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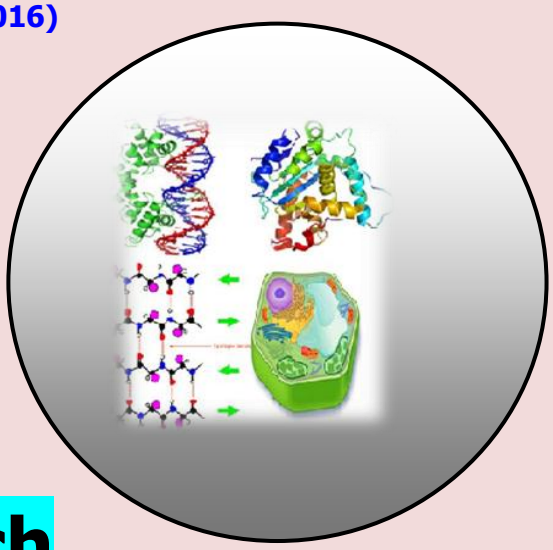

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RESEARCH PAPER

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Isolation and Characterization of Endophytic Bacteria from *Limonia acidissima* L. with Potential PGPA and Antimicrobial Activity

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ABSTRACT

Limonia acidissima* plant parts (root, stem bark and leaves) were investigated for the bacterial isolates having antibacterial and antifungal compounds producing ability. Total five isolates having potential plant growth promotion activity PGPA identified as Pseudomonas stutzeri and Aeromonas sobria from roots, Sphingomonas paucimobilis and Aeromonas sobria from stem bark, Aeromonas sobria from leaves. Results highlighted that large diversity prevails in the endophytic bacterial population and those can produce indole acetic acid (IAA) and ammonia which supports plant growth. In addition, they are also found to be promising candidate to produce several secondary metabolites which has ability to control human and plant pathogens for sure. These endophytes are recorded to be encouraging nominee for therapeutic studies in coming time.
Keywords: Limonia acidissima, Endophytic Bacteria, Plant Growth Promotion, Antibacterial and Antifungal, Phytochemical.

INTRODUCTION

Endophytes can be easily isolated from plant parts such as stem bark, petiole leaf blade (Hata and Sone, 2008), primordia, meristem and resin ducts (Pirttila *et al*. 2000; Pirttila *et al*. 2003), leaf segments with mid rib and roots (Hata *et al*., 2002). Endophytes as bacteria, fungi, and/or actinomycetes could be isolated from plant tissues by adopting number of techniques extensively used for the isolation of endophytes (Hallmann *et. al*. 1997; Reinhold-Hurek and Hurek, 1998). Generally, endophytes are isolated by surface sterilizing plant tissue and by culturing from ground tissue extract (Rai *et al*. 2007) and also by direct culturing of plant tissues (Hata and Sone, 2008) on media suitable for bacteria or fungi or actinomycetes growth. Endophytes are known to produce a wide range of plant growth promoting hormones, such as auxins, cytokinins and gibberellic acids. Besides that, endophytes also provide vital antimicrobial activity by which, it assists in controlling microbial pathogens in plants and/or animals. Mostly endophytes isolated from medicinal plants produces broad spectrum antimicrobials and remain effective to number of pathogenic microorganisms (Sette *et al*. 2006; Selim *et al*. 2011; Devaraju and Sathish, 2011). Some endophytes have unique ability to mimick properties of plants such as its ability to produce secondary metabolites (J. Zhao *et al*. 2010). *L. acidisimma* is the native plant of India and also present in Srilanka, Pakistan and Bangladesh (Bakshi *et al*. 2001). The plant is dominant in dry plains area. It grows well in a monsoon season and in distinct dry season also. The trees found to be reaching in height up to 450 meters especially in western Himalayas. It can sustain dry spell and better adapted to light soils (Vaidayaratnam Varier *et al*. 1995). Whole plant parts of Limonia were found to be useful in controlling number of diseases. As per Ayurveda, bark and leaves of *L. acidissima* are recommended for the treatment of two basic

ailments i.e. Vatta (wind) and Pitta (bile). Leaves are also astringent and carminative, effective against vomiting, hiccups, dysentery and indigestions. Leaves also found to be effective against hepatoprotective activity (Ilango *et al*. 2009). Ethanolic extract of leaves of *L. acidissima*, found to be possessing broad spectrum antibacterial activity against human pathogens of Gram positive and Gram negative nature. In contrast, methanolic extract exhibited good antibacterial activity; while chloroform extract reported to be having moderate zone of inhibition (Naidu, 2014). In the present study, endophytic bacteria of *L. acidissima* were investigated for plant growth promotion activity, their bioactive compounds producing features and possible antibacterial and antifungal activity against human and plant pathogens as new therapeutic agents.

