SUSTAINABLE PRODUCTION OF LIQUID BIOFERTILIZER: UTILIZING SPENT MUSHROOM SUBSTRATE AND WATERMELON PEELS

ABSTRACT

Biofertilizers are natural fertilizers that contains live biomass. This study demonstrates the production of Liquid biofertilizer through the use of spent mushroom substrate (SMS), and watermelon peels by liquid state fermentation technique. This is a result of contamination and lose of soil fertility faced by the environment and agricultural farm land as a result of incessant use of inorganic fertilizers that are persistent in the soil. The two waste materials of 5379g each were obtained and placed on a 35-litre drum, 27 litre of water was poured on the mixture after washing the watermelon peel and stirred evenly, then allowed to ferment for 3 weeks under anaerobic condition, then filtration of the mixture was done after complete fermentation, and microbiological and physicochemical analysis was carried out on the mixture before and after fermentation. Reduction in the concentration of total nitrogen, phosphorous, magnesium and potassium were observed after fermentation while iron content increased after fermentation. The following bacterial species were identified using 16S rRNA gene amplicons: *Pseudomonas aeruginosa*, *Stenotrophomonas, geniculata*, *Sphingobacterium daejeonense* and *Alcaligene faecalis.* A Pot experiment was carried out with a planting bag on three set ups in duplicates, (i) seed + liquid biofertilizer application, (ii) seed + chemical fertilizer application (iii) Seed alone. Then planting of bean seed (*Phasolous vulgaris L.*) and groundnut seed (*Arachis hypogea*) on each set up after physicochemical analysis of the planting soil. Growth comparison using germination test was done by measuring the shoot length, leaf length, leave number, leaf width, stem girth and number of branches, it was observed that the treatment with liquid biofertilizer application competed favourably with that of chemical fertilizer treatment and the biofertilizer also serves as a bio-control agent as the leaves of the biofertilizer set up was not eaten by caterpillar while that of inorganic (chemical fertilizer) was eaten up.

Key words: Liquid Biofertilizer, spent mushroom substrate, watermelon peels, chemical fertilizer, and fermentation.

Introduction

Organic fertilizers that include living biomass or dormant cells of beneficial microbial strains are known as biofertilizers. By populating the rhizosphere and making the nutrient readily accessible and useful to plant root hairs, they may increase nutrient digestion. Bio-fertilizer production is another way of practicing agricultural microbiology as it impacts on crop growth improvement, soil fertility through organic fertilizer, plant disease control through the process of antagonism. Soluble phosphate fertilizers are used for crop growth enhancement and these products or area of research has gained tremendous break through over the years, also the bio-fertilizer production has been commercialized for increasing soil fertility. The use of indigenous microorganism and fungi for this improvements are better in comparison to the introduced strains of microbes. These practices is to impede the harmful use of chemical fertilizers and then reduce the rate of pollution through this

natural approach.
The substrate used to grow mushroom is a combination of various natural materials that is prepared specifically for the cultivation of mushrooms. It is spent because it has already been used in the mushroom house after the harvest year, it is disposed.
Spent mushroom substrate is a waste material gotten after cultivation of oyster mushroom (*Pleurotus* Spp.). SMS refers to the residual substrates that remain after the crop has been harvested and composted. Mustapha et al.'s (2017) research found that SMS (spent mushroom substrate) retains certain residual nutrients that may still be used by mushrooms. However, from an economic standpoint, it is more cost-effective to replace the substrate and start a new crop. SMS is distinguished by its substantial organic matter content, which is beneficial and contains both macro and micro nutrients in total and accessible forms. Optimal pH, advantageous low C/N ratio, and minimal heavy metal concentration. The predominant focus of research on SMS has been on the enzymatic activity of the natural mycelium and its ability to produce lignocellulose enzymes, including laccase, XY lanase, lignin peroxidase, cellulose, and hemicellulose. This has been done with the aim of utilizing SMS as a cost-effective resource for bioremediation, animal feed, and energy feedstock production (Cezary et al., 2021).
Watermelon, scientifically known as *Citrullus lanatrus*, is a fruit of significant economic value. It is considered the second biggest fruit crop in the world, with a worldwide output of over 103 million tons. (Gupta et al., 2023) Approximately 25% of the watermelon peel, which makes up a significant portion of the fruit, is often disposed of or tossed into the environment. This practice poses environmental issues. The transformation of this waste in the environment into valuable chemicals and commodities is restricted owing to a lack of understanding in potential conversion methodologies.
The remnants of spent mushroom substrate (SMS) obtained from culture bags after the harvest of mushroom fruiting bodies are referred to as compost or spent mushroom substrate (SMS) (Martin et al., 2023;). SMS, or spent mushroom substrate, is a readily available biomass residue generated in large quantities after crop harvesting in mushroom farms. Approximately 5 kilograms of secondary metabolite substances (SMS) are generated every kilogram of freshly formed fruiting body. Following the collection of mushrooms, SMS debris is often discarded as refuse, posing a significant difficulty in waste management. In many countries, it is typically composted as a means of disposal (Anjali et al., 2021). In recent years, the mushroom farming business has seen significant growth, resulting in the yearly production of nearly 5 million tons of solid waste in SMS (Mohd et al., 2023).

