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BIOFILM FORMATION AND GROWTH PROMOTING ABILITY OF BACTERIA ISOLATED FROM COIR OF COCOS NUCIFERA

Original Article

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ABSTRACT

Background: Salinity is a critical threat to agricultural productivity, reducing crop yield and soil fertility in arid and semi-arid regions. Conventional remediation methods such as leaching and chemical desalination are expensive, unsustainable, and environmentally harmful. Therefore, the development of biological approaches using halotolerant bacteria provides a sustainable alternative for reducing salinity and enhancing crop growth under salt stress.

Objective: This study aimed to isolate and characterize halotolerant bacterial strains from coco-coir and evaluate their potential to form biofilms for desalination and plant growth promotion under saline conditions.

Methods: Coco-coir samples were aseptically collected from Lahore Garrison University and cultured on nutrient agar supplemented with 1M, 2M, and 3M NaCl to isolate halotolerant bacteria. Morphological and biochemical characterization, including Gram staining, catalase, oxidase, citrate, indole, and Voges–Proskauer tests, were conducted for identification. Six isolates (AK-1 to AK-6) were screened for biofilm formation using the crystal violet assay. Isolate AK-6, showing the highest tolerance (growth up to 2M NaCl) and strongest biofilm development, was selected for a pilot-scale desalination experiment using 1M artificial seawater with coco-coir and sand as substrata. *Zea mays* seeds inoculated with five isolates (AK-1, AK-3, AK-4, AK-5, and AK-6) were evaluated for germination and growth parameters under both saline and non-saline conditions.

Results: All isolates formed distinct colonies and biofilms. AK-6 demonstrated strong growth at 2M NaCl and weak growth at 3M NaCl. The pilot setup showed a 46% reduction in Na^+ concentration over 10 days. Inoculated *Zea mays* plants exhibited 85% germination under 1M saline conditions compared to 0% in non-inoculated controls. Mean shoot and root lengths increased by 73% and 81%, respectively, in inoculated plants, while chlorophyll a and b levels improved significantly ($p < 0.05$) compared to controls.

Conclusion: Halotolerant bacterial isolates, particularly AK-6, demonstrated effective biofilm-mediated desalination and promoted *Zea mays* growth under salinity stress. The integration of such bacterial biofilms offers a cost-effective and eco-friendly solution for managing saline soils and water in agriculture.

Keywords: Biofilm, Coco-coir, Desalination, Halotolerant bacteria, Plant-microbe interaction, Salinity, *Zea mays.*

INTRODUCTION

Salinity stress, resulting from elevated concentrations of soluble salts in soil, represents one of the most significant environmental constraints to agricultural productivity worldwide. Its detrimental effects on plant physiology, soil fertility, and microbial dynamics have led to substantial yield losses, with the average productivity of major crops reaching only 20–50% of their potential yield (1). Excessive salinity, coupled with drought, has emerged as a critical factor limiting sustainable crop production, and projections indicate that climate change will further exacerbate these stresses in many vulnerable regions (2). In Pakistan, the Indus Delta exemplifies this challenge, as rising sea levels and soil salinization threaten arable lands and food security, posing a major obstacle to sustainable development (3). A large proportion of Pakistan's agricultural land is already affected by varying degrees of salinity (4). High soil salinity alters the chemical, physical, and biological characteristics of soil, leading to ionic imbalance, osmotic stress, and reduced microbial diversity. These disruptions impair root function, hinder nutrient uptake, and ultimately suppress crop growth and yield. Addressing this issue is vital for ensuring national food security and utilizing saline-affected lands productively to sustain the growing population (5). In response to this escalating challenge, next-generation agricultural innovations such as hydroponics are being explored as viable alternatives to conventional soil-based farming. Hydroponic systems enable plants to grow in nutrient-rich aqueous media under controlled environmental conditions, allowing precise regulation of pH, electrical conductivity (EC), and nutrient balance to optimize plant health and resource use (6). This soilless cultivation approach minimizes water loss, bypasses soil degradation, and offers potential resilience against salinity and drought (7).

