UJMR, Vol. 8 No. 1, June, 2023, pp. 46 - 53 ***E-ISSN: 2814 – 1822; P-ISSN: 2616 – 0668***

https://doi.org/10.47430/ujmr.2381.005

Received: April 25, 2023 **Accepted:** June 20, 2023

Optımızatıon of Condıtıons for the Productıon of Indole Acetıc Acıd by *Azotobacter* spp.

Sule I.O.*, Agbabiaka T.O., Saliu B.K., Ajijolakewu K.A., Zakariyah R.F
Department of Microbiology, Faculty of Life Sciences, University of Ilorin,Ilorin, Nigeria
*Correspondence: suleism@unilorin.edu.ng; Phone number: +2348056663764

Abstract
Azotobacter spp. are known for their ability to fix nitrogen into the soil non-symbiotically. Their activities can be enhanced through the provision of optimum cultural conditions. Hence, this study aimed to isolate Azotobacter spp. and optimize their growth (medium and condtions) with a focus on pH, sucrose and indole acetic acid (IAA) concentrations. The counts of Azotobacter obtained from the rhizosphere of the crops ranged from 4.0 × 10^4 - 1.0 × 10^6 CFU/g. The three high IAA-producing Azotobacter spp. were identified as A. chroococcum, A. vinelandii and A. beijerinckii. They produced IAA in the absence and presence of 0.25 % tryptophan in the ranges of 0.20 - 0.36 and 604.5 - 1439.7 µg/mL respectively. However, under optimized conditions these isolates produced IAA in folds. Optimum IAA was produced by A. chroococcum, A. vinelandii and A. beijerinckii at pH, sucrose and tryptophan concentration ranging from 6.5 - 7.5, 2 - 3 % and 0.3 - 0.7 %. respectively. A. vinelandii produced a higher amount of IAA when compared with A. chroococcum and A. beijerinckii at the optimal conditions. These were 2001.1, 2541.1 and 2602.6 µg/mL at optimum pH 7.5, sucrose (2 %) and tryptophan (0.3%) respectively. It was concluded from these findings that, Azotobacter vinelandii is an excellent producer of plant growth promoting hormone, indole-3-acetic acid (IAA).
Key words: Optimization, Azotobacter, Indole acetic acid

INTRODUCTION

Nitrogen fixation is one of the most important microbial activities as it enhances the recycling of nitrogen on Earth and allows a balance and replenishment of nitrogen in the soil (Ladha *et al.*, 2022). Non-symbiotic or free-living nitrogen fixing bacteria include *Azotobacter, Clostridium, Azolla,* blue green algae (cyanobacteria) etc. According to Aquilanti *et al.* (2004), *Azotobacter* species do not depend on the root nodules of plants and are present in different environments such as soil, water and sediment. Earlier report (Khin *et al.*, 2012) has shown that they are good producers of plant growth stimulating hormones such as gibberellins, auxins, and cytokinins. Boiero *et al.* (2007) and Mohite (2013) reported that indole-3-acetic acid (IAA) can lead to an increase in the root length, creating large root surface area thereby making the plants more access to soil moisture and nutrients. Optimization of IAA production in culture media can be done by varying the precursor (L-tryptophan) and other constituents of the growth medium.

It is not an over statement that the genus *Azotobacter* plays a major role in soil productivity through different mechanisms including the production of IAA, but there is a dearth of information on the cultural growth conditions required for efficient indole acetic acid production by these isolates in tropical countries especially in Nigeria and particularly in Ilorin, Kwara State. This study therefore aimed to assess and optimize indole acetic acid production by *Azotobacter* spp. The objectives of this study were to isolate *Azotobacter* spp. from soils; screen and quantify IAA production by these isolates in the presence and absence of the precursor, tryptophan; characterize and identify the isolates, and optimize the effects of pH, sucrose and tryptophan on the production of IAA by the isolates.

MATERIALS AND METHODS

Collection of soil samples

Samples of soil (100 g each) were collected from the rhizosphere of different crops into sterile polythene bags and transferred immediately to the laboratory for microbiological analysis according to the method described by Pant and Agrawal (2014).

Isolation of *Azotobacter* from the soils

Ashby's Mannitol Agar (AMA) was prepared according to Ponmurugan *et al.* (2012) and used for the isolation and enumeration of *Azotobacter* spp. Ten grams of the soil sample was added into 90 mL of sterile distilled water to obtain 10^{-1} dilution. Thereafter, 1mL of aliquot from 10^{-1} dilution was added to 9 mL of sterile distilled water to obtain 10^{-2} dilution. Ten folds dilution of the soil sample was carried out until 10^{-4} dilution was obtained; the aliquot was transferred onto the surface of set plates of AMA; and incubated at 28 ˚C for 3 -7 days. At the end of incubation, the colonies were counted and expressed in CFU/g. Subculturing on AMA plates was carried out to obtain pure cultures of the isolates (Sulaimon *et al.*, 2019).

