https://doi.org/10.48047/AFJBS.6.13.2024.6321-6336

African Journal of Biological Sciences

Journal homepage: http://www.afjbs.com

Research Paper Open Access

Production and Optimization of Indole-3-Acetic Acid produced by Bacterial endophytes of Wheat and its effect on growth of *Triticum aestivum* and *Vigna radiata*.

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Volume 6, Issue 13, Aug 2024

Received: 15 June 2024

Accepted: 25 July 2024

Published: 15 Aug 2024

doi: 10.48047/AFJBS.6.13.2024.6321-6336

Abstract. Indole-3-acetic acid (IAA) is the type of auxin produced by plants as we l as endophytes present inside the plants. Bacteria do not utilize this hormone but IAA is essential for leaf, root initiation & development of lateral roots. In this study we used four bacterial endophytes *Enterobacter asburiae* (S2), *Bacillus licheniformis* (S4), *Achromobacter mucicolens* (S5) and *Pseudomonas fulva* (S8) which were isolated from germinating wheat (*Triticum aestivum)* and screened for plant growth promotional activity in our previous study. Now, these four strains were investigated for production and optimization of Indole-3-Acetic Acid (IAA). Initially IAA production was compared in Luria Broth (LB) and Yeast Extract Mannitol (YEM) media with 0.1% L-tryptophan, YEM media showed maximum production of IAA. To increase productivity of IAA, optimization studies were performed by varying different parameters like, incubation time, carbon source and substrate concentration (tryptophan). Based on the optimization study, yeast extract sucrose media (YES) supplemented 0.2% tryptophan showed higher amount of IAA production after 48 hrs of incubation. Strains *Enterobacter asburiae* (S2) and *Achromobacter mucicolens* (S5) gave yield of 251.5 µg/ml and 199.77 µg/ml of IAA respectively. Primary confirmation of IAA was done by thin layer chromatography and both the strains gave good color band & Rf value of samples were compared with standard IAA. FT-IR analysis and LC-ESI-MS of the samples further confirmed the presence of IAA. To check the effect of IAA on plant growth seed germina t ion study were performed. IAA produced by *Enterobacter asburiae* and *Achromobacter mucicolens* was checked on *Triticum aestivum* and *Vigna radiata,* where significant increase after 21 days in test samples was seen when compared with control. Result indicated that *Enterobacter asburiae* (S2) showed 68% increase in shoot length while *Achromobacter mucicolens* (S5) 90% increase in root system. This is a first report that reveals that endophytes of wheat *Enterobacter asburiae* and *Achromobacter mucicolens* are indole -3-acetic acid producers and that the IAA produced can be harnessed for increasing root system as we l as healthy growth of other plant.

Keywords: Indole -3-acetic acid, IAA, Optimization, endophytes, FT-IR, LC-ESI-MS, Wheat (*Triticum aestivum*), *Vigna radiata.*

1. INTRODUCTION

Due to rapid change in the environment, various direct and indirect environmental factors affect plant growth which causes loss in crop production (Matthews et al., 1984). The plant produces various types of phytohormones that play a major role in the development of healthy plants (Sabagh et al. 2021). Many phytohormones like auxin, cytokinin's, gibberellic acid, and abscisic acid are synthesized by plants to stimulate plant growth and help in nutrie nt acquirement (Luziatelli et al., 2021.

Endophytes are the microorganisms presents in plants that use the healthy nutrient form the root and produce different types of bioactive compound which enhances plant growth (Bharucha et al., 2013; Berendsen et al., 2012; Drake et al., 2013; Kampert et al., 1975). Several endophytes present in the seeds and other part of the plants show plant growth promoting (PGP) traits. Plant Growth Promoting Endophytes produce plant hormones such as IAA (Indole Acetic Acid) help plants to grow faster and healthier for example peanuts, corn, wheat, and sugarcane (Strzelczyk et al., 1984; Mattos et al., 2008; Herlina et al., 2017).

Indole-3-acetic acid (IAA) is the type of auxin produced by plants meristematic tissues as well as bacteria present inside the plants, bacteria do not utilize this hormone (Herlina et al., 2017; Chandra et al., 2018). However, is reported to provide a significant role for rapid growth of plants such as formation of leaf, root initiation & development of lateral roots, formation of fruits etc. IAA hormone supports and initiates branch formation in roots which help plants to take more nutrients from the soil (Datta et al., 2000; Spaepen et al., 2007).