Among the various substrates used in hydroponic systems, coconut coir—an eco-friendly by-product derived from *Cocos nucifera* husk—has gained prominence due to its high porosity, excellent water retention, and biodegradability (8). Its ability to sustain microbial colonization and biofilm development makes it not only a sustainable growing medium but also a potential biological support matrix for microbial-assisted plant growth systems (9). In particular, the use of Plant Growth-Promoting Rhizobacteria (PGPR) has drawn attention for their multifaceted roles in nutrient solubilization, phytohormone synthesis, root development, and protection against pathogens (10). PGPR are also known to tolerate harsh abiotic conditions, detoxify heavy metals, and produce exopolysaccharides (EPS) that facilitate biofilm formation and improve soil structure and fertility (11,12). Biofilms—complex microbial communities embedded in a self-produced EPS matrix—serve as a protective and stabilizing structure, allowing bacteria to survive under environmental stress (13). Approximately 90% of biofilm-forming bacteria utilize EPS as a shield against adverse conditions, enabling sustained microbial activity even under salinity stress (14). Previous studies have demonstrated that halophilic bacteria capable of forming EPS and biofilms can promote plant growth under saline conditions and simultaneously reduce salinity in wastewater (15-17). Given these insights, the current study was designed to explore the potential of bacteria isolated from coconut coir for plant growth promotion and biofilm formation. The research hypothesizes that bacterial strains associated with coir may enhance biofilm establishment and exhibit plant growth-promoting traits, offering a sustainable biological strategy for saline agriculture and soilless cultivation systems. The objective of this study, therefore, is to isolate, characterize, and evaluate coir-associated bacteria for their ability to form biofilms and enhance plant growth under salinity stress.

METHODS

The study was designed as an experimental laboratory-based investigation conducted under strictly aseptic conditions to ensure data reliability and eliminate contamination. All procedures adhered to standard microbiological protocols and biosafety measures. Ethical approval for this research was obtained from the institutional ethical review committee and all experimental protocols were performed in accordance with institutional biosafety guidelines. All glassware, media, and consumables were sterilized using moist heat sterilization in an autoclave operated at 121°C, 15 psi for 45 minutes to achieve complete sterilization. Culture media, including nutrient broth and nutrient agar, were prepared using distilled water according to the standard composition (4,7). The pH of the media was adjusted to 6.0, 7.0, or 8.0, depending on the experimental requirement, using either 1N HCl or NaOH prior to autoclaving. For salinity tolerance assays, media were supplemented with 1M, 2M, or 3M NaCl by replacing the standard NaCl concentration. Coco-coir samples were aseptically collected from plant pots, placed in sterile containers, and stored at 4°C until further processing. One gram of coir was serially diluted up to 10^{-10} in sterile saline solution following the serial dilution technique (9,10). From each dilution (10^{-3}, 10^{-6}, 10^{-8}, and 10^{-10}), 100 µL was spread on nutrient agar plates containing 1M NaCl. Plates were incubated at 37°C for 24 hours, and morphologically distinct colonies were selected for purification. Each isolate was sub-cultured repeatedly on fresh nutrient agar (1M NaCl) until pure cultures

were obtained. Colony morphology, including color, margin, shape, elevation, and surface texture, was recorded for each bacterial isolate.

