RESEARCH ARTICLE

Isolation of *Azospirillum spp* and Evaluation of its Plant Growth Promoting Abilities

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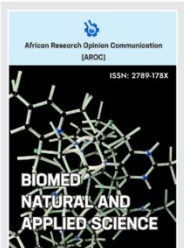

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ABSTRACT

Background: This study aimed to isolate Azospirillum species from the soil environment and assess their plant growth-promoting abilities. Soil samples were collected from 10 different agricultural sites. The bacteria were isolated by serially diluting the soil samples and each aliquot was transferred into a Nitrogen-free bromothymol blue medium and then incubated for 7 days at 37°C. The isolates were further screened based on their morphological, biochemical and plant growth phytohormone production. The Fourier Transform Infrared Spectrophotometer will be used to elucidate the functional groups present in the isolates. Furthermore, the isolates were subjected to molecular identification. The results showed that Seventeen (17) bacteria were isolated from the soil samples of different crop plants using nitrogen-free bromothymol blue medium with all forming a white subsurface pellicle and they were further screened to seven (7) isolates, based on their morphological, biochemical, and plant growth phytohormones production characteristics, they include A1, A2, A3, A4, A5, A6 and A7. Three of the isolates (A1, A3, and A6) were randomly selected for molecular identification. The Fourier Transform Infrared Spectrophotometer identified the functional groups that are present in the Azospirillum strains to be aromatic compounds. All isolates showed ammonium production potential with A7 having the highest production of 781.3 µg/ml. Only four (4) isolates showed the ability to solubilize Calcium triphosphate with A7 having the highest solubilization (776 µg/ml). Furthermore, all isolates exhibited the ability to produce Indole acetic acids in the growth medium with A6 having the highest production (798.5 µg/ml). The molecular analysis and the sequence analysis of the three isolates 16SrRNA showed two of the isolates to be *Azospirillum brasilense* (A1, A3) and the other Azospirillum lipoferum (A6). Therefore, this study shows that this strain of *Azospirillum* has a high ability to produce Indole Acetic Acids, which aids in the production of phytohormones such as Auxins, Solubilize Calcium triphosphate and hence proffer a sustainable solution to improving plant growth.

Keywords: Plant growth, *Azospirillum*, Phytohormones, and soil

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1.0 Introduction.

Azospirillum is a Gram-negative, microaerophilic, non-fermentative, and nitrogen-fixing bacterial genus from α-Proteobacteria, Order Rhodospirillales, Family Rhodospirillaceae, which are motiles, peritrichous, catalase and oxidase positives, containing Q-10 isoprenoidquinones as chemotaxonomic markers and C18:1ώ7C as predominant fatty acid [1].

It has been one of the most studied plant growth-promoting bacteria (PGPB) since its discovery by Martinus Beijerinckin the Netherlands in 1925. However, as a result of the research conducted by Johanna Döbereiner in Brazil in the 1970s, two main characteristics are used to define this bacterial genus: its ability to fix atmospheric nitrogen (N) [2] and produce several phytohormones, including auxins, cytokinins, and gibberellins [3].

Azospirillum spp. has been associated with several mechanisms to promote plant growth and a wide range of studies have detailed the beneficial effects of inoculation with these rhizobacteria [3]. The improvement of plant growth by *Azospirillum* spp. has been mostly attributed to their capacity to fix atmospheric N and to produce phytohormones; it is less attributed to the bio-disposition of nutrients, expression of enzymes, synthesis of compounds related to plant stress mitigation, and competition against phytopathogens, among other mechanisms. However, taken individually, none of these mechanisms is fully responsible for the changes observed in inoculated

plants [4]. *Azospirillum* spp. modes of action were initially explained by the additive hypothesis where the effects of small mechanisms operating either at the same time or consecutively create a larger final effect on plants [5].

Moreover, the high rates of application of chemical fertilizers have deleterious effects on the environment, contributing to the contamination of soils and water. An interesting option for the substitution of nitrogen fertilizers could be the exploitation of plant growth-promoting bacteria such as the genus *Azospirillum*, capable of affecting the growth and yield of many plants of agronomic and ecological significance [6]. Hence, there is a need to fill this gap in knowledge by exploring the diversity of *Azospirillum* spp. present in the soils with a view of being used as biofertilizers for better crop productivity.

