

# ISOLATION, MORPHOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF SUGARCANE RHIZOSPHERIC BACTERIA *AZOTOBACTER CHROOCOCCUM* OF TALUKA DAUND, M.S., INDIA

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(Received 23 October, 2024; Accepted 30 December, 2024)

**Key words:** *Azotobacter chroococcum*, Plant, Colonies, Bacteria, Culture.

**Abstract**– This study was carried out to isolate and characterize the rhizospheric soil bacteria *Azotobacter chroococcum*, which is free living aerobic nitrogen fixing rhizobacteria. Nine samples were collected from three villages of taluka Daund Yawat, Jawje Buwachiwadi and Bhandgaon, from each village three samples were collected. Out of nine only three samples showed the formation of milky white, translucent colonies and development of pigmentation in older colonies. Further they also showed positive results for various biochemical tests such as hydrogen sulfide production, catalase, gelatin liquefaction, starch hydrolysis and indole acetic acid production. Morphological and biochemical characteristics confirmed that these isolates are *Azotobacter chroococcum*.

## INTRODUCTION

Biofertilizers are composed of microorganism, which are used for enhancement of yield.

Biofertilizers act as a source of nutrients particularly for nitrogen and phosphorous Wani *et al.* (2013). Previous works have identified various free living, nitrogen fixing rhizobacteria as biofertilizer. Which can directly enhance the growth of plants by synthesizing growth hormones, nitrogen fixing or by solubilizing phosphorous Misbahud Din *et al.* (2019). To perform these activities bacteria utilizes root exudates as carbon and energy source Yoneyama *et al.* (2017).

These rhizobacteria can not only increases the growth but also reduces the pathogenic infections. It helps in dealing with biotic and abiotic stress, without causing infection to the plants. They can be used as a pesticide and also play an important role in phytoremediation (Compant *et al.*, 2010).

Roots of the Poaceae family plants such as wheat, rice, sugarcane shows association with nitrogen fixing and plant growth promoting rhizobacteria namely *Azotobacter*, *Azospirillum*, *Klebsiella*, *Zoogloea*, *Enterobacter*, *Bacillus*, *Acetobacter*, *Arthobacter*, *Azoarcus* and *Herbaspirillum* Mirza *et al.* (2001). Above mentioned all the microorganisms can form

symbiotic association except for *Azotobacter*. *Azotobacter* is a free living, nitrogen fixing, aerobic rhizobacteria. *Azotobacter* has an enzyme nitrogenase, which uses atmospheric nitrogen as a substrate and convert it into ammonium form, after lysis of the bacterial cell fixed nitrogen is released in the soil and absorbed by the plants.

Most commonly found species of *Azotobacter* is *A. chroococcum*, previous studies revealed the growth promoting activities such as production of growth hormones auxin, gibberellin, cytokinin, vitamins, siderophores which are natural iron chelating agent and also the antagonistic activities against pathogens Dasgupta *et al.* (2021). Auxin released by bacteria ensures fast proliferation of roots, increased root biomass also increases nutrient and water uptake. Hence, this investigation is planned to isolate efficient strain of *Azotobacter chroococcum* from the rhizosphere of sugarcane plant from taluka Daund.

## MATERIALS AND METHODS

### Sample collection

Soil samples were collected from the rhizospheric region of the villages Yawat, Jawje Buwachiwadi and Bhandgaon. Three samples were collected from each village. Sterile plastic bags were used for the

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collection of samples and labeled after collection. Samples were brought to the laboratory for further studies. Code is given to each sample and is given in Table 1.

**Table 1.** Collection of soil samples from different locations.

Sr. No.	Code Name	Location
1	Jaw 1	Jawje Buwachiwadi location-1
2	Jaw 2	Jawje Buwachiwadi location-2
3	Jaw 3	Jawje Buwachiwadi location-3
4	Yaw 1	Yawat location-1
5	Yaw 2	Yawat location-2
6	Yaw 3	Yawat location-3
7	Bha 1	Bhandgaon location-1
8	Bha 2	Bhandgaon location-2
9	Bha 3	Bhandgaon location-3

### Isolation

Ashby's mannitol agar media (Mannitol-20 g, Dipotassium phosphate-0.2 g, Magnesium sulphate-0.2 g, Sodium chloride-0.2 g, Potassium sulphate-0.1 g, Calcium carbonate-5 g, Agar-15 g.) is used to isolate *Azotobacter chroococcum* by spread plate method Basnett *et al.* (2024).

100ml of sterile distilled water is added and thoroughly mixed with 10 g of soil sample, this liquid suspension is left for some time and then filtered through Whatman filter paper. Residue is discarded and filtrate is used for serial dilution. In a sterile test tube 1ml of filtrate and 9ml of sterile distilled water is mixed and labeled as  $10^{-1}$ , 1ml of diluted sample from this tube is added to another tube with 9ml of distilled water and so on serially diluted upto 8 concentrations. Serially diluted samples were inoculated on Ashby's media by using spread plate method. Incubation was done at  $28 \pm 2$  °C for five to seven days. Colonies grown on the medium was critically observed and analyzed based on the characteristics defined by Bergey's manual (1994), similar colonies were pure cultured and used for morphological and biochemical characterization.

### Morphological characterization

Morphological characterizations of the cultured colonies were carried out for their identification. Grown colonies were critically analyzed for colour, shape, appearance, diameter and Gram nature to reveal their identity (Ahmad *et al.*, 2008).

