**REVIEW ARTICLE**

# THERMOSTABLE *AZOSPIRILLUM* SPP.: MORPHOLOGICAL, BIOCHEMICAL CHARACTERIZATION AND EFFECT ON SEED GERMINATION

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**DOI: https://doi.org/10.5281/zenodo.17569491**

**Abstract:**

Thermostable *Azospirillum* species were isolated from compost samples collected from Sunegao village and characterized for their morphological, physiological, and biochemical properties. All isolates were Gram-negative, vibroid or rod-shaped, and demonstrated catalase activity, nitrate reduction, and sugar fermentation, while showing variable responses in gelatin, amylase, and casein hydrolysis tests. Selected isolates were evaluated for biofertilizer production and their ability to enhance seed germination. Results indicated that inoculation with thermostable *Azospirillum* significantly increased root and shoot lengths, confirming their plant growth-promoting potential. These findings highlight the utility of thermostable *Azospirillum* as eco-friendly biofertilizers, offering a sustainable alternative to chemical fertilizers and improving soil fertility and crop productivity.

**Keywords:** *Azospirillum*, Thermostable Isolates, Nitrogen Fixation, Biofertilizer, Seed Germination.

**Introduction:**

*Azospirillum* is a genus of Gram-negative, nitrogen-fixing bacteria that act as plant growth-promoting rhizobacteria (PGPR). These bacteria are commonly found in the rhizosphere of grasses and cereal crops such as maize, wheat, and rice [1]. *Azospirillum* enhances plant growth not only by fixing atmospheric nitrogen into forms usable by plants but also by producing phytohormones like indole-3-acetic acid (IAA), cytokinins, and gibberellins [2]. Additionally, it improves nutrient uptake, stimulates root development, and increases plant tolerance to abiotic stresses such as drought and salinity [3]. Due to these multifaceted benefits, *Azospirillum* has emerged as a key component in sustainable agriculture, offering an eco-friendly alternative to chemical fertilizers while promoting soil health and crop productivity [4].

**Taxonomy of *Azospirillum***

Domain: Bacteria

Phylum: Pseudomonadota (formerly Proteobacteria)

Class: Alphaproteobacteria

Order: Rhodospirillales

Family: Azospirillaceae

Genus: *Azospirillum*

Type Species: *Azospirillum brasilense* [5]

**Notable Species:**

Several notable species of *Azospirillum* have been identified and studied for their plant growth-promoting abilities. Among them, *Azospirillum* brasilense and *Azospirillum* lipoferum are the most widely researched species, commonly associated with the roots of grasses such as maize, wheat, and sorghum [6]. A. brasilense is known for its high efficiency in nitrogen fixation and production of growth hormones like indole-3-acetic acid (IAA), which stimulate root elongation and branching [7]. A. lipoferum enhances plant nutrient uptake and improves stress tolerance in crops grown under nutrient-limited or drought-prone conditions [3]. Other species such as A. amazonense, A. halopraeferens, and A. oryzae have been isolated from diverse habitats and exhibit adaptations to varying environmental conditions, including salinity and temperature tolerance [8]. These species collectively contribute to improved crop productivity and soil fertility, making *Azospirillum* an important genus for biofertilizer formulation and sustainable agricultural practices.[9]

**Characteristics:**

*Azospirillum* species are Gram-negative, spiral-shaped, microaerophilic, and nitrogen-fixing bacteria predominantly found in the rhizosphere of grasses and cereal crops [6]. They play a vital role in promoting plant growth by fixing atmospheric nitrogen and producing phytohormones such as auxins, gibberellins, and cytokinins, which enhance root development and nutrient uptake [2]. These characteristics make *Azospirillum* an important plant growth-promoting rhizobacterium (PGPR) with significant potential in sustainable agriculture and biofertilizer development [3]. Morphologically, *Azospirillum* are Gram-negative, spiral or curved rod-shaped bacteria, typically measuring 0.2–0.9 μm in width and 1.0–3.0 μm in length [4]. They are motile due to the presence of one or more polar flagella.