2.0 MATERIALS AND METHODS

2.1 Collection of Samples

Soil samples were taken from the roots of various cultivated agricultural land within and outside the University of Ilorin in Nigeria. These include:

A. Rhizosphere of a pawpaw plant at agricultural farm, Oke-Odo, Tanke, Ilorin.

B. Rhizosphere of an Okro plant at Aliara village, Asadam road, Ilorin.

C. Rhizosphere of a rice plantation at Eyenkorin, Ilorin.

D. Rhizosphere of a Cassava plant at University of Ilorin, main gate.

E. Rhizosphere of a palm plant at aliara village, Asadam road, Ilorin.

F. Rhizosphere of a cashew plant at the University of Ilorin agricultural farm.

G. Rhizosphere of a cotton plant at Asa dam area, Ilorin.

H. Rhizosphere of a Mango plant at Aliara village, Asa dam road, Ilorin.

I. Rhizosphere of an Orange plant at Offa garage area, Ilorin.

J. Rhizosphere of a Millet plant at Eyenkorin village, Ilorin.

A total of 10 soil samples were collected from the rhizosphere of these plants: Cotton, Okro, Cassava, Cashew, Millet, Pawpaw, Rice, Orange, Mango, and Palm. The soil samples were collected into different sterile labelled polythene bags. The top layer of the soil was cleared of debris and a clean sterile trowel was used to dig into the rhizosphere around 20 cm deep, this was done carefully so as not to injure or damage the root of the plants or the plant itself. The samples were analyzed immediately after collection in the laboratory [7].

2.2 Microbiological analysis of the soil sample

The culture media used during this research included: Selective media which were Okon's agar media, Nitrogen-free bromothymol blue medium, and general-purpose media using Nutrient agar and Nutrient broth. The media were prepared according to standard procedures [7].

2.2.1 Isolation of Bacteria from the soils

This was done using soil dilution pour-plate method for the isolation of *Azospirillum* spp. from the soil samples. The collected soil sample 10 g each was added to 90 ml of sterile distilled water in a sterile conical flask and shaken well for proper mixing. This gave 1^{-1} dilution after which 1ml aliquot from 10^{-1} dilution was transferred to 9ml of sterile distilled water in a test tube and shaken properly to obtain 10^{-2}. The process of serial dilution was repeated to obtain 10^{-3} and 10^{-4} dilutions. The aliquot, 0.1ml was taken from the different dilutions and introduced into separate plates of Okon's agar medium and an aliquot of 1ml was introduced into Nitrogen-free Bromothymol blue medium in McCartney bottles. The inoculum was spread using an L-shaped glass spreader on the Okon's agar medium plates. The plates were inverted and together with the bottles incubated at 37°C for 7 days. The growths observed were counted.

2.3 Characterization and Identification of bacterial isolates

The bacterial isolates were characterized and identified based on their colonial characteristics and cellular morphology [7].

2.4 Biochemical Characterization of bacterial isolates

The biochemical characteristics of each bacterial isolate were done. These include oxidase, catalase, Urease, Citrate, Oxidative-Fermentative, Methyl Red-Voges Proskauer, sugar fermentation, nitrate reduction, and Starch Hydrolysis tests. [8];[7].

2.4.1 Determination of Ammonia Production

Production of ammonia was quantified by the method of Goswani [9]. Bacterial cultures grown in Ashby's Nitrogen-free liquid medium were centrifuged at 3000rpm for 10 min and 0.2ml culture supernatant was mixed with 1ml Nessler's reagent and the total volume made to 8.5ml by adding ammonia-free distilled water. The development

of a brown to yellow colour is indicative of ammonia production which was estimated spectrophotometrically at 410nm using a standard curve prepared with 0.1-2.0µg/ml ammonium nitrate

2.4.2 Determination of Indole acetic acid (IAA)

Production of indole acetic acid (IAA) was determined by the method of Patten and Glick [10]. Bacterial cultures were grown in Luria broth supplemented with tryptophan (1mg/ml) at 37C shaking at 120rpm for 2 days followed by centrifugation at 3000rpm for 10min. in total, 1ml of supernatant was combined with 2ml of Sakowski's reagent and incubated for 30 minutes at 25°C. The development of pink color indicated IAA production and its optical density was recorded at 530nm. The concentration of IAA was estimated using a standard curve

2.4.3 Quantitative Analysis for phosphate solubilization

This test was carried out to quantify the amount of phosphate the isolated organism solubilized. Each bacterial isolate was cultivated on an NA plate for 24 hours at 37°C. One hundred millilitre PVK broth containing 0.5g calcium phosphate was prepared in a 250 ml conical flask.