### Gram staining

Loopful of bacterial colony is smeared on glass

slide, slide is stained with crystal violet for one minute, wash using tap water gently, for one minute Gram's iodine is added, wash with tap water again, 95% ethanol is used for washing for not more than 10 seconds, wash with water and counter stain by using safranin, after a minute wash with tap water, keep the slide for air drying and use oil immersion microscope for observation Paray *et al.* (2023).

### Biochemical characterization

Different tests are used for biochemical identification such as gelatin liquefaction, starch hydrolysis, potassium hydroxide, hydrogen sulfide production, catalase, and indole acetic acid production Salle *et al.* (1967).

#### a) Hydrogen sulfide production test

Bacterial isolates were inoculated in sterile tube containing peptone water and with the help of sterile cotton plug lead acetate paper was held above the suspension, incubated for three days. Uninoculated tube represents control.

#### b) Catalase test

Hydrogen peroxide drop was taken on glass slide and a loop full of bacterial colony were mixed with it and observed for result.

#### c) Gelatin liquefaction test

Bacterial colony is inoculated using sterile needle by stab method in nutrient gelatin and uninoculated tube is considered as control.

#### d) Starch hydrolysis test

In this test inoculation of bacterial colony on starch agar was followed by seven days of incubation. Addition of Lugol's iodine will reveal the result. Uninoculated starch agar is used as control.

#### e) Indole acetic acid (IAA) test

Autoclaved 1% tryptone broth inoculated with bacterial culture and uninoculated broth is used as control. Incubation of 48-72 hrs followed by addition of 1 ml of Kovac's reagent will reveal the result.

#### f) Potassium hydroxide test

3% KOH drop was taken on glass slide and a loop full of bacterial colony were mixed with it and observed for result.

## RESULTS AND DISCUSSION

### Isolation of *Azotobacter chroococcum*

Ashby's media showed the growth of milky white, translucent, raised, smooth textured, shiny, 2-4 µm diameter bacterial colonies. Colonies with these characteristics were pure cultured. Only three culture plates showed the above mentioned characteristics, rest samples showed no formation of *A. chroococcum* colonies.

### Morphological characterization

Change in colour with age is a characteristic feature of *A. chroococcum* and the entire isolated milky white shiny colony showed change in colour from milky white to brown. Results are given in Table 2, which shows all three isolates are rod shaped and development of pink colour confirmed them as Gram negative bacteria.

**Table 2.** Morphological characterization of the isolates.

Sr. No.	Code Name	Gram Staining	Cell shape
1.	Jaw 3	- ve	Rod
2.	Yaw 3	- ve	Rod
3.	Bha 1	- ve	Rod

(+ve= Positive and -ve= Negative)

### Biochemical characterization

Lead acetate paper in inoculated peptone water tube turned black and the one inserted in control did not show any change in colour, which means hydrogen sulfide gas is produced by bacteria in inoculated tube which turned the colour of lead acetate paper from white to black and this result is observed in all three samples. Catalase is an enzyme which converts hydrogen per oxide to water and oxygen, when bacterial cell with catalase enzyme comes in contact with hydrogen per oxide it will start producing oxygen bubbles and all isolated bacterial colonies showed formation of bubbles, therefore they are catalase positive. In gelatin liquefaction test gelatinase enzyme produced by bacteria has the

ability to convert semi solid gelatin to liquid form and all the inoculated tube shows liquification of solidified nutrient gelatin. Starch hydrolysis test also detects the presence of bacterial enzyme amylase, when isolates grown on starch containing media they start to degrade starch and addition of Lugol's iodine shows formation of clear zone which is observed in all the isolates, therefore all of them are starch positive. Bacteria producing tryptophanase enzyme convert the tryptophan enzyme to indole, pyruvic acid and ammonia. To detect this ability in isolated bacterial colony, they were grown in tryptone broth and their activity is checked by addition of Kovac's reagent which showed formation of red coloured ring in the top layer after 10-15 minutes, hence all of them are indole positive bacteria. All the three isolates showed negative result for potassium hydroxide test (Patil *et al.*, 2014). All these results are given in Table 3.

## CONCLUSION

Result showed the formation of milky white colonies but as they get old started to produce brown pigmentation, which is a characteristic feature of *A. chroococcum* according to Bergey's manual. The isolates were also showing rod shaped cells and Gram negative bacteria when observed microscopically. Biochemical analysis for hydrogen sulfide production, catalase test, gelatin liquefaction test, starch hydrolysis and indole acetic acid production test were positive. Formation of indole compound confirmed the isolate as *A. chroococcum*. Earlier findings also supports the results that *A. chroococcum* has the ability to synthesize phytohormones one of them is indole acetic acid, which is auxin root promoting hormone. Auxin causes increased biomass of root which will directly influence its absorption function, more biomass more absorption and the crops whose rhizosphere lack *A. chroococcum* can be provided with the same to enhance their yield.

**Table 3.** Biochemical characterization of the isolates.

Sr. No.	Code Name	H <sub>2</sub> S	Catalase	Gelatin liquefaction	Starch hydrolysis	IAA	KOH
1.	Jaw 3	+ ve	+ ve	+ ve	+ ve	+ ve	- ve
2.	Yaw 3	+ ve	+ ve	+ ve	+ ve	+ ve	- ve
3.	Bha 1	+ ve	+ ve	+ ve	+ ve	+ ve	- ve

(+ve= Positive and -ve= Negative)

### Conflict of interest

No conflict of interest.

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