Characterization of the isolates

The isolates were characterized based on colonial and cellular morphology as well as biochemical tests (Vikram, 2011). The tests carried out for morphological identification were Gram staining and motility test, while the biochemical tests include oxidase, catalase, urease, citrate, methyl red, Voges-Proskauer, gelatin liquefaction, and nitrate reduction.

Molecular identification of the isolates

A Zymogen DNA extraction kit was used for the genomic DNA extraction following the instructions of the manufacturer. The extracted DNA was amplified using PCR protocol.

The sequence of the PCR product was obtained using the Big Dye Direct Cycle Sequencing Kit. The purified product was loaded on the ABI 3500 genetic analyzer using Applied Bio-systems to obtain the sequences of the organism. The sequences obtained were aligned and analyzed using the basic logic alignment (BLASTn) tools on the website of the National Center for Biotechnology Information (NCBI) in order to check the homology of the isolates with the existing one's database (Dashti *et al.*, 2009).

IAA standard curve

This involved preparation of Salkowski's reagent and different concentrations of IAA standard solutions. A 1ml of 0.5M $FeCl_3$ to was added to a 50 mL of 35% perchloric acid to prepare Salkowski's reagent (Kumari *et al.*, 2018). This solution was colourless. The absorbance of the different standard solutions was read at 530 nm over spectrophotometer and recorded (Chandra *et al.*, 2018). The graph of absorbance against the concentrations of the standard IAA solutions was plotted as presented in Figure 1.

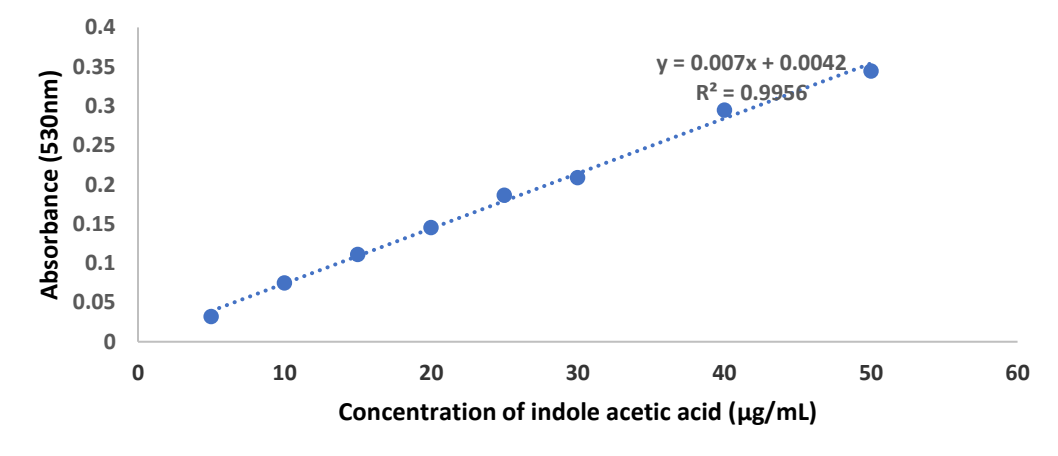


Figure 1: Indole acetic acid standard curve

IAA production by the isolates

Fifty milliliters of the Jensen's broth was dispensed into each conical flask and 0.125 g of tryptophan (0.25 % w/v) was added to each flask. After sterilization, the broth was cooled and a 2.5 mL (5.0 %) of the standardized culture was added to each flask. Shaking of the flasks was done at 120 rpm at 30 ˚C for 8 days.At the end of the shaking period, the culture broths were centrifuged at 3000 rpm for

1 hour. One milliliter of the supernatant was added to 7 mL of distilled water in a test tube, and 2 ml of Salkowski's reagent was also added. Thereafter, the mixture was shaken and covered with black polythene bag for 30 minutes. Absorbance was read at a wavelength of 530 nm to determine the degree of pink colouration developed in the broth. The amount of IAA produced by each isolate was extrapolated from the standard curve of IAA constructed (Sivasankari and Anandharaj, 2016).

