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Studies on the Application of Leaf Isolates of Lactic Acid Bacteria as PGPR

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ABSTRACT

The rhizosphere of a plant contains microorganisms that can be called plant growth-promoting rhizobacteria (PGPR), which play a crucial role in promoting plant growth and development. The beneficial effects of these bacteria on plant growth can be direct or indirect. The benefits provided by these bacteria can include increased nutrient availability, phytohormone production, and protection against several phytopathogens. PGPR has become an important strategy in sustainable agriculture due to the possibility of reducing synthetic fertilizers and pesticides, promoting plant growth, and enhancing soil quality. Lactic Acid Bacteria (LAB) are ubiquitous, Gram-positive, catalase-negative, and facultative aerophilic microorganisms. They are commonly found in a wide range of environments including food-rich environments, decaying plants, milk products, the human gut, vaginal flora, and on the skin of various living organisms. Their applications are seen in food fermentation, food preservation, the pharmaceutical industry, and dietary supplements. In addition, their presence in the rhizosphere expands their application as PGPR. The LAB isolated from soil and plant parts shows significant production of phytohormones like indole acetic acid, gibberellic acid, etc. They also show antimicrobial activity against phytopathogens. Their ability to fix nitrogen, iron chelation, phosphate solubilization, and ammonia production makes them the PGPR. In this paper, the PGPR characteristics of LAB and other reported PGPR are compared.

INTRODUCTION

Lactic acid bacteria (LAB) are ubiquitous, Gram-positive and facultative aerophilic, rod or cocci, non-spore-forming, catalase-negative, fermentative microorganisms. They are commonly found in a wide range of environments like food-rich environments, decaying plants, milk and milk products, human gut, vaginal flora, and on the skin of various living organisms. These bacteria produce lactic acid as a major by-product of carbohydrate metabolism hence they are named Lactic Acid Bacteria. The genera included under the group Lactic acid bacteria are *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Streptococcus*, *Pediococcus*, *Lactiplantibacillus*, *Leuconostoc*, *Weissella*, and *Bifidobacterium*.

US Food and Drug Administration has classified LAB as GRAS (Generally Recognized As Safe) which increases their demand in a variety of applications (2). They play a significant role in the food industry by exhibiting probiotic properties and can produce various biologically active metabolites such as γ-aminobutyric acid (GABA), exopolysaccharides (EPSs), conjugated linoleic acid (CLA), bacteriocins, reuterin, and reutericyclin, which provides enhanced nutraceutical properties to the final food products. They also produce several specific enzymes essential for producing substrate-derived bioactive compounds, such as polyphenols, bioactive peptides, inulin-type fructans and ß-glucans, fatty acids, and polyols. These compounds exhibit many health benefits, including better mineral absorption, oxidative stress protection, blood glucose, and cholesterol-lowering properties, prevention of gastrointestinal tract infections, and improved cardiovascular function (3).

The lactic acid produced by LAB has many industrial and medical applications such as pH regulator, cleaning agent, solvent, descaling agent, and neutralizer also used in minerals preparations, and dialysis solutions (4). Lactic acid exhibits properties that help in skin hydration, skin lightning, and antimicrobial activity which exhibits its use in the cosmetic industry for making moisturizers, skin lightening agents, skin rejuvenating agents, anti-acne agents, etc. (4). Microbial exopolysaccharides (EPS) produced by LAB play a pivotal role in heavy metal biosorption via keeping the producing cells vigorous. Specifically, EPS-producing LAB has been reported to show superior absorption, tolerance, and efficient abatement of the toxicity of heavy metals in vitro and/or in vivo to non-EPS-producing species (5).

The soil present in the vicinity of plants is known as rhizospheric soil. This soil is densely populated by diverse microorganisms including fungi, bacteria, protists, and viruses. There are some bacteria present in rhizospheric soil that directly or indirectly help plant growth hence are called plant growth-promoting rhizobacteria (PGPR). These are free-living bacteria that colonize plant roots and promote plant growth by using their metabolism (solubilizing phosphates, producing hormones, or fixing nitrogen), directly affecting the plant metabolism (increasing the uptake of water and minerals), enhancing root development, increasing the enzymatic activity of the plant, by "helping" other beneficial

microorganisms to enhance their action on the plant, or by suppressing plant pathogens (6). The presence of LAB in rhizospheric soil and on parts of plants expands their applications as plant growth-promoting bacteria.

