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Indole Acetic Acid Production and its Quantification from Microorganisms of *Rhizosphere*

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ABSTRACT

Plant Growth Promoting Rhizobacteria (PGPR) in the category of microbes that reside in the roots of the plant as well as free in the soil have plant growth-promoting capability. Moreover, that has characteristic properties that are helpful for better productivity of plants mainly including, plant protection against pathogens, root and shoot elongation, and works as fertilizers. This study aims to identify and characterize PGPR isolates highlighting their Indole Acetic Acid production capability. *Pseudomonas aeruginosa* and *Bacillus cereus* were characterized by 16S rDNA sequencing and phylogenetic analysis was performed. PGPR characteristics were tested by Indole acetic acid,hydrogen cyanide, siderophore, and 1-aminocyclopropane-1-carboxylic aciddeaminase test. Quantification of the IAA production by the two isolates was done using Reversed phase High Performance Liquid Chromatography against standard indolic compounds. Both the isolates showed a comparable number and quantity of indolic compounds in their supernatant. The area under the authentic IAA peak was 97% of the total peak area, while the IAA peak extracted from the culture of strain LC536053 was 30% of the total peak area. Once calculated back to the original concentration of the culture extract based on comparison with the known authentic IAA concentration, it was found that the strain LC536054 produced approximately 118 µmol/mL of IAA.

KEY WORDS: PGPR, IAA, PLANT GROWTH, HPLC, UV DETECTOR, RHIZOSPHERIC, CHROMATOGRAM.

INTRODUCTION

PGPR's have an immense role in enhancing the growth of plants by producing various products that help to influence natural characteristic properties of plants. These bacteria can colonize in both the roots of the plants as well as rhizospheric soil, they also reside freely in the soil. The properties include the production of auxin, gibberellin, ethylene, siderophores, HCN, and antibiotics (Arshad and Frankenberger, 1992) which directly affects plant growth. They belong to varied genera *viz: Azospirillum, Alcaligenes, Arthrobacter, Enterobacter, Erwinia, Acinetobacter, Bacillus, Burkholderia, Flavobacterium, Pseudomonas, Rhizobium and Serratia* (Tilak et al., 2005). The Plant Growth Promoting Rhizobacteria (PGPR) are of the great importance than other plant growth promoting microorganisms because they are associated to the plant via direct contact with their roots (Kumar et al., 2018). These microorganisms are capable of colonizing the roots and facilitate the growth of plant along with controlling various stress either directly or by producing the phytohormones (Patel, 2018).

One common commercial product of PGPR is Indole-3-acetic acid (IAA). IAA is one of the characterized plant hormone, auxin. It is produced by plants, algae, mosses, lichens and other variety of microorganisms. This metabolite is procured from tryptophan (Trp) following both Tryptophan dependent and independent pathways by a wide variety of plants and micro-organism

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(Pattern and Glick, 2002).It plays major role in growth and development of plants. IAA has since been implicated in virtually all aspects of plant growth and development (Teale et al., 2006).

Besides its integral role in the growth and development of plants, PGPR has other beneficial usages that directly or indirectly improve growth. These benefits include control of other microorganisms, antagonistic activity, quorum sensing, signal interference, inhibitory action against biofilm formation, elevation in the level of mineral by solubilization mechanism, systemic acquired resistance and induced systemic resistance (Patten and Glick, 1996). Commercialization of products like biofertilizers obtained from PGPR (Adesemoye and Kloepper, 2009) has increased these days as it is turning out to be the better substitute of chemical fertilizers and pesticides which have various side effects on plants as well as the farmers (Banerjee et al., 2006). Therefore this study aims to identify such microorganisms from rhizospheric soil and assess their property to produce a product like IAA and quantify the amount of IAA produced with the help of sophisticated instruments like HPLC.

The agricultural productivity is being negatively affected by the unpredictable climatic changes that are the result of this rapidly growing population leading to higher anthropogenic activities which in turn brings numerous unwanted environmental stresses (Pereira, 2016). The agricultural production of this era is very less due to all these combined environmental stress and above all the need for maintain good food security for this growing population has led the focus towards determining the measures that could enhance and improve the agricultural productivity without destroying the natural biomass of the soil. Looking forward to the PGPR organisms would surely give the better way to overcome this issue effectively. Within this context the present study was designed for the isolation of the bacterial isolates capable of producing IAA and their characterization for their PGPR potential by the qualitative analysis. Moreover, the study includes the molecular characterization of the isolated with good IAA production ability by 16srRNA sequencing.

