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Original Research Article

Preliminary Profiling of Extracellular Enzymes, Polysaccharide and Bioactive Secondary Metabolites of *Pseudomonas sp.* and *Lysinibacillus* sp. Isolated from Banana Cultivars of Assam

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Abstract: In this present study two banana endophytes; *Pseudomonas* and *Lysinibacillus* were studied for extracellular metabolites and enzyme. Endophytes were isolated from root of the Dwarfjehaji variety of Assam cultivars. The plate culture and detection method through colour development was used for extracellular enzyme, organic acid production and IAA production. Whereas, dual inoculation and solvent extraction method was followed for antimicrobial activity of these bacterial cultures. A preliminary study on extracellular production of enzymes, polysaccharides, organic acid, IAA and antifungal metabolites has been carried out on *Pseudomonas* sp. and *Lysinibacillus* sp. isolated from banana cultivar of Assam. *Lysinibacillus* sp. exhibited enhanced chitinase activity and good mineral solubilizing activity whereas *Pseudomonas* sp. did not show such chitinase activity on plate culture. Both the bacterial cultures produced exopolysaccharide and organic acids. No indication of IAA production by both the organisms has been found. However, both *Pseudomonas* sp. and *Lysinibacillus* sp. showed a wide range of antifungal potential. Data recorded on extracellular metabolic activity and antimicrobial activity of *Pseudomonas* sp. and *Lysinibacillus* sp. have been found very promising and may be useful parameter for selecting both the bacteria as bioinoculant for crop improvement of banana.

Keyword: Antifungal, Chitinase, *Lysinibacillus,* Phosphate solubilization, Polysaccharide, *Pseudomonas*.

Introduction

Bananas are considered a staple food and were ranked the 12th most important agricultural product in the world in 2017 (Garcia *et al.,* 2021). Bananas are the world's fourth most important cash crop. During the same time period, it reached the production benchmark of 116 million tonnes. Bananas are cultivated in over 135 countries, taking up a total area of 5.2 million hectares (Scott, 2021). Banana production is decreased due to the presence of plant diseases and mineral deficiencies. In these types of environments, bioinoculants that contain useful extracellular activity of enzymes, particularly hydrolases, secondary metabolites like organic acid that can leach out bound minerals, and bioactive antimicrobial compounds for the destruction of pathogens may be helpful in the development of bioinoculants.

Endophytes are normally defined as plant colonizing microorganisms, present in the host plant cell without causing any disease (Hyde and Soytong, 2008). Endophytes have the ability to produce useful enzymes and metabolites which could

promote resistance against disease causing organisms. Bacterial endophytic are present in the internal tissue of the plant. Most of the endophytes are maintains symbiotic bond with the host plant and plays a vital role in growth and development by the protection of damage caused by other pathogens (Vandana *et al.,* 2021). Endophytic bacteria are helpful in many aspects such as; regulate plant growth, promote yields capacity and most important that provides disease resistance capacity to the plant in adverse environmental condition (Beltran-Garcia *et al.,* 2014; Macedo-Raygoza *et al.,* 2019; Dini-Andreote, 2020; Bradshaw and Pane, 2020; Gupta *et al.,* 2020).

