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ISOLATION AND CHARACTERIZATION OF PLANT GROWTH-PROMOTING BACTERIA FROM DIFFERENT RHIZOSPHERIC SOILS

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Abstract

The production of indole acetic acid (IAA) is a significant property of rhizospheric bacteria to stimulate and promote plant growth. In this investigation, indole acetic acid-producing bacteria from the rhizosphere of five distinct plants (Wheat, Gulchen, Rose, Lady Finger, and Bouginvella) were isolated and characterized. Bacterial strains were isolated and identified by morphological characterization. Out of 30 bacterial isolates, 5 isolates were selected as efficient producers of Indole acetic acid. These selected bacterial isolates were subjected to several biochemical tests for identification at the genus level. After the determination of bacterial genus, antibiotic sensitivity test was performed to check the resistance pattern of each bacterial isolate against antibiotics. Selected bacterial isolates were characterized by using molecular techniques. DNA of five bacterial isolates was extracted by WizPrepTMg DNA mini kit and amplification of 16S rRNA gene was done by PCR. Amplification of PCR product was confirmed by agarose gel electrophoresis. From biochemical tests, *Klebsiella* spp., *E. coli*, and *Bacillus* sp. tested positive. *Klebsiella* spp. showed 87, 88, 81, 88, 91, 100, 90, and 87% resistance against enrofloxacin, gentamycin, penicillin, ampicillin, chlotetracyclin, tylosine, oxytetracycline, and amoxicillin, respectively. *E. coli* showed 80, 88, 79, 84, 79, 86, 82, and 89% against enrofloxacin, gentamycin, penicillin, ampicillin, chlotetracyclin, tylosine, oxytetracycline, and amoxicillin respectively whereas 87, 91, 100, 100, 93, 100, 89 and 100% resistance was shown by *Bacillus* sp. against enrofloxacin, gentamycin, penicillin, ampicillin, chlotetracyclin, tylosine, oxytetracycline, and amoxicillin, respectively. All these selected bacterial isolates were found

positive for the 16S rRNA gene and characterized as *Klebsiella pneumoniae* RY6, *Klebsiella oxytoca* strain RY8, *Klebsiella oxytoca* RY10, *Escherichia coli* RY20, and *Bacillus tequilensis* RY23. It is concluded that the property of synthesizing IAA is considered an effective tool for the isolation and characterization of bacteria that have a role in promoting plant growth.

Key words: Rhizosphere, Plant growth, PGPR, 16S rRNA ribotyping

INTRODUCTION

Indole acetic acid (IAA) is one of the most important active auxin (Kumar *et al*., 2020). It is a byproduct of L-tryptophan metabolism produced by a wide range of microorganisms, including plant growth-promoting rhizobacteria (PGPR) (Gupta & Pandey, 2019). PGPR bacteria are those that colonize the rhizosphere and roots of plants and stimulate plant development in any way (Santoyo *et al*., 2021). PGPR may exhibit various properties responsible for enhancing plant growth. Production of plant growth regulators (such auxin, ethylene, and gibberellin), siderophores, HCN, and antibiotics are common characteristics (Tyagi *et al*., 2022). Auxins are produced by bacteria to interfere with host physiological processes for their own gain (Kunkel & Johnson, 2021). Due to the abundance of substrates, microorganisms found in the rhizosphere of different crops can produce indole acetic acid as a secondary metabolite (Eichmann *et al*., 2021). IAA induces specific RXA and protein synthesis, increases cell osmotic content, and increases cell water permeability, decreases wall pressure, and increases cell wall synthesis to promote cell elongation (Zhang *et al*., 2019), promotes embial activity, inhibits or delays leaf fall, induces flowering and fruiting (Kumar *et al*., 2020).

IAA is derived from tryptophan by tryptophan independent and dependent pathways in bacteria and plants (Das *et al*., 2019). However, other pathways could also be present in bacteria. Tryptophan is converted into indole-3-acetamide (IAM) that is hydrolyzed to indole 3 acetic acid (IAA) by the action of IAM hydrolase in tryptophan dependent pathway. In indole independent pathway some bacteria grow aerobically showed maximum auxin production in the presence of ammonia (Cook & Ross, 2016). It seems to be of specific significance during the process of embryo formation in plants, when control over low levels of IAA is serious to polar development (Hu *et al*., 2021). Tryptophan independent pathway contributes considerably to the newly synthesized IAA; though, widespread Tryptophan to IAA transformation also occurs in such preparations (Abu-Zaitoon *et al*., 2022).