Tryptophan is the main precursor of synthesis of IAA in bacteria and in plants (Bhutani et al., 2018). In plant and bacteria various interrelated pathways are present for IAA biosynthes is, Mainly IAA has been produced by Tryptophan-Dependent IAA biosynthesis pathways in bacteria (Chandra et al., 2018; Zhao et al., 2010).

Most of the studies indicate that phytohormone IAA, work as a signal molecule among bacteria and plants (Spaepen et al., 2007), (Fu S.F et al. 2015; Bianco et al., 2006; Liu et al., 2006; Gilbert et al., 2018). IAA is commonly found phytohormone present in nature, around 80 % of endophytic bacteria of plants can produce IAA (Gilbert et al., 2018). IAA also supports colonization of bacteria which enhance plant growth (Spaepen and Vanderleyden 2011).

In the present study optimization of growth parameters for IAA production by previous ly isolated & characterized endophytes of germinating wheat (*Triticum aestivum*) isolated in our lab (Awasthi and Wadhwa, 2023). The aim of this study was to enhance the production of IAA by changing different parameters such as pH, temperature, carbon, and nitrogen sources, to find the best optimized media for higher IAA production. Further purification was done for IAA and identification and confirmation was done using FT-IR and LCMS analysis. Effect of the purified IAA was checked on germination of seeds of *Triticum aestivum* and *Vigna radiata*.

2. MATERIALS AND METHODS

Screening of endophytes for IAA production and optimization

In our previous study, 8 bacterial endophytes were isolated from germinating wheat (*Triticum aestivum*). Based on PGP potential of isolated endophytes, 4 bacterial endophytes were screened and identified by 16s rRNA sequencing (Awasthi and Wadhwa, 2023).

These four strains *Enterobacter asburiae* (OL_966967), *Bacillus licheniformis* (OL_966968), *Achromobacter mucicolens* (OL_966969) and *Pseudomonas fulva* (OL_966970) were used for this study.

Media preparation and production of IAA

Luria Broth (LB) and Yeast Extract Mannitol (YEM) media supplemented with 0.1% tryptophan were prepared (Gilbert et al., 2018; Spaepen et al., 2011; Patel et al., 2014). 24 hr grown bacterial strains S2-*Enterobacter asburiae*, S4- *Bacillus licheniformis,* S5-*Achromobacter mucicolens*, S8-*Pseudomonas fulva* were inoculated and media without inoculation was used as a control. All sets were in duplicate, incubated at 37^{o} C for 48 hr, after incubation all the media were centrifuged and supernatant were collected.

Quantitative Estimation IAA by colorimetric assay

The IAA production was analyzed by the colorimetric method using the Salkowski reagent (0.5M $FeCl_3$ + 70% perchloric acid). Cell free supernatant and Salkowski reagent were added in 2:1 ratio and incubated at room temperature for 30 min (Patten et al., 2002). The development of red color indicates the formation of IAA compounds and was measured by UV–vis spectrophotometer at 530 nm. The concentration of IAA in samples was determined with a standard graph of indole acetic acid (0-500 µg/ml).

Optimization of production parameters

The IAA production was optimized by changing various media conditions using OFAT approach, in this only factor changes at a time such as incubation time, composition of production media, concentration of tryptophan and different carbon source (Sachdev et al., 2009).

Effect of Incubation time on IAA production

In this test 24 hr, 48 hr and 72 hr incubation time was selected and YEM media with 0.1% L-tryptophan were inoculated and incubated at 37^{o}C for 24 hr, 48 hr and 72 hr separately and after incubation media were centrifuged and IAA concentration was measured by taking OD at 530 nm (Trivedi et al., 2020; Mohite, 2013).

Effect of carbon sources on IAA production

In this experiment three different media were prepared using three different Carbon sources like sucrose, dextrose, lactose to substitute maltose and add 0.1% tryptophan to obtain ideal IAA production (Spaepen et al., 2007; Bhutani et al., 2018). Media were incubated on a shaker at 37^{o}C for 48 hours and OD were taken after centrifugation.

