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Production of Indole Acetic Acid (IAA) by *Stenotrophomonas Maltophilia BE25* Isolated from Roots of Banana *(musa spp)*

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Abstract: ***In the present study based on morphological, cultural, biochemical and 16s rRNA gene sequencing a newly isolated endophytic bacterium from the root of the banana (musa spp) was identified as Stenotrophomonas maltophilia and was designated as BE 25. Twenty two isolates were screened out for their productivity of indole acetic acid (IAA) by salkowski's method on spectrophotometer. Out of these isolates BE-25 isolate showed high amount of indole acetic acid production supplemented with and without L-tryptophan in medium. IAA production of BE-25 was further confirmed by thin-layer chromatography (Rf-0.54cm) as well as by high-performance liquid chromatography (38.85, 39.72ppm at 280 nm) with and without L-tryptophan in medium.***

Keywords: Banana, 16s rRNA gene sequencing, IAA, HPLC

1. Introduction

Banana is the second major fruit crop in India in terms of acreage and production and is the second largest producer in the world. Banana has great socio-economic and religious significance and contributes to about 31% of the total food production in India. Bananas are a perishable and nutritious fruit that is grown mostly in tropical and subtropical regions of the world reported by **[13].** There is increasing commercial and scientific interest with endophytes due to their potential to improve quality and growth of plants through nitrogen fixation and Endophytic bacteria can be defined as those bacteria that colonize the internal tissue of the plant showing no external sign of infection or negative effect on their host **[32].** IAA (indole-3-acetic acid) is the member of the group of Phytohormones and is generally considered the most important native auxin **[4].** Indole acetic acid (IAA) is one of the most physiologically active auxin. IAA is a common product of L- tryptophan metabolism produced by several microorganisms including Plant Growth- Promoting Rhizobacteria (PGPR) **[22].** Indole acetic acid helps in the production of longer roots with increased number of root hairs and root laterals which are involved in nutrient uptake **[9].** *Stenotrophomonas maltophilia* is an endophyte, which plays important roles in agricultural production as a plant growth promoting bacterium **[7].** *Stenotrophomonas maltophilia*, formerly *Xanthomonas maltophilia* **[28]** is widely found on or in plants and has a worldwide distribution **[10].**

Though a variety of nitrogen fixing bacteria like *Acetobacter, Arthrobacter, Azoarcus, Azospirillum, Azotobacter, Bacillus, Beijerinckia, Derxia, Enterobacter, Herbaspirillum, Klebsiella, Pseudomonas and Zoogloea* have been isolated from the rhizosphere of various crops **[6], [17]** interest in the beneficial nitrogen fixing growth promoting rhizobacterial plant association has increased recently due to their potential use as biofertilizers **[37].** Within a few years, several species of endophytic diazotrophs were discovered including *A. diazotrophicus, Herbaspirillum seropedicae, Herbaspirillum rubrisubalbicans, Burkholderia sp., Enterobacter sp.* and *Klebsiella sp.* in sugarcane plants. Work has continued on these endophytes within sugarcane plants, but to date little success has been attained in elucidating which endophyte is responsible for the observed biological nitrogen fixation and other plant growth promoting traits **[8].** Our findings might be the first report about nitrogen fixing and IAA production abilities of *S. maltophillia* isolated from vetiver grass (AspAK5) in Thailand. Moreover, much evidence has indicated a potential role of such species in plant growth promotion properties as fungal biocontrol **[19], [39]** and IAA production **[29].** L-tryptophan serves as a physiological precursor for biosynthesis of auxin in plants and microbes. Auxins of microbial origin in the interior of plants could evoke a physiological response in the host plant **[27].** So that, screening of the endophytes for their *in vitro* potential of auxin production could provide a reliable base for selection of effective plant growth promoting bacteria. As per as literature survey lots of research work on the banana endophyte bacteria *Stenotrophomonas maltophilia*. However, very few reports reported for their ability to produce indole acetic acid (IAA). Assuming that, the present investigation was conducted to demonstrate the IAA production of *Stenotrophomonas maltophilia* BE-25 with and without l-tryptophan supplement in medium isolated from roots of banana.