IAA generating bacteria fuel seed incubation, intensification root development, plant development and expansion (Lastochkina *et al*., 2021). Distinctive plant seedlings respond in an alternate manner to variable auxin fixations and sort of microorganisms (Khan *et al*., 2020). The low measure of IAA delivered by plant development progressing rhizobacteria can vitalize the advancement of roots, however high IAA creation by bacterial strains controls the root improvement (Mirskaya *et al*., 2022). The first objective of this study was to isolate IAA producing bacteria from different rhizospheric plant soil. The second was to identify IAA producing bacteria by morphological, biochemical and molecular techniques.

MATERIAL & METHODS

Sample collection

Five soil samples were collected from different plants (Wheat, Gulchen, Rose, Lady Finger and Bouginvellain) in Lahore district. All samples were collected in the sterile falcon tubes and transported to Microbiology Lab of University of Central Punjab, Lahore. These samples were cultured on basal media by making serial dilutions. Dilution factor (10^{-6}) was cultured by pour plate method. Phenotypically diverse bacterial colonies were isolated by culturing on basal and selective media. Out of 30 (RY1, RY2, …. RY30) bacterial isolates, 5 (RY6, RY8, RY10, RY20 and RY23) were selected on the basis of indole positive test. These isolates were further confirmed by morphological, biochemical and molecular characterization.

Isolation and purification of IAA producing rhizobacteria

Luria broth (LB) agar was used as a basal media for the isolation of bacteria. LB was prepared by weighing 40g of media in 1000ml of distilled water. It was boiled and autoclaved at 121°C for 15 minutes. Dilution factor (10^{-6}) was inoculated on the agar plate by pour plate method and incubated for 24 hours at 37°C. After incubation, growth of bacterial colonies were observed and subjected to morphological characterization. *i.e.* Gram staining and growth on selective media.

Morphological and biochemical characterization IAA producing rhizobacteria

Isolated bacterial strains (RY1, RY2, …. RY30) were identified by Gram staining and selective media (MacConkey and MSA). Total 30 bacterial isolates (RY1, RY2, ….. RY30) were present in 5 soil samples. These bacterial isolates were subjected to indole test. Those isolates which had the ability to produce indole by the deamination of tryptophan amino acid were selected

and several biochemical tests (Methyl red, Catalase, Oxidase, Citrate, Urease and Voges Proskauer) were performed to identify them on genus level.

Antibiotics sensitivity test

To examine the sensitivity of bacterial isolates against antibiotics, Kirby Bauer disc diffusion method was used. A single colony was picked up with help of sterile loop and mixed in 2ml of normal saline. Turbidity of solution was adjusted to 0.5 Mcfarland standards. Muller Hinton agar was prepared and autoclaved at 121°C under 15 psi pressure for 60 minutes. Plates were labeled properly. Cotton swabs were dipped into 2ml of saline water containing bacterial culture and spreading was done on entire surface of agar. Antibiotics were applied on the agar plate with the help of forceps. Plates were incubated for 24 hours at 35ºC and zone of inhibition was calculated in millimeters (mm) with the help of Vernier calipers.

Molecular characterization of selected bacterial isolates

DNA extraction was done by WizPrepTMg DNA mini kit (https://www.wizbiosolution.com/) . C for further analysis. PCR amplification for the selected gene of microbial isolates was performed by using 16S-F (5'AGAGTTTGATCCTGGCTCAG3') 16S-R (3'AAGGAGGTGATCCAGCCGCA5') (Al-Nabulsi *et al*., 2015) for 16S rRNA gene. Conditions optimized for the amplification of gene as: Initial denaturation of strands at 94°C for 10 min; Denaturation at 94°C for 30 seconds; Annealing at 60°C for 1 min; Extension at 72°C for 1 min, Cycles repetition 35X; Final extension at 72°C for 10 min and lastly cooling at 4°C for 10 min. Amplified PCR products of 16S gene was sent for sequencing to Advance Bioscience International, Biotechnology Company Lahore, Pakistan. The sequences were blast against already submitted nucleotide sequences on NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and phylogenetic analysis was performed by constructing phylogenetic tree.

