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Investigating the Enhancement of Tolerance to Abiotic Stress and Improvement of Carbon Supply in Sugarcane Using, *Acidithiobacillus* sp.

Barkathulla Syed Nazeer, Vijaya Surya Velu and Dheepan George*

Deptartment of Microbiology, AVS College of arts and science (Autonomous), Salem India.

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To explore the potential of Plant Growth-Promoting Bacteria (PGPB) such as Acidithiobacillus sp. in improving sugarcane tolerance to abiotic stresses like drought, salinity, and nutrient deficiencies, and optimizing carbon supply for sustainable cultivation.1 The use of Plant Growth-Promoting Bacteria (PGPB) has the potential for enhancing agricultural sustainability and productivity. PGPB supports plants in a variety of ways, including nutrient absorption, hormone production, and disease prevention. However, strain variability, formulation and distribution concerns, regulatory barriers, and socioeconomic constraints all offer obstacles to their widespread use. Addressing these challenges requires collaborative efforts from researchers, policymakers, extension workers, and farmers to identify effective PGPB strains, develop stable formulations, navigate regulatory processes, and provide technical support for successful integration into agricultural practices.2 Sugarcane plantlets in tissue culture survived heat stress, suggesting the bioformulation induces abiotic stress tolerance and carbon content was higher in tissue culture-treated, bio-inoculated plants compared to pot culture plants and controls. Despite these challenges, using PGPB has the potential to boost crop yields, minimise chemical inputs, and promote sustainable agriculture, therefore contributing to global food security and environmental stewardship.3 The study To explore the potential of Plant Growth-Promoting Bacteria (PGPB) such as Acidithiobacillus sp. in improving sugarcane tolerance to abiotic stresses like drought, salinity, and nutrient deficiencies, and optimizing carbon supply for sustainable cultivation.1 The use of Plant Growth-Promoting Bacteria (PGPB) has the potential for enhancing agricultural sustainability and productivity. PGPB supports plants in a variety of ways, including nutrient absorption, hormone production, and disease prevention. However, strain variability, formulation and distribution concerns, regulatory barriers, and socioeconomic constraints all offer obstacles to their widespread use. Addressing these challenges requires collaborative efforts from researchers, policymakers, extension workers, and farmers to identify effective PGPB strains, develop stable formulations, navigate regulatory processes, and provide technical support for successful integration into agricultural practices.2 Sugarcane plantlets in tissue culture survived heat stress, suggesting the bioformulation induces abiotic stress tolerance and carbon content was higher in tissue culture-treated, bio-inoculated plants compared to pot culture plants and controls. Despite these challenges, using PGPB has the potential to boost crop yields, minimise chemical inputs, and promote sustainable agriculture, therefore contributing to global food security and environmental stewardship.3 The study suggests that bioformulation induces abiotic stress tolerance and improves carbon supply in sugarcane.

Keywords: Food security; Hormone synthesis; Plant Growth-Promoting Bacteria; Sustainable agriculture.

*Corresponding author E-mail: dheepanmicro2021@gmail.com

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The cultivation of *Saccharum officinarum*, commonly known as sugarcane, is vital for global sugar production and bioenergy. However, sugarcane faces numerous challenges, including abiotic stressors such as drought, salinity, and nutrient deficiencies, which cyanobacteria (PGPB) has emerged as a promising strategy to enhance plant growth, improve stress tolerance, and optimize nutrient uptake in various crops, including sugarcane.[4] Among the diverse array of PGPB *Acidithiobacillus sp* have shown potential in promoting plant growth and mitigating abiotic stress effects. *Acidithiobacilli* are a common group of microorganisms in acidic mines and are active in the degradation of a range of sulfide minerals. *Acidithiobacillus ferrooxidans* is a Gram-negative, Acidophilic bacterium, which thrives in harsh environments and exhibits a versatile energy metabolism. The most important one is *Acidithiobacillus ferrooxidans* which is a Gram negative chemolithotropic bacteria.[5]