Objectives of Study

- To isolate and characterize the microorganisms present in spent mushroom substrate (SMS) and watermelon peel before fermentation.
- To isolate and characterize plant growth promoting microorganisms in the liquid biofertilizer.
- To compare and contrast the productivity profiles of the liquid biofertilizer with that of inorganic fertilizer (chemical fertilizer).

Materials and method

Study Area

This study was carried out at Choba, Port Harcourt, Rivers State, Nigeria.

Sample collection

The spent mushroom substrate (*Pleurotus ostreatus*) was gotten from mushroom farm in Agric faculty of the University of Port Harcourt whereas the watermelon peel was gotten from Choba market roadside water melon sellers. Physicochemical analysis of the sample was determined before fermentation process.

Isolation of microorganism

One ml of well agitated mixture of the waste substrate was serially diluted in (10 fold) 9ml of normal saline. For phosphorous solubilizing bacteria, aliquots (0.1ml) of 10^{-3} was spread on pikovskaya media while for Nitrogen fixing bacteria, aliquots (0.1ml) of 10^{-3} was spread on Ashby's mannitol agar and also spreading was done on nutrient agar plate all spreading were in duplicates. The plates were incubated for 24 hours, after district colonies was observed on the plates, the plates were enumerated and then sub cultured into differential media such as MSA, Centrimide agar, mackonkey agar, Ashby's mannitol agar and Pikovskaya media. This was also carried out after the biofertilizer was produced.

Media preparation for nitrogen-fixing bacteria isolation

250ml of Mannitol Ashby agar media was formulated by measuring 5g of Mannitol, 0.05g of Potassium Hydrogen Phosphate (K_2HPO_4), 0.05g of Sodium Chloride (NaCl), 0.05g of Magnesium Sulphate Heptahydrate ($MgSO_4.7H_2O$), 0.025g of Potassium Sulphate (K_2SO_4), 1.25g of Calcium Carbonate ($CaCO_3$) 3.75g Agar-agar then dissolved in 250 ml of Distilled water. After 15 minutes in an autoclave at 121°C, the agar medium was placed onto a petri dish and allowed to harden at room temperature.

Media preparation for phosphate-solubilising bacteria isolation

250ml of Pikovskaya agar media was formulated by measuring 2.5g of Glucose, 1.25g of Calcium Phosphate ($Ca_3(PO_4)$ 0.05g of Sodium Chloride (NaCl), 0.025g of Magnesium Sulphate Heptahydrate ($MgSO_4.7H_2O$), 0.05g of Manganese (II) Sulphate Dihydrate ($MnSO_4.2H_2O$) 0.125g of Ammonium sulphate $(NH_4)_2 SO_4$, 0.125g of Yeast extract, 3.75g of Agar-agar then 250ml of normal saline was poured on the mixture. After 15 minutes in an autoclave at 121°C, the agar medium was placed onto a petri dish and allowed to harden at room temperature.

Identification of Isolates

The pure isolates were identified through their morphological, cultural, biochemical characteristics as stated in a manual of determinative bacteriology according to Bergeys. The biochemical tests that was conducted includes: catalase, oxidase, citrate, indole, TSIA, MR-VP, Sugars (lactose, fructose and sucrose utilization test). Azotobacter sp. were recognized by a clear zone around the colonies on Ashby mannitol agar. Cellular identification of bacterial isolates was carried out using gram staining method.

