

Isolation, Identification and Screening for Nitrogen Fixing Activities by *Azotobacter chroococcum* Isolated from Soil of Keffi, Nigeria as Agent for Bio-fertilizer Production

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Abstract: Free living nitrogen fixing bacteria are those that lives freely on rhizosphere of a young growing plant or those living in a close association with root of plants (Rhizobacteria) but not symbiotically. In most tropical soil, Nitrogen and other essential nutrients element are the most limiting nutrients which deficiencies could lead to slow growth and reduced crop production. Free living nitrogen fixing bacteria has the potential of secreting nitrogenase enzymes and produces organic acids such as glycolic, acetic, malic, succinic acids which fix atmospheric nitrogen directly into the soil for plants growth and development. To this end, the study on isolation, identification and screening of *Azotobacter chroococcum* from soil of Keffi, Nasarawa State, Nigeria was carried out from May to September 2018. Soil samples were collected from eight different locations and *Azotobacter* strains were isolated and identified using standard microbiological methods. The 16SrRNA gene sequence analysis of the strain showed maximum similarity of 96% with *Azotobacter chroococcum* of the reference type strain deposited in RDP Gen Bank database. *Azotobacter* strains isolated from four different locations showed coloured zone ranging between 16-10mm. Similarly, Percentage amount of nitrogen released by each *Azotobacter* strain in the culture broth ranging between 1.19% - 5.11% in an increasing order.

Keywords: Rhizobacteria, *Azotobacter chroococcum*, 16SrRNA Sequencing, Nitrogen-free Agar, Nitrogen-free Broth, Bio-fertilizer

1. Introduction

Rhizosphere, a zone of influence around plants roots harbors alot of microorganisms. Among these are the dominant rhizobacteria which prefer living close to vicinity of root plants or surface area and play a crucial role in plant growth [1]. Nitrogen (N) is a key plant nutrient required for plant growth. The elemental nitrogen is in abundant in the atmosphere [2]. However, most tropical soil are deficient in

soluble N and so is the most limiting nutrient element which deficiencies could led to slow growth and reduced crop production [3]. Nitrogen availability in agricultural soil comes either from inputs of atmospheric nitrogen by free living bacteria [4] or from biological nitrogen fixation [5]. Biological Nitrogen Fixation (BNF) is a one way process of converting elemental nitrogen from the atmosphere into plant useable form in the soil. *Azotobacter* and other group of rhizobacteria are actively involved in the process of BNF,

secreting nitrogenase enzyme, produces organic acids and siderophores which aid fixation of atmospheric dinitrogen directly into the soil, thus improving the soil fertility. Besides, symbiotic nitrogen fixation, the non-symbiotic nitrogen fixation is also known to be of great agronomic important [1]. BNF is an excellent, economically and environmentally sound approach to replace the use of chemical fertilizer to increase soil fertility and improve plant growth and crop production [6].

2. Materials and Methods

2.1. Sample Collection

Soil sample from eight different locations within Keffi, Nasarawa State-Nigeria were collected using hand shovel at 20mm depth [7] and transfer into sterile polyethylene bag before transported to Microbiology laboratory Nasarawa State University, Keffi for further study.

2.2. Isolation and Subculture of *Azotobacter* Species

Isolation was carried out using a method described by [8]. 1.0g of the soil sample was suspended in 9ml of sterile distilled water in a test-tube. 1ml was picked and transferred into another test tube containing 9ml of sterile water. The step was repeated to 10-fold dilutions. 0.5ml of the aliquot dilution was picked from the 6th-dilution tube using pipette and spread on prepared Burk's agar plate and incubated at 37°C for 48hrs. Discrete colonies were picked and re-streaked on Burk's agar slants for further study.

2.3. Identification of *Azotobacter chroococcum*

Cultural and morphological characteristics on Burk's agar were carried out by gram staining method as described [9]. Biochemical test such as methyl red, Voges-proskauer, indole, catalase, oxidase, urease, citrate utilization, motility, starch hydrolysis, gelatin hydrolysis, carbohydrate utilization and cyst formation were also carried out according to the method described in Bergey's Manual of Determinative Bacteriology 9th edition [10-12]. Amplification of the 16SrRNA gene from the extracted genome of the bacteria strain was carried out using the universal primer pairs 27F (5'-AGAGTTTGATCATGGCTCAG-3') as forward primer and 1492R (5'-GGTTACCTTGTACGACTT-3') as the reverse primer [13-15]. Full length sequencing of the amplified 16SrRNA gene product (amplicon) of the *Azotobacter chroococcum* was send to Epoch Life science (USA) for purification and Sanger's sequencing as outlined in QIAquick purification protocol (Qiagen, Crawley, UK) [16].

