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Isolation, Characterisation and Biofertilizer Potential of Halo-Alkaliphilic Bacteria from Lonar Lake, Maharashtra

*[1]Sonali Shrikant Patil, [2]Gutte Jaywant Vaijanath and [3]Bhosale Vishnu Suresh

*[1, 2, 3]Department of Microbiology, Rajarshi Shahu Mahavidyalaya, Latur (Empowered Autonomous Institution), Maharashtra, India.

Abstract

Lonar Lake, Maharashtra, is a unique meteorite-impact soda lake characterised by extreme alkalinity, elevated salinity, and diverse extremophilic microbial populations. The present study aimed to isolate and characterise halo-alkaliphilic bacteria from Lonar Lake and evaluate their plant growth-promoting potential under alkaline soil conditions. Water samples collected from the lake were enriched in alkaline Yeast Extract Mannitol (YEM) broth and cultured on selective media including YEM agar, Pikovskaya's agar, King's B agar, and Magnesium Solubilizing Medium (MgSM). Morphological, microscopic, and biochemical characterisation of the isolates was performed using standard microbiological methods. The majority of isolates were Gram-positive, rod-shaped, endospore-forming bacteria showing optimal growth at pH 9, indicating strong alkaliphilic adaptation. Positive catalase, amylase, Simmons citrate, methyl red, and Voges–Proskauer reactions demonstrated broad metabolic versatility and potential plant growth-promoting rhizobacterial (PGPR) activity. Several isolates exhibited phosphate and magnesium solubilisation abilities on selective media.

The biofertilizer potential of the isolates was evaluated using a fenugreek (*Trigonella foenum-graecum*) plant growth bioassay under alkaline soil conditions (pH 8.5). Biofertilizer-treated plants showed significantly enhanced growth compared with untreated controls ($p < 0.05$). Root length increased from 7.5 ± 0.5 cm in controls to 11.5 ± 0.6 cm in treated plants, while shoot length increased from 12.4 ± 0.4 cm to 15.8 ± 0.5 cm. Leaf number also increased significantly in treated plants, accompanied by earlier germination, denser root systems, and darker green foliage. These findings demonstrate the strong plant growth-promoting potential of halo-alkaliphilic bacteria isolated from Lonar Lake and highlight their possible application as biofertilizer inoculants for saline and alkaline agricultural soils. The study further emphasizes the importance of extremophilic ecosystems as reservoirs of agriculturally valuable microorganisms for sustainable agriculture.

Keywords: Halo-alkaliphilic bacteria, Lonar Lake, PGPR, Biofertilizer, *Bacillus* spp., Phosphate solubilisation.

1. Introduction

Extremophilic microorganisms are a diverse group of microbes capable of surviving and thriving under harsh environmental conditions such as extreme salinity, alkalinity, temperature, radiation, and desiccation. Among these, halo-alkaliphilic bacteria possess the combined ability to tolerate high salt concentrations and alkaline pH, making them ecologically and agriculturally important microorganisms. These bacteria have evolved specialized physiological and biochemical adaptations including Na^+/H^+ antiport systems, compatible solute accumulation, and alkaline-stable enzyme systems that enable survival under severe environmental stress conditions [1, 11]. The agricultural significance of halo-alkaliphilic bacteria has increased substantially due to the growing global problem of soil salinity and alkalinity, which affects nearly 831 million hectares worldwide and significantly reduces crop productivity by limiting nutrient availability and microbial activity [6]. In India, approximately 6.73 million hectares of land are affected by salinity and alkalinity, particularly in Maharashtra, Gujarat, and Rajasthan where soil pH frequently exceeds 8.5 [13]. Conventional biofertilizer strains developed for neutral soils often fail under such alkaline conditions because of poor survival and reduced metabolic activity. Therefore, stress-tolerant microbial inoculants adapted to alkaline environments are increasingly being explored as sustainable alternatives for improving soil fertility and crop productivity [8, 13]. Plant Growth-Promoting Rhizobacteria (PGPR) enhance plant growth through direct and indirect mechanisms including phosphate solubilisation, phytohormone production, siderophore secretion, nitrogen fixation, and induction of systemic resistance [5, 7, 19]. Among PGPR genera, *Bacillus* species are particularly important due to their endospore-forming ability, environmental tolerance, long shelf-life, and proven biofertilizer potential [7, 14]. Several studies have demonstrated that *Bacillus* spp. produce volatile compounds such as acetoin and 2,3-butanediol that stimulate plant growth and induce systemic resistance responses in plants [18].