Gram stain

Gram staining technique was employed to differentiate the bacteria into two distinct types of Gram +ve and Gram –ve organisms through the detection of the peptidoglycan which is a thick, tough and rigid layer in the cell wall of Gram +ve and tiny in Gram –ve bacteria cell wall. An uncontaminated, grease-free glass slide was used to make a smear of the

organism under study. After heat-fixing the slide, it was immersed in crystal violet solution at an angle for one minute, then rinsed under running water to remove the solution. The smear was then exposed to Gram's iodine for another minute, and finally, it was rinsed off with 75% alcohol for 30 seconds. Prior to viewing the glass slide under a light microscope with an oil immersion (x100) focal point, the smear was counter-stained with secondary dye (safranin) for one minute. The stain was thereafter rinsed off with running tap water. Gram +ve bacteria was identified through a purple coloration due to its retention of the primary dye (crystal violet) while Gram –ve bacteria was identified through a pink coloration due to its inability to retain the primary dye thereby taking up the secondary dye (Safranin). According to bergy's manual of determinative bacteriology (2013)

Catalase test

A drop of hydrogen peroxide (H2O2) was added to a 24-hour, pure isolate culture on a clean glass slide. Bubbles signify positive response, whereas no bubbles imply negative reaction. Ulrich et al., (2021)

Oxidase test

This test detects the final enzyme system in aerobic respiration, cytochrome C oxidase or cytochrome as3. For this test, filter paper was soaked in N,N,N-tetramethyl phenyldiamine (TMPD) and allowed to dry for a few seconds. Then, a 24-hour-old pure culture of the test isolate was collected with a wire loop and robbed on the filter paper. A deep/blue purple tint indicated a positive test, whereas no color change indicated a negative test. As stated by Okolie et al., (2019)

Citrate utilization test

This test verifies that bacteria may utilize sodium citrate as their only energy or carbon source. The test isolate cell culture was injected onto bromothymol blue-containing simon citrate agar in a vial with a sterile wire loop and incubated for 48 hours. Positive result was noticed by change in green colour of the citrate agar to blue, while negative result was identified those bottles that doesn't have any colour change. A pH greater than 7.6 causes the citrate agar to become blue, indicating an increase in the pH level. Martin et al., (2023)

TSIA test

The triple sugar iron agar test is a types of biochemical technique which is used to determine the ability of bacteria to ferment lactose, glucose and fructose and release acid and hydrogen sulfide. Culture of the test isolate is inoculated inside a bottle containing the TSI Agar in a slant form under sterile condition, then incubated for 48 hrs after which the bottle is observed for fermentation of these sugars then, the butt, slope, gas production and H_2S production in the media are observed. Glucose fermentation in the medium causes the slant butt area to change to a yellow color. Bacteria that cannot ferment lactose and sucrose are unable to use them as the only source of carbon. Consequently, after the glucose is depleted, the peptone in the slant will undergo oxidative metabolism, causing the pH of the medium to rise to an alkaline level and resulting in a color change from yellow to red. However, if the bacteria have the ability to ferment lactose and sucrose, they will break down these sugars and produce acid. This acid will lower the pH of the medium, causing the indicator to become yellow in both the butt and slant regions. In

contrast, if the bacteria do not undergo fermentation of any of these sugars, the medium will stay red (indicated as Red/Red or K/K). Additionally, when the bacteria create H2S, they metabolize the sodium thiosulfate present in the culture media, resulting in its depletion and the production of H2S gas. Berg. et al., (2013)

MR-VP Test

This test (methyl red Voges Poskaur test) is divided into two major parts; MR and VP. The MR test which is used to determine organisms that can produce stable acid end products from fermenting glucose methyl red indicator while the VP test is a technique used to detect organisms to specie level of gram –ve rods, *Aeromonas* and *Vibrio*, *Virdans* group *Streptococci*, and *Staphylococci*. The test was carried out using a universal bottle of MR-VP broth, and using a sterile wire loop, the test isolate from a 24 hrs old pure culture was inoculated in to the broth then incubated for 48 hours, after which the bottle was agitated rigorously and half of the broth was transferred into another sterile universal bottle.

For MR test, two drops of pH indicator methyl red were added to the bottle and swirled then the bottle was observed for an hour or two, positive results is identified by change in colouration to red, but if no change in colour, then the test is negative.