Being microaerophilic, they thrive in low-oxygen environments such as the rhizosphere. On solid media, their colonies usually appear round, smooth, and translucent [5]. Functionally, *Azospirillum* is a free-living, associative nitrogen fixer that converts atmospheric nitrogen ($N_2$) into ammonia ($NH_3$), making nitrogen accessible for plant uptake [10]. Unlike symbiotic nitrogen-fixing bacteria such as Rhizobium, *Azospirillum* colonizes the rhizosphere and root surfaces without forming nodules. This process is mediated by the nitrogenase enzyme complex, which remains active under microaerophilic conditions [11]. By fixing nitrogen, *Azospirillum* reduces the dependence on chemical fertilizers, enhances soil fertility, and contributes to increased crop productivity [12].

**Nitrogen Fixation by *Azospirillum***

*Azospirillum* species are capable of fixing atmospheric nitrogen into ammonia through the action of the enzyme nitrogenase under microaerophilic conditions [13]. This process provides an important source of biologically available nitrogen for plants, particularly in nitrogen-deficient soils [1]. The nitrogenase enzyme complex, composed of dinitrogenase and dinitrogenase reductase, catalyzes the reduction of atmospheric nitrogen ($N_2$) to ammonia ($NH_3$), which can then be assimilated by plants [14]. Since nitrogenase is highly sensitive to oxygen, *Azospirillum* maintains its activity by regulating respiration and forming protective structures around the enzyme system [15]. Although *Azospirillum* fixes less nitrogen than symbiotic bacteria such as Rhizobium, it significantly enhances plant growth by supplying a steady amount of nitrogen in the rhizosphere [16]. In addition, its association with plant roots improves the overall efficiency of nitrogen utilization, reducing dependence on synthetic nitrogen fertilizers and promoting sustainable agricultural practices [17].

**Review of Literature:**

*Azospirillum* spp. are widely recognized as plant growth-promoting rhizobacteria (PGPR) with multiple beneficial traits. Akbari *et al.* (2007) isolated indigenous *Azospirillum* strains from Iranian soils and found that IAA-producing strains significantly enhanced wheat root length, dry weight, and number of lateral roots in greenhouse assays [18]. Doroshenko *et al.* (2007) reported *Azospirillum* strains from a Sphagnum peat bog with nitrogen-fixing activity under acidic peat soil conditions [19]. Similarly, co-inoculation studies (e.g. *Azospirillum brasilense* + *Methylobacterium oryzae*) in Korean experiments showed improved growth and nutrient uptake of tomato, red pepper, and rice under greenhouse conditions [20]. Recent reviews also emphasize its multifaceted benefits in nitrogen fixation, phytohormone production, stress tolerance, and improved nutrient use efficiency. Collectively, these findings underscore the importance of *Azospirillum* as an eco-friendly biofertilizer and a sustainable tool for enhancing crop productivity under diverse environmental conditions.

**Materials and Methodology:**

Compost samples were collected using sterile spatulas and gloves and placed into sterile polythene bags to maintain aseptic conditions. The laboratory analysis was carried out using standard glassware and plasticware, including beakers, test tubes, Petri dishes, Erlenmeyer flasks, conical centrifuge tubes, and measuring cylinders. Microbiological manipulations were performed using pipettes, glass spreaders, and nichrome wire loops. An autoclave was used for sterilization of media and equipment, while an incubator facilitated bacterial culture. A spectrophotometer was employed to

measure optical density, and a compound microscope was used for morphological observations. All glassware and equipment were sterilized before use to ensure aseptic handling throughout the study. All glassware and equipment were sterilized before use to maintain aseptic conditions throughout the study.

**Materials required for Biochemical Analysis:**

The chemicals used for biochemical characterization included distilled water, Gram stain reagents, iodine solution for starch hydrolysis, and skimmed milk. Nitrogen-free bromothymol blue (Nfb) medium was used for the cultivation and growth studies. The test organism employed throughout the study was thermostable *Azospirillum* spp., isolated from compost samples. All chemicals and media were prepared using standard protocols and sterilized before use to maintain aseptic conditions during the experiments.

**Methodology:**

**Sample collection:**

The *Azospirillum* species studied in the present investigation were isolated from compost samples with a pH range of 5.5 to 7, collected from different regions of Sunegao village. The compost samples were collected in sterile plastic bags and transported to the laboratory for further processing. To maintain microbial viability and prevent contamination, the collected compost was preserved under refrigeration until use.