MATERIAL AND METHODS

Plant collection

In an order to detect endophytes and related studies, plant parts of *L. acidissima* such as roots, stem bark and leaves were freshly collected from Nagpur (Maharashtra, India) region and brought to the laboratory.

Isolation of bacterial endophytes from plant

In the present study, endophytic bacteria were isolated from the, root, stem bark and leaves parts of *L. acidissima* plant. In a process, freshly collected plant parts was washed in running water and checked for any injury as an exclusion criterion. After that, the parts were diced into pieces and then treated with surface sterilizing agent such as 70% ethanol for 30 seconds; it was then treated with 0.1% mercuric chloride for 2 minutes and once again three times washed with sterile distilled water. To confirm the disinfection protocol, final rinse of 0.5ml water was used as an inoculum on Soyabean casein agar (SCA) plate. All other sterilized plant parts then collected in sterile petri plates separately. Plant parts were then cut it into small transverse sections and plated on the SCA plates and incubated at 27^{o}C for 24 hrs. After incubation, all isolates were sub-cultured to purify on the same fresh medium.

Morphological characteristics

All bacterial isolates were Gram stained and checked for motility by standard methodology.

Plant Growth Promoting Activity (PGPA)

After successful isolation of bacterial species; all isolates were checked for the possible plant growth promoting activity by screening them with three tests such as Indole acetic acid, Phosphate solubilization and Ammonia production and promising isolates were selected for further study.

A. Indole 3 acetic acid production test

Quantitative estimation of IAA was performed according to Bric et.al. (1991). Sterilized L- tryptophan (1 mg/ml) was added to 25ml of sterile nutrient broth. Endophytic culture was then inoculated and kept for incubation at 30^{o}C for 72 hrs. After incubation broth was centrifuged at 3,000 rpm for 10 minutes. . For measuring the amount of IAA produced, 1ml of culture supernatant was pipetted into test tubes and mixed with 4ml of the Salkowsaki's reagent (50ml, 35% of perchloric acid, 1ml of 0.5M $FeCl_3$ solution) and 2 drops of *ortho* phosphoric acid. In a result, progression of pink color in tube indicated positive result. Formation of pink color was measured as 535nm by using spectrophotometer along with the control.

B. Phosphate solubilisation

Bacterial isolates were separately inoculated centrally on the Pikovskaya's medium and plates were incubated at 30^{o}C for 3-6 days. The clear zone around the colony considered as positive test for phosphate solubilization ability (Gaur, 1990).

C. Ammonia production

Bacterial isolates were tested for the production of ammonia. In requirement, peptone water (10ml) was inoculated with fresh culture and incubated at 30^{o}C for 48-72 hours. In peptone water, 0.5ml of Nessler's reagent was added and checked for brown to yellow color which has indicated positive test for ammonia production (Cappuccino and Sherman, 1992).

Bacteria identification by VITEK2 technique

All promising bacterial isolates were subjected to VITEK 2 analysis as per Gram nature and results were recorded to determine the species identity.

Molecular characterization

Total DNA isolation and extraction from bacterial cells for PCR analysis was done by Genomic CTAB protocol. PCR amplification of the 16S rRNA gene fragment was done by using 16s Forward (AGAGTTTGATCCTGGCTCAG), 16sReverse (AAGGAGGTGATCCAGCCGCA) primers.

The amplification mixture contains 32.0 µl nuclease free water, 5.0 µl PCR buffer 10x, 2.0 µl dNTP (10 mM), 0.4µl forward primer (10 µM), 0.4µl reverse primer (10 µM), 1.0µl Taq DNA polymerase enzyme (1U/ µl) and 200ng DNA template. PCR reaction was programmed as: Initial denaturation of 3 min. at 94ºC, denaturation of 1 min. at 94ºC, primer annealing for 1 min. at 94ºC, extension of 2 min. at 94°C, final extension for 5 min. at 72°C; total 30 cycles and stored at 4°C. Amplicon was sequenced and analyzed by BLAST to find the best-scored close homolog. In a phylogenetic analysis, top five best homologs were aligned in CLUSTALW.