Optimization of the conditions for IAA production

The compositions of the Jensen's medium were optimized using one factorial approach. Sucrose concentrations between 1.0 to 3.0 % were prepared. The pH of another set of the medium was varied between pH 5.5 to 8.5 while the tryptophan concentration of another set of the medium varied between 0.2 to 0.8 %. The agitation of the experimental conical flasks, temperature of incubation and duration of the experiment was at 120 rpm, 30 °C and 8 days respectively (Hasuty *et al.*, 2018; Kumari *et al.*, 2018).

RESULTS

Counts and characterization of *Azotobacter* spp. isolated from the soils

The population of *Azotobacter* across the rhizosphere soils of the different plants ranged from 4.0×10^4 – 1.0×10^6 CFU/g (Table 1). The characteristics of the isolates are presented in Table 2.

Table 1: Counts of *Azotobacter* isolated from the rhizosphere of some plants

Rhizosphere soils	Count (CFU/g)
Okro	6.0×10^5
Rice	1.0×10^5
Cassava	4.0×10^4
Moringa	2.5×10^5
Sorghum	7.0×10^5
Teak	1.0×10^5
Date palm	1.0×10^5
Pawpaw	1.5×10^5
Potato	1.0×10^6
Jatropha	1.2×10^5

Table 2: Characteristics of *Azotobacter* spp. isolated from the rhizosphere of some plants

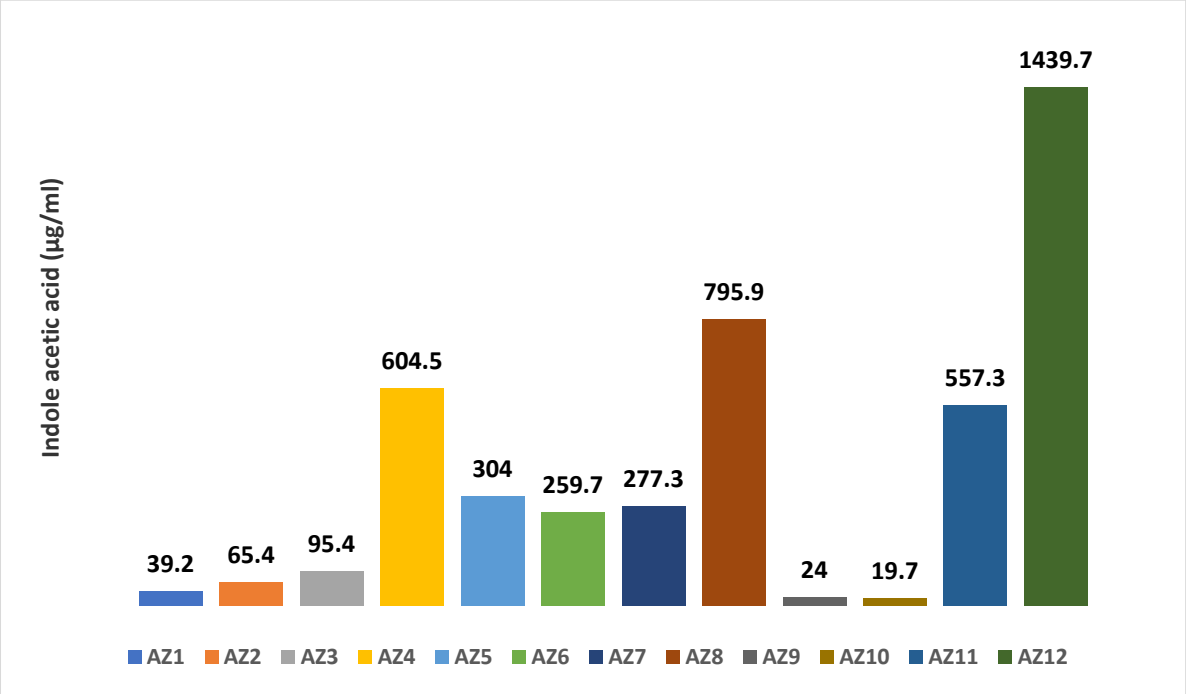
Isolates	GR	CS	OX	CA	GL	MT	NR	CT	AJ	UR	CR	MR	VP	PAM
AZ1	-	R	+	+	-	+	+	+	Y	+	+	ND	ND	WS
AZ2	-	R	+	+	-	+	+	+	Y	+	+	+	-	DB
AZ3	-	R	+	+	-	+	+	+	Y	+	+	-	-	DB
AZ4	-	R	+	+	-	+	+	+	Y	+	+	-	+	DB
AZ5	-	R	+	+	-	+	+	+	G	+	+	+	-	DB
AZ6	-	R	+	+	-	+	+	+	G	+	+	+	-	C
AZ7	-	R	+	+	-	+	+	+	G	+	+	ND	ND	LB
AZ8	-	R	+	+	-	+	+	+	Y	+	+	-	-	WM
AZ9	-	R	+	+	-	+	+	+	Y	+	+	+	-	W
AZ10	-	R	+	+	-	+	+	+	G	+	+	-	-	Y
AZ11	-	R	+	+	-	+	+	+	Y	+	+	-	-	WS
AZ12	-	R	+	+	-	+	+	+	Y	+	+	-	+	Y