Lonar Lake, located in the Buldhana district of Maharashtra, India (19°58'N, 76°31'E), is one of the world's unique meteorite-impact soda lakes formed nearly 50,000 years ago on the basaltic Deccan Plateau [1, 4]. The lake possesses highly alkaline water (pH 9.5–11.0), elevated salinity, and mineral-rich sediments containing sodium carbonate, bicarbonates, magnesium, calcium, and iron [2, 3]. These extreme physicochemical conditions support a unique microbial ecosystem dominated by halo-alkaliphilic bacteria and archaea. Previous investigations have reported the presence of *Bacillus*, *Alkalibacillus*, *Halomonas*, methanogenic archaea, and methylotrophic bacteria in Lonar Lake [1–4, 32]. However, despite extensive ecological studies, the agricultural and biofertilizer potential of these extremophilic microorganisms remains largely unexplored. Since halo-alkaliphilic bacteria naturally survive under conditions similar to saline and alkaline agricultural soils, they may serve as effective biofertilizer inoculants for stress-prone crop production systems. Therefore, the present study was undertaken to isolate and characterise halo-alkaliphilic bacteria from Lonar Lake and evaluate their potential as plant growth-promoting biofertilizers. The study specifically focused on isolation using selective alkaline media, morphological and biochemical characterisation of the isolates, assessment of phosphate and magnesium solubilisation abilities, and evaluation of biofertilizer potential through a fenugreek (*Trigonella foenum-graecum*) plant growth bioassay under alkaline soil conditions. The findings of this investigation may contribute to the development of stress-tolerant biofertilizer formulations suitable for saline and alkaline agricultural soils.

2. Materials and Methods

i). Study Site and Sample Collection: Water samples were collected aseptically from Lonar Lake, located in the Buldhana district of Maharashtra, India (19°58'N, 76°31'E), a hyper-alkaline meteorite-impact soda lake known for its extremophilic microbial diversity [1, 2]. Sampling was carried out during the post-monsoon season when alkalinity and dissolved salt concentrations are relatively high. Approximately 500 mL of surface water was collected in sterile glass bottles previously rinsed with 70% ethanol. The pH of the lake water was measured on-site and recorded between 9.5 and 10. Samples were transported to the laboratory on ice and processed within 24 h of collection.

ii). Enrichment and Isolation of Halo-Alkaliphilic Bacteria: For microbial enrichment, 5 mL of lake water was inoculated into 100 mL of Yeast Extract Mannitol (YEM) broth adjusted to pH 9.0 using sterile 1 M Na_2CO_3 and incubated at 37°C for 48 h under static conditions. Turbid enrichment cultures were subsequently streaked onto YEM agar (pH 8 and pH 9), Pikovskaya's agar, King's B agar, and Magnesium Solubilizing Medium (MgSM), followed by incubation at 30°C for 5–7 days. Morphologically distinct colonies were selected based on colony colour, shape, elevation, texture, and margin characteristics, purified through repeated sub-culturing, and maintained on agar slants at 4°C.

iii). Morphological and Microscopic Characterisation: Colony morphology of purified isolates was recorded after 48 h incubation. Characteristics including colony colour, form, elevation, texture, opacity, and margin were observed visually. Gram staining was performed using the standard crystal violet–iodine method followed by safranin counterstaining. Heat-fixed smears were examined under oil immersion microscopy (100×) to determine Gram reaction, cell shape, and endospore formation.