**Isolation and purification of thermostable *Azospirillum* species:**

Isolation of *Azospirillum* species from compost was carried out using the serial dilution method. One gram of compost was suspended in sterile distilled water and serially diluted up to $10^{-10}$. From each dilution ($10^{-1}$ to $10^{-10}$), 1 ml of the aliquot was inoculated into test tubes containing Okon's NFb (nitrogen-free bromothymol blue) semi-solid medium.The inoculated tubes were incubated at 40 °C for 48–72 hours and observed for growth. The development of a characteristic subsurface pellicle was considered positive for the presence of *Azospirillum*. Pellicles were further streaked onto Petri plates containing Okon's solid medium and incubated at 40 °C for 48–72 hours. Morphologically distinct *Azospirillum* colonies were observed. The colonies developed on Okon's agar medium (pH 6.8–8.0*)* were subsequently transferred to slants of the same medium and preserved at 4 °C for further study.

**Okon's media [29]:**

**Composition of Okon's NFb Medium:**

For the isolation and cultivation of *Azospirillum* species, Okon's NFb (Nitrogen-Free Bromothymol Blue) medium was prepared with the following composition (per 250 ml of distilled water):

Malic acid – 1.25 g

$K_2HPO_4$ – 0.125 g

KOH – 1.125 g

$FeSO_4 \cdot 7H_2O$ – 0.012 g

$MnSO_4 \cdot 7H_2O$ – 0.002 g

$MgSO_4 \cdot 7H_2O$ – 0.025 g

NaCl – 0.005 g

$CaCl_2$ – 0.0025 g
$Na_2MoO_4$ – 0.0005 g
Bromothymol blue – 0.5 ml
Agar – 5 g (for solid medium) or 0.5 g (for semi-solid medium)
$NH_4Cl$ – 0.25 g
Distilled water – 250 ml
The medium was sterilized by autoclaving at 121 °C for 15 minutes before use

**Cultural Characteristics:**

**Growth Pattern of Thermostable *Azospirillum* Isolates:**

Different isolates of thermostable *Azospirillum* species were cultured on respective standard media, and their characteristic growth patterns were observed. Serially diluted cultures of the isolates were inoculated onto Okon's agar medium in Petri plates and incubated at 40 °C for 72 hours. Distinct colony morphology and growth behavior of the thermostable isolates were recorded for further characterization.

**Identification:**

All the isolates obtained were then subjected for various various morphological and physiological analysis.

**Morphological Characteristics**

**Gram Reaction:** Smears were prepared from 48–72-hour-old cultures and Gram-stained. The slides were observed under a compound microscope using oil immersion.

**Shape:** Smears from 48–72-hour-old cultures were examined under oil immersion to observe cell morphology.

**Physiological Characteristics**

The following tests were performed to study the physiological behavior of the isolates.

**Enzyme Analysis:** Various enzymatic properties of the obtained isolates were studied, including catalase, gelatinase, starch hydrolysis, and casein hydrolysis tests.

**Catalase Test:** A loopful of 48-hour-old cultures was added to 10% $H_2O_2$. The production of gas bubbles was considered positive for catalase activity.

**Gelatin Hydrolysis Test:** Nutrient agar plates containing 0.4% gelatin (sterilized at 121 °C for 20 minutes) were streaked with respective isolates and incubated at 40 °C for 24–72 hours. The appearance of a yellow halo around the growth, after flooding with mercuric chloride solution, indicated gelatin utilization.

**Composition:** glucose 0.01 g, gelatin 0.8 g, $K_2HPO_4$ 0.3 g, $KH_2PO_4$ 0.1 g, nutrient agar 5.6 g, distilled water 200 ml

**Amylase Test:** Plates containing peptone, beef extract, agar, and starch (steam sterilized) were streaked with isolates and incubated at 40 °C for 24–48 hours. Starch hydrolysis was observed by flooding with dilute iodine solution; a colorless halo around the growth indicated starch utilization.

**Composition**: Peptone 1 g, beef extract 0.6 g, agar 4 g in 150 ml distilled water; starch 4 g in 50 ml distilled water

**Casein Test**: Plates containing nutrient agar with 2% skimmed milk (sterilized at 121 °C for 20 minutes) were streaked with isolates and incubated at 40 °C for 24 hours. The appearance of a clear zone around colonies indicated casein hydrolysis.