RESULTS

Sample collection

Five soil samples were collected from different plants in Lahore district (Wheat, Gulchen, Rose, Lady Finger and Bouginvellain). These samples were used by making serial dilutions. Dilution factor (10^{-6}) was cultured on basal media. (Table 1). Phenotypically diverse bacterial colonies were isolated by culturing on basal and selective media. Five bacterial isolates (RY6, RY8,

RY10, RY20 and RY23) were selected on the basis of positive indole test. These isolates were further confirmed by morphological, biochemical and molecular characterization.

Table 1: Collection of rhizospheric soil samples from different plants.

Sr. No	Plant Name	Scientific names	Specimen	Region
1	Wheat	*Triticum aestivum*	Soil	Lahore
2	Gulchen	*Plumeria alba*	Soil	Lahore
3	Rose	*Rosa rubiginosa*	Soil	Lahore
4	Lady Finger	*Abelmoschus esculentus*	Soil	Lahore
5	Bouginvella	*Bougainvillea glabra*	Soil	Lahore

Morphological characterization of bacterial isolates

Morphological characteristics of isolated bacterial strains (RY1, RY2,.… RY30) showed Gram positive cocci and Gram negative rods under microscope. Gram negative bacterial isolates were grown on MacConkey agar for selective growth of bacterial isolates primarily based on lactose and non-lactose fermentation. *E. coli* (RY20) and *Klebsiella* spp. (RY6, RY8, RY10) are lactose positive microorganisms and gave pink colored colonies on MacConkey agar. Gram positive bacterial isolates were grown on mannitol salt agar (MSA) for selective growth of mannitol fermenting and non-fermenting bacteria. The bacterial isolates that were grown on MSA gave large pink and smooth colonies which indicated suspected colonies of *Bacillus* sp. (RY23).

Biochemical characterization of bacterial isolates

Bacterial isolates were subjected to biochemical characterization for further confirmation. Indole test was performed for these bacterial isolates to find out the ability of bacteria to produce indole by the deamination of tryptophan amino acid. Those bacterial isolates were selected which gave red color ring upon addition of Kovac's reagent. Out of 30 bacterial isolates, 5 were indole positive and selected on the basis of indole production. Selected bacterial isolates *i.e.* positive for indole test were subjected to Methyl red, Catalase, Oxidase, Simmon citrate, Urease and Voges-Proskauer for further confirmation on genus level. (Table 2).

Table 2: Biochemical characterization of bacterial isolates.

	Selected Bacterial Isolates				
Biochemical tests	**RY6**	**RY8**	**RY10**	**RY20**	**RY23**
Indole	+	+	+	+	+
Catalase	+	+	+	+	+
Oxidase	-	-	-	-	+
Citrate	+	+	+	-	+
Urease	+	+	+	-	-
Voges Proskauer	-	-	-	-	+

Antibiotic Resistance Testing

Kirby Bauer disc diffusion method was applied to check the antibiotics susceptibility of selected bacterial strains. Antibiotic disc Gentamycin (CN10), Ampicillin (AM10), Chlotetracyclin (CHT30), Enrofloxacin (ENR10), Tylosine (TY30), Penicillin (P10), Amoxicillin (AX25) and Oxytetracyclin (T30) were applied for each bacterial isolate and zone of inhibition was measured after 24 hours of incubation. Pattern of resistance for each antibiotic is shown in Figure 1.

Molecular characterization of selected bacterial isolates

With the assistance of the WizPrepTMg DNA mini kit, Gram negative bacteria were subjected to molecular identification. 16S rRNA was amplified in the selected bacterial isolates through PCR. 16SrRNA gene was detected in all bacterial isolates and characterized as *Klebsiella pneumoniae* RY6, *Klebsiella oxytoca* strain RY8, *Klebsiella oxytoca* RY10, *Escherichia coli* RY20 and *Bacillus tequilensis* RY23. Product length of 16S rRNA gene was 1553 bp.