iv). Biochemical Characterisation: Biochemical characterisation of bacterial isolates was carried out using standard microbiological methods. Catalase activity was determined by the formation of oxygen bubbles following the addition of 3% hydrogen peroxide solution. Amylase activity was assessed on starch agar plates by observing clear halo zones after flooding with Gram's iodine solution. Citrate utilisation was evaluated using Simmons citrate agar slants, where blue colour development indicated positive utilisation. Methyl red (MR) and Voges–Proskauer (VP) tests were performed in glucose phosphate broth to determine mixed acid fermentation and acetoin production, respectively.

v). Preparation of Biofertilizer Inoculant and Seed Treatment: Purified bacterial isolates were inoculated into Nutrient Broth and incubated at 29 ± 2°C for 8–9 days to achieve maximum cell density and sporulation. The resulting bacterial suspension was used directly as the biofertilizer inoculant. Fenugreek (*Trigonella foenum-graecum*) seeds were surface sterilised with 70% ethanol and rinsed thoroughly with sterile distilled water. Seeds were coated with 5% jaggery solution as an adhesive agent and immersed in the bacterial suspension for 15–20 min. Control seeds were treated similarly using sterile distilled water instead of bacterial inoculum.

vi). Plant Growth Bioassay: Treated and untreated fenugreek seeds were sown in plastic cups containing unsterilised agricultural soil with pH 8.5. Each treatment consisted of two replicate cups containing 15 seeds each. The experimental setup was maintained under greenhouse conditions at 29 ± 2°C with regular watering. Germination percentage was recorded on the 7th day after sowing, and after 21 days, randomly selected seedlings were harvested for measurement of root length, shoot length, and total leaf number per plant.

vii). Statistical Analysis: All experiments were conducted in triplicate, and data were expressed as mean ± standard deviation (SD). Statistical significance between control and treated groups was analysed using Student's *t*-test, and differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Isolation and Colony Characteristics

Visible turbidity developed in all alkaline enrichment flasks after 48 h of incubation at 37°C, confirming the presence of viable halo-alkaliphilic bacteria in Lonar Lake water samples. Subsequent streaking on selective media yielded multiple morphologically distinct bacterial colonies. Colony abundance and morphological diversity were consistently greater on media adjusted to pH 9 compared with pH 8, indicating that the dominant cultivable bacterial population was strongly alkaliphilic in nature.

On YEM agar (pH 9), colonies appeared cream to off-white, circular to irregular in shape, convex, and smooth to chalky in texture. Several isolates formed dense, dry colonies suggestive of endospore-forming Bacillus-type bacteria. On Pikovskaya's agar, phosphate-solubilising isolates produced distinct transparent halo zones ranging from 8–22 mm around colonies, demonstrating active mineral phosphate solubilisation. King's B agar supported the growth of pigment-producing isolates, including yellow-green fluorescent colonies under UV illumination, suggestive of siderophore-producing Pseudomonas-like organisms.

Magnesium Solubilizing Medium (MgSM) also showed visible solubilisation halos around several isolates, indicating magnesium-solubilising capability under alkaline conditions.

Table 1: Summary of Colonial Morphology and Selective Medium Characteristics

Medium	Colony Colour	Morphology	Notable Feature	Optimum pH	Probable Genus
YEM Agar (pH 8)	Cream/White	Circular, raised	Moderate growth	pH 8	Rhizobium spp.
YEM Agar (pH 9)	Cream/Off-white	Circular–irregular, convex	Maximum growth, chalky colonies	pH 9	Bacillus spp.
Pikovskaya's Agar	White/Translucent	Circular, raised	Clear phosphate-solubilising halos	pH 8–9	Bacillus spp.
King's B Agar	Yellow/Green fluorescent	Circular, smooth	Pigment and fluorescence production	pH 8–9	Pseudomonas spp.
MgSM	White	Raised, dry colonies	Magnesium-solubilising halos	pH 9	Bacillus spp.