Bacterial colonies on nutrient agar were selected from plates and introduced into sterile normal saline, standardized using 0.5 MacFarland to compare the inoculum density. 2.5 ml of the inoculum which is equivalent to 5% was taken from the standardized culture in normal saline into each of the sterile 100ml PVK broth. The uninoculated PVK broth served as control. The setup was placed on a shaker incubator for 14 days.

The sample was then centrifuged at 3,000 rpm for 30 minutes to remove any suspended solids and the supernatant of each culture was decanted and filtered with Whatman filter paper No.11. The filtrate was used for the estimation of the amount of soluble phosphate using the Ascorbic acid method. One millilitre of the supernatant was added to 8 ml of combined reagent in a 50 ml volumetric flask. The flask was made to volume by adding distilled water. The flasks were allowed to stand for 30 minutes. The absorbance of the resulting blue solution was read using a spectrophotometer at 880nm. The amount of solubilized phosphate was extrapolated from the phosphate standard curve [3].

2.5 Determination of functional group of metabolites liberated by the bacterial isolates

The sample was taken from the shaker incubator after 14 days and centrifuged at 3000 rpm for 30 minutes to remove suspended solids. The filtrate was carefully collected and analyzed using a Fourier-transform infrared (FTIR) spectrometer for functional group identification and molecular structure [12].

2.6 Molecular identification of bacteria isolates and Sequencing

The selected bacterial isolates were inoculated in sterile nutrient broth and incubated at 37°C for 24 hours and then sent to the International Institute of Tropical Agriculture Laboratory, Ibadan, Oyo state, Nigeria for further identification using Molecular Technique.Fragments of the 16S rRNA gene was amplified from the genomic DNA of the isolates by using the forward primer 25f (5'-AACTKAAGAGTTTGATCCTGGCTC-3') and reverse primer 1492r (5'-TACGGYTACCTTGTTACGACTT-3') as described by Hurek [13]. The purified PCR products was sequenced using 35f (5'-CTKAAGAGTTTGATCMTGGCTCAGATTGAACG-3') 342f (5'-CTCCTACGGGAGGCAG-3') and 930f (5'-GGTTAAAACTYAAAKGAATTGACGGGGAC-3')

3.0 Results

3.1 Isolation of *Azospirillum*

After 72 hours of incubation, the Nitrogen free bromothymol semi-solid medium showed a white-coloured pellicle on the subsurface indicating the successful isolation of *Azospirillum*. The pellicles were then transferred to Nitrogen-free bromothymol solid plates and incubated for 48 hours. A total number of 7 isolates were obtained and they were preserved for further analysis.

3.2 Morphological characteristics of the isolates

The colonial and cellular morphology of the isolates were observed. The colonial morphology shows that the isolates are all rod-shaped and Gram-negative. The detailed report is presented in Table 1.

Table 1: Morphological Characteristics of the isolates

	Colonial morphology					Cellular morphology	
Isolate	Shape	Pigmentation of colony	Edge of colony	Optical characteristics	Consistency	Shape	Gram reaction
A1	Circular	Cream	Entire	Translucent	Mucoid	Rod	Negative
A2	Circular	Creamy green	Entire	Opaque	Mucoid	Rod	Negative
A3	Circular	Cream	Entire	Opaque	Mucoid	Rods	Negative
A4	Circular	Cream	Entire	Opaque	Mucoid	Rods	Negative
A5	Circular	Cream	Entire	Opaque	Mucoid	Rods	Negative
A6	Circular	Cream	Entire	Translucent	Mucoid	Rods	Negative
A7	Circular	Pink	Entire	Opaque	Mucoids	Rods	Negative

3.3 Biochemical characterization of the isolates

The result of the biochemical test shows that all the isolates utilized urease and glucose. And are oxidase and catalase positives. The detailed result is presented in Table 2.