Effect of Tryptophan Concentration on IAA production

Tryptophan is the precursor of IAA; thus, different concentration of tryptophan was used in the media. The tryptophan concentration on IAA production were 0.2 % and 0.3 %. The culture was incubated on a shaker at 37^{o}C for 48 hours (Mohite, 2013). IAA production was measured after incubation (Widawati et al., 2020).

Extraction and Purification of IAA

YES (Sucrose) media was prepared with 0.1 % tryptophan. Media were inoculated according to optimized conditions and incubated for 48 hrs after incubation, the media was centrifuged for 15 min, and the supernatant was acidified to pH 2.5 with 1 N HCl and extracted twice with an equal volume of ethyl acetate (Bhutani et al., 2018). The ethyl acetate fraction was evaporated at 40^{o}C using a rotatory evaporator. After drying, the powdery extract was dissolved in methanol and kept at 4^{o}C for further use (Sujithra et al., 2020; Lebrazi et al., 2020).

Primary Characterization of IAA by Thin Layer Chromatography

A purified from ethyl acetate extraction were used in TLC for primary characterization. Silica gel G plates was used for TLC, Crude extracts, and IAA standard (500 µg/ml) were spotted on Silica gel G plates and kept in Isopropanol: water (8:2) ratio (Bhutani et al., 2018; Patel et al., 2014). The Rf value of crude extract and IAA standard were calculated and compared (Lebrazi et al., 2020).

Characterization of IAA by FT-IR

IAA production by isolated bacterial endophytes was characterized based on chemical bonds and molecular structure, and FT-IR analysis was performed. (Gilbert et al., 2018; Patel et al., 2014). The purified extract dissolved in methanol and used for FT-IR analysis and bonds were compared with standard IAA and the transmission mode was 400–4000 cm^{-1} in (FTIR) Perkin Elmer LAMBDA spectrum IR Version 10.7.2 spectrophotometer was used. (Ravinder et al., 2022; Nithyapriya et al., 2021).

Detection of IAA by LC-ESI-MS

For more accurate validation of IAA produced by isolated bacterial endophytes was analyzed by LC-ESI-MS. IAA was analyzed with positive ionization mode (M+H+). Molecular weight of IAA is 175gm/l (Matsuda et al., 2005; Gang et al., 2019).

Effect of IAA on growth of *Triticum aestivum* and *Vigna radiata*

The seeds of *Triticum aestivum* and *Vigna radiata* were surface sterilized three times with distilled water than 0.1% mercuric chloride, washed again with distilled water and placed these seeds for germination in pots. After germination, extracted IAA was added in the soil and plant growth was observed at regular interval for 4 weeks and shoot length root and lateral root lengths were measured and compared with uninoculated control samples (Bhutani et al., 2018).

RESULTS AND DISCUSSION

Production of IAA in LB and YEM media

Initially, IAA production was checked in LB and YEM media supplemented with 0.1% Tryptophan, Incubation time was 48 hr. YEM media showed high amount of IAA compared to LB media (Table 1) in a l the bacterial strains S2, S4, S5, S8.

Table 1. Concentration of IAA produced by isolated strains of endophytic bacteria in LB and YEM media (0.1% L-tryptophan)

Isolated Endophytes	**Amount of IAA (µg/ml)**	
	LB	YEM
Enterobacter asburiae (S2)	86.64±0.001	135.81±0.03
Bacillus licheniformis (S4)	72.42±0.02	88.34±0.03
Achromobacter mucicolens (S5)	80.95±0.02	115.06±0.02
Pseudomonas fulva (S8)	83.79±0.009	94.60±0.005

*Values presented are mean ± SD

Optimization study for high amount of IAA production

Various conditions of the culture media with various parameters like incubation time, composition of production media, concentration of tryptophan and different carbon source and results obtained are discussed below.

Effect of Incubation time on IAA production

YEM media was selected for optimization study for a high amount of IAA. YEM media was incubated with different incubation Time (24hr, 48hr and 72hr). Concentration of IAA was checked in a l the experimental sets. Maximum amount was found in S2 and S5 at 48 hr incubation as shown in Table 2.

Table 2. Concentration of IAA produced by endophytes after 24 hr., 48 hr. & 72 hr.