Organic acids production by bacterial endophytes plays an important role in regulation of nutritional metabolism and physiology, while these can assist microbes in cycling of essential minerals from soil to plants, deactivating metal ions to stable chelating agents or complexes and regulating host–plant physiology (Barros *et al.,* 2013; Yadava *et al.* 2013). Bacterial endophytes are well known for production of bioactive compounds like: alkaloides, steroids, terpenoids, peptides, flavonoids, quinol, phenols etc. along with some natural insecticides are also produced. Endophyte bacteria are also capable for production of plant growth hormones, phosphate solubilization and nutrient acquisition (Liarzi *et al.,* 2016; Glick, 2012). These nutrients are nitrogen, phosphate, zinc etc. bacterial endophytes play a vital role in production of phytohormones such as indole acetic acid (IAA), cytokinin and abscisic acid (Cueva *et al.;* 2021; Saha *et al.,* 2016). Endophytes produce numerous hydrolytic enzymes, chitinases is one of them. Actinobacteria are well known for their chitinolytic enzyme production and activity. Microbial source of chitinase is resolve and capable for degrade the cell walls of many pathogens showing antibacterial, anti-fungal, insecticidal or nematicidal activity (Liu *et al.,* 2010; Beier and Bertilsson, 2013; Edreva, 2005). The endophytes are well source of production of biopolymers including extracellular polysaccharides (EPS) (Donot *et al.,* 2012). Microbial extracellular polymeric substances, the heterogenous matrix of polymers comprising of polysaccharides, proteins, nucleic acids, lipids etc. are produced by bacteria through intracellular or extracellular processes (Wingender *et al.,* 1999; Freitas *et al.,* 2011). Now a days, several types of structurally different EPSs with bioactive potentials have been reported from endophytes (Guo *et al.,* 2014; Mahapatra and Banerjee, 2016). In the present study a preliminary screening for enzyme, polysaccharide and secondary metabolites productions by *Pseudomonas* sp. and *Lysinibacillus* sp. isolated from banana cultivar of Assam has been reported.

Materials and methods

Collection of Sample, Isolation and Identification

Samples of different banana cultivar of Assam were collected during 2018-19 and brought to laboratory for isolation of endophytes. Here, root part of Dwarf jehaji variety was used for endophytes isolation. Surface sterilized plant samples were inoculated on Nutrient Hi-veg agar, incubated for seven days. From several microbial isolates obtained from mixed culture plate, two bacterial cultures were isolated, purified and identified at generic level according to Ganeshan *et al.* (2021).

Screening of extracellular enzyme

Five different plate culture techniques were followed for the screening of these bacteria for extra cellular enzyme production. Each enzyme was detected after growth of bacterial culture grown on specified media by using separate detecting agents and/or exhibition of clear zone around the growing colony (Aneja, 1993). Production of chitinase for both the bacteria was performed by the protocol proposed by Krithika and Chellaram, (2016). 10 g of chitin powder added 120 ml of conc. HCl, incubated at 37 °C, 180 rpm for 1 h. The mixture was transferred through glass wool to 50% ethanol and thoroughly mixed to obtain a homogenous suspension. This was further transferred through filter paper and washed with distilled water until the colloidal chitin reaches pH 7. Colloidal chitin was collected and stored at 4 °C until use. 1% colloidal chitin was added to nutrient agar and media plate. A normal pH (7.2±0.2) was maintained for bacterial growth. Bacterial endophyte *Lysinibacillus* sp. and *Pseudomonas* sp. were inoculated by single striking line and kept for an incubation of 72hr. Observation was taken after incubation.

Screening for mineral solubilization & secondary metabolites

Bacterial cultures were grown on Pikovaskaya and Zinc solubilising agar media and observed for clear zone formation around the growing colony, which was the indication of mineral solubilization by the organisms (Gupta and Das, 2008). Plate test on specific media used for organic acid and IAA production were performed. The clear zone around the grown colony and change of coloured plate due to change in pH was observed and noted. A quantitative analysis was carried out to evaluated the organic acid production by titration method for bacterial endophyte *Pseudomonas* sp. and *Lysinibacillus* sp. (AoAc, 1995). 10ml of culture broth was taken for each bacterial stain, 50ml of d/w added and 3 drops of phenolphthalein solutions (50% ethanol of phenolphthalein) also added. The total liquid system titrated with 0.1M NaOH to get a little pink colour solution by continuous staring. The amount 0.1M NaOH required to obtain the colour is expresses as titratable acidity (g/100ml). Apart from this, a standardized plate test method was also followed to verify the presence of organic acid production (Aneja, 1993).