Table 2: Biochemical characteristics of the bacterial isolates

	Isolates	Nitrate reduction	Urease	Methyl red	VogesProskauer	Lactose	Glucose	Maltose	Oxidase	Catalase	Simmon citrate	O-F TEST		TSI			
												Open	Close	Butt	Slant	Gas	H2S
4	A1	+	+	-	-	-	-	-	+	+	-	G	G	K	K	-	-
6	A2	+	+	-	-	-	+	-	+	+	-	G	G	K	A	-	-
11	A3	+	+	-	-	-	-	-	+	+	-	G	G	K	K	-	-
13	A4	+	+	-	-	-	-	-	+	+	-	G	G	K	K	-	-
14	A5	+	+	-	-	-	+	-	+	+	-	G	Y	K	K	-	-
15	A6	+	+	-	-	+	+	-	+	+	-	G	G	A	A	-	-
17	A7	+	+	-	-	-	+	-	-	+	-	G	G	A	A	-	-

KEYS: - = Negative, + = Positive, Y = Yellow, K= Alkaline, A= Acid, G= Green

3.4 Plants growth promoting characteristics of bacterial isolate

The production of indole acetic acid ranged from 16.80 to 798.48µg/ml, with isolate A6 showing the highest production. The production of ammonia ranged from 268.3 to 781.30µg/ml, with isolate A2, having the highest amount of production. The bacterial isolates were analyzed using PVK broth to quantify the amount of phosphate solubilized by each isolate. The highest amount of phosphate was solubilized by the A7 (776µg/ml), as shown in Table 3.

Table 3: Plant growth-promoting hormones

	Plant hormone-promoting properties (µg/ml)		
Bacterial isolates	**IAA production**	**Solubilized phosphate**	**Ammonia production**
Azospirillum brasilense	33.94	302.6	348.60
A2	174.48	0.354	781.30
Azospirillum brasilense	44.22	277.60	348.60
A4	500.16	0.802	657.20
A5	700.80	0.030	268.30
Azospirillum lipoferum	798.48	616.30	472.60
A7	141.84	776.00	490.90

3.5 Functional group of metabolites liberated by the bacterial isolates

The use of Fourier transform infrared spectrophotometer (FTIR) to analyze the liberated functional group by the bacterial isolates in the PVK broth resulted in peak values as shown in Figures 1 to 7. The bacterial isolates displayed a range of values between peak 3100-3000, overtones 2000-1665 (C-C stretch), 1500-1400 (C-C stretch in ring), and 900-675 (C-H "OOP") to confirm the presence of Aromatic compound.