Isolated Endophytes	**IAA Concentration (ug/ml)**		
	24 hr	**48 hr**	**72 hr**
Enterobacter asburiae (S2)	91.47±0.01	150.88±0.004	78.11±0.02
Bacillus licheniformis (S4)	76.69±0.02	87.49±0.01	85.5±0.004
Achromobacter mucicolens (S5)	81.52±0.007	127.57±0.007	81.24±0.008
Pseudomonas fulva (S8)	70.44±0005	87.49±0.01	68.45±0.003

Effect of carbon sources on IAA production

In basic Yeast extract mannitol (YEM) media, Mannitol was changed with dextrose, lactose and sucrose and a l other composition of the media was the same. IAA concentration was checked in a l the media. Strain S2 and S5 gives high amount of IAA in Yeast Extract Sucrose (YES) media, so this media was selected for purification steps.

Table 3. Concentration of IAA produced on varying Carbon sources (0.1% L-tryptophan)

Isolated endophytes	**IAA Concentration in Media with varying carbon sources (ug/ml)**		
	YED	**YEL**	**YES**
Enterobacter asburiae (S2)	89.48±0.008	76.12±0.01	202.9±0.04
Bacillus licheniformis (S4)	45.71±0.009	53.38±0.01	86.92±0.007
Achromobacter mucicolens (S5)	58.21±0.001	63.9±0.01	184.42±0.13
Pseudomonas fulva (S8)	50.54±0.001	84.62±0.02	69.3±0.001

*Values presented are mean ± SD

Effect of Tryptophan Concentration on IAA production

YES, Medium with varying concentration (0.2% and 0.3%) of tryptophan was incubated after inoculation and kept on a shaker at 37^{o}C for 48 hours. IAA production was measured in both medias, concentration of IAA is high in YES media when 0.2 % tryptophan was supplemented and when we increased the tryptophan amount 0.3%, concentration of IAA decreased. (Table 4).

Table 4. Concentration of IAA produced by varying the concentration of tryptophan (0.2% and 0.3%).

Isolated Endophytes	**IAA Concentration in YES (µg/ml)**	
	0.2% tryptophan	**0.3% tryptophan**
Enterobacter asburiae (S2)	251.5±0.04	115.06±0.05
Bacillus licheniformis (S4)	103.69±0.1	95.16±0.06
Achromobacter mucicolens (S5)	199.77±0.05	120.75±0.06
Pseudomonas fulva (S8)	92.89±0.06	80.38±0.08

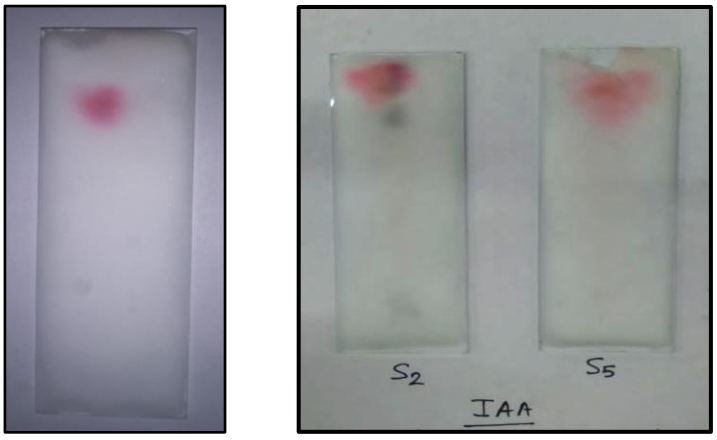
*Values presented are mean± SD

Extraction and Purification of IAA

For extraction & purification of IAA, YES media with the optimized conditions were prepared and strains S2 and S5 were inoculated. After 48 hr of incubation, equal volume of ethyl acetate was added in supernatant and solvent layer was collected after drying.

Primary Characterization of IAA by Thin Layer Chromatography

Pink colour spots of IAA were developed in Silica gel G plates with Isopropanol: water (8:2) as the mobile phase. The Rf value of crude extract and standard IAA were compared (Figure 1)**.**



IAA Standard **IAA produced by bacterial Strains**

Figure 1. TLC plates of IAA

Characterization of IAA by FT-IR analysis

In this result, absorption band was observed around wavelengths 3310 cm^{1}, 1450 cm^{1}, and 1020 cm^{1}, this indicates the presence of three main functional group N-H bond, C-N bond & C=O bond respectively. Thus, with this analysis of the functional group and characteristic peaks of IAA extracted from isolated endophytes *Enterobacter asburiae* and *Achromobacter mucicolens* confirms the presence of IAA (Table 5 and Figure 2). Report suggested that the

presence of characteristic N-H stretching of indole was observed at 3330 cm-1 and 3334 cm-1 respectively. C-N stretching of indole compound was observed at wavelength of 1450 cm-1 and 1403 cm-1 confirms IAA (Trivedi et al., 2020).