2.4. The DNA Extraction Procedures

500µl overnight culture broth of the bacteria strain was spun at 1000rpm for 5min. The pellet was resuspended in 300µl lysis buffer and 2µl RNaseA and later Vortex vigorously for 30-60sec. 8µl proteinaseK was added to the mixture by pipetting and then Incubated at 60°C for

10minutes and later cools down for 5minutes. Thereafter, 300µl binding buffer was added and vortex briefly before the tube was placed on an ice for 5minutes and later centrifuge for 5minutes at 10,000rpm. 40-50µl elution buffer was added into the centre of the column then incubated at room temperature for 1minute before it was centrifuged at 10,000g for 2minutes and the DNA pellet was stored at 4°C or -20°C as outlined in Jena Bioscience extraction protocol [17-18].

2.5. PCR Amplification of 16SrRNA Gene of the Bacteria Species Isolated

The 16SrRNA extracted genome DNA of the bacteria strained was amplified using Polymerase chain reaction (PCR) amplification processes by mixing a set of universal primers, 27F (5'-AGAGTTTGATCATGGCTCAG-3') as forward primer and 1492R (5'-GGTTACCTTGTACGACTT-3') as the reverse primer [13-15]. A typical PCR reaction mixture was prepared by adding 2µl of the template DNA with the 5X HOT FIREPol Blend Master mix (Solis Biodyne) containing 1.5µl of forward and 1.5µl reverse primers (BIOMERS, Germany), 5µl of 10X PCR buffer (Solis Biodyne), 1.5µl MgCl₂, 2.0µl mM of deoxynucleoside triphosphates (dNTP) (Solis Biodyne), 1.5µl Taq DNA polymerase enzyme (Solis Biodyne), and 5µl of nuclease free water alongside 5µl cells pellet of the extracted bacteria DNA and the entire reaction mixture was make up to 25µl by total concentration. The PCR reaction mixture tubes were placed on an Eppendorf vapo protect thermo cycler machine (Nexus series) to run 30 cycles programme under a given PCR conditions for an initial denaturation at 94°C in 2minutes (30cycles), follow by denaturation at 94°C for 45 seconds, annealing at 61°C for 1min, extension at 72°C for 1 minute 30 seconds (30cycles) and finally extension at 72°C for 5minutes (1cycle) and hold at 4°C. The amplified 16SrRNA gene product were run on a 1.5% agarose gel electrophoresis after staining with ethidium bromide standard solution (aliquot fraction of the PCR product and one DNA ladder were loaded per comb or gel well) for 1hour 30minutes at 80V. After electrophoresis, distinct DNA bands well separated of the bacteria species were compare with molecular ladder of the standard DNA of the gene size or molecular marker of 1500bp (Solis Biodyne) [19-20].

2.6. Sequencing of the Amplified 16SrRNA Gene

Fraction of the amplified genome of the bacteria strains were send (Epoch Life science (USA) for purification according to QIAquick purification protocol described by a study [16]. The purified product was send to Epoch Lifescience (USA) for sequencing (Sanger sequencer machine) and analysis using ABI 3100 soft ware module (version 5.2) [21] (Giovannoni *et al.*, 1990). Thus, the resulted sequenced obtained were compared with the published nucleotide sequences of close related bacteria type strain deposited in the GenBank National Centre for Biotechnology Information (NCBI) database and Ribosomal Database Project via BLAST searches (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) [22-23].