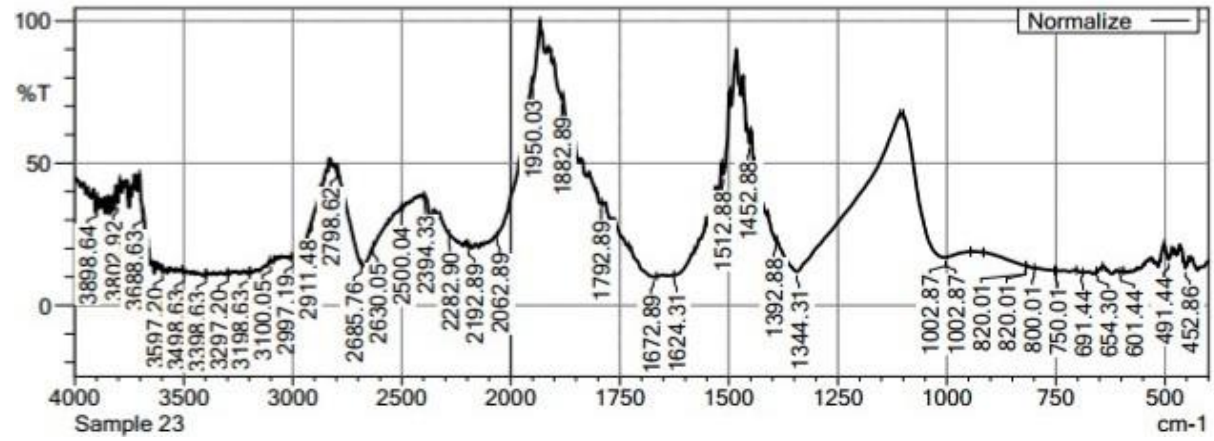

Figure 1: Fourier Transfer Infrared Spectra of A1

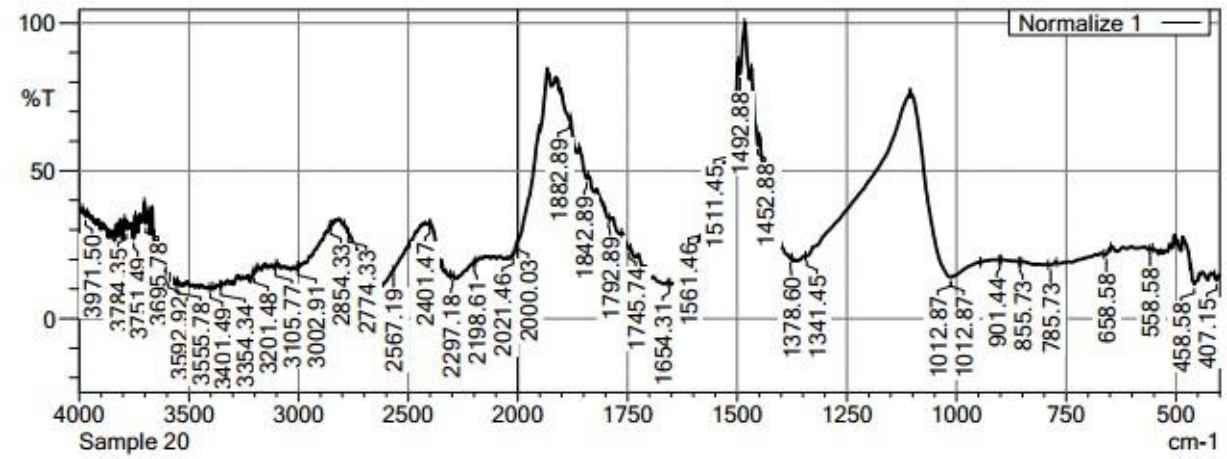

Figure 2: Fourier Transfer Infrared Spectra for A2

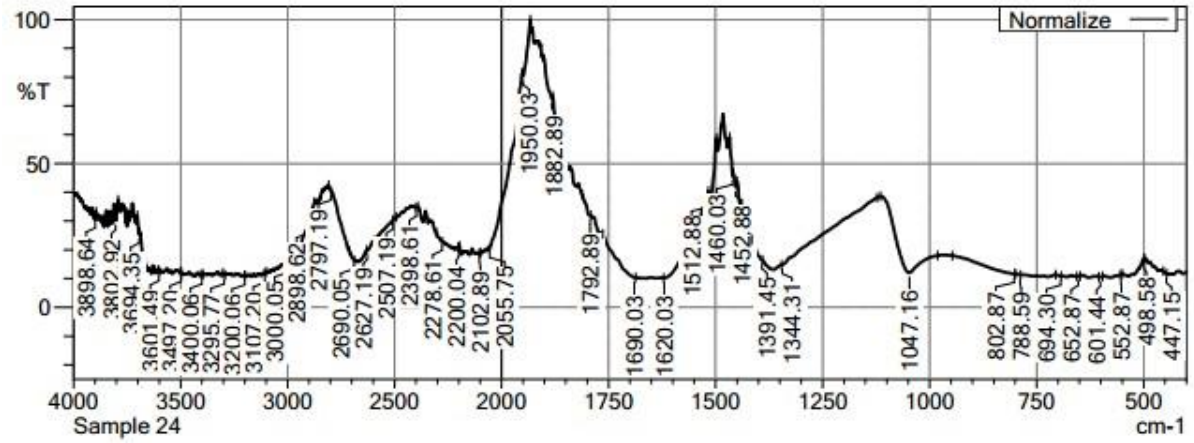

Figure 3: Fourier Transfer Infrared Spectra for A3

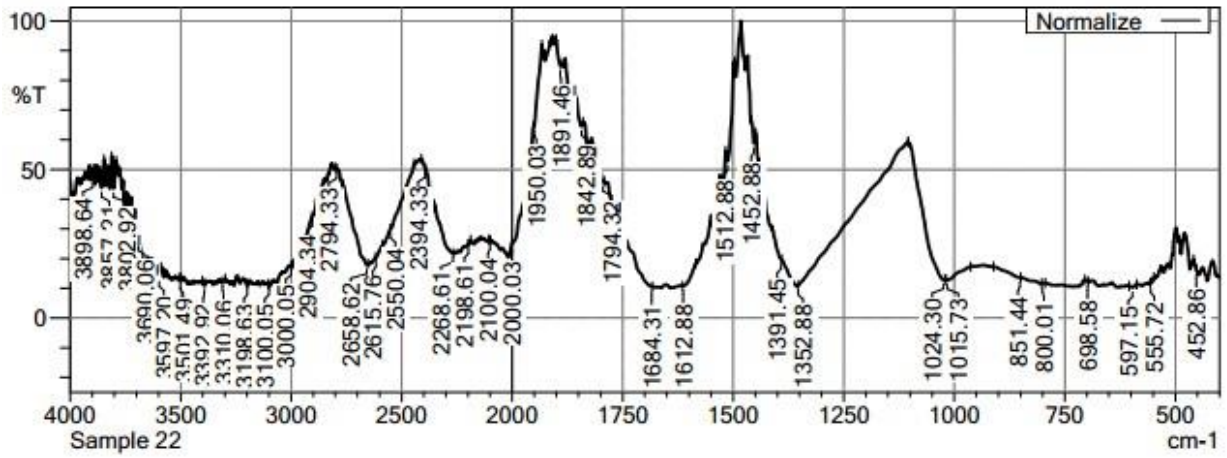

Figure 4: Fourier Transfer Infrared Spectra for A4