Phytochemical production by isolates

Based on PGPA, promising endophytic bacteria were selected to check the production of bioactive compounds. In this study, bacterial isolates were maintained on Soyabean casein broth. Bacterial isolates after inoculation incubated for 24 hours at 30^{o}C. After incubation, 1ml of the given medium for bacteria were inoculated to 10ml of M9 minimal media and incubated for 24 hours at 30^{o}C for bacteria. After incubation, once again 1ml of incubated broth was transferred to 10ml of M9 minimal media and allowed to incubate for 5-6 hours at 27^{o}C after that 1ml from this medium was transferred to 250 ml M9 medium and samples kept for growth on a rotary shaker at 27^{o}C at 150rpm. Optical density was measured every 2 hours at 620nm. Similarly change in pH was also recorded for the culture media three times in 24 hours. During study, peak point optical density was considered as the stage of life cycle where maximum Phyto-active compounds may get synthesized and hence for every sample peak point was considered as a stage of sample selection and withdrawal and those were further tested for preliminary phytochemical analysis. Antimicrobial study also been done with the same samples especially against plant and human pathogens.

Phytochemical analysis of fermented broth

Phytochemical analysis of fermented broth was also tested to detect Alkaloids, carbohydrates, Glycosides, Proteins and amino acids, Saponins, Phytosterols, fats, Phenolic compounds, flavonoids, gums and mucilages proposed by N. Raaman (Raaman N., 2006). Extracts were then subjected to antibacterial and antifungal assays.

Antimicrobial activity of the fermented broth against human and plant pathogens

Collected sample of fermented broth was centrifuged at 3000 rpm to obtain cell free supernatant on the same day.

Antibacterial and Antifungal activity

In an antibacterial and antifungal activity of filtrate, Hi-sensitivity agar medium was used and 0.1ml of culture having density equivalent to 0.5 O.D. McFarlands standard (1.5x 10^{8} CFU/ml) was inoculated on medium and spread evenly. Thereafter, 10mm wells were punctured with the help of sterile borer. In an empty well 100µl of cell free supernatant of each endophytic bacterial culture broth was added and allowed to diffuse for 1 hour in refrigerator at 8- 10^{0}C without disturbing. Plates were then incubated at 35^{o}C $\pm$ 2^{o}C for 24 hours and zone of inhibition was recorded in milli-meter (mm).

RESULTS

Plant collection

To investigate endophytic bacteria plant parts such as root, stem bark and leaves of *L. acidissima* were freshly collected.

Isolation of bacterial and fungal endophytes from plants

Presence of endophytic microorganisms was investigated when surface sterilized *L. acidissima* root, stem bark and leaves part were inoculated on to the soybean casein agar for bacterial isolation. After incubation, number of colonies was emerging out of the plant part. It is apparent that bacterial isolates which were endophytic in nature were prominently formed colony on the plate. All these isolates were sub cultured on the same media and finally these bacterial isolates were considered as endophytes.

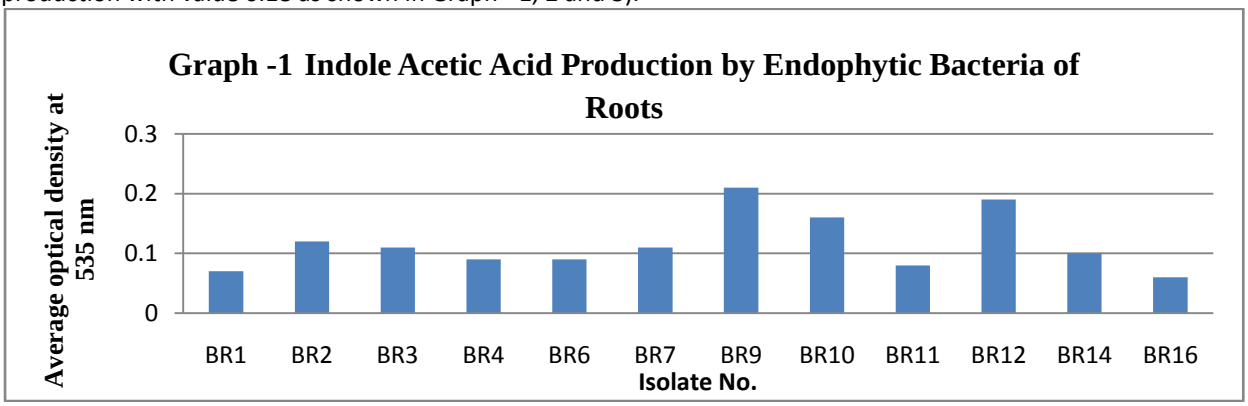
Morphology characteristics (Microscopic examination)