Estimation of exopolysaccharides (EPS)

The bacterial endophytes *Pseudomonas* sp. and *Lysinibacillus* sp. were inoculated separately in Nutrient Hi-Veg Broth media (Make-Himedia) and kept for a 72-hour incubation period at 30 °C. After incubation, broth cultures were filtrated, followed by a centrifugation process lasting 15 minutes at 5000 rpm. Pallets were discarded, and supernatant solutions were obtained. The volume of superpatriots was condensed using Soxhelt, and isopropanol was added at a ratio of 1:2 and kept for 48 hours. After 48 hr, this solution was again centrifuged, the pellets were collected, dried, and EPS was by the phenol sulfuric acid method (Dubois *et al.,* 1956; Hegde and Hofreiter, 1962). A sample was prepared by using the above protocol, absorbency was measured at 490 nm in a UV-spectrophotometer (Specord-50, Analytik Jena), and the results were compared with the standard value.

Screening for Antimicrobial activity

Co-incubation plate culture technique was followed for the screening of *Pseudomonas* sp. and *Lysinibacillus* sp. against 46 numbers of fungi (Balouiri *et al.,* 2015). Apart from this, secondary metabolites obtained for these two bacteria also evaluated for antimicrobial activity against *Fusarium* oxysporum. For crude secondary metabolites, Nutrient broth medium of 3lits. was prepared with pH-7.2. Bacterial endophytes *Lysinibacillus* sp. was inoculated and kept in incubation period of 72hr at 30°C (Talukdar *et al.,* 2021). After incubation period, broth culture was centrifuged, debris/pellets were removed and supernatant collected. Ethyl acetate was added to culture filtrates in 2:1 ratio and kept for 72hr. Ethyl acetate layer (upper layer) containing dissolved compounds was separated gently and kept in Hot air oven at temp. 50°C for evaporation. After complete evaporation, dried compounds were dissolved in methanol and passed through the Silica column (column make- Borosil, Bore diameter-18mm, legth-300mm; Silica make-Himedia, 60-120 mesh) by using standardised solvent system for partial purification. By the same method partially purified samples was obtained of *Pseudomonas* sp. dissolved in methanol and tested for antimicrobial activity.

Antimicrobial activity was carried out by some modification to the method proposed by Balouiri *et al.* (2015). NA and SDA media plate were used for bacteria and fungi respectively. The test was conducted with two controls; one is positive control and other with negative control. Test plate was prepared with 0.5ml of methanol dissolved sample, positive control plate congaing same volume of methanol and negative control plate congaing only medium. *Fusarium oxysporum* (disc of 8.0mm diameter) was inoculated on the three types of plate whereas bacterial strain was inoculated at the center of the media plate. After incubation period, colony growth was observed in diameter and analyzed.

Results

Extracellular Enzyme and Mineral Solubilization

Both bacterial species tested for extracellular enzyme, *Lysinibacillus* sp. has found positive for chitinase production,

Table 1. Extracellular useful metabolites and enzymatic activity of *Pseudomonas* sp. and *Lysinibacillus* sp.

Activity	*Pseudomonas* sp.	*Lysinibacillus* sp.
Amylase	-	-
Lipase	-	-
Cellulase	-	-
Xylanase	-	-
Chitinase	-	+
Mineral solubilisation	-	+
Organic acid production	+	+
IAA production	-	-

'+' Positive activity, '-' No activity

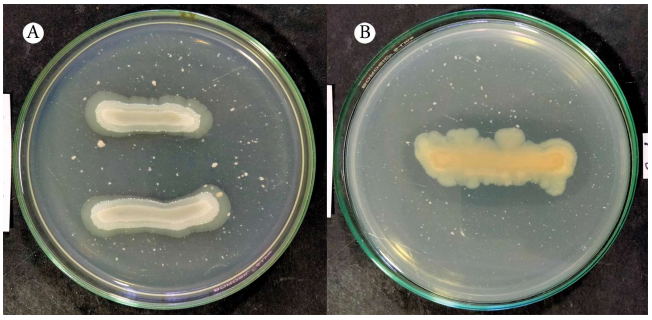

Fig. 1. Plate culture of *Pseudomonas* sp. (A) and *Lysinibacillus* sp. (B) showing chitinase activity.