2. Materials and Methods

2.1 Collection of Sample: Banana plant sample (Roots) was collected from village Chikhali, Tal: Kadegaon, Dist: Sangli, Maharashtra (India).

2.2 Isolation of endophytic bacteria from banana plant:

Surface sterilization: The banana roots samples were thoroughly washed in running tap water. They were then surface-sterilized using 70% ethanol for 2 min and immersed in 150 ml of 1.5% sodium hypochlorite plus a few drops of Tween 20 for 5 min with shaking. The samples were then

rinsed thoroughly in five changes of sterile distilled water and dried in sterile paper towels **[26].**

Isolation of Endophytic bacterial isolates:
Surface sterilized samples were macerated with a sterile mortar and pestle and then serially diluted in 12.5 mM potassium phosphate buffer at pH 7. For isolation of indole acetic acid producing endophytic bacterial isolates several types of media were used such as nitrogen-free media - NFb **[12]**, MacConkey's, Congo red **[31],** YEM agar **[38]** and nutrient agar **[11].** Isolation of *Stenotrophomonas* strains by using NFb medium. The medium contains malic acid - 0.5g,MgSO4.7H2O-0.2g ,Nacl - 0.1g , CaCl2 -0.02g ,Na2 MoO4 -0.002g ,MnSO4.H2O -0.01g ,EDTA 1.64% -4 ml ,Bromothymol blue 0.5% (W/W in ethanol) -3 ml , KOH - 4.5g,Biotin - 0.1mg ,distilled water -1000ml ,pH-6.8. A loopful of sample was spread on NFb medium and kept for incubation for 48 to 72hrs at room temperature. Well isolated colonies were observed for morphological and culture characterization. Total 22 isolates were obtained from roots of banana plant. The isolates were further checked for IAA production.

2.3 Morphological and cultural characterization of isolates

Morphological and cultural characterization was done on the basis of colony size, shape, color, margin, opacity, consistency, elevation, motility and gram staining, Endospore, capsule staining and based on the colony morphotypes selection of representative isolates was done.

2.4 Biochemical characterization of promising bacterial isolate

Biochemical tests like the tests involved, oxidase, amylase and gelatinase, catalase like enzyme production, citrate utilization, indole test, Vogus Proskaur test, methyl red test, H2S production, sugars (Glucose, Sucrose, Lactose, Xylose and Mannitol) fermentation, Triple sugar iron (TSI) test, nitrate reduction, urease test etc. **[2], [16], [35]** as well as forty seven biochemical testes on **VITEK-2 Compact Machine** in the Joshi Lab. Pune of selective endophytic bacteria was done. On the basis of morphological, cultural and biochemical examination N_2 fixing Endophytic bacterial isolate was identified.

2.5 16s rRNA gene sequencing

Bacterial genomic DNA was isolated using geneO-spin Microbial DNA isolation kit (geneOmbio technologies, Pune; India). Partially a 16S rRNA gene was sequenced. Bacterial 16S region gene was amplified using standard PCR (Machine by Applied Biosystems 2720) reaction. The primer pair 27F (AGAGTTTGATCMTGGCTCAG3) and 1492R (TACCTTGTTACGACTT) as a universal primer were used in a PCR reaction with an annealing temperature of 57°C. After amplification, products were purified by using a geneO-spin PCR product Purification kit (geneOmbio technologies, Pune; India) After PCR is completed, the PCR products were checked on 1% Agarose by Agarose Gel Electrophoresis and amplicon size was compared using reference Ladder. 1% agarose gel spiked with Ethidium bromide at a final concentration of 0.5 µg/ml was prepared using Agarose (LE, Analytical Grade, Promega Corp., Madison, WI 53711 USA) in 0.5X TBE buffer. 5.0 µl of PCR product was mixed with 1 µl of 6X Gel tracking dye. 5µl of gScale 100bp size standard *(geneOmbio technologies, Pune; India)* was loaded in one lane for confirmation of size of the amplicon using reference ladder. The DNA molecules were resolved at 5V/cm until the tracking dye is 2/3 distance away from the lane within the gel. Bands were detected under a UV Trans illuminator. Gel images were recorded using BIO-RAD GelDocXR gel documentation system. The PCR product of size 1450bp was generated through this reaction and directly sequenced using an ABI PRISM BigDye Terminator V3.1 kit (Applied Biosystems, USA). The sequences were analyzed using Sequencing Analysis 5.2 software. DNA sequencing was performed using one of the PCR primers **[18].** BLAST analysis was performed at BlastN site at NCBI server (http://ww.ncbi.nlm.nih.gov/BLAST) and similarity was checked.