**Composition:** Nutrient agar 5.6 g, skimmed milk 2 g, distilled water 200 ml

**Nitrate Reduction Test:** Media containing $KNO_3$ 0.2 g, peptone 1 g, beef extract 0.6 g, agar 0.6 g, and distilled water 200 ml were sterilized at 121 °C for 20 minutes. A loopful of isolates was inoculated and incubated at 40 °C for 48–72 hours. Nitrate reduction was confirmed by adding 1 ml each of sulphanilic acid and naphthylamine; pink color formation indicated nitrite presence.

**IMViC Tests**

**Methyl Red Test:**

Glucose-supplemented media were prepared in 10 ml test tubes, sterilized, cooled, and inoculated with isolates. After incubation at 40 °C for 24 hours, 3–4 drops of methyl red reagent were added. Red coloration indicated a positive result due to acid production.

**Composition:** Peptone 1 g, $K_2HPO_4$ 1 g, glucose 5 g, distilled water 200 ml

**Voges–Proskauer Test:** Glucose-supplemented media were inoculated and incubated at 40 °C for 24 hours. The test was performed by adding 1 ml KOH and 3 ml alpha-naphthol solution. Brownish-red or pink coloration indicated a positive result.

**Composition:** Peptone 1 g, $K_2HPO_4$ 1 g, glucose 5 g, distilled water 200 ml

**Citrate Utilization Test:**

Citrate agar plates were streaked with isolates and incubated at 40 °C for 24–48 hours. Blue coloration around colonies indicated citrate utilization.

**Composition:** Simmons' citrate 4.85 g, agar 4 g, distilled water 200 ml

**Sugar Utilization Tests:** Sucrose, maltose, and glucose were tested. Media were prepared with Durham tubes, sterilized at 121 °C for 20 minutes, and inoculated with isolates. Incubation was performed for 24–48 hours. Yellow color development and gas production in Durham tubes indicated sugar utilization.

**Composition:** Beef extract 0.6 g, peptone 3 g, phenol red solution until red, sugar 2 g, distilled water 200 ml

**Production of Biofertilizer**

Biofertilizer production is essential for enhancing soil nutrients critical for plant growth. Excessive use of chemical fertilizers degrades soil quality, whereas biofertilizers containing living microorganisms improve soil fertility and crop yield sustainably.

**Procedure:**

Nitrogen-free bromothymol blue (Nfb) medium was sterilized at 121 °C for 20 minutes. A loopful of the isolate showing the best nitrate reduction was inoculated into Nfb medium and incubated at 40 °C for 48–72 hours.

**Common Microbes Used as Biofertilizers:**

1. *Rhizobia*: Nitrogen-fixing bacteria in legume root nodules.
2. *Azotobacter*: Free-living nitrogen-fixing bacteria for non-legumes.

3. *Azospirillum*: Fixes atmospheric nitrogen and promotes root development.
4. *Mycorrhizae*: Symbiotic fungi that enhance nutrient and water absorption.
5. *Bacillus subtilis*: Improves soil structure, suppresses diseases, and produces growth-promoting compounds.
6. *Pseudomonas fluorescens*: Enhances plant growth and disease suppression through antibiotic and hormone production.

**Seed Germination Assay**

Microbial isolates were prepared in sterile Nfb broth. Seeds were soaked in the microbial inoculum for 30 minutes, while controls were soaked in sterile distilled water. Seeds were incubated on moist, sterilized filter paper in Petri plates at room temperature for 7 days. Root and shoot lengths were measured at the end of the assay.

**Results and Discussion:**

**Morphological Characteristics:**

All isolates were Gram-negative and vibroid or rod-shaped.

**Table 1: Colony count**

| Time | 10-1 | 10-2 | 10-3 | 10-4 | 10-5 | 10-6 | 10-7 | 10-8 | 10-9 | 10-10 |
|---|---|---|---|---|---|---|---|---|---|---|
| 24 hr | - | - | - | - | - | - | - | - | - | - |
| 48 hr | - | - | - | - | - | - | - | - | - | - |
| 72 hr | 15 | 20 | 6 | - | - | 3 | - | 2 | 2 | - |

