can be used as a best sustainable alternative to recalcitrant inorganic fertilizers, chemical fungicides and chemical ion chelators. This potential of *Enterobacter* sp. PR14 could be further explored for enhancement of its biocontrol activity and tolerance to abiotic stresses such as heavy metals and trace elements. This multi-potent isolate can be used as stress and heavy metal tolerating bioinoculant-cum-BCA and as an agent for bioremediation of heavy metal polluted agricultural soil.

Acknowledgment

Authors acknowledge Dr. Thomas Abraham, SHUATS, Allahabad, India for providing the soil samples of an organic farm for the present study, and also Dr. Noshin Ilyas, PMAS University of Arid Agriculture Rawalpindi, Pakistan for language and editing.

Conflict of Interest

All authors declare no conflicts of interest.

References

1 Sayyed RZ, N Ilyas, B Tabassum, A Hashem, EF Abd_Allah & HP Jadhav, Plausible role of plant growth-promoting rhizobacteria in future climatic scenario, In: *Environmental Biotechnology: For Sustainable Future*, (Ed. Sobti RC, Arora NK & Kothari R; Springer, Singapore), 2019 pp. 175.

2 Glick BR, Bacteria with ACC deaminase can promote plant growth and help to feed the world. *Microbiol Res* 169 (2014) 30.

3 Kumar P, Thakur S, Dhingra GK, Singh A, Pal MK, Harshvardhan K, Dubey RC & Maheshwari DK, Inoculation of siderophore producing rhizobacteria and their consortium for growth enhancement of wheat plant. *Biocatal Agric Biotechnol*, 15 (2018) 264.

4 Mukhtar S, Mehnaz S, Mirza MS & Malik KA, Isolation and characterization of bacteria associated with the rhizosphere of halophytes (*Salsola stocksii* and *Atriplex amnicola*) for production of hydrolytic enzymes. *Braz J Microbiol*, 1 (2019)13.

5 Melo J, Carolino M, Carvalho L, Correia P, Tenreiro R, Chaves S, Meleiro AI, de Souza SB, Dias T, Cruz C & Ramos AC, Crop management as a driving force, of plant growth-promoting rhizobacteria, Physiology. *Springerplus*, 5 (2016) 1574.

6 Sayyed RZ & Patel PR, Biocontrol potential of siderophore based heavy metal resistant *Alcaligenes* sp. And *Acinetobacter* sp. vis-à-vis organophosphorus fungicide. *Indian J Microbiol*, 51 (2011) 266.

7 Singh RP & Jha PN, The PGPR *Stenotrophomonas maltophilia* SBP-9 augments resistance against biotic and abiotic stress in wheat plants. *Front in Microbiol*, 8 (2017) 1.

8 Goswami D, Thakker JN & Dhandhukia PC, Portraying mechanics of plant growth-promoting rhizobacteria (PGPR): A review. *Cogent Food Agric*, 2 (2016) 1127500

9 Dutta J, Handique PJ & Thakur D, Assessment of culturable tea rhizobacteria isolated from tea estates of Assam, India for growth promotion in commercial tea cultivars. *Front Microbiol*, 6 (2015) 1252. doi: 10.3389/fmicb.2015.01252

10 Jadhav HP, Shaikh SS & Sayyed RZ, Role of hydrolytic enzymes of rhizoflora in biocontrol of fungal phytopathogens: An overview In: *Rhizotrophs-Plant Growth Promotion to Bioremediation*, (Ed. Samina Mehnaz, Springer-Nature Publication, Singapore) 2017, pp. 183.

11 Wani SJ, Shaikh SS & Sayyed RZ, Statistical-based optimization and scale-up of the siderophore production process on laboratory bioreactor. *3 Biotech*, 6 (2016) 69.