2.7. Screening for Nitrogen Fixing Activities by *Azotobacter* Strain Isolated on Nitrogen-free Agar

Eight *Azotobacter* species each was inoculated at the centre of an agar plate and incubated at 37°C for 3-5 days by a method described by [24]. On the third day blue coloured zone producing strains were marked as nitrogen fixers on the agar plate and the colouring zone formed was calculated each by deducting colony diameter from the coloured zone diameter.

$$\text{Coloured zone index} = \frac{\text{Colony diameter} + \text{colour zone}}{\text{Colony diameter}}$$

2.8. Quantitative Estimation of the Amount of Nitrogen Content (%) Released by *Azotobacter* Strains on Nitrogen-free Culture Broth

To determine whether these *Azotobacter* strains previously screened qualitatively are truly nitrogen fixers, they were further inoculated on a Nitrogen-free broth to quantify the amount of nitrogen produced by each strain quantitatively using Kjeldahl method described by [25]. *Azotobacter* strains were inoculated each on a 25ml conical flask containing nitrogen-free broth (Composition/L; 2.5g glucose, 0.20g K₂HPO₄, 0.05g KH₂PO₄, 0.1g NaCl, 0.01g CaCl₂, 0.1g MgSO₄·7H₂O, 0.005g FeSO₄·7H₂O, 0.005g MnSO₄·5H₂O, 0.5g Na₂MoO₄·2H₂O, 0.5g CaSO₄·2H₂O in 250ml sterile distilled water with pH adjusted to 7.0 before sterilization). The flasks were then incubated for 9 days on a continuous air flows shaker incubator for steady oxygen supply. After the 9th day, fermented cultures broth were harvested, centrifuged at 10,000rpm for 15min, to remove dead cells. Using Kjeldahl method as described by [25], the total Nitrogen content captured into the culture flask by *Azotobacter* species each was quantitatively calculated. 10ml supernatant (filtrate) was mixed with 20ml concentrated H₂SO₄ (0.01N) along with 0.1g CuSO₄ catalyst before digestion. Mixture was heated (digested) at a high temperature 550-600°C until white foams are seen. The digest was allowed to cool and the volume make up to 10ml with sterile water. The 10ml aliquot was then mixed with 50ml of 5N concentrated NaOH solution to neutralize pH and convert probable nitrogen bonds to soluble

nitrate. The mixture was condensed by distillation process, and a total of 50ml distillate (Ammonia gas) was capture into a distillation flask containing 4% boric acid solution with 4 drops of mixed indicator (methyl red and methylene blue) till the solution change from pink to green on completion of the process. Thereafter 2 drops of the mixed indicator was added to the distillate on the flask and titrated against HCl acid till the colour change from green to slight violet colour. The titre value for each of the experiment performed was recorded and the % nitrogen content produced was calculated using the formula below.

Calculations

% Total Nitrogen released on the fermented culture broth is calculated as

$$\%N = \frac{T_s \times N \times M \times V_d \times 100}{W_i \times V_b}$$

Where,

T=Titre value = 0.7, and 0.8

N = normality of the HCl (0.01N)

M= Mass of nitrogen in the evolved ammonia (14)

V_d = volume of the digest=250ml

V_b = volume of Aliquot filtrate (base) = 50ml

W_i = volume of the broth (10cm=10g)

2.9. Statistical Analysis

Data obtained were analysed using R Console software (Version 3.2.2). Pearson's Chi-square test was used to compare the proportion of activity and ability of the bacterial strains in fixing nitrogen in relation to locations where soil samples was collected. P<0.05 showed no significant difference ($\chi^2=5.1017$, df=7, P=0.6475).

3. Results

3.1. Isolation and Identification of *Azotobacter* Species

Cultural, morphology and biochemical characteristics of *Azotobacter* species isolated is given in Table 1.

Table 1. Cultural morphology and biochemical characteristics of the bacterial species.

| Cultural | Morphology | | Biochemical characteristics | | | | | | | | | | | Inference |
|--------------------|------------|---|-----------------------------|---|---|----|----|---|-----|----|----|----|----|-----------------------|
| | G | S | Ca | O | I | MR | VP | M | CUT | SH | UH | GH | CF | |
| D/B on Burk's Agar | - | C | + | + | - | - | - | + | + | + | - | - | + | <i>Azotobacter</i> sp |

G = Gram reaction, S = shape, Ca = catalase, O = Oxidase, I = indole, MR = Methylred, VP = Voges-proskauer, M = Motility, CUT = Citrate utilization test, SH = Starch hydrolysis, UH = Urease hydrolysis, GH = Gelatin hydrolysis, CF = Cyst formation, - negative, + positive, P = Polymorphic, D/B = Dark/brown

3.2. Percentage Occurrence of *Azotobacter* Species Isolated from Different Locations in Keffi

Percentage occurrence of the *Azotobacter* species isolated from different locations within keffi showed that Angwan lambu (AL) and pyanko had the highest occurrence of *Azotobacter* isolates with 100% while High court, Kofar hausa, Old barrack, BCG, pyanko, and Angwan mada had 50% occurrence as given in Table 2.