2.6 Quantification of Indole Acetic Acid (IAA) of isolates by Spectrophotometric Method

To determine the amounts of IAA produced by each isolate, a Colorimetric/Spectrophotometric technique was performed with Van Urk Salkowski's reagent using the Salkowski's method **[14], [15].** The isolates were grown in yeast malt dextrose broth ± 0.1% L-tryptophan (YMD± 0.1% L-tryptophan broth) (Himedia, India) and incubated at 28 °C for 4 days. The broth was centrifuged at 3000 rpm for 30 min. after incubation. Two milliliters of the supernatant was mixed with 2 drops of orthophosphoric acid and 4 ml of Salkowski's reagent (50 ml, 35% perchloric acid; 1 ml 0.5 FeCl3) and kept in the dark. The optical density (OD) was recorded after red colour development on spectrophotometer **(Systronics – 119)** at 530 nm after 30 min and 120 min. High amount of indole acetic acid (IAA) producing bacterial isolate was selected for their purification indole acetic acid by thin layer chromatography and Fractionation and Quantification by HPLC method.

2.7 Extraction and purification of Indole acetic acid (IAA)

The isolates were grown in yeast malt dextrose broth (YMD + 0.1% tryptophan broth) (**Himedia, India**) and incubated at 28 °C for 4 days on a shaker incubator. Bacterial cells were separated from the supernatant by centrifugation at 10,000 rpm for 30 min described by **[5].** The supernatant was acidified to pH 2.5 to 3.0 with 1 N HCl and extracted twice with ethyl acetate at double the volume of the supernatant. Extracted ethyl acetate fraction was evaporated to dryness in a rotator evaporator at 40 oC. The extract was dissolved in 300 ml of methanol and kept in Refrigerator **[5].** Thin layer chromatography (TLC) slide was prepared with silica gel G and calcium carbonate. Propanol: Water (8:2) was used as Solvent system. The extracted sample and standard IAA (10mg/100ml) were spotted on TLC plate. Spots with Rf values identical to authentic IAA were identified by spraying the plates with the Salkowski's reagent **[20].**

2.8 Fractionation and Quantification of Indole Acetic Acid (IAA) by High Performance Liquid Chromatography (HPLC) Method

This ethyl acetate extract was evaporated to dryness at 35°C, and the residue was dissolved in 1,500µl of pure methanol **(Fisher Scientific, India)**. The samples were analyzed on HPLC **(Chemito 6600 isocratic)** using UV detector and C18 column (39× 300 mm). For identification of hormones, a 100-µl sample was filtered through 0.45 Millipore filter and 20 µl of the filtered extract injected into a 5µm reverse phase column. Pure IAA **(Sisco Laborarory, India)**, dissolved in HPLC grade methanol, was used as standards for identification and quantification of bacterial hormone (IAA). Growth hormones were identified on the basis of retention time and peak area of the standard. The solvent system used to separate IAA was water: acetonitrile [76:24 (v/v)] used as mobile phase. Flow rate was adjusted at 2 ml/min, with an average run time of 20 min/sample. The wavelength used for detection of IAA was 280 nm **[34].**