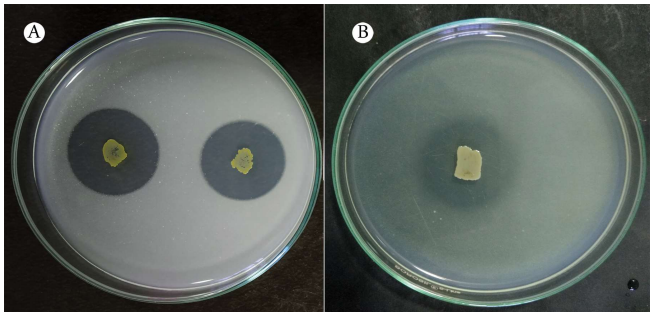

Fig. 2. Halo zone formation by *Lysinibacillus* sp. on Zinc agar medium (A) and Phosphate agar medium.

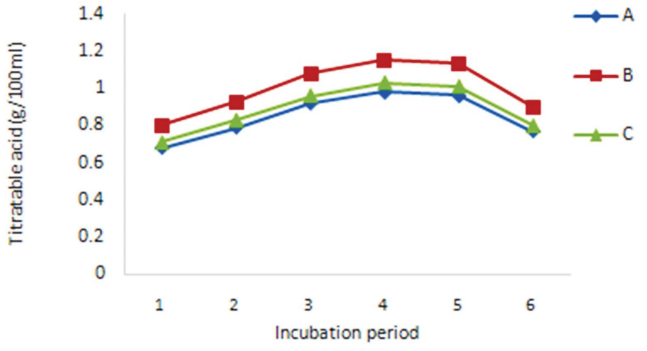

Fig. 3. A- Periodical observation of organic acid production by *Lysinibacillus* sp. Abbreviation: 1, 24hr; 2,48hr; 3, 72hr; 4, 96hr; 5, 168hr; A-Citric acid, B-Tartaric acid, C- Malic acid.

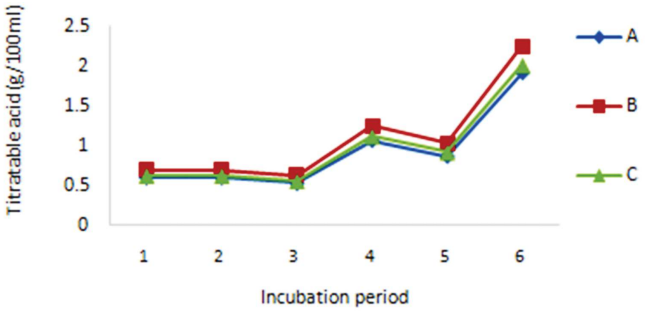

Fig. 3. B- Periodical observation of organic acid production by Pseudomonas sp. Abbreviation: 1, 24hr; 2,48hr; 3, 72hr; 4, 96hr; 5, 168hr; A-Citric acid, B-Tartaric acid, C- Malic acid.

whereas *Pseudomonas* sp. was negative in this regard (Table. 1 & Fig. 1). Findings of clear zone around growing colony of *Lysinibacillus* sp. in zinc agar and phosphate agar indicates the mineral solubilising property of the *Lysinibacillus* sp. (Fig. 2).

Organic acid production

Pseudomonas sp. and *Lysinibacillus* sp. have been observed as organic acid producer. Data recorded on periodical observation of organic acid production and calculated value of citric acid, tartaric acid and malic acid revealed the highest production of organic acid at 96 hr by both the bacterial species [Fig. 3(A&B)].

Production of exopolysaccharides

1.250 g and 0.750 g of crude EPS samples were obtained from *Lysinibacillus* sp. and *Pseudomonas* sp., respectively, from a total of six litters liquid culture for each species by following a standard laboratory procedure. After determining the amount of exopolysaccharide produced by crude samples, it was determined that *Lysinibacillus* sp. and *Pseudomonas* sp. produced 0.815±0.09 (mg/ml) and 0.666±0.23 (mg/ml) EPS per ml, respectively.

