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SOIL-BASED ISOLATION AND MASS CULTURE OF AZOTOBACTER: A STEP TOWARD SUSTAINABLE AGRICULTURE

Khushbu Sengar[1], Vaidehi Desai[2], Dishaben Dave[3]

[1] Department of Biotechnology, Faculty of Science, Monark University, Vahelal, Ahmedabad, Gujarat, India.
[2] Department of Microbiology, Faculty of Science, Monark University, Vahelal, Ahmedabad, Gujarat, India.
[3] Department of Microbiology, Faculty of Science, Monark University, Vahelal, Ahmedabad, Gujarat, India.

ABSTRACT

This study focuses on the isolation, identification, and mass cultivation of Azotobacter spp., a free-living nitrogen-fixing bacterium widely recognized for its biofertilizer potential. Soil samples were aseptically collected from the Vastral region of Ahmedabad and subjected to serial dilution followed by primary isolation using Nutrient Agar. Distinct bacterial colonies were purified and characterized through Gram staining and selective media growth on Ashby's and Mannitol agar, both of which are nitrogen-free. Biochemical tests, including catalase activity, ammonia production, nitrate reduction, starch hydrolysis, and indole production, were conducted to confirm the identity of Azotobacter species. Positive results across these tests supported the classification of the isolates as Azotobacter. The confirmed strains were then cultivated in nitrogen-free liquid media under controlled conditions for mass production. The study demonstrates a systematic approach to isolating and confirming Azotobacter spp. and highlights its potential application in sustainable agriculture as an eco-friendly alternative to chemical fertilizers.

KEYWORDS: Azotobacter, Biofertilizer, Sustainable Agriculture, PGPR, Nitrogen Fixing Bacteria

ABBREVIATIONS: PGPR- Plant Growth-Promoting Rhizobacteria, NA- Nutrient Agar, CFU- Colony Forming Unit

1. INTRODUCTION

The term *biofertilizer* is often broadly used to refer to a range of natural substances, including organic manures and plant-based extracts. However, in a more precise context, biofertilizers are defined as preparations containing living microorganisms that, when applied to seeds, plant surfaces, or soil, colonize the rhizosphere and enhance the availability or uptake of essential nutrients, thereby promoting plant growth. Biofertilizers serve as cost-effective and renewable sources of plant nutrients, offering a sustainable alternative that complements the use of chemical fertilizers. (Itelima, 2018).

A wide variety of soil bacteria inhabit the rhizosphere and are known as plant growth-promoting rhizobacteria (PGPR). Among their many beneficial roles, some PGPR function specifically as biofertilizers by improving nutrient availability(Sumbul et al., 2020). The most common microbial groups used as biofertilizers include nitrogen-fixing bacteria, phosphate-solubilizing microorganisms, and mycorrhizal fungi, all of which contribute significantly to sustainable agriculture by enhancing plant nutrient uptake and reducing the dependency on chemical fertilizers.(Bhattacharjee & Dey, 2014)

In addition to their ability to fix atmospheric nitrogen, *Azotobacter* species also produce plant growth-promoting substances, including phytohormones such as auxins, gibberellins, and *cytokinins*, which contribute to enhanced plant development and productivity.(Das, 2019). Strains of Azotobacter have demonstrated significant positive impacts on plant growth, crop yield, and nitrogen requirements in various economically important cereal and pulse crops, with reported yield increases of up to 40%.(Aasfar et al., 2021). Azotobacter: belongs to family Azotobacteriaceae, aerobic, free living, and heterotrophic in nature(Upadhyay et al., 2015). Azotobacters are present in neutral or alkaline soils and A. chroococcum is the most commonly occurring species in arable soils. A. vinelandii, A. beijerinckii, A. insignis and A. macrocytogenes are other reported species(Rajvir & Sudhir Kumar, 2013). To enhance their effectiveness and shelf life, biofertilizers are commonly formulated with suitable carrier materials that support the survival and activity of the microbial inoculants. (Itelima, 2018).

2. MATERIAL AND METHOD

2.1 Collection of Soil Sample

Soil was aseptically collected from the Vastral region of Ahmedabad approximately 1kg. The sample was transferred into a sterile plastic bag and promptly transported to the laboratory for the purpose of bacterial isolation.