3. Results and discussion

3.1 Morphological and cultural characterization of isolates

The isolation of bacteria from surface sterilized roots of banana normally allows the recovery of putative Endophytic bacteria. Growth in semisolid nitrogen free medium has been a useful strategy to select for nitrogen fixing bacteria. All isolates, obtained from banana, selected by these combined criteria were grouped by morphological similarities and phenotypic characteristics **[21].** In the present study total 22 N_2 fixing Endophytic bacterial isolates were isolated from roots of banana plant which showed different culture characteristics as well as sixteen isolates showed gram negative rods in nature, three isolates showed gram positive rods in nature and remaining isolates showed gram positive and negative cocus in nature, isolates showed Sporulation (4), capsulation (10) and 16 isolates were motile in nature.

3.2 Biochemical characterization of bacterial isolate BE 25

Maximum Indole acetic acid producing isolate (BE -25) was identified based on morphological biochemical characterization (Table 1, 2) and 16s rRNA gene sequencing. Bergey's manual of determinative of bacteriology was used as a reference to identify the isolates **[24].**

Table 1: Forty seven biochemical tests:

S. N.	Biochemical Tests	Isolates BE - 25
1	Ala-phe-Pro Arylamidase (APPA)	+
2	ADONITOL (ADO)	-
3	L-Pyrrolydonyl-ARYLAMIDASE (PyrA)	-
4	L-ARABITOL (IARL)	-
5	D-CELLOBIOSE (dCEL)	-
6	BETA-GALACTOSIDASE (BGAL)	-
7	H_2S Production (H_2S)	-
8	BETA-N-ACETYL-GLUCOSAMINIDASE (BNAG)	-
9	Glutamyl Arylamidase pNA (AGLTp)	-
10	D-Glucose (dGLU)	-
11	GAMMA-GLUTAMYL-TRANSFERASE (GGT)	+
12	Fermentation/Glucose (OFF)	-
13	BETA-GLUCOSIDASE (BGLU)	+
14	D-MALTOSE (dMAL)	-
15	D-MANNITOL (dMAN)	-
16	D-MANNOSE (dMNE)	-
17	BETA-XYLOSIDASE (BXYL)	-
18	BETA-Alanine Arylamidase pNA (BAlap)	-
19	L-PROLINE ARYLAMIDASE (ProA)	+
20	LIPASE (LIP)	+
21	PALATINISE (PLE)	-
22	Tyrosine Arylamidase (TryA)	-
23	Urease (URE)	-
24	D-SORBITOL (dSOR)	-
25	SACCHAROSE/SUCROSE (SAC)	-
26	D-TAGATOSE (dTAG)	-
27	D-TREHALOSE (dTRE)	-
28	Citrate (CIT)	+
29	MALONATE (MNT)	-
30	5-Keto-D-GLUCONASE (5KG)	-
31	L-LACTATE-alkalinisation (ILATK)	+
32	Alpha-glucosidase (AGLU)	+
33	Succinate (SUCT)	+
34	BETA-N-ACETYL-GALACTOMINIDASE (NAGA)	-
35	Alpha-galactosidase (AGAL)	-
36	Phosphate (PHOS)	-
37	Glycine-Arylamidase (GlyA)	-
38	Ornithine Decarboxylase (ODC)	-
39	Lysine Decarboxylase (LDC)	-
40	L-Histidine assimilation (IHISa)	-
41	COUMARATE (CMT)	-
42	BETA-GLUCURONIDASE (BGUR)	-
43	Resistance Comp.vibrio (O129R)	-
44	Glu-Gly-Arylamidase (GGAA)	+
45	L-malate-ACETYL-galactominidase (IMLTa)	-
46	ELLMAN (ELLM)	-
47	L-Lactate Assimilation (ILATa)	-

Table 2: Biochemical tests:

S. N.	Biochemical Tests	Isolates BE- 25
1	Oxidase test	-
2	Amylase test	+
3	Gelatinase liquification test	+
4	Catalase test	-
5	Indole test	+
6	Methyl red test	-
7	Vogus Proskaur test	-
8	Citrate utilization	-
9	H_2S production	+
10	Glucose	-
11	Sucrose	-
12	Xylose	-
13	Mannitol	-
14	Lactose	-
15	Triple sugar iron (TSI) test	+
16	Nitrate reduction	+
17	Urease test	-