3.2. Gram Staining and Microscopic Morphology

Microscopic examination following Gram staining revealed that the majority of isolates recovered from alkaline selective media were Gram-positive, rod-shaped, and endospore-forming, characteristics typical of Bacillus and related alkaliphilic genera. Endospores were observed in central and sub-terminal positions in several isolates.

A smaller proportion of isolates obtained from King's B agar were Gram-negative, non-spore-forming rods exhibiting fluorescence under UV illumination, consistent with Pseudomonas-type bacteria. The predominance of Gram-positive endospore-forming bacteria indicates strong adaptation to the alkaline and saline environmental conditions of Lonar Lake.

Table 2: Gram Staining and Microscopic Characteristics

Isolation Medium	Gram Reaction	Cell Shape	Spore Formation	Probable Genus
YEM Agar	Gram-negative	Rods	Absent	Rhizobium spp.
Pikovskaya's Agar	Gram-positive	Rods	Present	Bacillus spp.
MgSM	Gram-positive	Rods	Present	Bacillus spp.
King's B Agar	Gram-negative	Rods	Absent	Pseudomonas spp.

3.3. Biochemical Characterisation

All bacterial isolates demonstrated positive reactions for catalase, amylase, Simmons citrate utilisation, methyl red (MR), and Voges–Proskauer (VP) tests, indicating broad metabolic versatility and adaptation to nutrient-variable alkaline environments. Catalase positivity confirmed oxidative stress tolerance, while amylase activity demonstrated extracellular starch degradation capability. Positive citrate utilisation suggested metabolic flexibility under nutrient-limited conditions.

MR positivity indicated the production of organic acids during glucose metabolism, which may contribute to phosphate and mineral solubilisation in alkaline soils. VP-positive reactions further confirmed acetoin and 2,3-butanediol production, metabolites associated with plant growth stimulation and induction of systemic resistance in plants.

Table 3: Summary of Biochemical Test Results and Their Agricultural Significance

Test	Result	Enzyme/Pathway	Agricultural Significance
Catalase	Positive (+)	H_2O_2 decomposition	Oxidative stress resistance and root colonisation
Amylase	Positive (+)	Starch hydrolysis	Soil carbon cycling and alkaline enzyme potential
Simmons Citrate	Positive (+)	Citrate utilisation	Metabolic adaptability in rhizosphere
Methyl Red	Positive (+)	Mixed acid fermentation	Organic acid-mediated mineral solubilisation
Voges–Proskauer	Positive (+)	Acetoin production	Plant growth stimulation and ISR induction

3.4. Plant Growth Promotion Outcomes

Fenugreek seeds treated with the bacterial biofertilizer inoculant exhibited significantly enhanced growth and vigour compared with untreated controls under alkaline soil conditions (pH 8.5). Germination in treated groups occurred earlier (48–60 h) than in control groups (72–84 h), indicating improved seedling establishment and metabolic activation. Statistical analysis using Student's *t*-test demonstrated that all measured growth parameters were significantly different between treated and control groups ($p < 0.05$).

At 21 days after sowing, treated plants showed substantial increases in root length, shoot length, and leaf number. Mean root length increased from 7.5 ± 0.5 cm in controls to 11.5 ± 0.6 cm in treated seedlings, representing a 53.3% increase. Shoot length increased from 12.4 ± 0.4 cm to 15.8 ± 0.5 cm (27.4% increase), while leaf number increased from 8.0 ± 0.7 leaves per plant in controls to 12.1 ± 0.8 leaves per plant in treated seedlings, corresponding to a 51.2% improvement.

Treated seedlings also exhibited darker green foliage, denser root systems, and improved overall plant vigour, suggesting enhanced nutrient uptake and chlorophyll development. These findings strongly support the plant growth-promoting potential of halo-alkaliphilic bacteria isolated from Lonar Lake and indicate their possible application as biofertilizer inoculants for alkaline and saline agricultural soils.