2.2 Serial Dilution of Soil Samples

A 1-gram portion of the collected soil was taken and suspended in 10 ml of sterile distilled water in a test tube to serve as the stock solution. Subsequently, nine additional sterile test tubes were each filled with 9 ml of sterile distilled water. From the initial stock solution, 1 ml was aseptically transferred into the first dilution tube to obtain a 10^{-1} dilution. This serial dilution procedure was continued sequentially up to a final dilution of 10^{-9}. All steps were performed under aseptic conditions within

a laminar airflow cabinet to prevent contamination and ensure sterility.

2.3 Primary Isolation of Bacteria

For bacterial isolation, 100 ml of Nutrient Agar (NA) medium was prepared and poured into four sterile Petri dishes under laminar airflow conditions. Once solidified, 100 µl aliquots from the 10^{-2}, 10^{-4}, 10^{-6}, and 10^{-8} dilutions were spread uniformly onto the surface of the NA plates using a sterile glass spreader. Plates were inverted and incubated at 37°C for 24 hours.

Following incubation, the plates were examined for bacterial growth. Morphologically distinct and well-isolated colonies were selected for further purification. Individual colonies were aseptically picked using a sterile inoculating needle and streaked onto six separate nutrient agar slants to obtain pure cultures. Each slant was labeled based on its source colony, and the tubes were incubated overnight at 37°C.

Post incubation, the growth of uniform and morphologically consistent bacterial colonies on the slants confirmed successful isolation of pure bacterial cultures for subsequent biochemical and morphological characterization.

2.4 Gram Staining of Azotobacter

Gram staining was performed at various stages of the study to assist in the identification of *Azotobacter* colonies. All procedures were conducted under sterile conditions using a Laminar Air Flow cabinet to prevent contamination.

To begin, pure culture plates were placed inside the laminar hood, and the target colonies were marked on the bottom of the Petri dishes using a permanent marker. Clean microscope slides were prepared by washing them thoroughly with soap and water, followed by rinsing with alcohol to eliminate any residual grease or contaminants. After air drying, a drop of sterile distilled water was placed at the center of each slide.

A sterile inoculating loop was then used to collect a small portion of the selected colony, which was gently mixed into the water drop to form a thin, even smear. The prepared smear was heat-fixed by carefully passing the slide over a flame two to three times until the sample dried completely and adhered to the glass surface.

Following this, the Gram staining process was carried out. The slides were removed from the laminar cabinet, and the smear was first flooded with Crystal Violet stain for one minute. After staining, the slides were gently rinsed under running tap water. This was followed by the application of Gram's iodine, which was also left on the smear for one minute before being rinsed again. To decolorize the smear, 95% ethanol was applied for a few seconds until the runoff became clear, and the slides were immediately washed with distilled water.

Next, Safranin was used as the counterstain and allowed to act for one minute, after which a final rinse with distilled water was performed. The slides were then left to air dry.

Once completely dried, the stained smears were observed under a compound microscope—initially at 40X magnification and subsequently under 100X oil immersion. The Gram reaction and morphological characteristics of the bacteria were noted for the identification of Azotobacter species.

2.5 Growth of Azotobacter sp. on Selective Media

After confirming bacterial morphology through Gram staining, the presence of Azotobacter species was further validated by culturing the isolates on selective nitrogen-free media—specifically Ashby's medium and Mannitol Agar. The ability of *Azotobacter* to grow on these nitrogen-deficient media serves as a distinctive characteristic due to its free-living nitrogen-fixing capacity.

2.5.1 Preparation of Selective Media

The media were prepared by accurately weighing and dissolving all components in 100 ml of distilled water, following standard formulations:

Ashby's Medium Composition:

- Mannitol – 1.5 g
- Magnesium sulfate ($MgSO_4$) – 0.02 g
- Dipotassium hydrogen phosphate (K_2HPO_4) – 0.02 g
- Calcium chloride ($CaCl_2$) – 0.02 g
- Ferric chloride ($FeCl_3$) – 0.005 g
- One drop of 10% molybdenum trioxide (MoO_3) solution
- Agar-agar – 1.8 g

Mannitol Agar Composition:

- Mannitol – 1.5 g
- Magnesium sulfate ($MgSO_4$) – 0.02 g
- Dipotassium hydrogen phosphate (K_2HPO_4) – 0.05 g
- Calcium sulfate ($CaSO_4$) – 0.01 g
- Calcium carbonate ($CaCO_3$) – 0.5 g
- Sodium chloride (NaCl) – 0.02 g
- Agar-agar – 1.8 g

The prepared media were sterilized by autoclaving at 121°C for 15 minutes. Once sterilized, the media were poured into sterile Petri plates and allowed to solidify under aseptic conditions inside a Laminar Air Flow chamber.