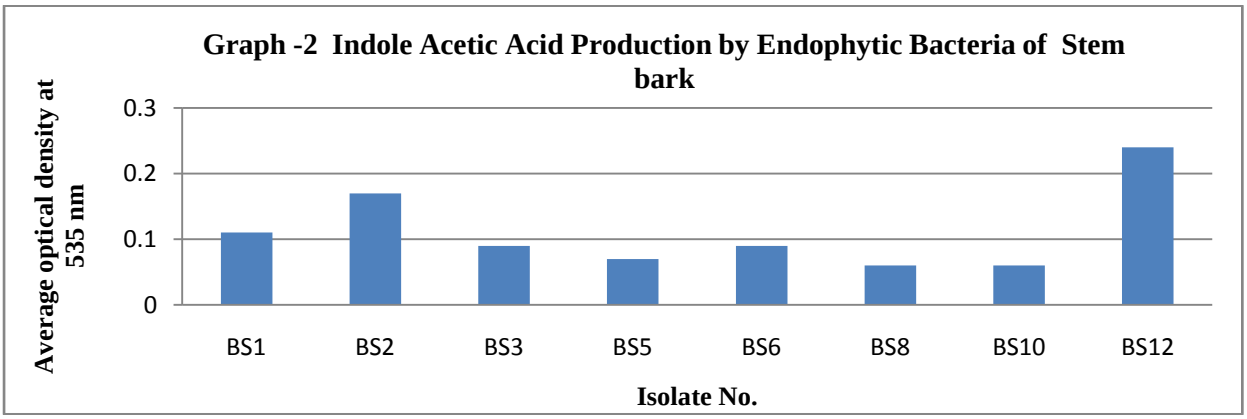
Based on endophytic isolation protocol, twenty seven bacterial isolates were purified. On the basis of microscopic examination, out of 27 bacterial isolates 6 of roots, 6 of stem bark and 4 of leaves were found to be Gram positive. Whereas 6 of roots, 2 of stem bark and 3 of leaves were found to be Gram negative. These isolates were then subjected to screening for efficient isolates on the basis of their ability to exhibit PGPA and also for identification by Vitek 2.

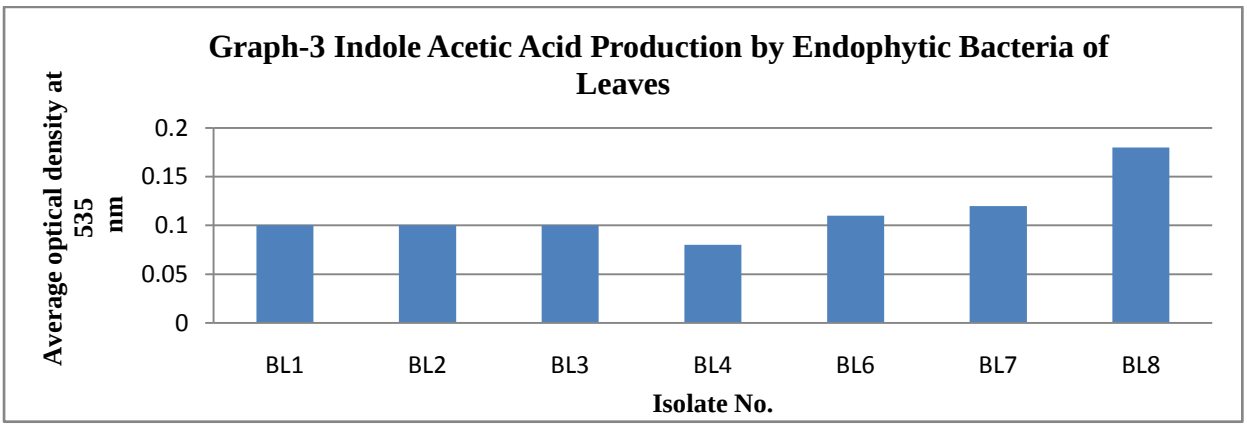
Plant growth promoting activity (PGPA)

Among root isolates when those were tested for indole acetic acid (IAA) presence, top two isolates with better IAA production were recorded as BR9 and BR12 with value of 0.21 and 0.19 respectively.