In recent decades, the global agricultural sector has faced escalating challenges due to climate change, dwindling natural resources, and increasing demand for food and bio energy. Among the crops vulnerable to these challenges is *Saccharum officinarum*, or sugarcane, a primary source of sugar and bio ethanol. By harnessing the synergistic interactions among these PGPB, this study aims to explore their collective potential in improving sugarcane tolerance to abiotic stress and optimizing carbon supply, thereby contributing to sustainable sugarcane cultivation and global food security.[6] As the global agricultural sector grapples with the dual challenges of ensuring food security and environmental sustainability, there is a pressing need to explore innovative strategies for enhancing crop resilience and productivity.

The intricate interplay between plants and beneficial microorganisms offers a promising avenue for bolstering crop resilience and productivity in the face of mounting environmental challenges. Among the diverse array of beneficial microbes, plant growth-promoting bacteria (PGPB) have garnered significant attention for their ability to enhance nutrient uptake, improve stress tolerance, and stimulate plant growth. *Acidithiobacillus sp.,* are three such PGPB that have shown considerable promise in enhancing the performance of various crops, including sugarcane. By leveraging the unique capabilities of these microbes, this study aims to explore novel strategies for optimizing sugarcane growth and productivity under adverse environmental conditions. Through a comprehensive investigation of the interactions between sugarcane and these PGPB, this research seeks to unlock new insights into microbial-assisted crop management and contribute to the development of sustainable agricultural practices for the future.

MATERIALS AND METHODS

Isolation of *Acidithiobacillus* from soil

Collect soil samples from desired locations known to contain sulfur-rich environments, such as mine tailings or acidic soils. Collect samples from different areas to ensure diversity.[7] Prepare a sulfur medium such as modified 9K medium or 9K-S according to manufacturer instructions. Sterilize the medium by autoclaving at 121°C for 15-20 minutes. Allow the medium to cool to approximately 45-50°C before use.[8] Weigh out a suitable amount of soil sample (e.g., 1 g) and add it to a sterile container with 9 mL of sterile water or saline solution. Mix the soil and solution thoroughly to create a soil suspension. Perform serial dilutions by transferring 1 mL of the soil suspension to 9 mL of sterile water or saline solution, and repeat as necessary to achieve dilutions of 10^-1 to 10^-5.[9] Take 100 µL aliquots from appropriate dilutions (e.g., 10^-3 to 10^-5) and spread them evenly onto the surface of selective agar plates (e.g., 9K-S agar) using a sterile loop or spreader. Incubate the plates inverted (lid side down) in an incubator set to 30-37°C for 3-7 days. Using a sterile loop or swab, select individual colonies of *Acidithiobacillus* from the plates and streak them onto fresh selective agar plates to obtain isolated colonies. Incubate the plates as before and observe for pure cultures of *Acidithiobacillus.* To confirm the identity of the isolated colonies, perform biochemical tests such as oxidase and catalase tests, as well as molecular techniques like PCR amplification and sequencing of 16S rRNA gene. Once pure cultures are obtained and confirmed, store them at -80°C in suitable cryoprotective medium (e.g., glycerol stock) for long-term storage.[10]

Tissue culture of sugarcane

Prepare the desired tissue culture medium,

such as MS basal medium, according to the manufacturer's instructions. Adjust the pH of the medium to around 5.8 using a suitable pH buffer. Add agar or a gelling agent to the medium at the recommended concentration (0.7-0.8% w/v) to solidify it. Optionally, supplement the medium with sucrose as a carbon source and plant growth regulators (auxins and cytokinins) at appropriate concentrations to promote shoot initiation and growth.[11] Obtain sugarcane explants from healthy and disease-free plants. Nodal segments or meristematic tissue are commonly used. Wash the explants thoroughly under running tap water to remove any debris or contaminants. Surface sterilize the explants by immersing them in a disinfectant solution (70% ethanol or a bleach solution) for a few minutes, followed by rinsing with sterile distilled water. Trim the explants to the desired size (1-2 cm) under sterile conditions using a scalpel or razor blade.[12]