MATERIAL AND METHODS

Isolation of bacteria from rizospheric soil: Bacterial isolation was done from Rizospheric soil collected from the region around wheat plant roots. The soil was collected in zipped plastic bags and brought to the laboratory for isolation. The soil sample was serially diluted up to 10-8 dilution. 50 µl of the dilution (10-7) was spread on the freshly prepared nutrient plates. The plates were incubated at 37˚C for 48 hours and observation was recorded after completion of the incubation period. The colonies observed were streaked on different fresh plates for testing of various PGPR characteristics.

Characteristic PGPR production Assay Production of indole acetic acid (IAA): The detection of IAA by the isolates was done using Salkowski reagent (2% FeCl3 (0.5M), 35% perchloric acid) (Bric et al., 1991). The method could be briefly described as tryptic soy agar (TSA) media supplemented with 0.05% tryptophan was prepared and a membrane (nitrocellulose) was placed onto it. Suspension of the bacterial culture (20 µl) was added on the membrane and incubated at 28°C. This membrane was then placed on a filter paper soaked with Salkowski reagent. IAA production was detected by the development of pink halos near bacterial colonies.

Production of hydrogen cyanide (HCN): The detection of hydrogen cyanide (HCN) production by the bacterial isolates was done using sodium carbonate (2%) and picric acid solution (0.5%) (Bakker and Schippers, 1987). TSA media incorporated with 4.4 g/l glycine streaked with target isolates were sealed with paper inside it, the paper was earlier soaked with the above-mentioned reagent. Change in color to red indicates positive for HCN Production. Production of siderophores:Siderophore test was done using CAS (chrome azurol S) media (Schwyn and Neilands, 1987). The media was flooded with CAS medium at the top of TSA streaked with target microorganism. A positive test was indicated by an orange halo region around the streak.

Aminocyclopropane-1-carboxylate (ACC) deaminase activity: Aminocyclopropane-1-carboxylate deaminase activity was screened for target isolates on the sterile minimal DF (Dworkin and Foster) salts media with modification of 3 mM ACC added instead of (NH4)2SO4 (nitrogen source) (Dworkin and Foster, 1958; Penrose and Glick, 2003). Test samples were observed time to time for incubation period of 72 hours.

Molecular Characterization of isolates: DNA extraction for the isolates was done using kit Nucleo-pore gDNA Bacterial Mini Kit (Cat. NP-7006D). Quantification of the extracted DNA was performed using UV-Visible Double Beam Spectrophotometer (PC based Systronic 2202) observing absorbance at 260nm and 280nm. The quantification was recorded in ng/µl and purity ratio at 260/280nm was the record to estimate any contamination of protein or RNA. PCR amplification of 16S rRNA gene was done using universal primers 27F (5' AGAGTTTGATCCTGGCTCAG 3') and 1392R (5' TACGGTTACCTTGTTACGACTT 3') to identify species. PCR reactions for the detection of the bacterial species were set up with reactions mixture of 20 µl volumes containing 2 µl of the genomic DNA sample, 1× PCR buffer containing; 0.16 mM dNTP Mix; 20 pmol of forward and reverse primers and 0.75 U Taq DNA polymerase (MBI, Fermentas, Lithuania).

Amplification was carried out in a thermal cycler (Eppendorf Mastercycler) with the PCR conditions as follows: Initial denaturation at 95°C-6 min, denaturation at 95°C- 30 sec, annealing at 50°C-1 min, and extension at 72°C-1 min , final extension was performed at 72°C-10 min. Polymerase Chain Reaction was performed till 40 cycles. PCR products were analyzed using 1% agarose gel electrophoresis. The samples were then sequenced using Sanger's Method.