2.5.2 Inoculation and Incubation

Using a sterile inoculating loop, a small amount of bacterial culture from the previously isolated pure *Azotobacter* slants was streaked onto the surface of both selective media plates. The inoculated plates were then incubated at 28°C for a period of three days.

2.6 Confirmation of Azotobacter Through Biochemical Characterization

To further confirm the identity of the *Azotobacter* isolates, a series of biochemical tests were conducted. These assays targeted characteristic enzymatic and metabolic activities commonly associated with *Azotobacter* species, aiding in their differentiation from other soil-dwelling bacteria. The tests were selected based on standard bacteriological methods outlined in (*Experiments In Microbiology, Plant Pathology And Biotechnology - K. R. Aneja - Google Books, n.d.*)

1. Catalase Activity
The catalase test was performed to determine the isolate's ability to break down hydrogen peroxide into water and oxygen—a typical trait of aerobic bacteria. A drop of 3% hydrogen peroxide was placed on a smear of the culture on a clean glass slide. Immediate effervescence, seen as the formation of bubbles, confirmed the presence of the catalase enzyme.

2. Ammonia Production
To evaluate ammonia production, the isolate was inoculated into peptone water and incubated at 37°C for 3 to 5 days. After the incubation period, a few drops of Nessler's reagent were added. A positive result was indicated by the development of a yellow to brown coloration, suggesting the release of ammonia due to protein degradation.

3. Nitrate Reduction Test
This test was used to assess the ability of the bacterium to reduce nitrate to nitrite or other nitrogenous compounds. The culture was inoculated into nitrate broth and incubated at 37°C for 24 to 48 hours. Following incubation, sulfanilic acid and α-naphthylamine were added. A red color indicated a positive nitrate reduction result. If no color appeared, zinc powder was introduced to detect residual nitrate. No color change even after zinc addition confirmed complete nitrate reduction.

4. Starch Hydrolysis
Starch hydrolysis was assessed by streaking the isolate on starch agar plates, followed by incubation at 37°C for 24 to 48 hours. After incubation, the plates were flooded with iodine solution. A clear halo around the bacterial colonies indicated positive starch hydrolysis, confirming the presence of amylase activity.

5. Indole Production
For the indole test, the culture was inoculated into tryptone broth and incubated at 37 ± 1°C for 48 hours. After incubation, 4–5 drops of Kovac's reagent were carefully added along the inner wall of the test tube. The development of a distinct red-colored ring at the liquid interface indicated a positive result, confirming the organism's ability to break down tryptophan into indole.(*Bergey's Manual of Determinative Bacteriology - Google Books, n.d.*)

2.7 Cultivation and Mass Production of Azotobacter spp.
2.7.1 Starter Culture Development
Pure cultures of *Azotobacter* were initially isolated and maintained on slants. Selected colonies were aseptically transferred into liquid production media to initiate growth. The liquid medium served both as a nutrient-rich environment for bacterial proliferation and as a starter culture for subsequent large-scale production.

For nitrogen-fixing *Azotobacter*, nitrogen-free Ashby's medium was employed, following established biofertilizer production protocols. Two sterile 100 ml conical flasks were filled with the prepared medium, pH-adjusted and autoclaved to ensure complete sterility.

Under aseptic conditions in a laminar airflow chamber, loopfuls of *Azotobacter* culture were transferred into the sterile broth using a flame-sterilized inoculation loop. The inoculated flasks were incubated in a B.O.D. rotary shaker at 28–30°C for 7 days to support optimal bacterial growth and multiplication.

After the incubation period, the broth culture typically reached a population density of approximately 10^9 CFU/ml, forming a dense starter inoculum suitable for scale-up operations in biofertilizer production.

2.7.2 Large-Scale Cultivation
For large-scale cultivation, 1000 ml conical flasks containing Ashby's nitrogen-free broth were inoculated with a calculated volume of the starter culture. The inoculation was carried out under strict aseptic conditions to avoid contamination.

The flasks were then incubated at 28–30°C for 7 days under constant agitation to ensure adequate oxygenation and homogenous distribution of microbial cells. Periodic monitoring was conducted to assess bacterial growth and ensure sterility.