Table 4: Growth Parameters of Fenugreek Seedlings at Day 21

Growth Parameter	Control (Mean ± SD)	Biofertilizer-Treated (Mean ± SD)	% Improvement	Significance
Root Length (cm)	7.5 ± 0.5	11.5 ± 0.6	53.3%	$p < 0.05$
Shoot Length (cm)	12.4 ± 0.4	15.8 ± 0.5	27.4%	$p < 0.05$
Leaf Number per Plant	8.0 ± 0.7	12.1 ± 0.8	51.2%	$p < 0.05$
Germination Time (h)	78 ± 4	54 ± 3	Reduced by ~24 h	$p < 0.05$
Leaf Colour	Light green	Dark green	Improved chlorophyll appearance	—

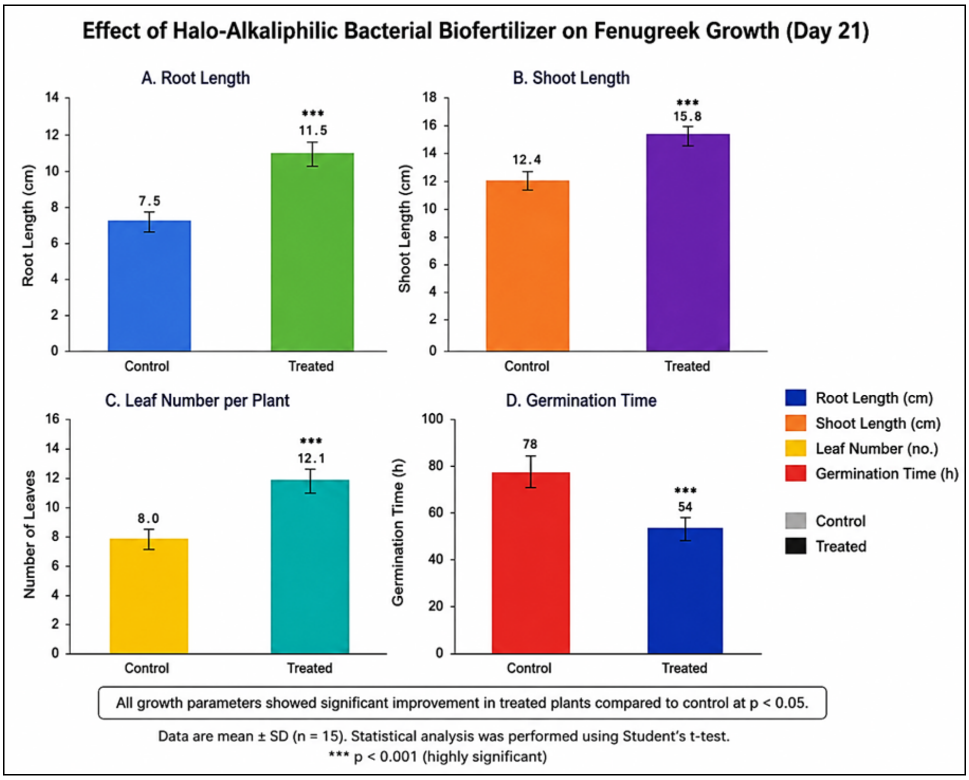

Fig 1: Effect of Halo-Alkaliphilic Bacterial Biofertilizer on Fenugreek Growth Parameters at Day 21.

Bar graphs represent the comparative effects of biofertilizer-treated and untreated control plants on (A) root length, (B) shoot length, (C) leaf number per plant, and (D) germination time under alkaline soil conditions. Data are expressed as mean ± standard deviation (SD) (n = 15). Statistical analysis was performed using Student's *t*-test, and significant differences between treatments were observed at $p < 0.05$. Error bars indicate standard deviation, while asterisks (***) denote highly significant differences between control and treated groups. Biofertilizer-treated plants exhibited significantly improved growth performance, enhanced root development, increased leaf production, and earlier germination compared with untreated controls.

Plate A and B: Comparison of fenugreek growth between untreated control and biofertilizer-treated plants at day 7 and day 21.