Quantification of IAA using HPLC Sample preparation:Sample preparation consisted of a single centrifugal filtration step using 3-kDa cut-off membrane centrifugal filters. For this purpose, 0.5 mL of bacterial culture supernatants or spiked sterile bacterial broths were transferred to the sample chamber of a 0.5 mL centrifugal filter tube and centrifuged at 14,000×g (relative centrifugal force) at 4 °C for 30 min. The filtrates were directly analyzed by HPLC

Instrumentation and chromatographic conditions: Eluent A consisted of 2.5: 97.5 % (v/v) acetic acid: H_2O, pH 3.8 (the pH was adjusted by addition of 1 mol L−1 KOH) and eluent B consisted of 80: 20 % (v/v) acetonitrile: H_2O. The mobile phase started with eluent A: eluent B at 80: 20 %, changing to 50: 50 %, 0: 100 % and 80: 20 % in 25, 31 and 33 min, respectively. The total run time was 20 min. The flow rate of the mobile phase was 1 mL min−1, the injection volumes were 20 µL, and the detector was set to excitation and emission wavelengths of 280 and 350 nm, respectively.

RESULTS AND DISCUSSION

Isolation of bacteria and screening for PGPR: Isolates obtained from serial dilution were then tested separately for PGPR activity. The results of the isolates are summarized in the table 1. In the two isolates positive results were observed as indication of the development of pink halos colonies on the addition of Salkowski's reagent. HCN production was observed for isolate 1 with the color change of the paper to red and no change in case of isolate 2 indicating a negative result. ACC deaminase was observed negative in both cases. Siderophore test was also observed positive in both Isolates tested on CAS media with an orange halo region around the test organism.

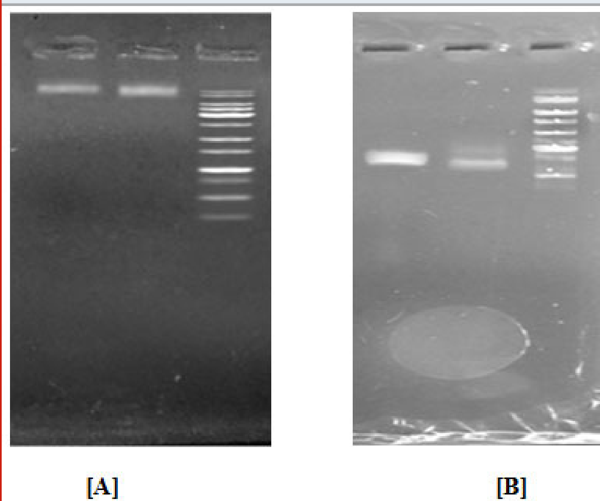
Table 1. Summarized result of PGPR for isolates

S.No.	Test	Isolate 1	Isolate 2
1	IAA Production	+	+
2	HCN production	+	-
3	ACC deaminase	-	-
4	Siderophore	+	+

*Positive result: (+) and Negative result: (-)

Molecular Characterization of Isolates: Molecular Characterization of two PGPR positive isolates was performed. The extracted DNA was observed on 0.1% agarose gel in the presence of UV light. The orange color bands confirmed the presence of DNA (Figure 1[A]). The concentration of the extracted DNA was determined using the spectrophotometric method. The purity ratio of 260/280 nm for Sample 1 and 2 were 1.809 and 1.689 respectively and the concentration of DNA calculated to be 7060 ng/µl and 6605 ng/µl. The concentration of DNA confirms its suitability for amplification, thus universal primers 27F and 1392R were used and PCR products of 1.4 kb -1.5kb amplicon size was observed as shown in figure 1[B].

Figure 1[A]: Genomic DNA at 0.8% Gel; Lane 1: Sample 1; Lane 2: sample 2 (bacterial sample); Lane 3: Ladder (1Kb) [B] PCR Product at 1.2 % Gel; Lane 3: Ladder (1Kb); Lane 1: Sample 1 (PCR Product); Lane 2:Sample 2 (PCR Product); Band Size: Approx 1.2-1.5 Kb