Table 2. Percentage occurrence of the bacteria species from different locations in keffi.

| Locations | No. Sample | % <i>Azotobacter</i> isolates |
|-----------|------------|-------------------------------|
| AL | 2 | 2(100) |
| HC | 2 | 1(50) |
| KH | 2 | 1(50) |
| GRA | 2 | 1(50) |
| OB | 2 | 1(50) |
| BCG | 2 | 1(50) |

| Locations | No. Sample | % <i>Azotobacter</i> isolates |
|-----------|------------|-------------------------------|
| PYANKO | 2 | 2(100) |
| AM | 2 | 1(50) |

AL=Angwan lambu, KH = Kofar hausa, Pyanko, HC = Highcourt, OB = Oldbarrack, AM = Angwan mada, % = sPercentage

3.3. 16SrRNA Sequenced Gene of the *Azotobacter* Strain

16SrRNA gene sequence of the selected *Azotobacter* strain with hyper activity is given in Figure 1. The sequences obtained was compared with 16SrRNA gene sequences of reference type strain available in the RDP Genbank database ([http://11rdp\(me.msu.edu/](http://11rdp(me.msu.edu/)).

GGAACGGGTACACTAGAGTTTGTATCCTGGCTCAGAAAACCTCAAAACGGGGACCCACAAATTT
TTGCCCCGGCCACAAAAAGCCCCAAAGAAGGCCCGTAGTTTTTTTTTTTACAACCCTACCG
AGCCCCGGGGGAACCCCTTGGTTTTTTTTTTTAAACAAATCACCCGAGCGGGGAAAAACACC
TTTGGTTTTTTTTTTTAAACCTTCCCGGGCGGGGAAAACCCCCCTTTTTTTTTTTTTTAAAC
TTCTAGGGGGGGGGAGGAACCCCTTGTTTTTTTTTTTTCATCTCTTCAAGCCCGGGGAAAA
CCCCCATTTGTTTTGGGCTTTTATCGTTTGGGGGGGGGAAGAAAACCAACTTGGCTTTTTTTTT
TTTAAAAACCTCGAGAGGGGGAGCAGACCTTTTTTTTTTTTTTTTTTTCACAAACAATCGGAGGG
GGAAGGCAAAATTTTATCTCCTTTTCTTCCATCAGAGGGGGGGGGGGGGGAAGGCTGAGT
GTTTATAAAAGAACAGACGAAGGCTCAAGAAAAAAGATGGAGTTTTTACTTCATATGCATGT
CAGGCCGGCGCAACAGAAATATAGCTTTTAAGAACGACTAACAAGCTACACGACCGAGAGCG
AGAAGTGCGGACAGCGTCAGGAACAGATGGGGCAGCTGC

Figure 1. Fulllength 16SrRNA gene sequence of *Azotobacter chroococcum*.

3.4. Rate of Nitrogen Fixing Activity of *Azotobacter chroococcum* on Nitrogen-free Agar

Figure 2 shows the rate of nitrogen fixing activity of *Azotobacter* strains were the highest coloured zone was observed in *Azotobacter* species isolated from Angwan lambu (AL) with 16 mm followed by pyanko with 14mm, GRA with 12mm and the least was observed from isolate from Highcourt (HC) with 10mm.