MATERIALS AND METHODS

1. Isolation, Characterization and Identification of the Isolates:

The isolates of Lactic Acid Bacteria were isolated from soil, and aerial parts of plants like leaves, flowers, and stems. Here plants like Guava, Custard apple, Onion, Banana, Double beans, and Pomegranate were used. The morphological, physiological, and biochemical tests were performed according to Bergy's Manual of Determinative Bacteriology. Each isolate was grown on a sterile MRS medium plate under microaerophilic conditions for 48 hours and sent for 16S rRNA identification.

2. Plant growth-promoting traits:

Production of plant growth-promoting hormones viz., indole acetic acid, and gibberellic acid was checked. Activities like Phosphate solubilization, nitrogen fixation, iron chelation (siderophore production), and ammonia production were observed.

A. Indole acetic acid production:

Production of IAA was determined with Salkowski's method using Salkowsky reagent. The isolates were grown in Nutrient broth medium with 500 µg/ml tryptophan and incubated for three days on a shaker incubator at 150 rpm. After incubation, the culture was centrifuged at 3,000 rpm for 30 minutes. 1 ml of supernatant was transferred into a sterile test tube and 4 ml of Salkowski reagent was added, then incubated in the dark for 30 min. A qualitative test was done by observing the color change of the solution to pink and the quantitative test was done by measuring the absorbance value of this solution using a spectrophotometer at a wavelength of 530 nm compared with the IAA standard curve. For the standard curve, Synthetic IAA was made serially, where 5 mg was dissolved into 50 ml of ethanol to obtain a concentration of 70 ppm and methanol was added to 1,000 µl, then 4 ml Salkowski reagent was added. The solution was incubated in the dark for 60 min and absorbance was taken (8).

B. Gibberellic acid production:

Isolates were inoculated in deMann Rogosa Sharpe broth and incubated for 10 days at 25 °C at 100 rpm. After 10 days, a cell-free supernatant was obtained to measure the amount of Gibberellic acid produced by bacterial isolates by the DNPH (2, 4 -Dinitrophenyl hydrazine) method. For qualitative estimation, an equal volume of cell-free extract and ethyl acetate was taken in a test tube and shaken vigorously for 10 min. The ethyl acetate layer was taken in a separate tube, this was repeated thrice. Ethyl acetate was allowed to evaporate at room temperature. The remaining contents were dissolved in absolute alcohol. 2 ml of this suspension was mixed with 1 ml of DNPH incubated at 100°C for 5 min and cooled in the water bath. To this, 5 ml of 10% potassium hydroxide was added and allowed to stand till the red wine color developed. 15 ml of sterile distilled water was added and finally, the content was diluted to 1:2 using sterile distilled water. Color intensity was measured at 430 nm. Quantitative estimation was done by comparing the absorbance value with the standard curve. For the standard curve, different aliquots of standard gibberellic acid (0.8 mg/ml) were prepared using absolute alcohol and estimated similarly.

C. Phosphate solubilization:

The phosphate solubilization ability of isolates was checked using Pikovskaya's medium. Isolates were streaked on sterile Pikovskaya's agar plate and incubated at 25 °C for 3 days. After incubation zone of clearance was observed.

D. Nitrogen fixation:

Ashby's mannitol agar was used to check the nitrogen fixation ability of isolates. This medium is nitrogen free so organisms have to use atmospheric nitrogen as a nitrogen source. The growth of organisms on this medium indicates the nitrogen-fixing ability of organisms.

E. Iron chelation (siderophore production):

Iron is an essential micronutrient for plants. It is present abundantly in nature but it is in insoluble form. Siderophores are small molecules that microorganisms secrete to acquire iron from their environment. The siderophore production ability of isolates was checked using Chrome Azurol S medium (CAS)-agar medium. Yellow-orange halos around the colonies on blue agar are indicative of siderophore excretion.

F. Ammonia production:

Bacterial strains were checked for ammonia production ability using Nessler's reagent. The Nessler's reagent is named after the German chemist Julius Nessler (1827–1905). 0.5 mL/1 mL Nessler's reagent was added to 10 mL bacterial culture grown in peptone water, then allowed to react for 10 minutes at room temperature and observed for development of yellow to brown color, which indicates the production of ammonia. Nessler's reagent was prepared by dissolving.: a) 5 g of potassium iodide (KI) in 5 mL of cold water (5–7℃), then b) 2.2 g of mercury dichloride ($HgCl_2$), was dissolved in 35 ml distilled water. The obtained solutions from Step a and step b were mixed and 20 mL of KOH solution (5M) was added slowly under constant stirring until the mixture became colorless. This reagent consists of a highly alkaline solution of Potassium mercuric iodide (K_2HgI_4). Under alkaline conditions, the potassium, mercury, and iodine react in proportion to the concentration of ammonia to create a yellow-brownish to reddish-brown colored complex (17).