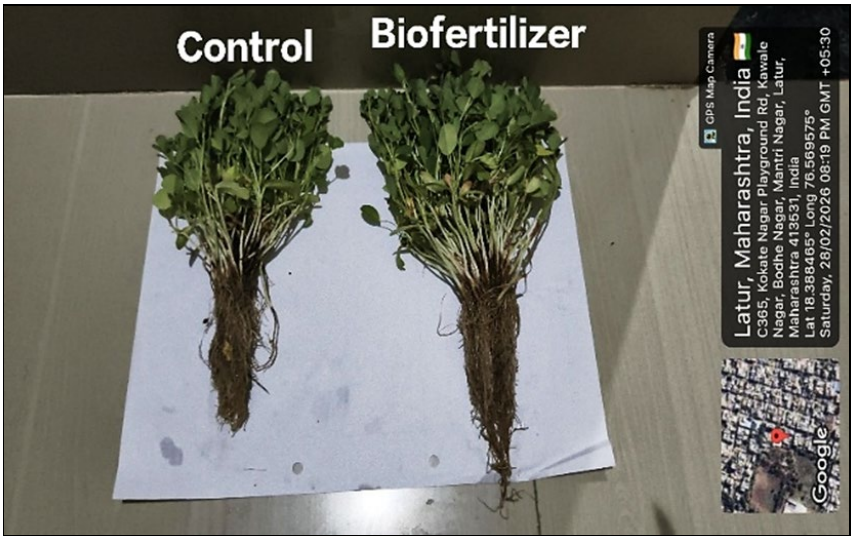

Plate C: Harvested fenugreek seedlings showing enhanced root development and shoot biomass in biofertilizer-treated plants compared with untreated controls.

4. Discussion

The present investigation demonstrated that Lonar Lake harbours diverse halo-alkaliphilic bacteria possessing significant plant growth-promoting potential under alkaline soil conditions. The successful recovery of bacterial isolates showing optimal growth at pH 9 confirms the dominance of true alkaliphilic microorganisms adapted to the extreme physicochemical environment of Lonar Lake. Similar findings have previously been reported from Lonar Lake and other soda lake ecosystems, where *Bacillus*, *Alkalibacillus*, and *Halomonas* species were identified as dominant microbial groups adapted to high salinity and alkalinity [1–4, 24, 25]. The ability of these microorganisms to survive under alkaline stress conditions makes them particularly suitable candidates for development of stress-tolerant biofertilizer formulations for saline and alkaline agricultural soils.

The predominance of Gram-positive, rod-shaped, endospore-forming isolates observed in the present study strongly suggests the presence of *Bacillus*-type bacteria. Endospore formation is an important ecological and commercial characteristic because spores provide resistance against desiccation, osmotic stress, high temperature, and nutrient limitation [7, 14]. These characteristics increase the shelf-life and field survival of microbial inoculants, making *Bacillus*-based biofertilizers more commercially viable than many non-spore-forming PGPR strains [21]. Previous reports have similarly demonstrated the agricultural importance of stress-tolerant *Bacillus* spp. for sustainable crop production under adverse environmental conditions [8, 13].

The biochemical properties exhibited by the isolates further support their plant growth-promoting potential. Universal catalase positivity among isolates indicates strong oxidative stress tolerance, which is essential for successful rhizosphere colonisation because plant roots often generate reactive oxygen species during microbial interaction [8]. Positive amylase activity demonstrates the capability of the isolates to hydrolyse starch and utilize complex carbon substrates present in soil organic matter, thereby improving microbial survival and nutrient cycling. Similarly, positive citrate utilisation reflects metabolic versatility under nutrient-limited conditions. The observed methyl red positivity suggests organic acid production, which may contribute to phosphate and mineral solubilisation under alkaline conditions where nutrient availability is often restricted due to precipitation of phosphate complexes [16].