For VP test, Barritt's reagent (5% alpha-napthol solution) and Barritt's reagent (40% potassium hydroxide solution) is added to the other bottle and vigorously agitated. Positive result is identified by change in colouration to pink or red while no colour change indicates negative result.

Biofertilizer Production

The watermelon peel was washed and cut into smaller fragments then weighed using the weighing machine and also, the spent mushroom substrate was weighed and scattered into the drum, both waste materials had equal weight of 5379.00g and 27 litre of distilled water was poured on the mixture and stirred evenly then covered airtight to ensure anaerobic condition, the essence of the high quantity of water was to allow free movement of microorganisms and for fermentation to take place faster (Hallsworth, 2023). The substrate was left for fermentation to take place and on weekly intervals, the mixture was checked to observe if absolute fermentation has taken place. After 3 weeks fermentation period, the mixture was sieved using a sterile sieve into 20 litres gallon and kept under room temperature.

Molecular Identification

DNA Extraction

The ZR fungal/bacterial DNA miniprep kit (manufactured by Zymo Research) was used to extract DNA.

DNA Quantification

Nanodrop spectrophotometry was used to determine the concentration of the DNA and

access its purity. Agarose Gel Electrophoresis for DNA and PCR was carried out.

DNA Amplification

Sequencing and Sequence Analysis

The fragments were sequenced using the Nimagen, Brilliant Dye™ Terminator Cycle Sequencing Kit V3.1, BRD 3-100/1000. The cycles that was conducted were denaturation temperature and times, Annealing temperature and time and Elongation temperature and time. The used Bacterial Primer sequences that were used includes:27F with the sequence 5' AGAGTTTGATCMTGGCTCAG '3 and 907R with the sequence 5' CCGTCAATTCMTTTRAGTTT '3.

Evolutionary Relationships of Taxa

The evolutionary history was deduced using the Neighbor-Joining approach (Saitou et al. 1987). The tree that provides the best or most favorable outcome is shown. The bootstrap test (with 1000 repetitions) reveals the proportion of duplicate trees where the related taxa grouped together, as shown by the branches (Felsenstein 1985). The tree is accurately shown, with branch lengths measured in the same units as the evolutionary distances used to represent the phylogenetic tree. The Jukes-Cantor technique (Jukes et al., 1969) was used to record the evolutionary distances, which are measured in terms of the number of base substitutions per site. There were 10 nucleotide sequences included in this investigation. The included codon positions are the first, second, third, and noncoding locations. All instances of unclear places were eliminated for each pair of sequences using the paired deletion option. A total of 521 places were documented in the final dataset. The evolutionary studies were performed using MEGA11 software (Tamura et al., 2021).

Physicochemical Analysis

Physicochemical analysis of the mixed substrate before fermentation and after fermentation with that of the planting soil was conducted in a pharmaceutical chemistry laboratory, located in University of Port Harcourt where analysis of the nitrogen content, phosphorous, magnesium, iron content, potassium, pH value and temperature value was ascertained.

Magnesium Determination

Three conical flasks were each filled with 25 ml of standard zinc solution. Then, 20 ml of pH 10 buffer was added to each flask, followed by 15 ml of distilled water. The mixture was swirled, and enough eriochrome black T indicator was added to provide a light red-wine color. The solution was titrated using an EDTA solution until the color changed from wine-red to a clear blue. The outcome was used to determine the molar concentration of the EDTA solution that would be employed in the titration of your unidentified magnesium solution. Next, the unidentified (sample) is acquired and put into a volumetric flask with a capacity of 100 ml, where it is then diluted to the desired level. Subsequently, 10 ml of the created unidentified solution is transferred to three conical flasks. Each flask was supplemented with 15ml of pH 10 buffer and 20ml of distilled

water. A little amount of eriochrome black T indicator was applied until a faint wine-red color was achieved. The standard EDTA solution was used to titrate each solution until it reached a distinct blue color.