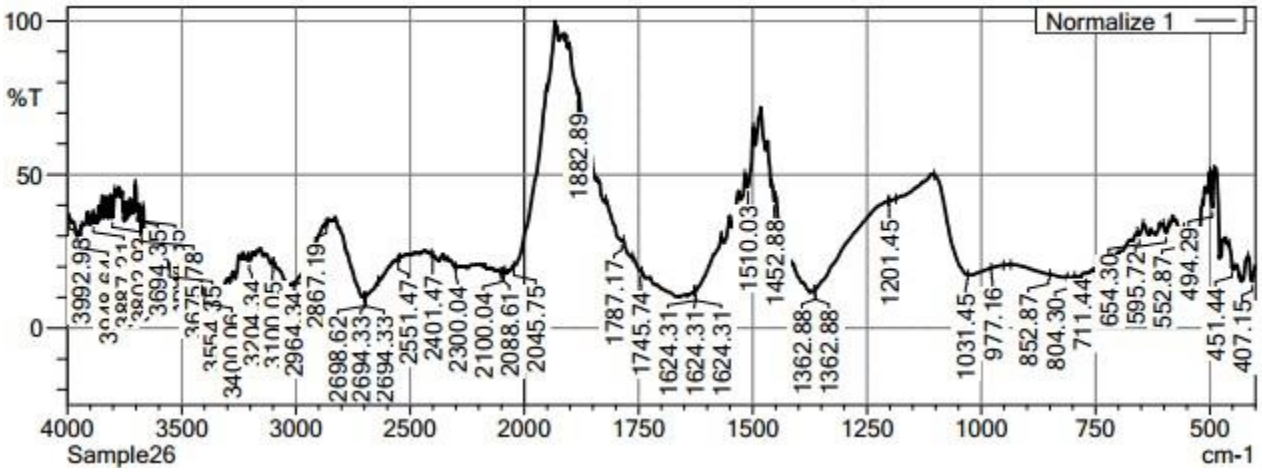

Figure 5: Fourier Transfer Infrared Spectra for A5

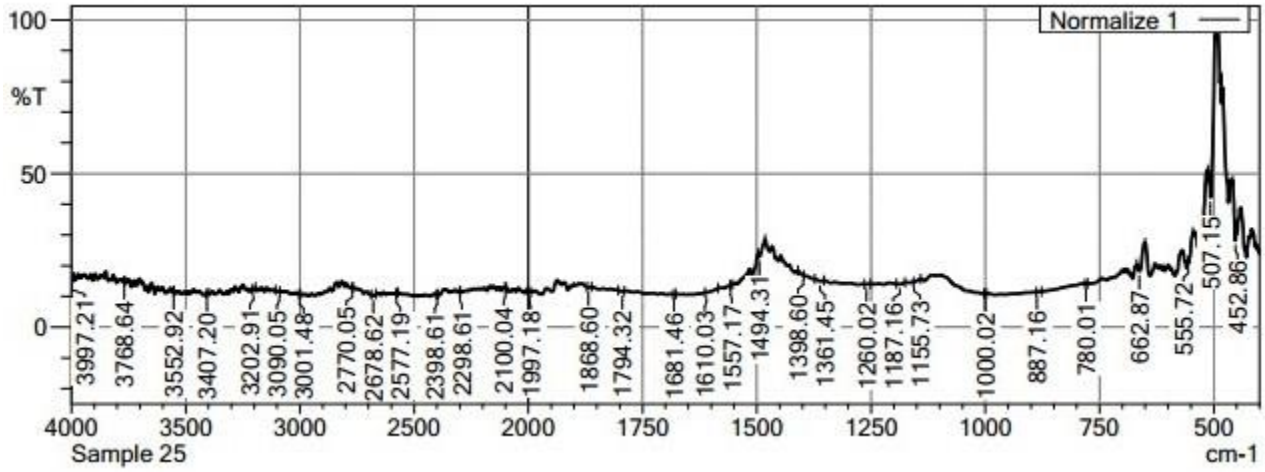

Figure 6: Fourier Transfer Infrared Spectra of A6

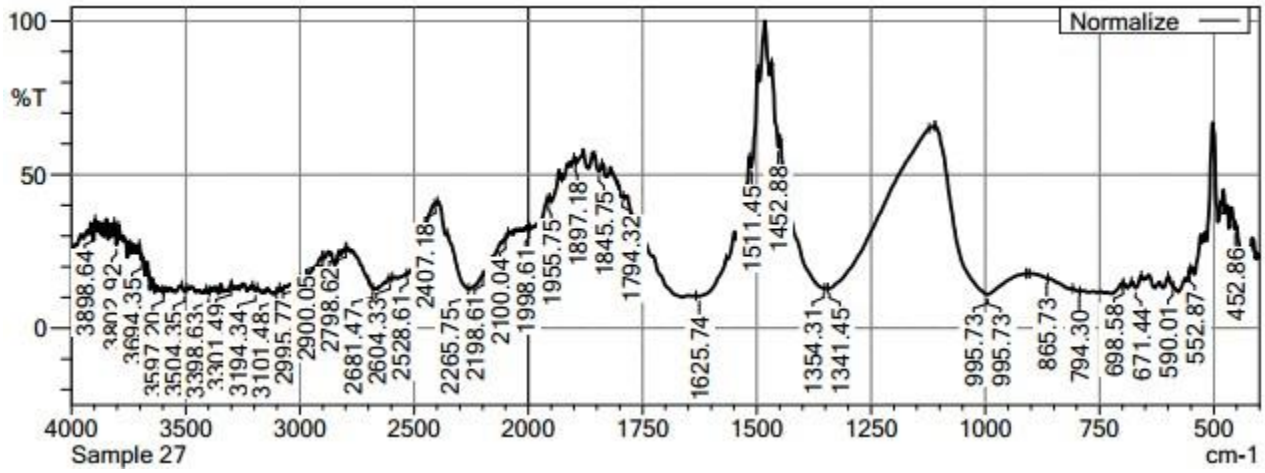

Figure 7: Fourier Transfer Infrared Spectra of A7

3.6 Molecular identification of the bacterial isolate

The three best bacterial isolates were selected for molecular analysis based on their biochemical characterization and they include A1, A3, and A6. The extracted DNA was sequenced and it is as shown in Appendix 1-3. The nucleotide sequences were submitted to GeneBank to obtain percentage similarity and accession number as shown in Table 4.