Among stem bark isolates, two isolates recorded to be better performer (BS2 and BS12) with value of 0.17 and 0.24, respectively. Finally, among leaves isolates, only one isolate as BL 8 was recorded with better IAA production with value 0.18 as shown in Graph - 1, 2 and 3).







Phosphate solubilization

All twenty seven endophytic bacterial isolates of roots, stem bark and leaves showed negative result and represented lack of such ability.

Ammonia production

All isolates was prominently recorded positive for the production of ammonia.

Based on these features, total five isolates (BR9, BR12, BS2, BS12 and BL8) found to be best performer and considered as a candidate for PGPA and further evaluated

Identification of endophytic bacteria

The promising bacterial isolates having number of PGPA features were identified as per Vitek 2 test. The results are highlighted below:

BR9 as Unidentified isolate, BR12, BS12 and BL8 identified as *Aeromonas sobria and* BS2 as *Sphingomonas paucimobilis.*

Here after BR12 will be referred as *Aeromonas sobria* BR12, BS12 as *Aeromonas sobria* BS12, BL8 as *Aeromonas sobria* BL8 and BS2 as *Sphingomonas paucimobilis* BS2.

16s rRNA gene sequencing

Isolates BR9 was selected for the phylogenetic analysis and the criterion for selection was incapability of Vitek 2 to identify this isolate. After the phylogenetic study, the bacterial strain was identified and was found to be having close sequence alignment with *Pseudomonas stutzeri* strain H8 (accession number JF 727663.1) with 100 % query coverage and 99% identify and hence identified as *Pseudomonas Stutzeri* (accession number LC 431194) as shown in Fig. 1. Here after, BR9 isolate will be referred as *Pseudomonas stutzeri* BR9.

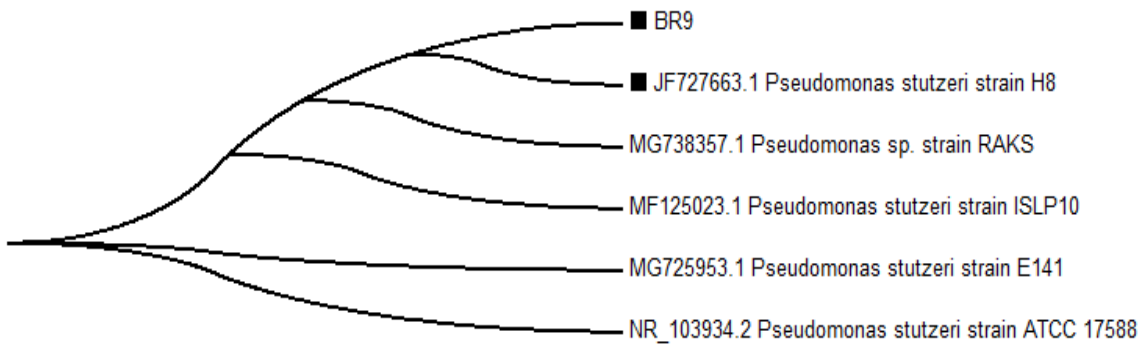


Figure 1. Phylogenetic tree for multiple sequence alignment of BR9

Phytochemical production by isolates

The ability of five bacterial isolates to grow in a medium was found to be proficient as the optical density (O.D.) was on increasing logarithmic pattern. During recording a peak point, O.D. was considered as a factor where maximum bioactive compounds are produced. Accordingly, isolate *Pseudomonas stutzeri* BR9 showcased its maximum growth at 7^{th} day with optical density 0.69. In case of isolates *Aeromonas sobria* BR12, *Sphingomonas paucimobilis* BS2, *Aeromonas sobria* BS12, and *Aeromonas sobria* BL8 the optimum growth in the form of peak point was recorded on 9^{th} day with O.D. value as 0.64, 0.69, 0.71, and 0.71 respectively.

Phytochemical analysis

Ability of bacterial species those have been promising for PGP activity were tested further for the production of bioactive Phyto-chemical compounds when allowed to grow on M9 Minimal media. Samples were withdrawn and tested at a peak point of cellular growth. The extracts under study are rich in phytochemical content except glycosides which are not found in any extracts. The details of the result have been showcased below Table no 1: -

Foot note: "+" positive result; "_" negative result; Increasing + number indicate higher degree of reaction.