Upon completion of incubation, the culture typically attained a density of ~10^9 CFU/ml. The flasks were then transferred to a cooler environment to decelerate metabolic activity and preserve the culture until further processing.

2.8 Preparation and Selection of Carrier Material
The efficacy of a microbial inoculant significantly depends on the quality of the carrier material used. An ideal carrier should have the following attributes:

1. High organic matter content
2. Water holding capacity >50%
3. Non-toxic to microbes
4. Good friability and uniform particle size for ease of processing

In this study, a composite carrier blend was formulated using lignite, cow dung compost, and vermicompost. Initially, lignite was pulverized manually using a mortar and pestle and then finely ground using a mechanical grinder to achieve uniform texture. To this, 1% calcium carbonate ($CaCO_3$) and activated charcoal were added to maintain pH stability and minimize microbial contamination.

Dried cow dung was similarly ground into a fine powder, and vermicompost was prepared for blending. These three components—lignite, cow dung, and vermicompost were mixed in a 1:2:1 ratio, respectively. The composite carrier mixture was then transferred to sterilizable glass containers and autoclaved for complete sterilization before inoculation.(Pal et al., 2017)

2.9 Inoculation of Carrier Material with Azotobacter Culture
The refrigerated Azotobacter culture was allowed to reach room temperature before use. Simultaneously, the sterilized carrier material was cooled under sterile conditions within a Laminar Air Flow cabinet.

Once at ambient temperature, the carrier material was moistened with 10 mL of sterile distilled water. The prepared broth culture was then added to the carrier to reach a moisture level of over 60%, and the mixture was thoroughly blended to ensure even distribution of microbial cells. A thin film of liquid was observed on the surface, aiding in rapid bacterial proliferation.

The inoculated carrier was left overnight at room temperature to allow microbial establishment. The next day, approximately 200 g of sterile soil was incorporated to lightly dampen the mixture. The resultant biofertilizer was then cured under shaded conditions for 7 days.

An alternate approach involved directly mixing the inoculum into the sterilized carrier until 60% moisture was achieved, followed by packing into sterilized, breathable packets. These packets were also cured under shade for 7 days to stabilize the final biofertilizer formulation prior to field use.

3. RESULT

3.1 Primary Isolation of Bacteria

The data from Table 1. Provide insights into the morphological characteristics of bacterial colonies observed at various dilution levels. The primary isolation reveals diverse bacterial morphologies, particularly in form, margin, color, and size. This diversity indicates the presence of multiple bacterial species or strains in the original sample.

Dilution	No of colonies	Form	Elevation	Margin	Colour	Transparency	Size
10^{-2}	15	Circular	Flat	Curly	Milky white	Translucent	Moderate
10^{-4}	7	Irregular	Flat	Entire	White	Translucent	Small
10^{-6}	5	Circular	Flat	Entire	Cream	Translucent	Medium
10^{-8}	2	Circular	Flat	Curly	Clear white	Translucent	Small

Table 1: Result of Primary Isolation of Bacteria

3.2 Gram Staining

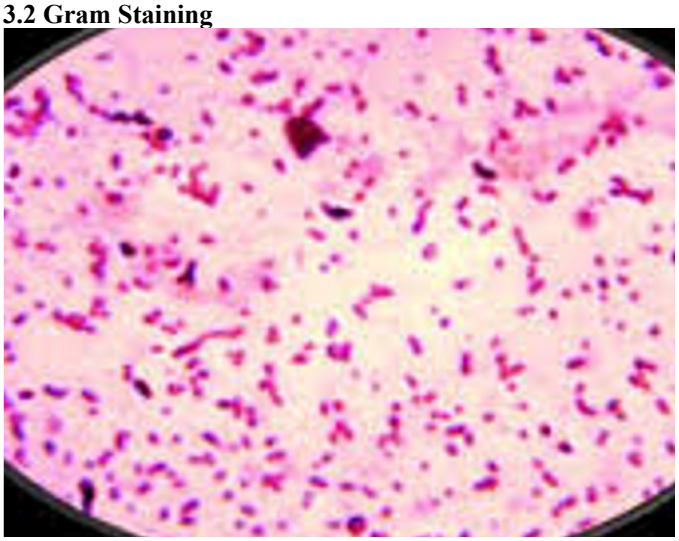

Figure 1: Microscopic view of Azotobacter after gram staining

Figure 1. shows the microscopic examination of *Azotobacter* appeared as Gram-negative, large, fluffy rod-shaped cells.