Inoculation and Culture Initiation

Transfer the sterilized explants to the culture vessels containing the prepared tissue culture medium. Seal the vessels with sterile closures (cotton plugs or plastic caps). Incubate the culture vessels in a growth chamber or incubator with controlled temperature (25-28°C) and light conditions (16-hour photoperiod with fluorescent lights).[13] Check the cultures regularly for contamination and growth of new shoots. Subculture the actively growing shoots onto fresh medium every 4-6 weeks to prevent overcrowding and maintain healthy growth. Monitor the cultures for shoot proliferation and elongation, and adjust the growth regulator concentrations as needed to optimize growth.[14]

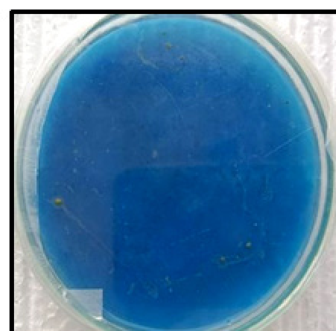
Rooting and Acclimatization

Once shoots have developed and reached an appropriate size, induce rooting by transferring them to a rooting medium containing suitable

A

B

C



D

Fig. 1. Isolation of *Acidithiobacillus ferrooxidans* by 9K medium

auxins (indole-3-butyric acid, IBA). After roots have developed, carefully transfer the plantlets to pots or trays containing a sterile potting mix. Harden off the plantlets by gradually exposing them to ambient environmental conditions (reduced humidity, increased light intensity) over a period of 1-2 weeks before transferring them to the greenhouse or field.[15]

Preparation of Bioformulation Preparation of Bacterial Inoculum

Start by growing the desired bacterial strain in suitable liquid culture medium until they reach the desired cell density (usually mid-log phase). Harvest the bacterial cells by centrifugation or filtration, and wash them with sterile water or saline solution to remove any residual medium.[16]

Preparation of Carrier Material

Sterilize the carrier material such as peat, vermiculite by autoclaving or other suitable

Table 1. Bio chemical test for *Acidithiobacillus ferrooxidans*

Biochemical Test	*Acidithiobacillus ferrooxidans*
Indole test	(-) ve
Methyl Red Test	(+) ve
Voges Proskauer (VP) Test	(-) ve
Citrate Utilization Test	(+) ve
Catalase	(+) ve

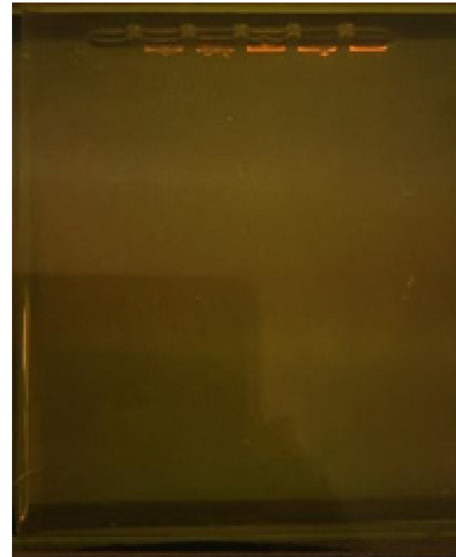


Fig. 2. PCR products of the ITS region of *Acidithiobacillus ferrooxidans*

A

B

C

D

Fig. 3. Mustard seed processing with consortia

sterilization method to ensure it is free from contaminants. If using alginate beads, prepare the alginate solution and sterilize it separately before encapsulating the bacterial cells.[17]

Incorporation of Bacterial Cells into Carrier

Mix the washed bacterial cells with the sterile carrier material in a suitable ratio to achieve the desired cell concentration in the final formulation. Optionally, add nutrients or growth-promoting substances to enhance bacterial viability and activity in the formulation. Ensure thorough mixing to achieve uniform distribution of bacterial cells within the carrier material.[18]

Addition of Additives

Incorporate any desired additives into the formulation to improve stability, protect the bacterial cells, and enhance performance. Common additives include protective agents such as trehalose, stabilizers such as carboxymethyl cellulose, polyvinyl alcohol, and adhesives such as guar gum, polyacrylic acid.