GR = Gram reaction; CS = Cell shape; OX = Oxidase; CA = Catalase; GL = Gelatin liquefaction; MT = Motility; NR = Nitrate reduction; CT = Cyst; AJ = Acid production in Jensen medium; UR = Urease; CR = Citrate utilization; MR = Methyl Red; VP = Voges-Proskauer; PAM = Pigment in Ashby's benzoate agar; Y = Yellow; W = White; WS = White and shiny; DB = Dark brown; WM = White and

mucoid; LB = Light brown; C = Cream; G = Green; + = Positive reaction; - = Negative reaction; ND = Not determined; AZ = *Azotobacter* isolate

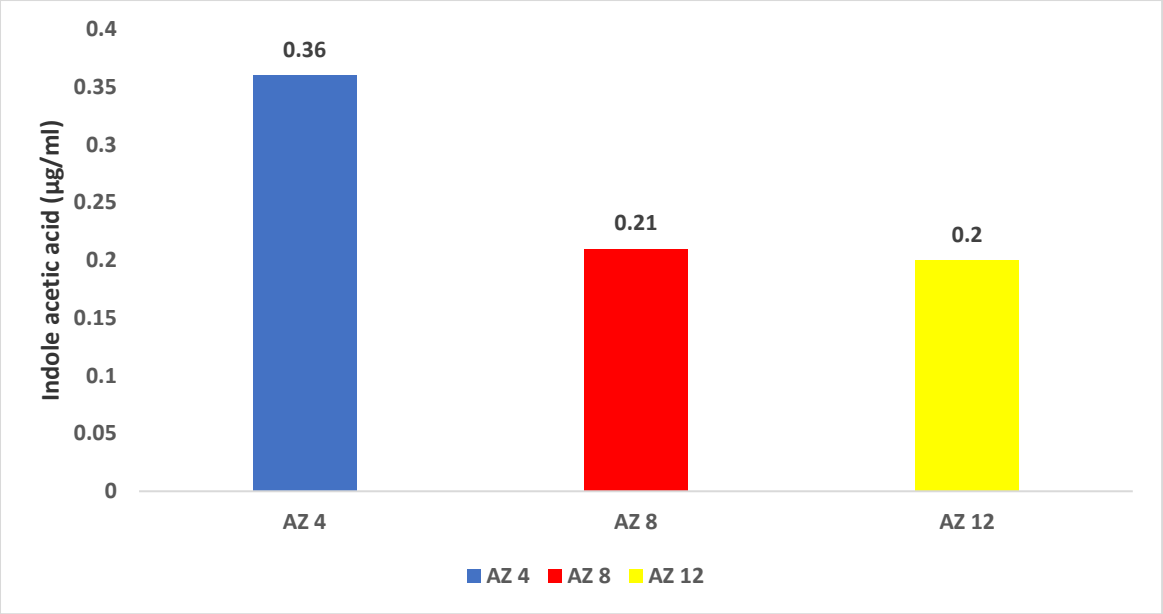
IAA production by the isolates

The IAA produced by the isolates ranged from 19.7 – 1439.7 µg/mL (Figure 2). The three highest -ranking IAA producers (AZ4, AZ8 and AZ12) were selected for molecular identification and optimization of conditions for IAA production. These selected isolates produced IAA in the presence and absence of tryptophan ranging from 604.5 to 1439.7 (Figure 2) and 0.20 to 0.36 µg/mL respectively (Figure 3).



AZ = *Azotobacter* sp.

Figure 2: Indole acetic acid production by *Azotobacter* spp. in the presence of 0.25% tryptophan



AZ=*Azotobacter* sp.

Figure 3: Indole acetic acid production by selected *Azotobacter* spp. in the absence of tryptophan

Identification of bacterial isolates

The three high-ranking IAA producers, AZ4, AZ8 and AZ12 were identified as *A. chroococcum*, *A. beijerinckii* and *A. vinelandii* respectively (Table 3).