3.3 Molecular characterization (16s rRNA gene sequencing) of isolate BE 25

As described in more detail in Methods we devised and implemented a method to extract the bacterial SSU rRNA 27f and 1492r primer-binding site sequences from the data in the

RDP and the Sargasso Sea metagenomic data. A key point of the method is that it assumes only a _50% sequence identity between the region containing the primer-binding site in the sequence being analyzed and at least one member of a diverse set of "reference" sequences. The method is general and can be used to extract any portion of a sequence that is sufficiently conserved or at least is flanked by conserved sequences **[18].** As per partially 16s rRNA gene sequencing of the isolate 768 bases sequenced are as follows:

(AAGAAGGCCTTCGGGTTGTAAAGCCCTTTTTTGGG
AAAGAAATCCAGCTGGTTAATACCCGGTTGGGATG
ACGGTACCCAAAGAATAAGCACCGGCTAACTTCGT
GCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGT
TACTCGGAATTACTGGGCGTAAAGCGTGCGTAGGTG
GTCGTTTAAGTCCGTTGTGAAATCCCTGGGCTCAAC
CTGGGAACTGCAGTGGATACTGGGCGATTAGAGTGT
GGTAGAGGGTAGCGGAATTCCTGGTGTAGCAGTGA
AATGCGTAGAGATCAGGAGGAACATCCATGGCGAA
GGCAGCTACCTGGACCAACACTGACACTGAGGCAC
GAAAGCGTGGGGAGCAAACAGGATTAGATACCCTG
GTAGTCCACGCCCTAAACGATGCGAACTGGATGTTG
GGTGCAATTTGGCACGCAGTATCGAAGCTAACGCGT
TAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTG
AAACTCAAAGGAATTGACGGGGGCCCGCACAAGCG
GTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGA
ACCTTACCTGGTCTTGACATGTCGAGAACTTTCCAG
AGATGGATTGGTGCCTTCGGGAACTCGAACACAGGT
GCTGCATGGGTGTCGTCAGCTCGTGTCGTGAGATGT
TGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCT
TAGTTGCCAGCACGTAATGGTGGGAACTCTAAGGA
GACCGCCGGTGACAAACCGGA) After NCBI BLAST analysis these bases 99% matched with *Stenotrophomonas maltophilia* belong the family *Xanthomonadaceae* and hence this isolate was identified and confirmed as *Stenotrophomonas maltophilia* based on Bergey's manual, 47 biochemical test and 16s rRNA gene sequencing and was designated as *BE 25.*

3.4 Quantification of Indole Acetic Acid (IAA) of isolates by Spectrophotometric Method

Table 1. Quantification of Indole Acetic Acid (IAA) produced by N_2 fixing Endophytic Bacterial isolates isolated from roots of Banana Plant.

Twenty two isolates were screened out for their productivity of indole acetic acid (IAA) on spectrophometer. out of these 22 isolates seven isolates (BE-25, BE-28, BE-30, BE-35, BE-37, BE-39 and BE-42) showed ability to produce indole acetic acid (IAA) without L-tryptophan supplement in medium at 30 and 120minutes as well as nine isolates (BE-25, BE-27, BE-28, BE-30, BE-33, BE-35, BE-37, BE-39 and BE-42) showed ability to produce indole acetic acid with 0.1% L-tryptophan supplement in medium at 30 and 120minutes. The synthesis of indole-3-acetic acid (IAA) in the presence of L-tryptophan was detected in 40% of the isolates. The isolate EB-40 (Bacillus sp.) produced the highest amount of IAA (47.88 µg/ml) in medium supplemented with L-tryptophan and was able to synthesize IAA in the absence of L-tryptophan **[1].**