**- : No growth**

**White colonies of *Azospirillum***

**Table 2: Colony characteristics**

| | **10-1 coded 1** | **10-2 coded 2** | **10-3 coded 3** | **10-6 coded 4** | **10-8 coded 5** | **10-9 coded 6** |
|---|---|---|---|---|---|---|
| **Shape** | Vibroid | Vibroid | Vibroid | Vibroid | Vibroid | Vibroid |
| **Size** | 1 mm | 1mm | 3mm | 2mm | 1 mm | 3mm |
| **Margin** | Entire | Entire | Entire | Entire | Entire | Entire |
| **Suface** | Smooth | Smooth | Smooth | Smooth | Smooth | Smooth |
| **Colour** | White | White | White | White | White | White |
| **Consistency** | Sticky | Sticky | Sticky | Sticky | Sticky | Sticky |
| **Elevation** | Raised | Raised | Flat | Raised | Flat | Raised |
| **Opacity** | Opaque | Opaque | Opaque | Opaque | Opaque | Opaque |
| **Gram's nature** | Gram negative | Gram negative | Gram negative | Gram negative | Gram negative | Gram negative |
| **Motility** | Motile | Motile | Motile | Motile | Motile | Motile |

**Physiological Characteristics:**

Catalase Test: All isolates were positive.

Gelatin Hydrolysis Test: All isolates were negative.

Amylase Test: All isolates were negative.

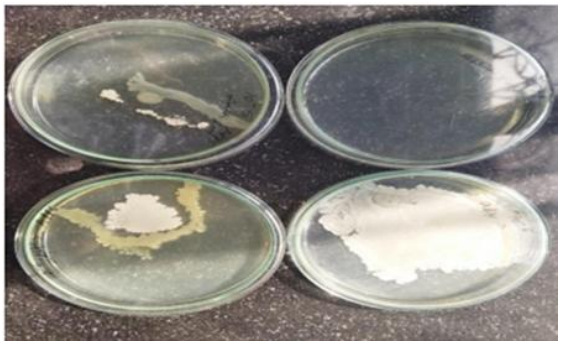
Casein Hydrolysis Test: Only one isolate hydrolyzed casein.

Nitrate Reduction Test: All isolates reduced nitrate.

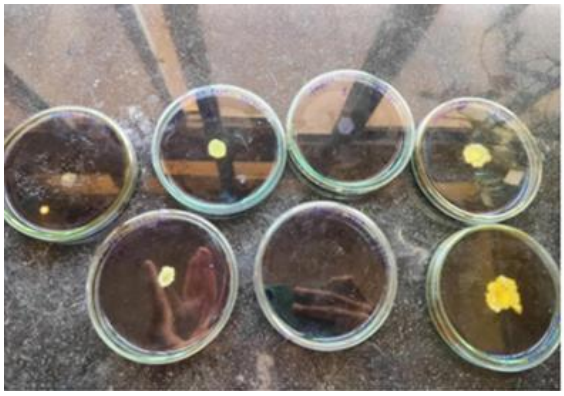
**Table 3: Enzyme profile test**

| | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Catalase | + | + | + | + | + | + |
| Amylase | - | - | - | - | - | - |
| Gelatinase | - | - | - | - | - | - |
| Casein | - | - | - | - | - | - |

**Catalase test**



**Gelatine hydrolysis test**



**Amylase test**

**Caesin hydrolysis test**

**MViC Tests:**

Methyl Red: Four isolates positive

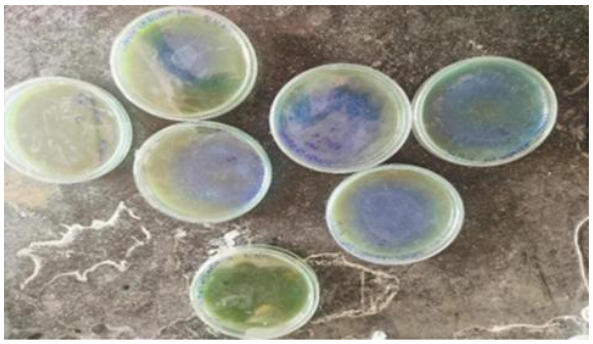
Voges–Proskauer: All negative

Citrate Utilization: Five isolates positive

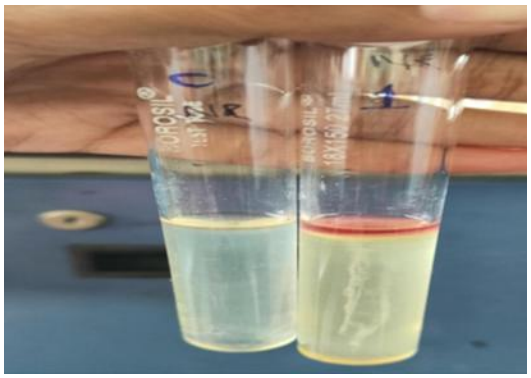
Nitrate reduction test: All Positive

Sugar Fermentation: All isolates utilized sucrose, maltose, and glucose; gas and acid production observed