Table 3: Identification of selected *Azotobacter* isolates

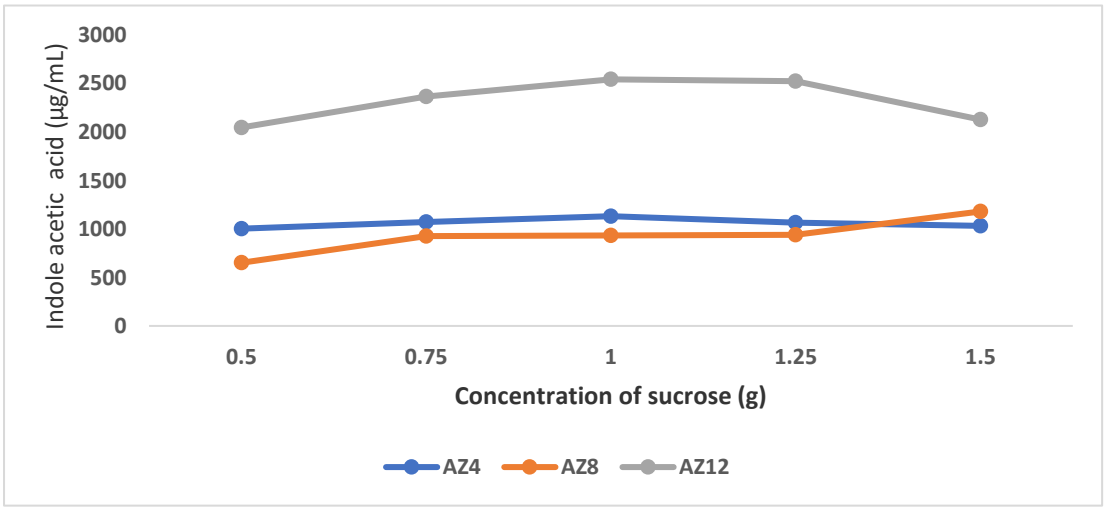
Code	Identification	Percent Identity	Accession number
AZ4	*Azotobacter chroococcum*	93.79	MH249629.1
AZ8	*Azotobacter beijerinckii*	93.37	MN340240.1
AZ12	*Azotobacter vinelandiii*	95.30	LN874283.1

Optimization of conditions for the production of IAA

A. vinelandii produced the highest amount of IAA at the optimum concentration of sucrose when compared with *A. chroococcum* and *A. beijerinckii*. Both *A. chroococcum* and *A. vinelandii* produced their optimal IAA at 1 g (2.0 % concentration of sucrose). However, *A. beijerinckii* required a higher amount of sucrose 1.5 g (3 %) to produce IAA (Figure 4).

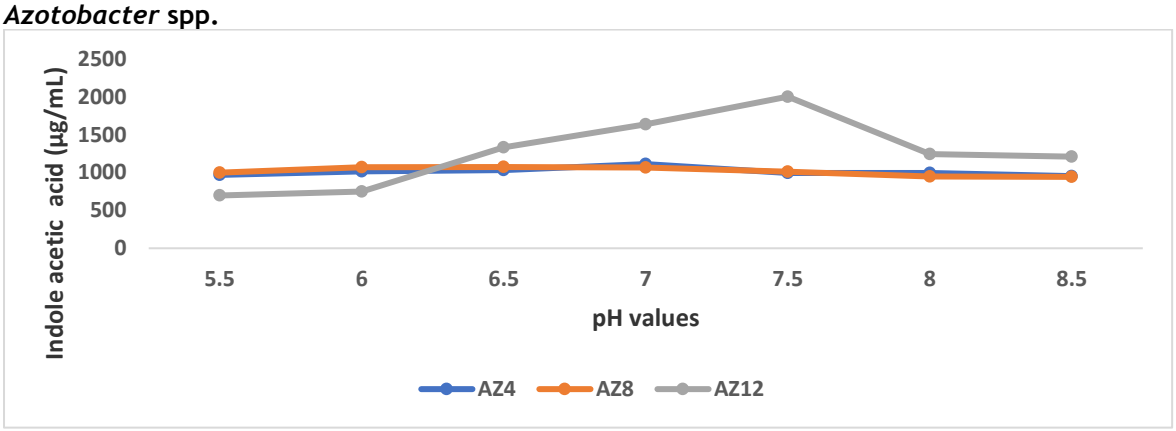
A. vinelandii and *A. chroococcum* produced the highest amount of IAA at neutral to slightly alkaline pH 7.0 – 7.5 while *A. beijerinckii* produced optimum IAA at slightly acidic pH 6.5 (Figure 5).