Table 5. FT-IR analysis result of IAA

Samples	**FT-IR spectra of IAA extracted from bacteria**		
	N-H bond	**C-N bond**	**C=O bond**
IAA Standard	3308.58 cm^{-1}	1449.15 cm^{-1}	1022.11 cm^{-1}
IAA by *Enterobacter asburiae* (S2)	3309.97 cm^{-1}	1449.09 cm^{-1}	1021.95 cm^{-1}
IAA by *Achromobacter mucicolens* (S5)	3309.64 cm^{-1}	1449.01 cm^{-1}	1021.87 cm^{-1}

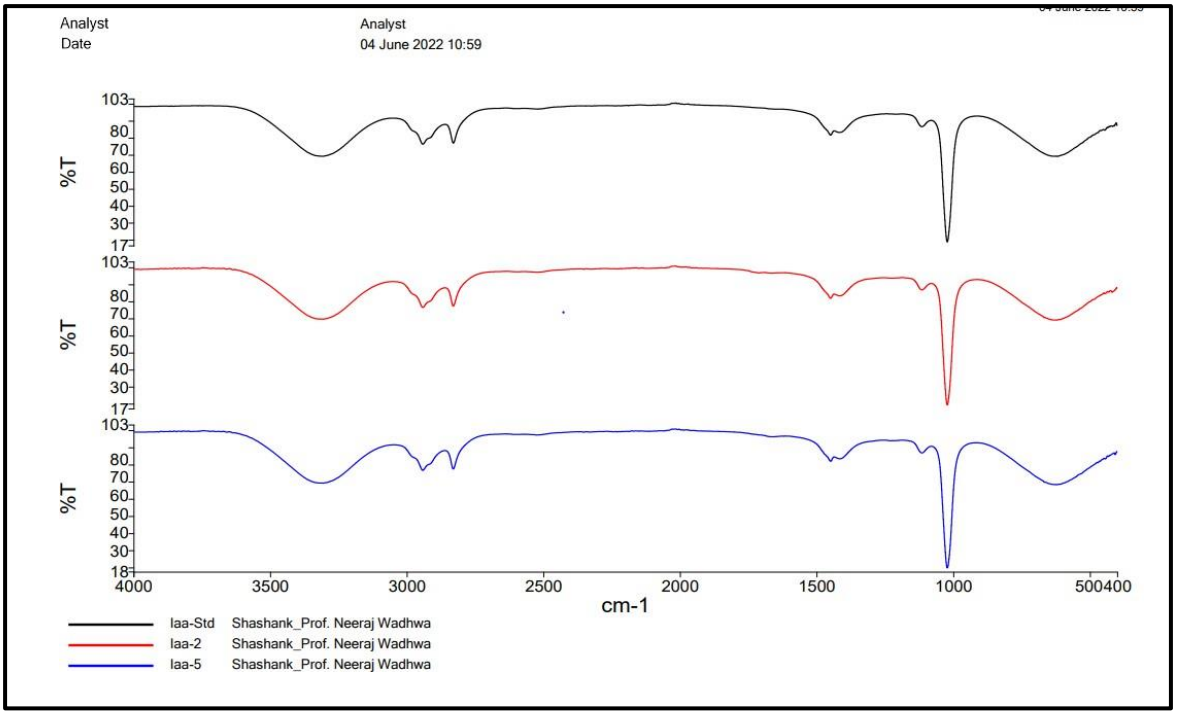


Figure 2. FT-IR analysis of IAA compared with standard

Detection & Confirmation of IAA by LC-ESI-MS analysis

IAA extracted from endophytes was identified by LC-ESI-MS analysis, samples are dissolved in methanol after ethyl acetate extraction procedure and these sample were run in LCMS and compared with standard IAA. Molecular weight of IAA is 175.18 g/mol and in standard, IAA peak was present at 175.6 and present in samples. Clear peak was observed at retention time 4.74 min in strain S2 *Enterobacter asburiae* and IAA extracted from *Achromobacter*

mucicolens showed peak at 4.72 min. Retention time in both the samples were compared with retention time of standard (Figure 3,4 and 5) and a fragmented at m/z 130, suggesting presence of free IAA as Gilbert et al. (2018) also reported.