3. Antifungal Activity against fungal phytopathogen:

Fusarium sp. and *Aspergillus sp.* were selected as fungal phytopathogens. The antifungal activity was studied using the agar overlay method (10).

RESULTS

1. Isolation, Characterization and Identification of the Isolates:

A total of 25 isolates were isolated from soil and parts of plants. Among them, 2 leaf isolates were selected for further studies. The isolates were Gram-positive and non-motile. They were catalase-negative and produced acid and gas in the case of glucose, sucrose, fructose, and mannitol sugars (Table 1).

Table 1. Fermentation of Sugars.

Isolates	Sugars			
	Glucose	Sucrose	Fructose	Mannitol
BL1	Acid production	Acid-Gas production	Acid production	Acid production
OL2	Acid production	Acid-Gas production	Acid-Gas production	Acid production

Both the isolates showed acid production for glucose and mannitol and no gas production was observed even after a prolonged incubation. For sucrose acid and gas production was observed and for sucrose, BL1 showed acid production whereas OL2 showed acid and gas production.

Identification:

Identification of the isolates was done by 16S r RNA sequencing (Table 2).

Table 2. 16S rRNA sequencing of the isolates.

Isolates	Organism	Accession number
BL1	*Enterococcus mundtii*	PQ659186
OL2	*Leuconostoc mesenteroides*	PQ659286

The obtained nucleotide sequence of these isolates was deposited in the GenBank nucleotide sequence data library with the given accession numbers.

2. Plant Growth Promoting Traits of the Isolates:

Table 3. Plant growth promoting traits.

PGP traits	Isolates	
	BL1	OL2
IAA (µg/ml)	30.837	7.898
Gibberellic acid (µg/ml)	19.898	6.206
Phosphate solubilization	-	-
Nitrogen Fixation	+	-
Iron chelation	-	+
Ammonia production	+	+

IAA: Indole Acetic Acid, +: Positive, -: Negative.

Both the isolates are showing significant production of IAA and gibberellic acid. There was no phosphate solubilization. Nitrogen fixation was observed for BL1 and iron chelation i.e. siderophore production was observed for OL2. Ammonia production was observed for both the isolates.

3. Antifungal Activity of Lactic Acid Bacteria against *Fusarium sp*. and *Aspergillus sp*.

Table 3. Antifungal activity of Lactic Acid Bacteria against *Fusarium sp*. and *Aspergillus sp*.

Isolates	Zone of inhibition (mm)	
	Fusarium sp.	***Aspergillus sp.***
BL1	15±0.00	No zone observed
OL2	5±0.00	7±0.00

OL2 showed antifungal activity against *Fusarium sp.* and *Aspergillus sp.* Whereas BL1 showed antifungal activity against *Fusarium sp.*

DISCUSSION

Indole acetic acid (IAA) is the main auxin in plants, regulating growth and developmental processes such as cell division and elongation, tissue differentiation, and apical dominance. IAA serves as a signaling molecule necessary for the development of plant organs and coordination of growth. According to *Jain et al.*, the *Azotobacter* strains isolated from semi-arid regions of India were able to produce IAA ranging from 16.5 to 34 µg/ml in media supplemented with tryptophan (12). IAA production by Indigenous isolates like *Pseudomonas* and *Proteus* was observed by Cavalcante *et al.*, and the amount of IAA produced was 18.37 µg/mL and 16.53 µg/mL respectively (11). If we compare the IAA production values of reported organisms, the BL1 isolate is showing significant production.

The gibberellic acid (GA) is a phytohormone, also referred to as gibberellins, regulates almost all processes of plant development and growth, such as seed development and germination, stem and root growth, cell division, and flowering time. The *Azotobacter* strains produced significant quantities of GA varying from 9.5-36.7 µg/mL in Gayan *et al.*, In a study by Desai (2017), GA was found to be secreted around sugarcane rhizosphere by as many as 60 different bacterial isolates, and one of the isolates, viz., K8 (identified as *Pseudomonas*) produced 24 µg/mL of GA. Here, the BL1 isolate is showing 19.898 µg/mL of GA production which is significant as compared to other reported organisms.