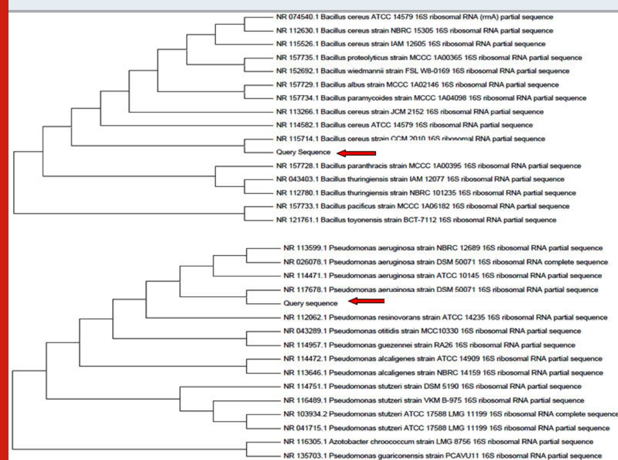
[A] [B]

The sequence obtained from sequencing was processed using a biological sequence alignment editor (BioEdit 7.2). The subsequent analysis had been done using NCBI-BLAST (National Centre for Biotechnology Information- Basic Local Alignment Search Tool) https://blast.ncbi.nlm.nih.gov/Blast.cgi and MEGA X (Molecular Evolutionary Genetics Analysis). The Sample 1 species was identified as *Bacillus cereus* as it was found 98.78% similar to *Bacillus cereus* strain CCM 2010 16S ribosomal RNA, partial sequence (NR_115714.1) (shown in figure 2). The phylogenetic tree constructed using the maximum parsimony method shows other closely related species. Sample 2 was identified as *P. aeruginosa* as it was found to be 99.23% similar to *Pseudomonas aeruginosa* strain DSM 50071 16S ribosomal RNA, partial sequence (NR_117678.1). Sequence submitted in the DNA Data Bank of Japan (DDBJ) with the accession number LC536053 and LC536054. The phylogenetic tree constructed using the maximum parsimony method shows other closely related species.

Quantification of IAA using HPLC: Earlier studies on Indole Acetic acid have revealed that it consist of acidic (IAA, ILA), amphoteric (Trp), basic (TAM) or essentially neutral (IAN, IAM, TOL) (Liu et al., 2019). Thus selection of pH range for mobile phase plays an important role in proper separation, retention time and peak shape of ionizable compounds (Espinosa et al., 2002; Chandrul and Srivastava, 2010). Here, a gradient for mobile phase was chosen and proportions of various solvents were set accordingly to obtain better separation. The selected eluents: Eluent A consisted of 2.5: 97.5 % (v/v) acetic acid: H_2O, pH 3.8 (the pH was adjusted by addition of 1

mol L−1 KOH) and eluent B consisted of 80: 20 % (v/v) acetonitrile: H_2O.

Figure 2: Phylogenetic Tree constructed using MEGA X by Maximum Parsimony method [A] Sample 1 [B] Sample