3.3 Biochemical Characterization of *Azotobacter* spp.

The isolated bacterial strains were subjected to a series of biochemical tests to confirm their identity as Azotobacter species. The results of these tests are given in Table 2. and supported the classification based on standard references such as **Bergey's Manual of Determinative Bacteriology**.

1. Catalase Activity

The isolates demonstrated strong catalase activity, as evident from the immediate effervescence upon the addition of hydrogen peroxide. This confirms the presence of the catalase enzyme, which protects aerobic bacteria from oxidative damage by degrading hydrogen peroxide into water and oxygen.

2. Ammonia Production

The production of ammonia was confirmed by the development of a yellow to brown coloration upon the addition of Nessler's reagent. This trait is significant, as ammonia production contributes to nitrogen enrichment in the soil, enhancing plant growth.

3. Nitrate Reduction

A red coloration developed after the addition of sulfanilic acid and α-naphthylamine, indicating the reduction of nitrate to nitrite. In some cases where no color change was observed initially, the addition of zinc did not result in a red hue, suggesting complete nitrate reduction to gaseous forms. This confirms the denitrifying potential of the isolates.

4. Starch Hydrolysis

Clear halo zones around the colonies on starch agar plates after iodine application indicated positive starch hydrolysis, confirming the presence of amylase activity. This enzymatic function aids in the breakdown of complex carbohydrates and supports the nutritional versatility of *Azotobacter*.

5. Indole Test

A red ring developed at the interface of the culture broth and Kovac's reagent, confirming the ability of the isolates to produce indole from tryptophan. This is consistent with previous reports describing indole production by *Azotobacter*, which may play a role in plant growth promotion.

Sr. no	Biochemical test	Results
1.	Catalase activity	+
2.	Ammonia production	+
3.	Nitrate reduction test	+
4.	Starch hydrolysis	+
5.	Indole test	+

Table 2: Result of Biochemical test

These results collectively support the successful isolation and identification of *Azotobacter* spp. with characteristic biochemical traits aligning with documented standards for this genus.

3.4 Cultivation and Mass Production of Azotobacter

The cultivation process was initiated using Ashby's nitrogen-

free medium, which selectively supports the growth of nitrogen-fixing Azotobacter species. After 7 days of incubation in a rotary shaker at 28–30°C, the bacterial density reached approximately 10^9 CFU/ml, indicating robust proliferation.

The scale-up phase using 1000 ml flasks showed consistent growth, and no contamination was observed throughout the incubation period, demonstrating the effectiveness of aseptic handling and sterilization protocols. The achieved population density validated the suitability of Ashby's medium and the incubation conditions for mass multiplication.

3.5 Carrier Material and Inoculant Formulation

The composite carrier material (1:2:1 ratio of lignite, cow dung, and vermicompost) demonstrated excellent physical properties, including high organic matter content and water holding capacity, essential for microbial viability. The inclusion of 1% $CaCO_3$ and activated charcoal helped maintain pH stability and reduced the risk of microbial competition or contamination.

Upon inoculation with the broth culture, the carrier material reached the targeted >60% moisture content, and thorough mixing ensured uniform microbial distribution. After incubation and curing, the biofertilizer retained good texture, odor, and visible microbial colonization without contamination.

The addition of sterile soil during curing further mimicked natural conditions, allowing microbial adaptation before field application. The alternate method—direct packaging into breathable, sterilized bags also preserved microbial viability, confirming both techniques as viable for practical application.

4. DISCUSSION

The successful isolation, identification, and multiplication of Azotobacter spp. underscore its potential as an effective biofertilizer for sustainable agriculture. The organism's ability to fix atmospheric nitrogen, produce growth-promoting substances, and survive in nitrogen-deficient media makes it an ideal candidate for enhancing soil fertility without chemical inputs.

The biochemical traits confirmed the metabolic versatility and stress tolerance of the strains, while the mass cultivation and carrier formulation methods demonstrated practical feasibility for commercial-scale production. The use of organic carrier materials further enhances the ecological value of the inoculant.This study aligns with existing literature on Azotobacter applications in agriculture and provides a replicable methodology for biofertilizer production with a focus on quality, safety, and microbial efficiency.

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