Phosphorous Determination

A volume of 7 milliliters of bray solution was introduced to a 1 milliliter sample in a centrifuge tube. The tube holding the bray solution serves as the blank. Subsequently, the tube was forcefully shaken for a duration of 1 minute and then placed in the centrifuge, where it was spun at a speed of 6000 revolutions per minute for a period of 5 minutes. Next, 0.5 ml of the liquid remaining after centrifugation, known as the supernatant, was combined with 2.0 ml of reagent C in a tube used for measuring color, called a colorimeter tube. The mixture was well mixed and left undisturbed for a duration of 30 minutes. A reference standard was created using a solution containing 2.5 mg/L of phosphorus. The spectrophotometer was calibrated to zero using the blank solution. The standards and sample were assessed for absorbance at a wavelength of 882 nm. Then a graph of standard data of phosphorous concentration against absorbance. The graph was used to ascertain the phosphorous concentration in the sample.

Iron content Determination

Ten, twenty, thirty, and Fifty mls of a standard solution were pipetted into conical flasks with a capacity of 100 ml. Subsequently, 1 ml of hydroxylamine solution and 1 ml of sodium acetate solution were added to each flask. Each flask was diluted to a volume of 75 ml using distilled water. Then, 10 ml of phenanthroline solution was added to each flask, followed by the addition of distilled water to bring the total volume to 100 ml. The mixture was then left undisturbed for a duration of 10 minutes. An additional 50ml of distilled water was introduced into a separate conical flask, and steps 2 and 4 were performed as previously stated (this refers to the blank). Subsequently, the spectrophotometer was used to measure the absorbance of each solution at a wavelength of 508 nm, relative to the blank. A calibration curve was generated by plotting the absorbance on the x-axis and the concentration of a standard iron solution on the y-axis. To ensure a virtual comparison, maintain the solution as outlined in steps 1-4. Subsequently, the sample was vigorously blended and 5 ml of the resulting mixture was precisely transferred into a conical flask. Then, 1 ml of HCL solution with a concentration of 1 ml and 0.5 ml of hydroxylamine solution were introduced. Additionally, glass beads were included. The combination was then heated to the point of boiling until the volume was decreased to 5 ml, ensuring complete dissolution. Subsequently, the flask was allowed to cool to the ambient temperature and then carefully moved into a 100ml nessler tube. First, 2 ml of ammonium acetate buffer solution and 1 ml of phenolphthalein solution were diluted with distilled water to the 50 ml mark. The mixture was then carefully mixed and left undisturbed for 10-15 minutes to get the highest level of color intensity. The solution's absorbance was measured using a spectrophotometer. The iron concentration (in mgFe) was determined using the calibration graph. Next, in order to do a virtual comparison, the

color of the sample was aligned with the color of the standard that was created in steps 1-4. Please be aware that the colorimetric standard will provide the iron content in the samples, measured in micrograms per iron (µg/Fe).

pH Determination

The electrode was immersed in a buffer solution with a predetermined pH, after which the power supply was activated and a measurement was recorded. The instrument underwent standardization. The electrode was immersed in a buffer solution with a pH of 7, after which the pH of the samples was measured and documented.

Nitrogen Content Determination

Two ml of sample was transfered into 1lit. round bottom flask.

To avoid foaming, 1 milliliter of liquid paraffin and 2 or 3 glass beads were added to the mixture to create bumping. To the flask, 100 milliliters of freshly made potassium permanganate and 100 milliliters of sodium hydroxide solutions were added. After distillation, the resulting liquid was transferred to a beaker that already contained 20 milliliters of boric acid working solution.

After collecting around 150 ml of distillate, it was titrated with standard H_2SO_4 0.02N until the color changed from green to red. The burette reading was recorded at that point.

Collection of Planting Soil Sample

The experimental soil sample was gotten from the university of Port Harcourt Delta campus on a randomized sampling method with the use of trowel and was filtered with a large basket to remove debris such as stones, pieces of plastics etc. then the soil was taken to the laboratory for physicochemical analysis.

Pot Experiment set up

The experimental soil was transferred into garden planting polythene bags of 24.1 length, and 22.2 width, each contained about 2723.25g of soil, then seedlings of beans and groundnut were rinsed with a distilled water and planted on the soil in duplicate set ups. For the biofertilizer application soil, the soil was divided into two and packed in duplicate bags also. The beans, groundnut and experimental soil sample were all identified in the department of crop and soil science, faculty of agriculture, university of Port Harcourt. The seeds of beans and groundnut for soil and foliar application were soaked in 5ml of the liquid biofertilizer and chemical fertilizer according to Shikha et al (2023). For each crop, three set ups were considered in duplicates which includes: liquid biofertilizer + soil + seed, chemical fertilizer + soil + seed (positive control) and soil + seed only which is serves as the negative control. The seeds were distributed in each set up randomly after been subjected to viability treatment.