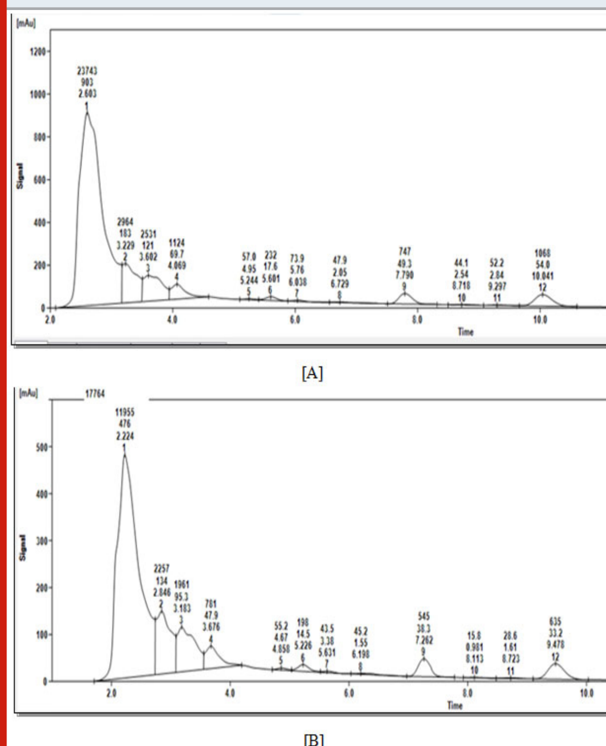


The mobile phase started with eluent A: eluent B at 80: 20 %, changing to 50: 50 %, 0: 100 % and 80: 20 % in 25, 31 and 33 min, respectively. The total run time was 20 min. The flow rate of the mobile phase was 1 mL min−1, the injection volumes were 20 µL, and the detector was set to excitation and emission wavelengths of 280 and 350 nm, respectively. Thus, following above mentioned conditions, 7 major well separated peaks, but not symmetrical, were observed in each sample. Retention times (min) of the peaks were 2.603, 3.229, 3.602, 4.069, 5.601, 7.790, 10.041 and 2.224, 2.846, 3.183, 3.676, 5.226, 7.262, 9.478 for sample 1 and sample 2 respectively were observed.

The linearity and range of the analysis was assessed by preparation and analysis of different concentrations of the standard solutions of the IAA. Calibration curves of standards were plotted for the stock solutions. The range of concentration for IAA standard was kept between 0.05µg/ml-0.025 µg/ml. Our results showed presence of numerous indolic compounds which was in accordance to work discussed by different authors Ahmad et al. (2005), Khakipouret al. (2008) and Chaiharn and Lumyong (2011).Thus bacterial supernatant have presence of load of such compounds, Production of such compounds by bacterial species does not require supplemented enriching compounds. And as mentioned in Szkop and Bielawski (2013) centrifugal filtration method was followed to reduce analytes with high molecular weight (> 3 kDa), our study followed to remove contaminants at initial stage.

This also ensures purity of the sample with less numbers of noise and detectable compounds in chromatogram. The aim of this study was to estimate qualitatively and Quantitatively IAA production by rhizospheric soil microbes. Several strains of genus *Bacillus, Azotobacter, Pseudomonas* were reported to produceIAA (Cassán et al., 2014; Verma et al., 2018). The evaluationof bacterial isolates for production of IAA have revealed that both are significant producers of IAA hence they could be used as PGPR.The identified bcaterial species were B.cereus and P.aeruginosa. IAA production was quantified by HPLC technique and both isolates showed comparable quantity of IAA in the supernatant. Earlier studies have enlightened the role of IAA produced by rhizospheric soil microbes (Wahyudiet al., 2011).

Figure 3: Chromatograms obtained in the analysis of IAA [A] Chromatogram of B.cereus culture supernatants after 72 hrs incubation period in King B medium supplemented with 3.5 mM Trp. [B] Chromatogram of P.aeruginosa culture supernatants after 72 hrs incubation period in King B medium supplemented with 3.5 mM Trp.



Some reported species for IAA production, root elongation in *Sesbaniaaculeata* by inoculation with *Azotobacter* spp. and *Pseudomonas* spp., in *Brassica campestris* by *Bacillusspp* (Ghosh et al., 2003), in Vigna radiate by *Pseudomonas putida* (Pattern and Glick, 2002) and in *Pennisetumamericanum* by *Azospirillumbrasilense*(Tien et al., 1979).Effect of IAA producing isolate was also observed in *Solanum lycopersicum*, (Khan et al., 2016) where it significantly increased the shoot and root biomass and chlorophyll (a and b) contents as compared to control plants.Plant roots secrete tryptophan in the rhizosphere which is utilized by rhizobacteria as a precursor for IAA biosynthesis (Shameer & Prasad, 2018).The IAA producing bacteria are known to assist the plant growth and they can even effectively protect them from the various environmental stress inclusing the salinity stress as reported the IAA producing microrganism promote plant growth both under normal and saline conditions (Gupta and Pandey, 2019; Kang et al., 2019).

CONCLUSION

PGPR is being studied by researchers to explore products that are yet unexplored or to identify sources of microbes with a maximum yield of these products. The microbes that colonize the roots of the plants or are freely available in the soil can produce a wide range of products that contributes to plant growth promotion, biocontrol agents, disease resistance agents, insecticidal effect and many more. We aimed to isolate and characterize such PGPR isolates and emphasize on their IAA producing capability. The quantity of IAA was also estimated using the technique of HPLC. This method could prove as a tool to quantify IAA or other PGPRs between different bacterial species.

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