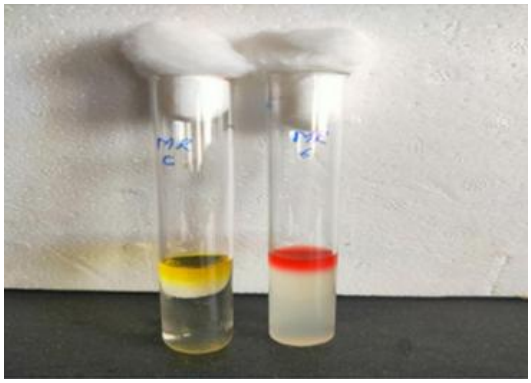
**Voges–Proskauer test**



**Citrate utilization test**



**Nitrate reduction test**



**Methyl red test**

**Table 4: IMVIC test**

| | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| **MR** | + | + | - | + | - | + |
| **VP test** | - | - | - | - | - | - |
| **Citrate test** | + | + | + | + | + | - |
| **Nitrate Reduction test** | + | + | + | + | + | + |

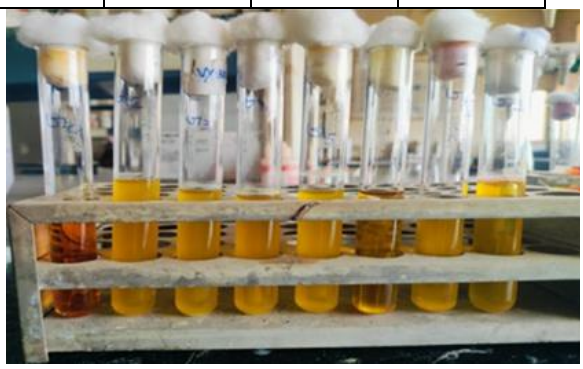
**Table 5: Sugar test acid production**

| | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| **Glucose** | + | + | + | + | + | + |
| **Sucrose** | + | + | + | + | + | + |
| **Maltose** | + | + | + | + | + | + |

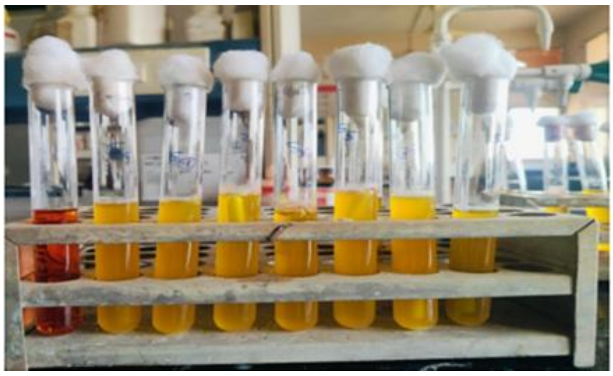
**Table 6: Gas production**

| | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| **Glucose** | + | + | - | + | - | + |
| **Sucrose** | - | - | - | - | - | - |
| **Maltose** | - | + | + | - | - | + |