Table 4: Molecular Characterization of bacterial isolate

Isolate code	Identity	Percentage (%) Identity	Accession number
A1	*Azospirillum brasilense*	95.05	LN874276.1
A3	*Azospirillum brasilense*	95.03	LN874276.1
A6	*Azospirillum lipopferum*	89.54	MH388302.1

4.0 DISCUSSION

The ability to form plant hormones is the major property of many microorganisms and Plant Growth Promoting Bacteria in general and specifically, species of *Azospirillum* that stimulates and facilitates plant growth [14]. A total of 7 bacteria were isolated using the Nitrogen free bromothymol blue all forming a white subsurface pellicle and turning the olive green colour of bromothymol blue to brilliant blue. This pellicle indicates the isolates to be likely *Azospirillum* spp. which is in line with the work of Okon [15]. All the isolates are rod-shaped and Gram-negative.

The sugar fermentation test showed that only four isolates (A2, A5, A6, and A7) produced acid in glucose peptone broth while 3 isolates (A1, A3, and A4) failed to produce acid. Also, all seven isolates didn't produce acid in lactose and maltose peptone broth.

The oxidative and fermentative (O-F) utilization of sugars indicated that all seven isolates didn't produce acid in both sealed and open tubes which are characterized by an unchanged colour of the medium from blue to yellow and suggests no fermentation reaction. There was no production of acid in any open tubes only which suggests that there was no oxidative utilization of the sugar. *Azospirillum* is known to be catalase and oxidase positives and it was discovered that all seven isolates are oxidase and catalase positives.

The ability to produce phytohormones such as indole acetic acid was assayed with A6 having the highest production rate of 798.48µg/ml and followed by A5 with 700.80µg/ml which is greater than the 2.90 to 10.80mg/l produced by two agronomically important *Azospirillum* strains reported by Perrig [16], 19.40 to 30.20mg/l in *Azospirillum brasilense* cultures as reported by Fatima [17] and the 29.80 and 194.80mg/l produced by two *Azospiillum zeae*strains from the rhizosphere of wheat as reported by Venieraki [18]. The effects of indole acetic acid production have been attributed to its ability to aid in the production of longer roots with an increased number of root hairs and laterals which are involved in nutrient uptake [19]. This attribute will confer on most plant crops especially grains/cereals to compete favorably with other plants for the available nutrients in soils. Indole acetic acids also, stimulate cell elongation by modifying certain conditions like, an increase in osmotic contents of the cell, an increase in permeability of water into the cell, and a decrease in wall pressure. It promotes cambial activity, inhibits or delays the abscission of leaves, and induces flowering and fruiting [20].

On the other hand, all the isolates were found to release a high amount of ammonia in the ammonium release quantification assay (Table 4). This assay analyzed the concentration of ammonium excreted by the bacterial isolates in the growth medium when cultured. It was found that the concentration of ammonium release ranged from 268.30µg/ml to 781.20µg/ml. The highest concentrated ammonium was released by A2.

Phosphate solubilization potential was observed in all the seven isolates with the highest discovered in A7 (776µg/ml) followed by A6 (616µg/ml) while A3 had the lowest with 277.6µg/ml. The noticed reduction in pH and its associated phosphate solubilization agrees with several studies [21-23] who have all reported that phosphate solubilizing activity is always associated with a drop in the medium pH. This drop in pH according to Mohammed [24] can be attributed to the varying diffusion rates of different organic acids secreted by tested organisms.

The 7 isolates were subjected to Fourier transform infrared spectrophotometer to determine the functional group liberated and the analysis revealed the liberation of aromatic compound. The molecular analysis and the sequence analysis of their 16SrRNA of the three most efficient isolates A1, A3, and A6 identified them as *Azospirillum brasilense* (A1), *Azospirillum brasilense* (A3) and *Azospirillum lipoferum*(A6).

5.0 CONCLUSION

Therefore, this study shows that this strain of *Azospirillum* has a high ability to produce Indole Acetic Acids, which help in the production of phytohormones such as Auxins, Solubilize Calcium triphosphate and hence proffer a sustainable solution to improving plant growth that can be used in the cultivation of grains in Sub-saharan Africa.

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Conflicts of Interest: The authors declare no conflict of interest.

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