Antimicrobial activity

Antimicrobial screening was performed by using two bacterial strain *Lysinibacillus* sp. and *Pseudomonas* sp. against 47 fungal endophytics (Table 2). *Lysinibacillus* sp. found with wide spectrum antifungal activity, whereas *Pseudomonas* sp. exhibited poor inhibitory activity against fungal species tested.

Table 3. Antibacterial screening of fungal endophytes using two bacterial strains.

Sl. No.	Test Fungi	Inhibitory Activity of Bacteria	
		Lysinibacillus sp.	*Pseudomonas sp.*
1.	Aspergillus sp1	-	-
2.	Aspergillus sp1	-	-
3.	Aspergillus sp1	+	-
4.	Aspergillus sp1	-	-
5.	Aspergillus sp10	+	-
6.	Aspergillus sp11		
7.	Aspergillus sp2	+	-
8.	Aspergillus sp3	+	-
9.	Aspergillus sp4	-	-
10.	Aspergillus sp5	+	-
11.	Aspergillus sp6	-	-
12.	Aspergillus sp7	+	+
13.	Aspergillus sp8	-	-
14.	Aspergillus sp8	+	-
15.	Aspergillus sp9	-	-
16.	Cladosporium sp1	+	+
17.	Cladosporium sp2	+	-
18.	Cladosporium sp2	+	-
19.	Cladosporium sp3	+	-
20.	Curvularia sp1	+	-
21.	Curvularia sp2	-	-
22.	F. oxysporum	+	+
23.	Fusarium sp 13	+	-
24.	Fusarium sp1	+	-
25.	Fusarium sp10	+	-
26.	Fusarium sp11	+	-
27.	Fusarium sp12	+	-
28.	Fusarium sp2	+	-
29.	Fusarium sp3	+	+
30.	Fusarium sp4	+	
31.	Fusarium sp5	+	+
32.	Fusarium sp6	+	-
33.	Fusarium sp7	+	-
34.	Fusarium sp8	+	-
35.	Fusarium sp9	+	-
36.	Hemicola sp 1	-	-
37.	Hemicola sp 2	+	-
38.	Mucor or Rhizopus	-	-
39.	Sterile 1	-	-
40.	Sterile 2	+	+
41.	P. digitatum	-	-
42.	Penicillium sp	+	-
43.	Penicillium sp1	-	-
44.	Penicillium sp2	+	-
45.	Penicillium sp3	+	-
46.	Penicillium sp 4	-	-
47.	Tricoderma sp	-	-

'+' Positive inhibition, '-' No growth inhibition

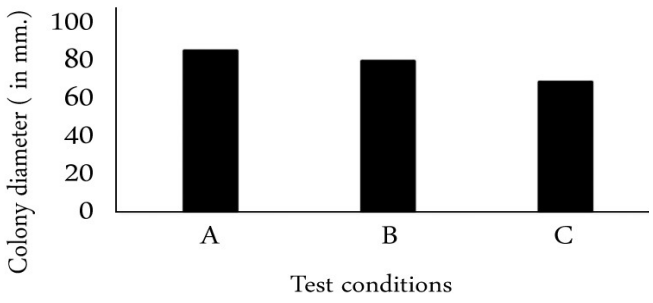

Fig. 4. A- Growth of regression of *Fusarium oxysporum* in the presences of bioactive extracts (*Lysinibacillus* sp.) under plate culture condition.
Abbreviation: A– medium + organism; B – medium + methanol+ organism; C – methanolic bioactive extracts + organism.