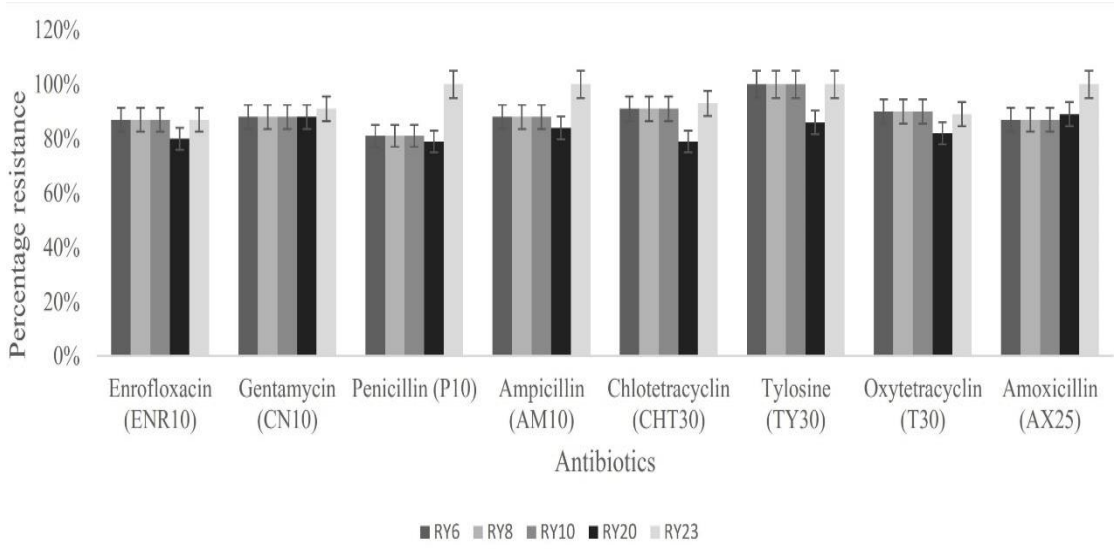

Figure 1: Antibiotic sensitivity pattern of selected bacterial strains against antibiotics.

Phylogenetic analysis was performed by comparing sequences of selected 16S rRNA gene. All the selected bacterial isolates showed significant similarity with other type strains (https://lpsn.dsmz.de/) (Figure 2).

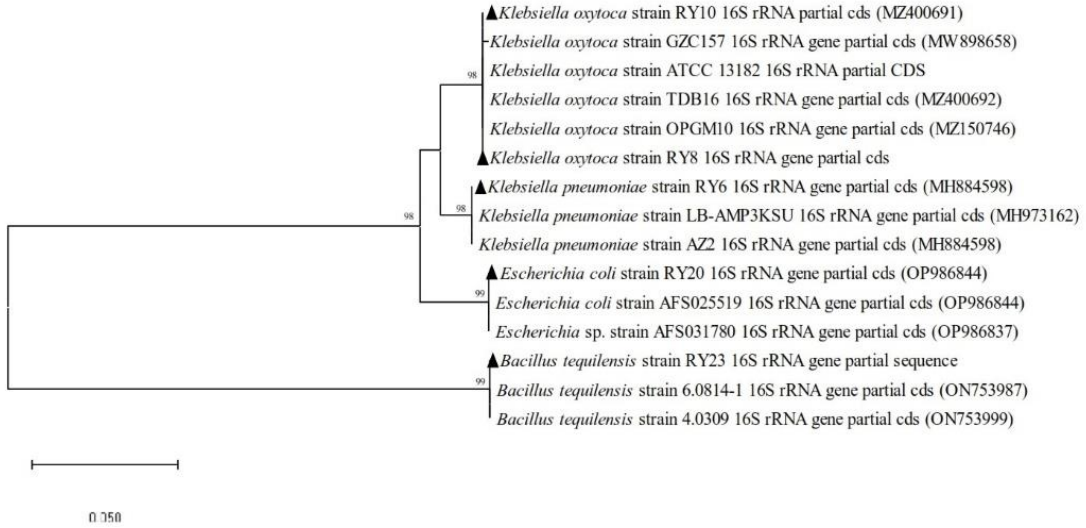

Figure 2: The Neighbor-Joining method was used to infer the evolutionary history. The tree is drawn to scale, with branch lengths measured in the same units as the evolutionary distances used to infer the tree. The evolutionary distances were calculated using the Maximum Composite Likelihood method and are in base substitutions per site.