Adjustment of pH and Moisture Content

Measure the pH of the formulation using a pH meter and adjust it to the desired range of pH 6-8 using sterile solutions of acids or bases as needed. Adjust the moisture content of the formulation to ensure adequate hydration of the bacterial cells without excess moisture that could lead to microbial contamination or degradation of the formulation. Store the bioformulation under appropriate conditions to maintain bacterial viability and stability until use.

Determination of organic carbon in samples by potassium dichromate oxidation methods

The detection method works on the basis of organic matter extraction from the soil. The extraction is again enhanced by addition of chemicals provided in the kit. The colour developed after extraction can be compared with chart provided for estimation of organic carbon content of the soil. The analysis is easy to perform, requires only 15-20 min.[19]

Fig. 4. Friable Callus Induction of Sugarcane

RESULTS

The isolation of Acidithiobacillus ferrooxidans involves collecting samples from environments rich in sulfide minerals, such as acid mine drainage, coal mines, or bioleaching sites (Fig: 1), (Table: 1).

- Sample Collection
- Enrichment Culture
- Isolation and Purification
- Identification

DNA Sequencing

The ITS region of total isolates was successfully amplified by universal primer pairs (ITS1) and generated PCR products with a size of 1496 bp. Bioinformatics analysis, which was performed by comparing the sequence results

Table 2. Callus induction from nodal explants *Saccharum officinarum cultured* on MS medium supplemented with various concentrations of growth regulators

Concentrations of Plant Growth Regulations	No. of explants inoculated	No.of explants forming callus	Morphology of callus
MS + bap 1.5 mg/l + 2,4-D 1.5 mg/l	20	15 ± 0.49	G, N, F
MS + BAP 2.0 mg/l + 2,4-D 2.0 mg/l	20	10 ± 0.99	G, N, F
MS + IAA 1.0 mg/l + BAP 1.0 mg/l	20	5 ± 1.48	YG, M, F
MS + IBA 0.5 mg/l + 2,4-D 0.5 mg/l	20	10 ± 1.48	YG, M, F

F = Friable, G = Greenish, M = Massive, N=Nodular, YG = Yellowish Green

of the ITS region with sequence data registered in the NCBI nucleotide Blast https://www.ncbi.nlm.nih.gov/nuccore/PV030920.1, indicated that the ITS identified the species *Acidithiobacillus ferrooxidans* (Fig : 2).

Molecular sequencing confirmed that isolated species was belongs to the Acidithiobacillus ferrooxidans.

Preparation of Bioformulation

Bioformualtion was prepared with addition of calcium carbonate and carboxy methyl cellulose. After preparation of bioformulation with three bacteria, it was applied to the plantlets of mustard to analyse the vigour index. In that the growth was induced in the treated plantlets (Fig:3).

Plant Tissue Culture for Sugarcane *(Saccharum spp.)*

In tissue culture, the meristem shoot tip culture was done on MS media. The plantlets were yet to grow on media. The heat stress was given to the plantlets by keeping in higher temperature like 40-50 degree heat. After 15 days of observation, the plants survived no physical symptoms happened due to heat stress. This proves that the bioformulation induce abiotic stress tolerance to the paddy plants. It can further used in sugar cane cultivating field for large scale trails.(Fig: 4), (Table: 2), (Table: 3), (Table: 4).

Carbon estimation in both TC and pot culture test plants

Carbon was higher in TC treated bio inoculated plants whereas the estimation found less in pot culture compared to control.