**Sugar fermentation test (Maltose)**



**Sugar fermentation test (Glucose)**

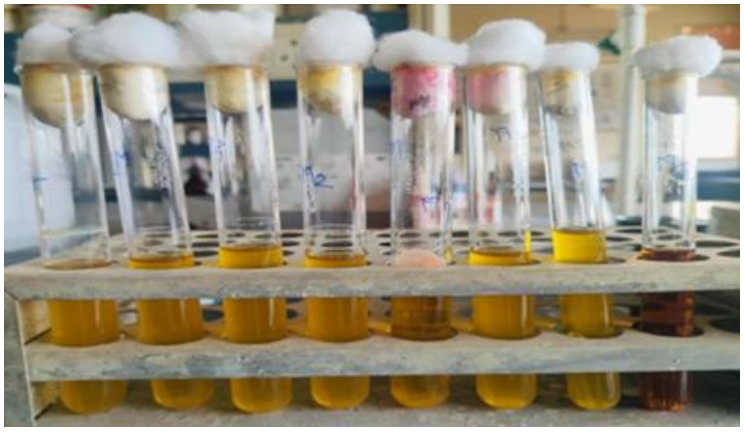


**Sugar fermentation test (Maltose)**

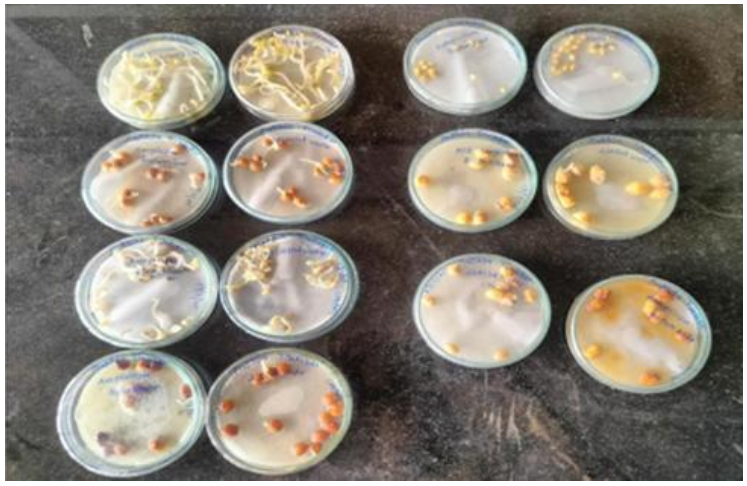
**Seed Germination Assay:** Thermostable *Azospirillum* isolates significantly increased root and shoot lengths compared to control seeds, indicating their potential as biofertilizers under environmental stress conditions.

**Table 7: Seed germination Assay (After 4 day incubation)**

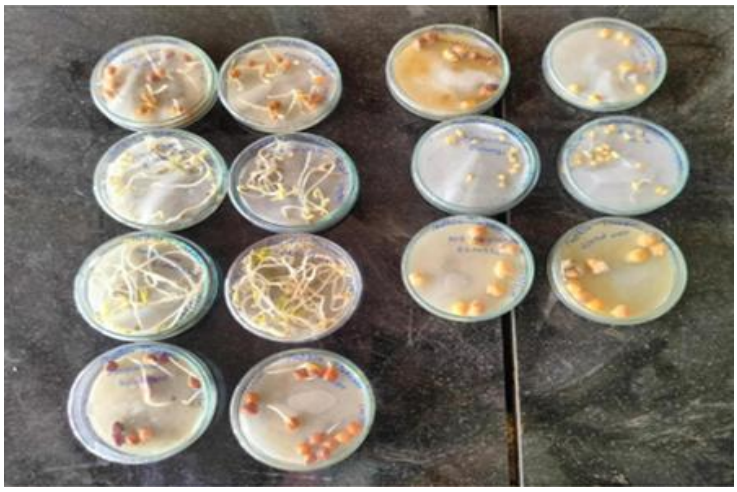
| | **Control** | | **Biofertilizer** | |
|---|---|---|---|---|
| | **Root length** | **Shoot length** | **Root length** | **Shoot length** |
| Mug beans (*vigna radiata*) | 2.5 cm | 7 cm | 7cm | 8.5 cm |
| Moth Beans (*Vigna Aconitifolia*) | 1.5 cm | 5 cm | 3 cm | 6.5 cm |
| Brown chickpea (*Cicer arietinum*) | 0.5 cm | 2.5 cm | 1.5 cm | 6.5 cm |
| Pigeon Pea (*Cajanus cajan*) | 2.5 cm | 3 cm | 3 cm | 4.5 cm |



**2 days of discussion**



**4 days of discussion**



**7 days of discussion**

**Conclusion:**

The present study successfully isolated and characterized thermostable *Azospirillum* species from compost samples. Morphological and physiological analyses confirmed that all isolates were Gram-negative, vibroid or rod-shaped bacteria, capable of nitrate reduction and sugar fermentation. Selected isolates demonstrated potential for nitrogen fixation and biofertilizer production. Seed germination assays further indicated that these isolates significantly enhanced root and shoot growth,

highlighting their role in promoting plant development. Overall, thermostable *Azospirillum* species represent an eco-friendly and sustainable alternative to chemical fertilizers, contributing to improved soil fertility, crop productivity, and environmentally safe agricultural practices.

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