DISCUSSION

An indole acetic acid bacterium belongs to phytohormone group of bacteria that is considered as a most important essential and natural auxin. It is a derivative of indole, colorless and polar organic solvent (Simon & Petrášek, 2011). In the current study, different IAA bacterial isolates

were studied. A total 5 isolates were isolated from 5 soil samples and identified as 1 Gram positive and 4 Gram-negative isolates. The same Gram negative isolates were isolated in the study of B. Mohite 2013 (Mohite, 2013). Five bacterial varieties were isolated and classified as *E. coli*, (RY20); *Klebsiella* spp., (RY6, RY8, RY10) and *Bacillus* sp. (RY23) from soil throughout this research. Various methods were used to analyze bacterial colony features. Eventually, colony morphology was achieved with the Gram staining technique, which is a standard characterization approach adapted by several field of study (Kaur at al., 2014).

In the current study, Gram-positive isolate was belonging to *Bacillus* sp. and isolated on the basis of IAA production (Wahyudi, 2013). It has been reported that production of IAA by bacterial isolates vary amongst diverse classes and strains. They are swayed by culture condition, substrate, and growing stage (Mutluru and Konada, 2007). In my study, *Klebsiella* spp. (RY6, RY8, RY10) are the most important isolate that was associated for IAA in rhizosphere. According to the study of Fatima *et al*., 2009 revealed that IAA producing bacteria enhanced the germination rate, growth of roots, shoot growth of the plants.

IAA is considered as synthesizing property and effective tool for IAA generating bacteria and beneficial microorganisms that effect on the growth of plant (Wahyudi *et al*., 2011). Indole acetic acid bacterial strains stimulated the propagation of sidelong root's hairs and roots. According to this study 5 isolated bacteria were identified as the main source of IAA producing bacteria that enhanced the growth of plants. Another study showed that *Bacillus* sp. (RY23) was identified, and they enhanced the growth of seeds. It was difficult to identify the optimum concentration of indole acetic acid (Aris *et al*., 2011).

Another study showed that only *Bacillus* sp. was isolated from the rhizosphere of the soybean plant. Few segregates showed IAA producing activity, phosphate solubilization, and siderosphore production, inhibit the development of plant microorganisms like fungi and many other pathogenic bacteria (Bano & Fatima, 2009). In this study, Gram-positive bacterium *Bacillus* sp. (RY23) was isolated showed the same properties of indole acetic acid bacteria. Isolates were studied for their outcome on plant physiology. Indole acetic acid had significant effect on the growth of plants. Isolated bacteria were studied for the effect on the plants. Plants showed significant change in the roots and the elongation of shoots and chlorophyll material in plants. Indole acetic acid bacteria also effected positively on growth and germination of seeds. It is also considered as plant promoter (Sadaf *et al*., 2009; Wahyundi *et al*., 2009).

One of the most essential secondary metabolites revealed by bacteria are antibiotics. More than 80 percent of antibiotics obtain from soil-isolated bacteria and are for medicinal usage. In future, the very next hurdle in the treatment of communicable diseases treatment is the resistance developed by bacteria against antibiotics (Marston *et al*., 2016). Additionally, we are concerned about whether chemicals that prevent the development of other bacteria could be produced by either of the soil bacterial isolates. In contrast to the microbial pathogens, bacterial soil isolates reported bactericidal activities. These implications suggested that isolates develop bacteriostatic components for various microorganisms.

CONCLUSION

Production of IAA is the major characteristic of rhizospheric bacteria. It is concluded that bacterial species that have a role in promoting growth of plants were isolated from different rhizospheric soil. These bacterial isolates were characterized by morphological, biochemical and molecular techniques. In this study, property of synthesizing IAA is considered as an effective tool for isolation and characterization of beneficial bacteria *i.e. Klebsiella* spp., *E. coli* and *Bacillus* sp. in promoting plant growth.

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