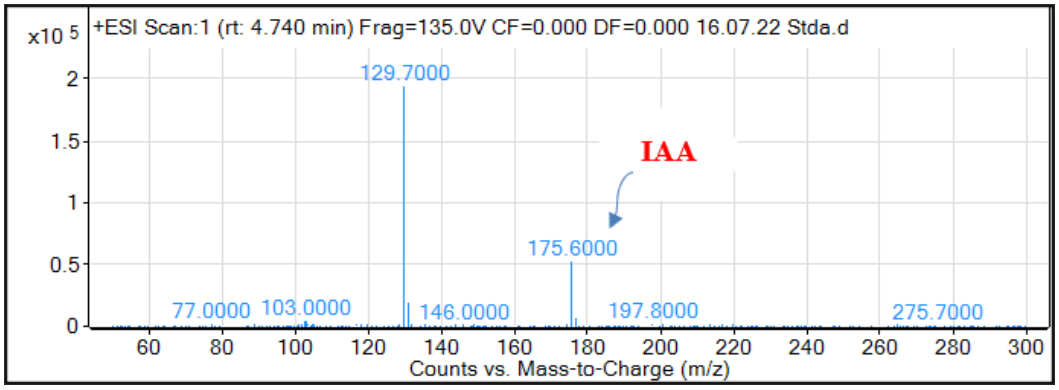


Figure 3. LC-ESI-MS peak of Standard IAA

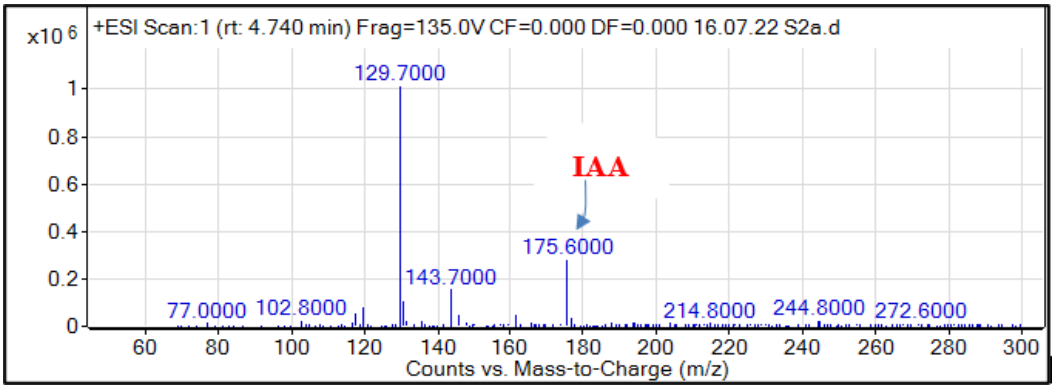


Figure 4. LC-ESI-MS peak of IAA extracted from S2 *Enterobacter asburiae*

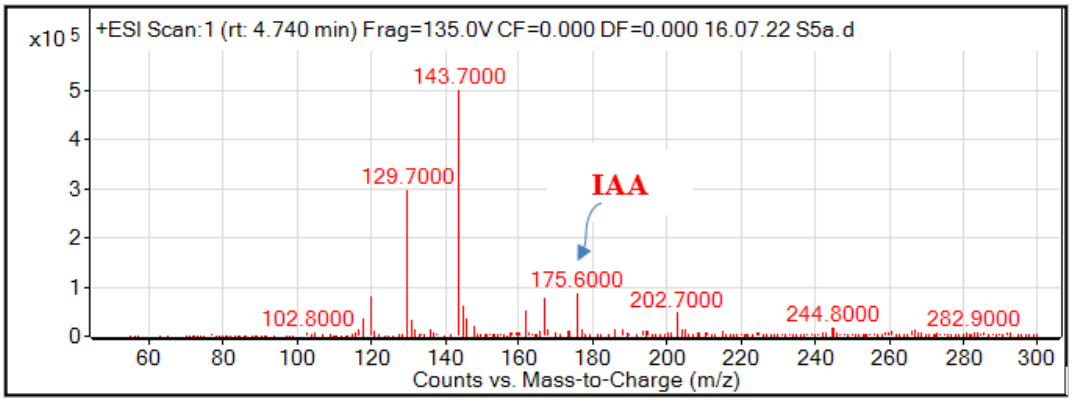


Figure 5. LC-ESI-MS peak of IAA extracted from S5 *Achromobacter mucicolens*

Effect of extracted IAA on growth of plants

For this, sterilized seeds were dipped in purified IAA & sowed in pots and observe the effect. After four weeks of germination, Morphological studies like root length, shoot length and lateral roots were measured and significant increase was observed when compared with uninoculated control (Table 6) (Figure 6,7 and 8). Growth parameters were favourably influenced in presence of the IAA producing endophytes.