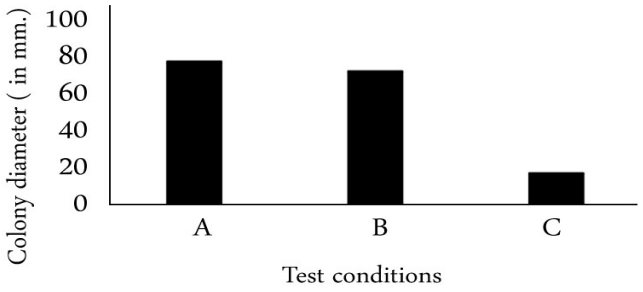

Fig. 4. B- Growth of regression of *Fusarium oxysporum* in the presences of bioactive extracts (*Pseudomonas* sp.) under plate culture condition.
Abbreviation: A– medium + organism; B – medium + methanol+ organism; C – methanolic bioactive extracts + organism.

Antifungal activity of partially purified metabolites of *Lysinibacillus* sp. and *Pseudomonas* sp. was well differentiated with fungal growth pattern on media plates. Under plate culture conditions, bioactive extracts from *Pseudomonas* sp. showed 18.82% growth inhibition of *Fusarium oxysporum*, whereas 75.65% growth inhibition was seen for bioactive extracts from *Lysinibacillus* sp. In the presence of bioactive extracts, *Fusarium oxysporum* exhibited regression in growth [Fig. 4(A&B)].

Test organism *Fusarium oxysporum* was tested for antibiotic sensitivity towards Clotrimazole (CC10), Itraconazole (IT30), Miconazole (MIC 30), Nystatin (NS50), Fluconazole (FLC10), Amphotericin-B (AP20) and Ketoconazole (KT30) and it was found sensitive towards Clotrimazole (CC10), Itraconazole (IT30) and Miconazole (MIC 30). As both bacteria performed well against test organism *Fusarium oxysporum,* we may predict the production of such type bioactive compounds by these bacterial cultures.

Discussion

Bacterial endophytes belonging to genus *Pseudomonas* and *Bacillus* are well known for production of wide range of secondary metabolic such as; antibiotics, anticancer compounds, volatile organic compounds, antifungal, antiviral, insecticidal and immunosuppressant agents. Apart from this, a broad range of biologically active compounds have been isolated from these endophytes (Lodewyckx *et al.,* 2002; Miller *et al.,* 1998). In the present study, both bacterial endophytes *Pseudomonas* sp. and *Lysinibacillus* sp. exhibited good bioactive potential. Both the bacteria, found with noticeable antifungal activity against *Fusarium oxysporum. Fusarium oxysporum* have also found to be sensitive against soneantifungal agents like; Clotrimazole (CC10), Itraconazole (IT30) and Miconazole (MIC 30), which clearly reflecting that; both the endophytes have the antifungal activity. This finding concluded that, these endophytes can be used as broad-spectrum antifungal agent in future. Bacterial endophytes also reported for the production of different enzymes; ACC deaminase, cellulases, protease, amylase, pectinase, esterase, lipase, protease, asparaginase, phytase, etc. (Sturz *et al.* 2000; Carrim *et al.* 2006). In our study, *Lysinibacillus* sp. have the capacity for chitinase production which can be promoted to large scale production. Endophytes also promote plant growth through number of mechanisms like; indole acetic acid production (Lee *et al.,* 2004) phosphate solubilization activity (Verma *et al.,* 2001; Wakelin *et al.,* 2004). Exopolysaccharides from intracellular and extracellular region also been reported from endophytes (Donot*et al.,* 2012). In this assessment, a notable indication of exopolysaccharide production by both bacterial cultures has been obtained in the present work. remarkable organic acid production was observed in *Pseudomonas* sp. than *Lysinibacillus* sp. *Pseudomonas* sp. found with high-rate production of tartaric acid of 1.09±0.62 (g/100ml). Bacterial endophytes are responsible for production of the plant growth-promoting abilities, regulate plant growth through cycling of nutrients and minerals such as nitrogen, phosphate and other nutrients. Now a days, these plant growth-promoting bacterial endophytes are being introduced for developing areas of forest regeneration and phytoremediation of contaminated soils. All the screening made for the both endophytes and metabolites obtained from this is remarkable. Future study on these endophytes and their metabolites may come with new useful finding for society.

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