In this study, it was found that *A. vinelandii* required the least quantity of tryptophan, 0.15 g (0.3%) to produce the optimum amount of IAA while *A. chroococcum* and *A. beijerinckii* required more amount of tryptophan to produce optimum IAA which were 0.3g (0.6%) and 0.35g (0.7%) respectively (Figure 6).



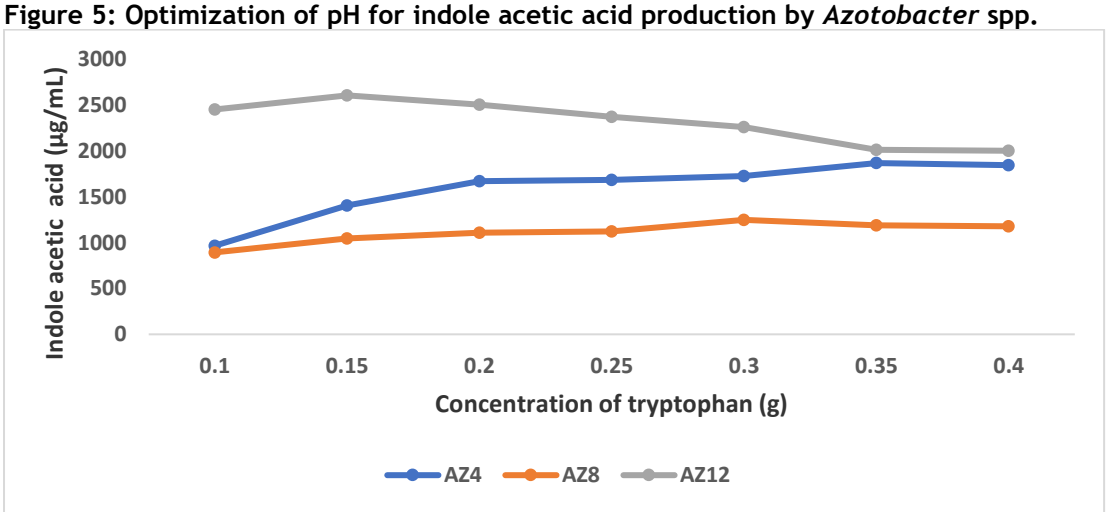
AZ4 = *A. chroococcum*; AZ8 = *A. beijerinckii*; AZ12 = *A. vinelandii*

Figure 4: Optimization of concentration of sucrose for indole acetic acid production by *Azotobacter* spp.



AZ4 = *A. chroococcum*; AZ8 = *A. beijerinckii*; AZ12 = *A. vinelandii*

Figure 5: Optimization of pH for indole acetic acid production by *Azotobacter* spp.



AZ4 = *A. chroococcum*; AZ8 = *A. beijerinckii*; AZ12 = *A. vinelandii*

Figure 6: Optimization of concentration of tryptophan for indole acetic acid production by *Azotobacter* spp.

DISCUSSION

The highest counts of *Azotobacter* obtained in this study 10^6 CFU/g was above the 10^4 CFU/g obtained by Martyniuk and Martyniuk (2003) and Sivasankari Anandharaj (2016). Purwaningsih *et al.* (2022) obtained counts of *Azotobacter* in the range of 1.1×10^6 – 4.9×10^6 CFU/g from the rhizosphere of rice.

The *Azotobacter* spp. isolated in this study showed the characteristic pigments in Ashy's benzoate agar. These were brown/black, yellow green and whitish for *A. chroococcum*, *A. vinelandii* and *A. beijerinckii* respectively (Jimenez *et al.*, 2011). In a study by Chen *et al.* (2018) *A. tropicalis, A. chroococcum*, *A. vinelandii* and *A. beijerinckii* were isolated from rice rhizospheric soils.

El-Mahrouk and Belal (2007) reported that *Azotobacter* spp. were unable to produce IAA in the absence of tryptophan; in contrast to this study where they do. However, in the presence of tryptophan (0.1 g/l) their *Azotobacter* spp. produced IAA in the range of 12 – 54 mg/mL. Karthikeyan and Sakthivel (2011) reported that *Azotobacter chroococcum* produced 7.8 µg/mL of IAA in the absence of tryptophan and 40 µg/mL in the presence of 5mg/ml of tryptophan. Chennappa *et al.* (2016) reported that in the presence of 1 mg/mL of tryptophan, *Azotobacter tropicalis* and *Azotobacter vinelandii* produced 15.5 and 25.5 µg/mL of IAA respectively. Sivasankari and Anandharaj (2016) obtained IAA in the absence of tryptophan in the range of 4.49 – 7.48 µg/mL while 52.80 µg/mL of IAA was the highest obtained in the presence of 5 mg/mL of tryptophan in their study. Furthermore, Dashti *et al.* (2021) reported IAA production by bacterial isolates in the absence and presence of 0.5 mg/mL tryptophan that ranged from 14.3 – 65.9 and 103.5 – 111.6 µg/mL respectively.