Table 6. Effect of purified IAA on growth of *Triticum aestivum* and *Vigna radiata*

Isolate	**Shoot Length (cm)**		**Root Length (cm)**		**Lateral roots (cm)**	
	Triticum aestivum	***Vigna radiata***	***Triticum aestivum***	***Vigna radiata***	***Triticum aestivum***	***Vigna radiata***
Control	3.23±0.2*	3.6±0.43	2.23±0.55	2.73±0.2	4.56±0.35	4.8±0.36
Enterobacter asburiae (S2)	4.96±0.2	6.06±0.25	3.46±0.35	4.43±0.2	6.63±0.41	7.43±0.56
% increase in length	53.56	68.33	55.15	62.27	45.39	54.79
Achromobacter mucicolens (S5)	5.53±0.3	4.9±0.4	4.36± 0.3	4.7±0.52	8.03±0.56	9.16±0.3
% increase in length	71.20	36.11	95.51	72.16	76.09	90.83

*Values presented are mean± SD

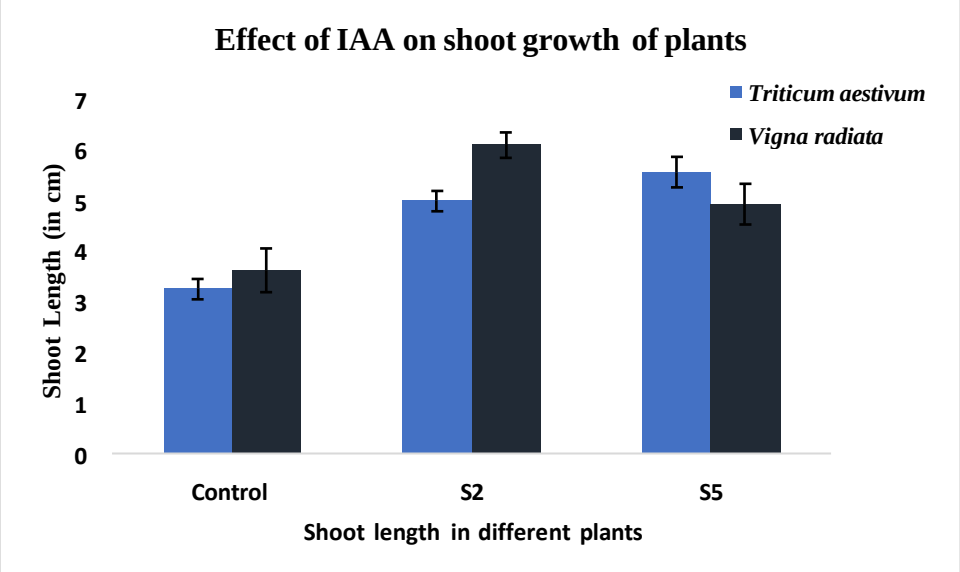


Figure 6. Effect of purified IAA on shoot growth of *Triticum aestivum* and *Vigna radiata*