12 Behnsen J & Raffatellu M, Siderophores: More than stealing iron. *mBio*, 7 (2016) e01906, doi:10.1128/mBio.01906-16.

13 Reshma P, Naik MK, Aiyaz M, Niranjana SR, Chennappa G, Shaikh SS & Sayyed RZ, Induced systemic resistance by 2,4 diacetylphloroglucinol positive fluorescent *Pseudomonas* strains against rice sheath blight. *Indian J Expl Biol*, 56 (2016) 207.

14 Sayyed RZ & Chincholkar SB, Chemical characterization, cross-feeding and iron uptake studies on hydroxamate siderophores of *A. faecalis*. *Indian J Microbiol*, 51 (2011) 176.

15 Trung NT, Hieu HV & Thuan NH, Screening of strong 1-aminocyclopropane-1-carboxylate deaminase producing bacteria for improving the salinity tolerance of cowpea. *App Microbiol*, 2 (2016) 1.

16 Han Y, Wang R, Yang Z, Zhan Y, Ma Y, Ping S, Zhang L, Lin M & Yan Y, 1-aminocyclopropane-1-carboxylate deaminase from *Pseudomonas stutzeri* A1501 facilitates the growth of rice in the presence of salt or heavy metals. *J Microbiol Biotechnol*, 25 (2015) 1119.

17 Holt JG, Krieg NR, Sneath PHA, Staley JT & Williams ST, *Bergey's Manual of Determinative Bacteriology*, 9th edn. (Williams and Wilkins Co, Baltimore), 1994, pp. 272.

18 Sambrook J & DW Russel, Molecular Cloning: A Laboratory Manual, 3rd ed. (Cold Spring Harbor Laboratory Press, New York, USA), 2001, pp. 94.

19 Dey R, Pal KK, Bhatt DM & Chauhan SM, Growth promotion and yield enhancement of peanut (*Arachis hypogaea* L.) by application of plant growth-promoting rhizobacteria. *Microbiol Res*, 159 (2004) 371.

20 Castric P, Hydrogen cyanide, a secondary metabol*ite of Pseudomon*as *aeruginosa*. *Can J Microbiol*, 21 (1975) 613.

21 Meyer JM & Abdallah MA, The Fluorescent pigments of *Fluorescent Pseudomonas*: Biosynthesis, purification and physicochemical properties. *Microbiology*, 107 (1978) 319.

22 Patel PR, Shaikh SS & Sayyed RZ, Modified chrome azurol S method for detection and estimation of siderophores having affinity for metal ions other than iron. *Environ Sustain*, 1 (2018) 1.

23 Schwyn B & Neilands JB, Universal chemical assay for the detection and determination of siderophores. *Anal Biochem*, 160 (1987) 46.

24 Payne SM, Detection, isolation, and characterization of siderophores In:*Methods Enzymol* (Ed. Clark VL & Bovill PM, Academic Press, New York), 1994, 329.

25 Safronova VI, Stepanok VV, Engqvist GL, Alekseyev YV & Belimov AA, Root-associated bacteria containing 1-aminocyclopropane-1-carboxylate deaminase improve growth and nutrient uptake by pea genotypes cultivated in cadmium supplemented soil. *Biol Fertil Soils*, 42 (2006) 267.

26 Penrose DM & Glick BR, Methods for isolating and characterizing ACC deaminase-containing plant growth-promoting rhizobacteria, *Physiol Plant*, 118 (2003) 10

27 Das SN, Dutta S, Anil K, Neeraja Ch, Sarma PVSRN, Srinivas V & Podile AR, Plant growth-promoting chitinolytic *Paenibacillus elgii* responds positively to tobacco root exudates. *J Plant Growth Regul*, 29 (2010) 409.

28 Velusamy P & Kim KY, Chitinolytic activity of *Enterobacter* sp. KB3 antagonistic to *Rhizoctonia solani* and its role in the degradation of living fungal hyphae. *Int Res J Microbiol*, 2 (2011) 206.