Antimicrobial activity of Bacterial filtrate

Ability of Bacteria to produce number of bioactive compounds as an antibacterial and antifungal agent has been systematically investigated in Hi-sensitivity agar medium against six bacterial pathogens such as *E. coli* (NCIM- 2065), *Bacillus subtilis* (NCIM- 2063), *S. aureus* (NCIM- 5345), *Shigella boydii* (NCIM- 5644), *Pseudomonas aeruginosa* (NCIM- 2200) and *Salmonella abony* (NCIM- 2257) and in case of fungi it was *Aspergillus brasiliensis* (NCIM- 1106) and *Candida albicans* (NCIM- 3471). In other approach plant pathogens like bacteria *Xanthomonas campestris 1 (Xac 1), Xanthomonas campestris 2 (Xac2),* along with fungi as *Rhizoctonia bataticola* and *Sclerotium rolfsii* were tested successfully for the antimicrobial effect.

Human pathogens antimicrobial activity

As per antibacterial testing, human pathogens *E. coli, Shigella boydii,* found to be totally resistant to all five filtrates of bacterial species. In an effective inhibition, *S. aureus* only found to susceptible with *Aeromonas sobria* BL8 filtrate with 28mm zone of inhibition. Similarly, *Bacillus subtilis* also recorded to be growth inhibited with 15mm and 11mm of inhibition which is of intermediate type with filtrate of *Pseudomonas Stutzeri* BR9 and *Sphingomonas paucimobilis* BS2. In an antifungal assay, *Aspergillus brasiliensis* and *Candida albicans* did not found to be inhibited in growth by any filtrate and remained resistant in all sense.

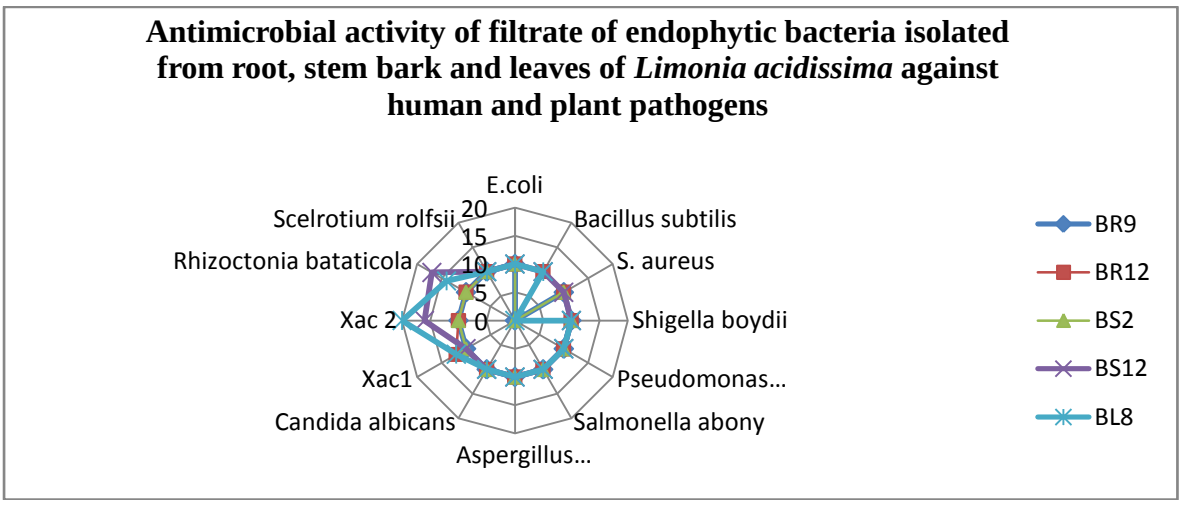
Plant pathogen antimicrobial activity

Ability to inhibit plant bacterial pathogens, *X. campestris 1* and *2* along with plant fungal pathogens, *R. bataticola* and *S. rolfsii* was found to be promising with the bacterial fermentation filtrate. As per data recorded *Aeromonas sobria* BL8 filtrate able to control *X. campestris 1* and *2* along with *R. bataticola* with 12mm, 20mm, and 14mm zone of inhibition, respectively but failed to inhibit *S. rolfsii*. *X. campestris 1* showcased growth inhibition with *Aeromonas sobria* BR12, and *Aeromonas sobria* BL8 filtrates with 12mm each zone of inhibition.