In this study out of the 12 *Azotobacter* spp. isolated from the rhizosphere of plants, 3 of them were able to produce high amount of IAA and were identified as *A. chroococcum*, *A. beijerinckii*, and *A. vinelandii*. In another study, Jimenez *et al.* (2011) isolated *A. vinelandii, A. nigricans, A. chroococcum,* and *A. paspali* from the rhizosphere of vegetables. *A. chroococcum* and *A. vinelandii* were isolated by Torres-Rubio *et al.* (2000) from the rhizosphere of rice cultivated.

Further, the isolates in this study produced IAA in slightly acidic to slightly alkaline condition and sucrose concentration between 2 - 3%. El-Mahrouk and Belal (2007) reported that *Azotobacter* spp. were able to produce optimum IAA at pH 7.0 in their study. Tryptophan is one of the compounds present in exudates produced by several plant species. It can also be produced by *Azotobacter* in the rhizosphere of plants to enhance the plant growths (Hasuty *et al.*, 2018). In this study, it was observed that *A. vinelandii* required less amount of tryptophan (0.3% or 3 mg/mL to produce IAA when compared with *A. chroococcum* and *A. beijerinckii*. Vikram (2011) observed an increase in IAA production when the amount of tryptophan of their medium increased from 1 to 5 mg/mL.

CONCLUSION

In this study, it has been shown that *Azotobacter* spp. were able to initiate indole acetic acid production in the absence of tryptophan precursor but high quantities were produced in its presence. Optimum IAA was produced by *Azotobacter* spp. at pH, sucrose and tryptophan concentration ranging from 6.5 – 7.5, 2 - 3% and 0.3 – 0.7 % respectively. *A. vinelandii* produced the highest amount of IAA when compared with *A. chroococcum* and *A. beijerinckii* at the optimal conditions. These were 2001.1, 2541.1 and 2602.6 µg/ml at optimum pH 7.5, sucrose (2%) and tryptophan (0.3 %) respectively. It is concluded from this study that *Azotobacter vinelandii* is an excellent producer of plant growth promoting hormone, IAA.

ACKNOWLEDGEMENT

This study was funded by Tertiary Education Trust Fund (TETFUND), Nigeria, under the 2016 – 2017 Institutional Based Research Fund (IBRF) award. We also appreciate Centre for Research Development and In-House Training (CREDIT), University of Ilorin for shortlisting of our proposal.

REFERENCES

Aquilanti L., Favilli F., Clementi F. (2004). Comparison of different strategies for isolation and preliminary identification of *Azotobacter* from soil samples. *Soil Biology and Biochemistry* 36: 1475 – 1483. **[Crossref]**

Boiero L., Perrig D., Masciarelli O., Penna C., Cassán, F., Luna V. (2007). Phytohormone production by three

strains of *Bradyrhizobium japonicum* and possible physiological and technological implications. *Applied Microbiology and Biotechnology* 74(4): 874 – 880. **[Crossref]**

Chandra S., Askari K., Kumari M. (2018). Optimization of indole acetic acid production by isolated bacteria from *Stevia rebaudiana* rhizosphere and its effects on plant growth. *Journal of Genetic Engineering Biotechnology* 16(2): 581 – 586. **[Crossref]**

Chen S.L., Tsai M.K., Huang Y.M., and Huang C.H. (2018). Diversity and characterization of Azotobacter isolates obtained from rice rhizosphere soils in Taiwan. *Ann Microbiol*. 68:17 – 26. **[Crossref]**

Chennappa G., Naik M.K., Adkar-Purushothama C.R., Amaresh Y.S., Sreenivasa M.Y. (2016). Plant Growth Promoting potential, abiotic stress tolerance and antifungal activity of *Azotobacter* strains isolated from paddy soils. *Indian Journal of Experimental Biology* 54: 322 – 331.

Dashti N., Khanafer M., El-Nemr I., Sorkhoh N., Ali N.*et al*. (2009). The potential of oil utilizing bacterial consortia associated with legume root nodules for cleaning oily soils. *Chemosphere* 74(10): 1354 – 1359 **[Crossref]**

Dashti N.H., Nedaa Y., Al-Sarraf A., Cherian V.M., Montasser M.S. (2021). Isolation and characterization of novel plant growth promoting rhizobacteria (PGPR) isolates from tomato (*Solanum lycopersicum*L.) rhizospheric soil: A novel IAA producing bacteria. *Kuwait J. Sci*. 48(2): 1 -18. **[Crossref]**

El-Mahrouk M.E., Belal E.B.A. (2007). Production of indole acetic acid (bioaxin) from *Azotobacter* sp. isolate and its effect on callus induction of *Dieffenbachia maculata* cv. Marianne. *Acta BiologicaSzegediensis* 51(1): 53 – 59.