29 Miller GL, Use of dinitrosalicylic acid reagent for determination of reducing sugar, *Anal Chem*, 37 (1959) 426.

30 Lowry OH, Rosebrough NJ, Farr AL & Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem*, 193 (1951) 265.

31 Lauritsen I, Kim SH, Porse A & Nørholm MHH, Standardized cloning and curing of plasmids. In: *Synthetic biology methods in molecular biology*, (Ed. Braman J, Humana Press, New York, USA), 2018, pp. 479.

32 Parker RE, Continuous distribution: Tests of significance, In: *Introductory Statistics for Biology, 2nd edn* (Ed by Arnold, Cambridge University Press, London), 1979, pp. 18.

33 Joseph B, Patra RR & Lawrence R, Characterization of plant growth-promoting rhizobacteria associated with chickpea (*Cicer arietinum* L.). *Int J Plant Prod*, 1 (2007) 141.

34 Dutta J &Thakur D, Evaluation of multifarious plant growth-promoting traits, antagonistic potential and phylogenetic affiliation of rhizobacteria associated with commercial tea plants grown in Darjeeling, India. *Plos One*, 12(2017) e0182302.

35 Rijavec T & Lapanje A, Hydrogen cyanide in the rhizosphere: not suppressing plant pathogens but rather regulating the availability of phosphate. *Front Microbiol*, 7 (2016) 1785.

36 Shaikh SS, Patel PR, Patel SS, Nikam SD, Rane TU & Sayyed RZ, Production of biocontrol traits by banana field fluorescent *Pseudomonas* and their comparison with chemical fungicides. *Indian J Expl Biol*, 52 (2014) 917.

37 Bal HB, Nayak L, Das S & Adhya TK, Isolation of ACC deaminase producing PGPR from rice rhizosphere and evaluating their plant growth-promoting activity under salt stress. *Plant Soil*, 366 (2013) 93.

38 Kruasuwan Worarat & Thamchaipenet Arinthip, 1-Aminocyclopropane-1-carboxylate (ACC) deaminase-producing endophytic diazotrophic *Enterobacter* sp. EN-21 modulates salt–stress response in sugarcane. *J Plant Growth Reg*, 10 (2018) 1007.

39 Gupta S & Pandey S, ACC Deaminase producing bacteria with multifarious plant growth promoting traits alleviates salinity stress *in* french bean (*Phaseolus vulgaris*) plants. *Front Microbiol*, 10 (2019) 1506.

40 Shaikh SS, Wani SJ, Sayyed RZ, Thakur R & Gulati A, Production, purification and kinetics of chitinase of *Stenotrophomonas maltophilia* isolated from rhizospheric soil. *Indian J Expl Biol*, 56 (2018) 274.

41 Woo CJ & Park HD, An extracellular *Bacillus* sp. chitinase for the production of chitotriose as a major chitinolytic product. *Biotechnol Lett*, 25 (2003) 409.

42 Dahiya N, Tewari R & Tiwari RP, Production of an antifungal chitinase from *Enterobacter* sp. NRG4 and its application in protoplast production. *World J Microbiol Biotechnol*, (2005) 21.

43 Sayyed RZ, Patel PR & Shaikh SS, Plant growth promotion and root colonization by EPS producing *Enterobacter* sp. RZS5 under heavy metal contaminated soil. *Indian J Expl Biol*, 53 (2015) 116.

44 Srinivasan R, Mageswari A, Subramanian P, Maurya VK, Sugnathi C, Amballa C, Sa Tongmin & Gothandam KM, Exogenous expression of ACC deaminase gene in psychrotolerant bacteria alleviates chilling stress and promotes plant growth in millets under chilling conditions. *Indian J Expl Biol*, 55 (2017) 463.

45 Tom JM & Kay WW, A simple and rapid method for the elimination from enteric bacteria. *Curr Microbiol*, 11 (1984) 155.

46 Letchumanan V, Chan KG & Lee HL, An insight of traditional plasmid curing in *Vibrio* species. *Front Microbiol*, 6 (2015) 735. doi: 10.3389/fmicb.2015.0073.