Table no. 1. Phytochemical analysis of endophytic bacterial extracts.

Sr no.	Phytochemicals test	*Pseudomonas stutzeri* BR9	*Aeromonas sobria* BR12	*Sphingomonas paucimobilis* BS2	*Aeromonas sobria* BS12	*Aeromonas sobria* BL8
1	Alkaloids	++	++	++	++	++
2	Carbohydrates	+	+	+	+	+
3	Glycosides	-	-	-	-	-
4	Proteins & amino acid	+	++	++	++	++
5	Saponins	+	+	+	+	+
6	Phytosterols	+	+	+	+	+
7	Fats	+	+	+	+	+
8	Phenolic compound & flavonoids	++	++	++	++	++
9	Gum and Mucilages	+	+	+	+	+

Another bacterium, *X. campestris 2* was found sensitive to filtrate of *Aeromonas sobria* BS12 and *Aeromonas sobria* BL8 with 16 mm and 20mm zone of inhibition, respectively. Plant pathogenic fungi, *R. bataticola* also registered wide growth inhibition with filtrate *Aeromonas sobria* BS12 and *Aeromonas sobria* BL8 with 17mm and 14mm of inhibition, respectively. In all aspect *S. rolfsii* was not getting inhibited by any of the filtrate tested and remained resistant to all of them.



DISCUSSION

Since, we were able to culture most of the endophytes on the given media we confirmed that endophytes are prominently present in *Limonia acidissima* root, stem bark and leaves as bacteria.

With the successful isolation of endophytic bacteria, ability to provide essential growth factors/nutrients to the parent plant has been investigated. In first set of study, root, stem bark and leaves originated isolates as two, two and one respectively in numbers found to be better producer for indole acetic acid with the value of in the range of 0.1-0.24.

Similar manner, by involving traditional methodologies indole acetic acid (IAA) producing bacteria are widely associated with many plants. Jiang X *et al*. isolated as many as 66 isolates belonging to 26 species of 15 genera of 5 Phyla. Majority of them belongs to *Staphylococcus, Microbacterium, Rhizobium* and *Methylobacterium* (Jiang, X *et al*. 2013). Jasim *et al*. (2014) reported IAA production of bacterial species with important gene *ipdc* which was very well correlated with the relation to improve plant production.

In the present study twenty seven bacterial isolates able to produce indole acetic acid but completely remain devoid of phosphate solubilization. In contrast number of endophytes as plant growth promoting *Rhizobacteria* such as *Pseudomonas putida* able to carry out phosphate solubilization when investigated with *Mentha piperita* (Santoro *et al*. 2015); *Bacillus sp.* and *Paenibacillus sp.* also been able to carry out phosphate solubilization (Khalaf and Raizada, 2016).

Whereas ammonia production was found to be positive for all the bacterial isolates. In a similar report, endophytic bacteria when isolated from date palm (*Phoenix dactylifera L.*) root part. They were able to produce ammonia, and can chelate ferric iron (Fe^{3+}) and solubilize potassium (K^{+}). They were identified as *Paenibacillus xylanexedans* and *Enterobacter cloacae*. All these isolates found positive for PGP activity (Yaish *et al*. 2015).

In a similar way number of ammonia producing endophytes were recorded earlier such as Anc*yclobacter sp.; Azorhizobium sp.; Sinorhizobium sp.; Novosphingobium; Burkholderia sp.; Acinetobacter* (Banik *et al*. 2016); *Sphingomonas sp., Bacillus sp. and Enterobacter sp.* (Li *et al*. 2016).