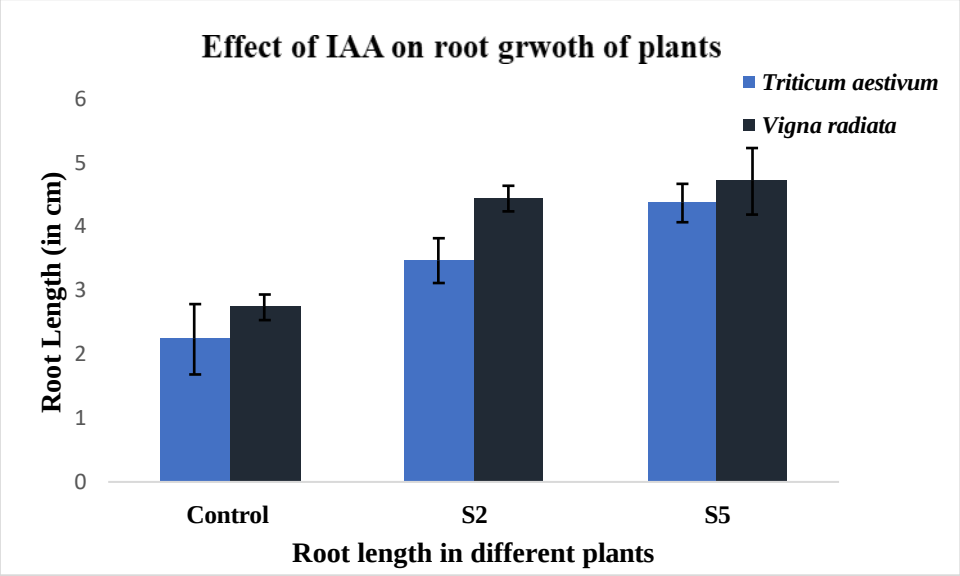


Figure 7. Effect of purified IAA on root growth of *Triticum aestivum* and *Vigna radiata*

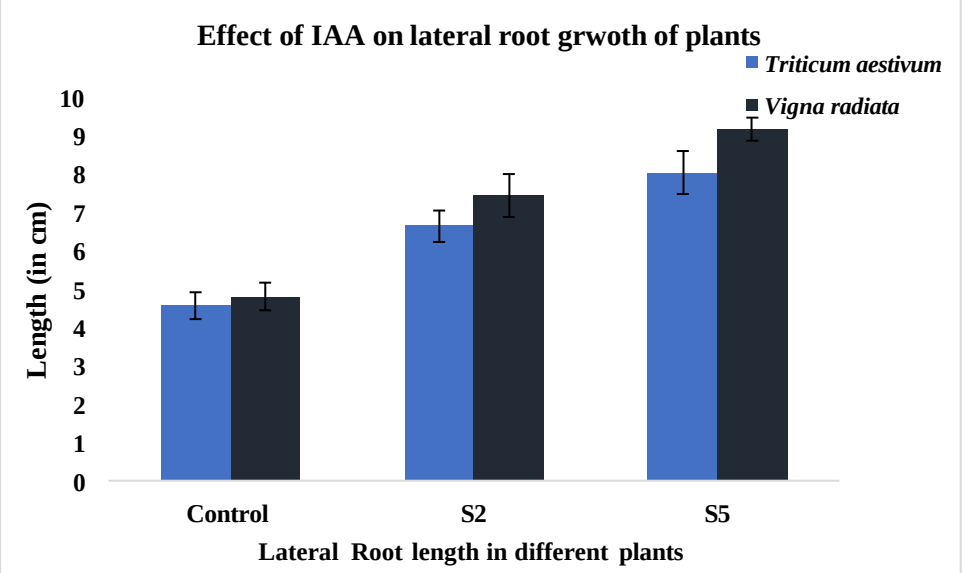


Figure 8. Effect of purified IAA on lateral root growth of *Triticum aestivum* and *Vigna radiata*

4. CONCLUSION

In this study, bacterial endophytes isolated in the previous study were screened for their ability for IAA production and its effect on root system of different crops. To increase the production of IAA different parameters like different media, Incubation time, different carbon source, different concentration of tryptophan was optimized.

Hence these strains had potential to produce increased amount of IAA in the modified media, can be purified easily from the culture broth. IAA of endophyte can positively impact the development of the plant and it is suggested that these endophytic strains *Enterobacter asburiae* (S2) and *Achromobacter mucicolens* (S5) can be effectively used to develop a chemical free bio formulation.

Acknowledgments

All the authors are thankful to Jaypee Institute of Information Technology, Noida, India for providing the laboratory, technical support, and encouragement and authors also acknowledges the Central Sophisticated Instrumentation Facility (CSIF), BITS Pilani KK Birla Goa Campus for providing LC-ESI-MS analysis of the samples.

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