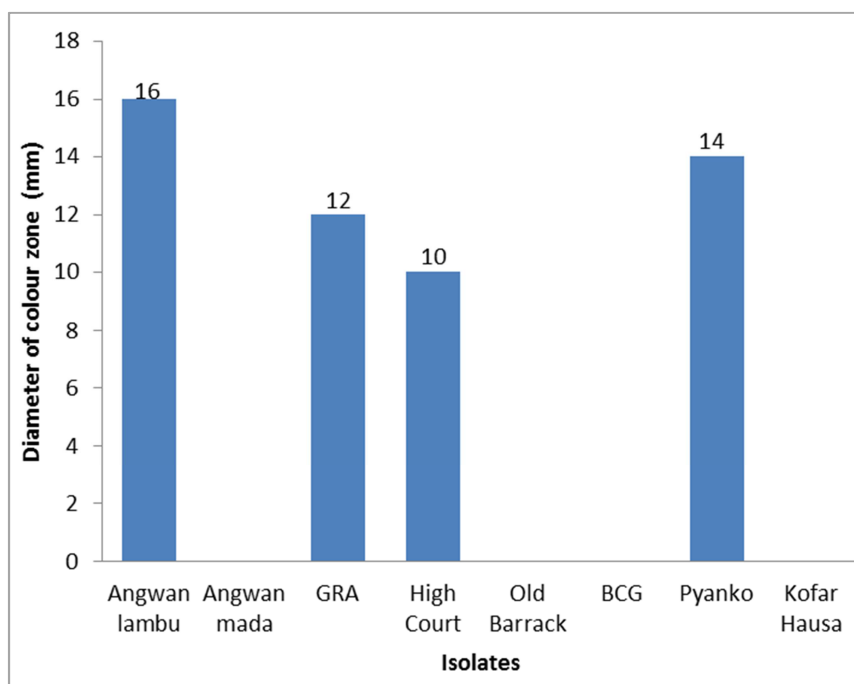


Figure 2. Nitrogen fixing ability of *Azotobacter chroococcum* on Nitrogen-free agar.

3.5. Quantitative Estimation of Percentage Amount of Nitrogen Produced by *Azotobacter* Isolates in the Nitrogen-free Broth

Figure 3 shows the percentage amount of nitrogen capture by *Azotobacter* strain were the highest amount was observed in *Azotobacter chroococcum* isolated from Angwan-lambu (AL) with 5.11 %N, followed by pyanko with 4.20 %N followed by GRA with 3.71 %N and Highcourt (HC) with 3.01 %N respectively while low amount was observed in *Azotobacter* isolates from Angwan mada(AM) with 2.31 %N, followed by Old barrack (OB) with 1.61 %N, followed by BCG with 1.4 %N and the least amount was observed in isolate from Kofar Hausa (KH) with 1.19 %N respectively.