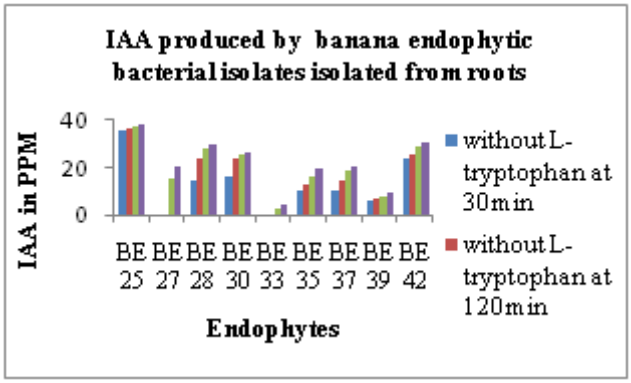

Figure 1: Indole acetic acid production by banana Endophytic bacterial isolates with and without L-tryptophan supplement in medium.

Indole acetic acid (IAA) production of diazotrophic *Stenotrophomonas maltophilia* is 2.6 mg ml^{-1} reported by **[30].** Some microorganisms produce auxins in the presence of a suitable precursor such as L-tryptophan. The effects of auxins on plant seedlings are concentration dependent, i.e. low concentration may stimulate growth while high concentrations may be inhibitory **[3].** From these isolates indole acetic acid producing isolates BE-25 produced high amount of indole acetic acid (IAA) supplemented with and without L-tryptophan in medium **(Fig.2).**

3.5 Extraction and purification of Indole acetic acid (IAA)

On the basis of IAA production level centrifuged culture of *Stenotrophomonas maltophilia BE 25* was used to extract IAA for characterization by TLC. The spots of ethyl acetate extracts of the respective culture and standard IAA were tested in solvent systems Propanol: Water (8:2). Chromatograms of culture spots and standard IAA, sprayed with Ehmann's/ Salkowski's reagent, showed almost the same Rf values. Our TLC findings are in agreement with reports by other scientists **[40].** Purified Indole acetic acid (IAA) sample was compared with standard IAA on TLC chromatograms. TLC of ethyl acetate extract showed pink colour spot at the Rf corresponding to the authentic IAA (0.54cm). It confirmed IAA producing potential of Endophytic bacterial isolate isolated from roots of banana plant.

3.6 Fractionation and Quantification of Indole Acetic Acid (IAA) by High Professional Liquid Chromatography (HPLC) Method

The concentration of indole acetic acid (IAA) produced by Endophytic bacterial isolate (BE-25) without and 0.1% L-tryptophan supplement in the medium was also analyzed by HPLC. Organism produced Indole acetic acid (IAA) 38.85 ppm without L-tryptophan supplement as well as 39.72ppm with 0.1% L-tryptophan supplement in the medium (Figure 2,4).

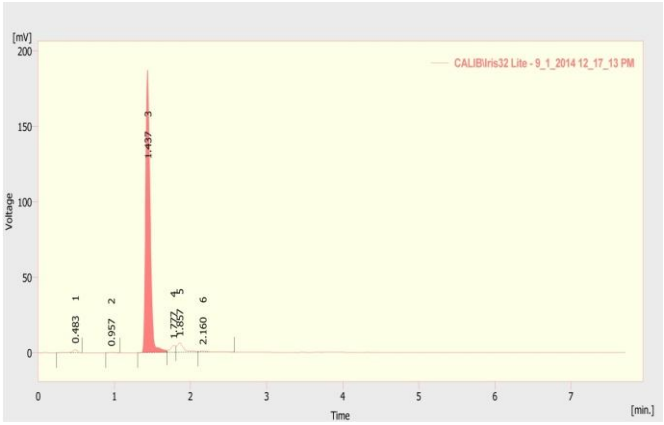

Figure 4: Chromatogram of Indole acetic acid (IAA) produced by banana Endophytic bacterial isolate (BE-25) without tryptophan in medium:

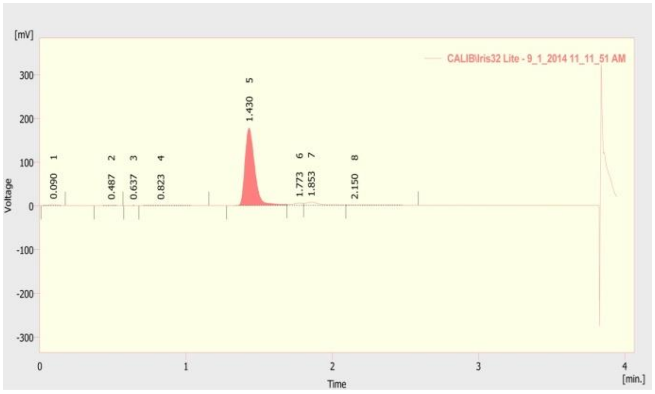

Figure 5: Chromatogram of Indole acetic acid (IAA) produced by banana Endophytic bacterial isolate (BE-25) with 0.1% tryptophan in medium:

The production of substantial amounts of IAA during growth in nutrient broth in IAA production in the presence of tryptophan such as 13.8 and 21.0 µg ml^{-1} of IAA in medium containing 250 and 500 µg ml^{-1} of tryptophan, respectively 11 µg ml^{-1} IAA in the absence of tryptophan **[33].** It has been reported that IAA production by bacteria can vary among different species and strains, and it is also influenced by culture condition, growth stage and substrate availability **[25].** The use of the technique for the detection of IAA using the Van Urk Salkowski's reagent is an important option for qualitative and semi-qualitative determination that assure the presence of the hormone in the supernatant of bacterial cultures or liquid formulations of biological inoculants. The amount of IAA produced by the bacteria was within the detection limits of Salkowski's reagent (Ehmann's, 1977). The reagent gives reaction with IAA and does not interact with L-tryptophan and Na-acetyl-L-tryptophan and used by and large **[36].** As per HPLC analysis in this study *Stenotrophomonas maltophilia BE. 25* produced high amount of IAA with 0.1 % L-tryptophan supplement in medium comparative to other researcher.

4. Conclusions

From this study, it is clear that Endophytic Bactria isolated from roots of banana can provide a rich source of Indole Acetic Acid (IAA) production and has the ability to produce a significant amount of IAA in a tryptophan-supplemented medium. Furthermore, the concurrent Phytohormones (IAA) production suggested its auxiliary beneficial role as plant growth promoter even under stressed environmental conditions. Out of these 22 isolates seven isolates showed ability to produce IAA without L-tryptophan supplement in the medium and nine showed ability to produce IAA with 0.1% L-tryptophan supplement in the medium. BE-25 showed high amount of IAA producer which are estimated on Spectrophotometer, HPLC as well as confirmed with Thin Layer Chromatography (TLC). It is concluded that presence of such growth promoting Endophytic bacteria (*Stenotrophomonas maltophilia BE 25*) accountable for the beneficial effects on crop growth and yield. review has been summarized the current information on PGPR, its application on bananas, as well as different crop plants and indicates the potential of PGPR as a bioenhancer and biofertilizers for banana cultivation. Nitrogen fixation, plant growth promotion and improved nutrient absorption are important criteria for achieving a sustainable banana production system. During the last four decades, PGPR research has brought together scientists from multiple disciplines endeavoring a wide range of topics including: discovery of novel PGPR strains and traits; performance in greenhouse and field trials; production, formulation and delivery of inoculum; mechanisms of growth promotion and biocontrol and their molecular and biochemical basis; root colonization and rhizosphere competence traits; role of PGPR in suppressive soils; plant, pathogen and rhizosphere community responses to PGPR and recombinant PGPR and risk assessment **[23].** The significance of the study could be stated as the potential of these IAA producing isolate will flourish the growth and ultimately IAA production in the field and prevent environmental pollution by avoiding excessive applications of chemical fertilizers. These Endophytic bacteria also helpful to development of liquid bioinoculant for economically convenient and sustainable agriculture.

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