Hasuty A., Choliq A., Hidayat I. (2018). Production of indole acetic acid by *Serratia marcescens* subsp. *marcescens* and *Rhodococcus aff. Oingshengii*. *International Journal of Agricultural Technology* 14(3): 299 -312.

Jimenez D.J., Montana J.S., Martinez M.M. (2011). Characterization of free nitrogen fixing bacteria of the genus *Azotobacter* in organic vegetable grown Colombian soils. *Brazilian Journal of Microbiology* 42(3): 846 – 858. **[Crossref]**

Khin M.L., Moe-Moe M., Tar –Tar W., Zin M.A. (2012). Isolation of Plant Hormone (Indole-3-Acetic Acid -IAA) Producing Rhizobacteria and Study on their Effects on Maize Seedling. *Engineering Journal*. 16(5):137-144. **[Crossref]**

Karthikeyan A., Sakthivel K.M. (2011). Efficacy of *Azotobacter chroococcum* in rooting and growth of Eucalyptus camaldulensis stem cuttings. *Research Journal of Microbiology* 6(7): 618 -624. **[Crossref]**

Kumari S., Prabha C., Singh A., Kumari S., Kiran S. (2018). Optimization of indole-3-acetic acid production by diazotrophic *B. subtilis* DR2 (KP455653), isolated from rhizosphere of *Eragrostis cynosuroides*. *International Journal of Pharmaceutical and Medical Sciences* **[Crossref]**

Ladha J.K., Peoples M.B., Reddy P.M., Biswas J.C., Bennett A., Jat M.L. and Kruonik T.J. (2022). Biological nitrogen fixation and prospects for ecological intensification in cereal-based cropping systems. Field Crops Research 283: 108541**[Crossref]**

Martyniuk S., Martyniuk M. (2003). Occurrence of *Azotobacter* spp. in some Polish soils. *Polish Journal of Environmental Studies* 12(3):371 – 374.

Mohite B. (2013). Isolation and characterization of indole acetic acid producing bacteria from rhizospheric soil and its effect on plant growth. *Journal of Soil Science and Plant Nutrition* 13(3): 638 – 649**[Crossref]**

Pant G., Agrawal P.K. (2014). Isolation and characterization of indole acetic acid producing plant growth promoting rhizobacteria from rhizospheric soil of

Withania somnifera. *Journal of Biological and Scientific Opinion* 2(6): 377 – 383. **[Crossref]**

Ponmurugan K., Sankaranarayanan A., Al-Dharbi N.A. (2012). Biological activities of plant growth promoting *Azotobacter* sp. isolated from vegetable crops rhizosphere soils. *Journal of Pure and Applied Microbiology* 6(4): 1689 – 1698

Purwaningsih S., Mulyani N., Nugroho A.A., Suriani N.L. (2022.) Effectiveness of rhizosphere *Azotobacter* bacteria in promoting rice growth and yield in a green house. In: First Asian PGPR Indonesian Chapter International e-Conference 2021, *KnE Life Sciences* 328 – 339. **[Crossref]**

Sivasankari B., Anandharaj M. (2016). Indole-3-acetic acid production and enhanced plant growth promotion of *Vigna unguiculata* (L.) Walp by *Azotobacter* sp. *International Journal of Advanced Research* 4(5): 1445 – 1450. **[Crossref]**

Sulaimon K.H., Al-Barakah F.N., Assaeed A.M. and Dar B.A. (2019). Isolation and identification of *Azospirillum* and *Azotobacter* species from *Acacia* spp. at Riyadh, Saudi Arabia. *Bangladesh J. Bot*. 48(2): 239 – 251**[Crossref]**

Torres-Rubio M.G., Valencia-Plata S.A., Bernal-Castilo J., Martienez-Nieto P. (2000). Isolation of enterobacteria, *Azotobacter* sp., and *Pseudomonas* sp., producers of indole-3-acetic acid and siderophores from Colombian Rice rhizosphere. *R L de Microbiologica*, 42: 171 – 176.

Vikram P. (2011). Production of indole acetic acid by *Azotobacter* sp. *Recent Research in Science and Technology* 3(12): 14 – 16