Seed Viability Test

The both seed of groundnut (*Araichis hypogea*) and beans (*Phaseolus vulgaris*) were subjected to viability test by spreading up to 30 pieces each of the test crop on a tray slightly covered with soil and covered with a polybag to create humidity (Ibiene et al 2012). After 4-5 days of planting, over 90% of the seeds germinated which is indicative

that the seeds are viable.

Application of Chemical Fertilizer
The chemical fertilizer were applied according to manufacturer's instruction, the seeds of beans and groundnut were firstly rinsed using distilled water and sundried, after which soaked in 1.5ml of the chemical fertilizer for 30 minutes before planting on PE5 and PE6 accordingly, foliar and soil application started 10 days after germination of crops. The application continued within the interval of 7 days according to Okoli et al (2019).

Application of Liquid Biofertilizer
The seeds of beans and groundnut for this application were also firstly rinsed using distilled water and sundried after which they were soaked in 5ml of liquid biofertilizer and 5ml of the liquid biofertilizer was sprayed on the sterilized soil to enhance the microbial activities of the soil. Since our biofertilizer contains live organisms that are capable of bringing about aeration and accessibility to some essential nutrients due to their presence in the soil. The soil application and foliar applications commenced after 10 days of seed germination and continued at an interval of 2 days. This application was done and monitored for 5 weeks.

Growth Parameters measurement
The growth parameters for germination test analysis were measured using a tape in centimeter according to Criollo et al (2011) which included the plant height (cm), the number of leaves, leave length (cm) and the stem girth.

RESULTS and DISCUSSION

Table 1: Biochemical test results for isolation before fermentation (BF)

Sample code	Morphology & colour	Shape	Gram stain	Catalase	oxidase	Citrate	lactose	glucose	Sucrose	Slope	butt	gas	H_2S	MR	VP	Tentative identity
MP1. CENTRI.	pink	rod	-	+	+	+	-	+	-	R	R	-	-	+	-	*Pseudomonas* sp.
SSA.	pink	rod	-	-	-	+	-	+	-	R	R	-	-	-	-	*Bacillus* sp.
MSA.	purple	+	+	+	-	+		+	-	R	R	-	-	+	-	*Staphylococcus* sp.
EMB (2).	pink	rod	-	+	-	+	-	+	+	R	R	-	-	+	-	*Pseudomonas* sp.
EMB(3).	pink	rod	-	+	-	+	-	+	-	R	R	-	-	+	+	*Pseudomona* sp.

EMB1.	Pink	rod	-	-	-	+	+	+	-	R	R	-	-	+	+	*Pseudomonas* sp.
NA1.	pink	rod	-	+	-	+	-	-	+	R	R	-	-	+	-	*Pseudomonas* sp
NA2.	pink	rod	-	-	+	+	-	-	-	R	R	-	-	-	+	*Pseudomonas* sp.
NA 3.	pink	rod	+	+	-	+	+	-	-	R	R	-	-	-	-	*Baccillus* sp
NA 4.	Pink	rod	-	+	-	+	+	-	-	R	R	-	-	+	-	*Pseudomonas* sp.

Table 2 Biochemical characterization of isolates after fermentation (AF)

Sample code	Morphology & colour	Shape	Gram stain	Catalase	oxidase	Citrate	lactose	glucose	Sucrose	Slope	butt	gas	H_2S	MR	VP	Tentative identity
PIV1.	pink	rod	-	+	-	+	+	+	+	R	R	-	-	+	-	*Azotobacter* sp.
PIV2	pink	rod	-	+	+	+	+	+	+	R	R	-	-	-	+	*Pseudomonas* sp.
PIV3.	pink	rod	-	+	+	+	-	+	-	R	R	-	-	-	+	*Clostridium* sp.
AM1	pink	rod	-	+	+	+	-	-	-	R	R	-	-	+	+	*Clostridium* sp.
AM2	pink	rod	-	+	+	+	-	+	-	R	R	-	-	+	+	*Azotobacter* sp.

Bacterial isolation

maintained during the fermentation period. The nitrogen fixing species and *Azotobacter* species were recognized with a clear zone around the colonies on the growth medium.

MOLECULAR RESULTS