DISCUSSION

The study identifies strain variability, formulation and distribution concerns, regulatory barriers, and socioeconomic constraints as potential obstacles to the widespread use of PGPB16. Optimizing bacterial inoculation methods and ensuring bacterial compatibility are also noted as challenges. The use of plant growth-promoting bacteria (PGPB) like Acidithiobacillus, in sugarcane cultivation has shown potential for sustainable production.[20] PGPB can enhance plant tolerance to abiotic stress, such as drought, salinity, and heavy metal toxicity, by modulating hormone levels, increasing antioxidant activity, and improving water use efficiency.[21] The application of the bacteria may result in synergistic effects, but

Table 3. Shoot induction from nodal explants of *Saccharum officinarum cultured* on MS medium supplemented with various concentrations of growth regulators

Concentrations of Plant Growth Regulations	No. of explants inoculated	No.of explants produced shoots	No.of shoots developed
MS + BAP 3.0 mg/l + Kin 0.5 mg/l +IAA 0.5 mg/l	20	4 ± 0.49	1
MS + BAP 3.0 mg/l + Kin 0.5 mg/l + IBA 0.5 mg/l + NAA 0.5 mg/l	20	18 ± 0.49	2
MS + BAP 3.0 mg/l + Kin 0.5 mg/l +IBA 0.5 mg/l	20	10 ± 1.48	2

Value represents means ± SD from 20 ruplicates

Table 4. Root induction from nodal stem explants of *Saccharum officinarum cultured* on MS medium supplemented with various concentration of growth regulators

Concentrations of Plant Growth Regulator	No. of shoot and clones inoculate	No.of clones produced roots	No.of roots Developed
MS + IAA 0.5 mg/l + NAA 0.5mg/l + IBA 0.5 mg/l + BAP 1.0 mg/l	20	12 ± 0.99	2
MS + IBA 0.5 mg/l + NAA 0.5mg/l + BAP 1.0 mg/l	20	6 ± 0.99	2

Value represents means ± SD from 20 ruplicates

challenges include optimizing bacterial inoculation methods, ensuring bacterial compatibility, and understanding the mechanisms underlying plant-bacteria interactions.[22] Future research should focus on elucidating the specific mechanisms by which these bacteria confer stress tolerance and improve carbon supply in sugarcane, involving molecular and physiological studies. Field trials and large-scale implementation studies are needed to assess the efficacy and economic feasibility of these bacteria in sugarcane cultivation under diverse environmental conditions.

CONCLUSION

The application of PGPB shows promise for enhancing crop production and sustainability, and continued research and innovation are essential for realizing the full potential of PGPB in agriculture. The study suggests that bioformulation induces abiotic stress tolerance and improves carbon supply in sugarcane. In conclusion, the application of plant growth-promoting bacteria (PGPB) presents a promising avenue for enhancing crop production and sustainability. Despite facing challenges such as strain variability, formulation issues, regulatory barriers, and socio-economic constraints, the potential benefits of PGPB in agriculture cannot be overlooked.[23] By leveraging advancements in microbial ecology, genetics, and biotechnology, along with collaborative efforts among researchers, policymakers, and agricultural stakeholders, it is possible to address these challenges and realize the full potential of PGPB in crop production. Moving forward continued research, innovation, and adoption of PGPB-based technologies are essential for overcoming existing limitations and harnessing the benefits of these beneficial microorganisms to meet the increasing demand for food while minimizing environmental impact.

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The authors do not have any conflict of interest.

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This statement does not apply to this article.

Ethics Statement

This research did not involve human participants, animal subjects, or any material that requires ethical approval.

Informed Consent Statement

This study did not involve human participants, and therefore, informed consent was not required.

Clinical Trial Registration

This research does not involve any clinical trials.

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Not Applicable.

Authors' Contribution

Barkathulla Syed Nazeer: Data Collection, Analysis, Writing – Review & Editing; Vijaya Surya: Visualization, Supervision, Project Administration; Dheepan George: Methodology, Writing – Original Draft, Visualization, Supervision, Project Administration.

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