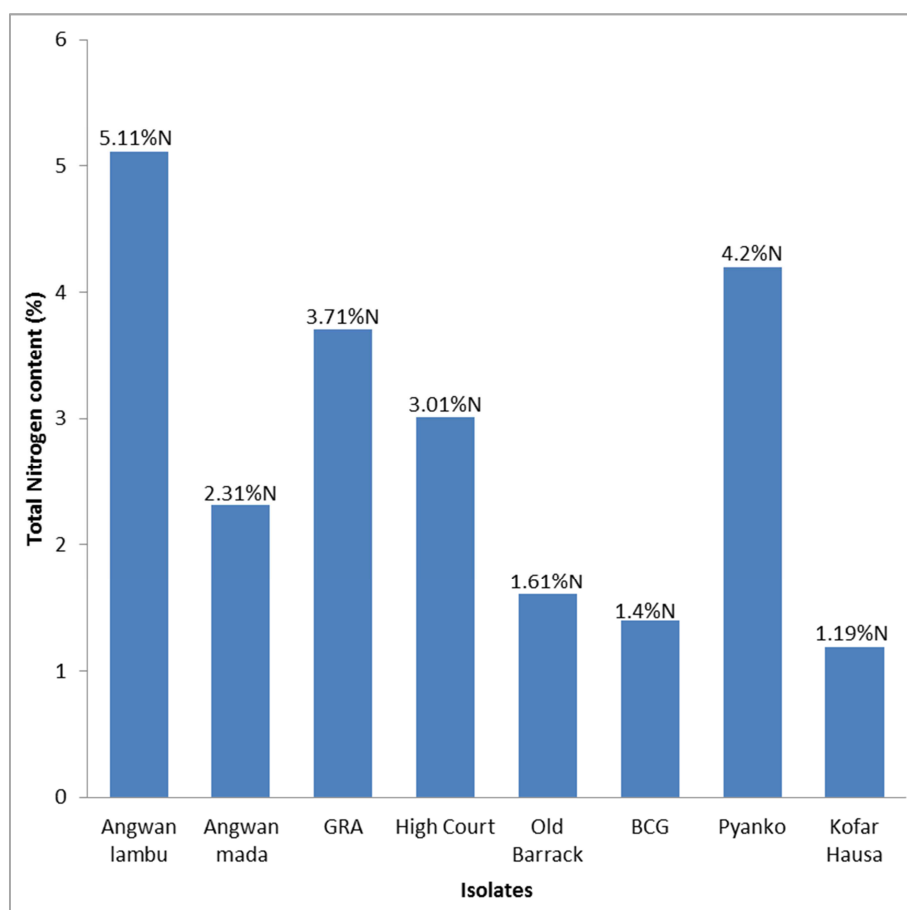


Figure 3. Percentage amount of Nitrogen produced by *Azotobacter chroococcum* in the Nitrogen-free broth.

4. Discussion

Eight *Azotobacter* species were isolated and their cultural features was observed and identified by various biochemical characteristics and 16SrRNA sequencing analysis. Microscopic examination of the strains showed that they are gram negative, short rod and polymorphic in nature. The 16SrRNA sequence analysis of the selected strain showed maximum similarity of 96% with *Azotobacter chroococcum* of the reference type strain. Out of the eight *Azotobacter* isolates tested on nitrogen free agar for their nitrogen fixing activities. The highest nitrogen fixing activity by coloured zone formation was observed in *Azotobacter* species isolated from Angwan lambu (AL) with 16mm followed by pyanko with 14mm, GRA with 12mm, and the least was observed in isolate from High court (HC) with 10mm. Thus, the coloured zone formed by *Azotobacter* strains on nitrogen free agar plate indicates their nitrogen fixing ability and this is due to the secretion of nitrogenase enzyme. Formation of coloured zone by *Azotobacter* strains is due to secretion of nitrogenase enzyme or production of organic acids by the strains [26], [1]. The coloured zone formed by the strains in relation to locations showed a very high significant difference ($\chi^2=55.077$, $df=7$, $P=0.000000001439$).

Similarly, *Azotobacter* species with the highest percentage nitrogen was observed in *Azotobacter* strain isolated from

Angwan-lambu (AL) with 5.11 %N followed by Pyanko with 4.2 %N, GRA with 3.71 %N, High-court (HC) with 3.01 %N, Angwan mada (AM) with 2.31 %N, Oldbarrack (OB) with 1.61 %N, BCG with 1.4 %N and the least was observed in isolate from Kofar Hausa (KH) with 1.19 %N as presented in figure 3 above. Nitrogen fixation occurred due to the production of organic acids or secretion of nitrogenase enzyme by the *Azotobacter* species in the medium and this is the main principal mechanism of nitrogen fixations [9].

These findings agreed with previous studies [8], [27], [4]. That oxygen supply aid fixation of atmospheric nitrogen into a broth medium devoid of nitrogen source but with little amount of carbon source to be effective. High oxygen supply into the culture medium aid the secretion of nitrogenase enzyme that speed up the process of nitrogen fixation on the medium [9]. However, variability in the percentage nitrogen produced by each *Azotobacter* strain on the broth medium could be due to climatic factors such as oxygen supply and the nutrient composition of the broth. Statistically there was no significant difference in the amount of nitrogen produced in the medium $P>0.05$

5. Conclusion

The highest nitrogen fixing activity was observed in *Azotobacter* species isolated from Angwan lambu (AL) with

16mm followed by pyanko with 14mm, GRA with 12mm, and the least was observed in isolate from Highcourt (HC) with 10mm. Similarly, the highest percentage total nitrogen capture by *Azotobacter* strain on culture broth was observed in *Azotobacter* strain isolated from Angwan-lambu (AL) with 5.11 %N, followed by pyanko with 4.20 %N followed by GRA with 3.71 %N and Highcourt (HC) with 3.01%N respectively while low amount was observed in *Azotobacter* isolates from Angwan mada (AM) with 2.31%N, followed by Oldbarrack (OB) with 1.61%N, followed by BCG with 1.4%N and the least amount was observed in isolate from Kofar Hausa (KH) with 1.19%N respectively.

Thus, *Azotobacter chroococcum* isolated from Angwan lambu (AL) is considered a potential candidate for bio-fertilizer production to enhance plant nutrition especially in soil with low nutrients for an improve crop productivity.

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