As per overall results in PGPA study, successfully isolated and selected bacterial endophytes from *L. acidissima* were BR9, BR12, BS2, BS12, and BL8. They were tested for their biochemical features successfully by involving Vitek2 analysis method. The methods found useful to characterize number of positive markers available in each isolates and it also finds its utility in identification of each isolate tested. Here with Vitek2 technique,BR9 as unidentified*,* BR12 as *Aeromonas sobria*; BS2 as *Sphingomonas paucimobilis*, BS12 as *Aeromonas sobria* and*.* BL5 identified as *Aeromonas sobria* According Gashgari and Selim (2015) *Aeromonas sobria* exhibits natural antimicrobial features and by which it can be 100% resistant to the nalidixic acid, cephalothin, carbenicillin, erythromycin, kanamycin, tetracycline and trimethoprim-sulfamethoxazole. Probably this is the reason why these bacteria able to survive as endophytes in plant which always remain surrounded by many antibiotic producing microorganisms. In the present study another bacterium identified as per Vitek2 was *Sphingomonas paucimobilis* found to be isolated from the stem part of the plant. In a similar report, plant *Corchorus capsularis* found to have association with *S. paucimobilis* and able to reduce anthracene. Hence by using *Glomus mosseae,* and *Glomus intraradices* in a jute inoculation, it improves plant growth especially when enhance anthracene removal occurred in presence of *S. paucimobilis* (Cheung *et al*. 2008). In the study, along with Vitek identity of isolates, we used 16s rRNA gene sequencing methodology which is a common approach for bacterial identification as reported earlier. By 16s rRNA gene sequence isolate BR9 identified as *Pseudomonas stutzeri* which was reported to be promising plant growth isolates as recorded in number of researches. Babaei *et al*. (2015) reported the promising feature of *P. stutzeri* in nitrogen fixation ability and phytohormone production. They genome sequenced the *P. stutzeri* and recorded 890 genes, 1135 reactions and 813 metabolites and confirmed the potential of *P. stutzeri* as plant growth promoting agent. Culture broth of all endophytic bacteria in M9 minimal medium, *Aeromonas sobria* BR12, *Sphingomonas paucimobilis* BS2*, Aeromonas sobria* BS12*, Aeromonas sobria* BL8*, Pseudomonas Stutzeri* BR9 were found to be contained alkaloids, reducing sugars (carbohydrates), proteins and amino acid, saponins, Phytosterols, fat, phenolic compounds, gums and mucilage, whereas glycoside was not evident in any of the fermented broth of endophytic bacteria of plant parts. Study further recorded the potential of producing antibacterial and antifungal compounds by the isolates. In M9 minimal culture broth of *Pseudomonas stutzeri* BR9 and *Sphingomonas paucimobilis* BS2 were found to inhibit *B. subtilis* with zone of inhibition with 15mm and 11 mm respectively. *Aeromonas sobria* BL8 was highly effective against *S. aureus* with zone of inhibition of 28mm. *Aeromonas sobria* BR12 and *Aeromonas sobria* BS12 were found to be ineffective against any test organism As it has been recorded that bacterial endophytes able to produce bioactive compounds which are ultimately remain associated with plant with which they grow. We can certainly use plant or the endophytes as a novel antimicrobial compound producer. According to Alvin *et al*. (2014) natural products drug discovery providing the best platform for new cost-effective concept and it can treat multiple diseases with same formulation. They hypothesized that plant/endophytes association produces polyketides and peptides which remains antibacterial in nature and needs further investigation. Ellsworth *et al*. (2013) isolated number of endophytes and prepared sixty-two crude extract from spent fermentation broth of liquid cultures for antifungal and antibacterial activity. Similar to our result, they reported twenty-two extracts able to inhibit *Staphylococcus aureus* and twelve found to effective against *Candida albicans*.

In a further study, promising antibacterial filtrate of all isolates failed to showcase any antifungal activity when tested against *Aspergillus braseiliensis* and *Candida albicans*. In a similar approach, ability to control bacterial plant pathogens, (*X. Campestris* 1 and 2) and fungal pathogens (*R. bataticola* and *S. rolfsii*) found promising in its action where *Aeromonas sobria* BL8 filtrate successfully controlled growth of *X. campestris* 1 and 2 along with fungi *R. bataticola* with 12, 20- and 14-mm zone of inhibition respectively. Similarly, *Aeromonas sobria* BS12 and *Aeromonas sobria* BL8, able to control bacterial and fungal species conferring this feature in controlling bacterial and fungal diseases in plants.

CONCLUSION

L. acidissima, being a medicinal plant able to provide number of antibacterial and antifungal compounds to the nature as its own. In addition, here we proposed that, plant also posses' number of bacterial and fungal endophytes producing several bioactive compounds which was guaranteed to control the growth of human or plants bacterial and fungal isolates with a great success. Here, we confirmed that every plant part (stem bark, root and leaves) must be receiving these biomolecules as we confirmed them in *invitro*. These isolates also assured to be imparting plant growth promoting features by producing Indole acetic acid and ammonia production, making them complete plant growth supporting microbes.

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Competing Interests

The authors declare that they have no competing interests.

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