Particularly important was the consistent Voges–Proskauer positivity among isolates, indicating the production of acetoin and related volatile compounds through the 2,3-butanediol pathway. Previous studies have shown that volatile metabolites such as acetoin and 2,3-butanediol produced by *Bacillus* spp. significantly stimulate plant growth and induce systemic resistance responses in plants [18, 19]. Therefore, the VP-positive reaction observed in the present isolates may partially explain the enhanced growth performance of fenugreek plants treated with the bacterial biofertilizer.

The plant growth bioassay demonstrated that biofertilizer-treated fenugreek plants showed significantly greater root length, shoot length, leaf number, and earlier germination compared with untreated controls ($p < 0.05$). Root length increased by approximately 53%, while shoot length and leaf number increased by nearly 27% and 51%, respectively. Enhanced root development is particularly important because larger root systems improve nutrient absorption, water uptake, and plant stability under stress conditions [27]. The darker green foliage observed in treated plants may indicate improved chlorophyll synthesis and nutrient acquisition, possibly resulting from phosphate and magnesium solubilisation activities of the isolates. Similar improvements in plant growth parameters following PGPR inoculation have been reported in fenugreek, soybean, pea, and other crop species [23, 27, 28, 33].

The faster germination observed in treated seeds further suggests that the isolates may produce growth-promoting metabolites such as indole-3-acetic acid (IAA), gibberellins, or other phytohormones that stimulate early seedling development [17, 20]. Although phytohormone production was not directly quantified in the present study, the observed plant growth responses strongly indicate the presence of multiple PGPR mechanisms operating simultaneously. The ability of halo-alkaliphilic bacteria to function efficiently under alkaline conditions represents a major advantage over conventional biofertilizer strains developed for neutral soils, which often exhibit poor survival and reduced activity in saline or alkaline environments [13].

Overall, the present study provides preliminary evidence supporting the agricultural potential of halo-alkaliphilic bacteria isolated from Lonar Lake as biofertilizer inoculants for alkaline and saline soils. However, the study was limited to morphological, biochemical, and preliminary plant growth analyses. Further investigations involving molecular identification through 16S rRNA sequencing, quantification of IAA and siderophore production, evaluation of salt tolerance ranges, and large-scale field trials are necessary to validate their commercial applicability. Nevertheless, the findings highlight the importance of extremophilic ecosystems such as Lonar Lake as reservoirs of agriculturally and biotechnologically valuable microorganisms for sustainable agriculture.

5. Conclusion

The present study successfully demonstrated the isolation and characterisation of halo-alkaliphilic bacteria from the extreme alkaline environment of Lonar Lake, Maharashtra, and evaluated their potential as plant growth-promoting biofertilizers under alkaline soil conditions. The isolates exhibited optimal growth at pH 9 and showed important plant growth-promoting characteristics including phosphate and magnesium solubilisation, catalase activity, amylase production, citrate utilisation, and positive methyl red and Voges–Proskauer reactions, indicating strong metabolic adaptability and rhizospheric competence. Morphological and microscopic observations revealed the predominance of Gram-positive, rod-shaped, endospore-forming bacteria, suggesting the presence of *Bacillus*-type organisms capable of surviving under harsh environmental conditions.

The fenugreek plant growth bioassay demonstrated significant enhancement in germination, root length, shoot length, leaf number, and overall plant vigour in biofertilizer-treated plants compared with untreated controls ($p < 0.05$). Treated plants exhibited approximately 53% increase in root length, 27% increase in shoot length, and 51% increase in leaf number under alkaline soil conditions, highlighting the strong plant growth-promoting potential of the isolates. These findings suggest that halo-alkaliphilic bacteria from Lonar Lake may serve as promising biofertilizer candidates for saline and alkaline agricultural soils where conventional microbial inoculants often perform poorly.

Present study highlights Lonar Lake as a valuable natural reservoir of agriculturally important extremophilic microorganisms with significant biotechnological potential. Further studies involving molecular identification, phytohormone quantification, field-scale evaluation, and formulation development are required to validate their commercial applicability and support the development of sustainable stress-tolerant biofertilizer technologies for future agriculture.

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