Naturally, phosphate is present in inorganic or organic forms in soil. Inorganic phosphate includes orthophosphate anions, orthophosphate minerals, etc. Organic phosphate includes phosphate atoms covalently bonded to carbon and it is derived from soil organic matter, crop residue, or manures. Phosphate plays a major role in cell division. The microorganisms present in soil solubilize phosphate so that plants can easily absorb the phosphate. Both the isolates in my study are unable to solubilize phosphate.

To fulfill the nitrogen requirement of plants various chemical fertilizers like urea, ammonium nitrate, and anhydrous ammonia are used. However, the use of these chemical fertilizers causes soil acidification, reduction of soil fertility, and pollution of soil and water. Biological fertilizers serve as an effective and eco-friendly solution to this problem. Often the nitrogen-fixing bacteria are used as biological fertilizers. *Azospirillum* and *Azotobacter* can colonize at roots of diverse plants, provide nitrogen, and hence improve plant growth (21). Diverse species of *Bacillus* comprising *B. cereus*, *B. circulans, B. firmus, B. licheniformis, B. megaterium, B. subterraneous, B. aquimaris, B. vietnamensis,* and *B. aerophilus* are known to fix atmospheric nitrogen (22). The Ashby's mannitol agar is used for the isolation of nitrogen-fixing bacteria. This media lacks nitrogen. For growth, organisms have to use mannitol as a carbon source and atmospheric nitrogen as a nitrogen source. BL1 isolate showed growth on Ashby's mannitol agar which indicates the nitrogen-fixing ability of this isolate.

PGPR isolates are also known to release iron-chelating compounds that increase the availability of iron to plants in iron-limiting soil. Iron is present in complex form and siderophores play important role in making iron available to plants. The isolate OL2 has shown a notable yellow-orange halo around the colony which demonstrates the production of a significant amount of siderophore. Siderophore production was seen in both bacteria and fungi and it's antimicrobial role was also studied. *Pseudomonas sp.* and *Bacillus sp.* were shown to produce siderophores and induce *Cephalosporium maydis* disease resistance in maize crops (19). Martina *et al.*, say *Pseudomonas aeruginosa* and *Acinetobacter baumannii* use iron chelation as an antibacterial strategy (18). Fungus like *Penicillium chrysogenum and Aspergillus* were reported for siderophore production (20).

Ammonia (NH_3) is one of the reactive Nitrogen forms. It could be produced from the degradation of different amino acids coming from food proteins or complex media. The organic ammonia present in biological tissues (dead organisms, excreted wastes, etc.) undergoes decomposition by fungi and prokaryotes to produce ammonia. This is the ammonification process (24). The presence of ammonia-producing Plant Growth Promoting bacteria (PGPB) and other prokaryotes is indicative that the ammonification process was taking place in the plant rhizosphere (25). Interestingly, this process not only improves plant growth, through the supply of nitrogen but also indirectly influences plant development by inhibiting plant pathogenic microbes (16). Prajka reported ammonia production in *Bacillus sp* (23). Abdelwahed reported *Bacillus*, *Rhizobium*, *Pseudomonas,* and *Azotobacter* for the production of ammonia (16). In my study, both the isolates have shown brown color which indicates significant production of ammonia.

In the rhizospheric soil, there are many organisms present (mainly fungi) that can act as pathogens. Hence antifungal activity of the isolates was checked against the

phytopathogens *Fusarium sp.* and *Aspergillus sp.* The fungus *Fusarium* is one of the major pathogens that cause different disease symptoms in almost all plant parts, which may result in significant economic losses. *Aspergillus* is a very prominent and widely reported fungal pathogen. It easily attacks plants at different stages including seed germination, seedling, and storage of grains. To overcome this problem the organisms having strong antifungal activity can be used. The antifungal activity of *A. xylosoxidans*, *Bacillus sp.*, and *P. aeruginosa* against *Fusarium sp.* was reported (26,27). The antifungal activity of *Lactiplantibacillus plantarum* isolated from herbal juice was observed against mycotoxigenic *Aspergillus species* (28). Li *et al.*, state that the *Bacillus velezensis* isolated from a paddy field has been shown to have the potential to control *A. flavus* contamination (29). In the present study, BL1 isolate showed a significant zone of inhibition against *Fusarium sp.,* and no zone was observed for *Aspergillus sp.* OL2 isolate showed a small zone of inhibition against both *Fusarium sp.* and *Aspergillus sp.* But the zone was not disturbed even after prolonged incubation which signifies strong antifungal activity against fungus.

CONCLUSION

From the above data, it can be said that the leaf isolates of Lactic acid bacteria have the potential to be used as PGPR.

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