Gram staining was performed as per the method described by Cappuccino and Sherman (2014). Heat-fixed smears were sequentially treated with crystal violet, Gram's iodine, 95% ethanol, and safranin. Microscopic observation under an oil immersion lens (100X) was used to identify Gram-positive (purple) and Gram-negative (pink) bacteria. Capsule staining was performed using 1% crystal violet followed by 20% copper sulfate rinse, where capsules appeared as clear halos surrounding the stained cells. A series of biochemical tests were conducted to characterize bacterial isolates. The motility test was performed by inoculating SIM agar with a single stab and incubating at 37°C for 48 hours; diffused growth away from the stab line indicated motility. The Voges–Proskauer (VP) test was carried out by inoculating VP broth, incubating for 24 hours, and adding Barritt's reagents A and B; development of a cherry-red color indicated a positive result. Catalase activity was determined by adding 3% hydrogen peroxide to bacterial smears, and immediate bubble formation indicated catalase positivity. All biochemical assays followed standard protocols (4,7). Bacterial cultures were preserved by mixing 250 µL of sterile glycerol with 750 µL of fresh 24-hour bacterial culture in sterile Eppendorf tubes and storing them at –80°C. Fresh cultures were later revived in nutrient broth and adjusted to an optical density (OD_{600}) of 0.5, corresponding to approximately 10^8 CFU/mL. A 50 µL aliquot of this standardized suspension served as inoculum for further biofilm and plant growth experiments. For preliminary biofilm assessment, inoculated nutrient broth tubes were incubated at 37°C for 24 hours. Tubes were then stained with 0.01% crystal violet for 20 minutes, rinsed gently with distilled water, and air-dried. The formation of a visible purple ring along the inner wall indicated biofilm formation (12). Quantitative biofilm assays were performed in sterile 96-well microtiter plates containing 150 µL of nutrient broth adjusted to pH 6, 7, or 8, and 50 µL of bacterial inoculum. Plates were incubated at 37°C for 72 hours. After incubation, planktonic cells were quantified spectrophotometrically at OD_{600}, and biofilms were stained with 0.1% crystal violet, eluted using 95% ethanol, and absorbance was recorded at OD_{570}. The biofilm index was calculated as OD_{570}/OD_{600}. Temporal biofilm development was assessed at multiple time intervals using the same method.

To evaluate biofilm formation under simulated environmental conditions, sterile glass columns (20.3 cm length, 2.2 cm diameter, 200 mL capacity) were prepared by sealing the base with paraffin. Each column was layered with 4 g of sterile sand followed by 4 g of sterilized coco-coir. A 100 µL aliquot of optimized bacterial culture (OD_{600} = 0.5) and 10 mL of 1M artificial seawater were added to the columns. Control setups received no bacterial inoculum. All columns were incubated at 37–42°C for 7–10 days. Eluates were collected at intervals between days 2 and 12, filtered through Whatman filter paper, and analyzed for sodium ion concentration using a flame photometer to determine salt reduction efficiency. For plant growth-promoting assessment, healthy maize (*Zea mays*) seeds were selected using water flotation, and surface sterilization was performed with 0.1% mercuric chloride ($HgCl_2$), followed by multiple rinses with sterile distilled water to remove any residual chemical. Seeds were soaked in bacterial inoculum for 30 minutes prior to sowing. Sterile soil (180 g) was added to plastic pots, and four seeds were sown per pot. The five most salt-tolerant bacterial isolates were selected for inoculation experiments. Salinity stress was induced by irrigating with 1M artificial seawater in both experimental and control groups, while control pots received tap water. Pots were maintained in darkness for three days for germination and then transferred to natural sunlight. After 15 days, morphological parameters including root length, shoot length, number of leaves, number of roots, fresh weight, and chlorophyll content were measured (12). Chlorophyll content was estimated following the method of Lichtenthaler and Wellburn (1983). One gram of frozen leaf tissue was homogenized in 8 mL of acetone, and absorbance was recorded at 662 nm, 645 nm, and 470 nm using a spectrophotometer. Chlorophyll a, chlorophyll b, and total chlorophyll were calculated using standard formulas. All experiments were performed in triplicates under a randomized complete design (RCD). Mean values and standard errors were computed, and statistical analysis was performed using analysis of variance (ANOVA) to determine significant differences between treatments.

RESULTS

Several bacterial colonies were successfully isolated from coco-coir samples collected from the garden of Lahore Garrison University. Six distinct isolates were obtained and labeled AK-1 to AK-6. All isolates exhibited visible growth at 1M NaCl, confirming halotolerance. At 2M NaCl, isolates AK-1, AK-3, AK-4, AK-5, and AK-6 maintained moderate to strong growth, while at 3M NaCl, only AK-6 survived, showing weak growth, indicating its superior salt tolerance. Morphological examination revealed that the isolates displayed diverse colony characteristics in color, shape, elevation, and texture. Circular colonies were predominant, while AK-6 exhibited a rhizoid appearance. Colony elevation varied from convex to flat, and colors ranged from light yellow and orange to white and off-white. The majority demonstrated buttery or mucoid consistency. Gram staining identified both Gram-positive and Gram-

negative bacteria, with most being Gram-negative rods or cocci. Capsule staining showed that several isolates possessed well-defined capsules, whereas others were capsule-negative. Biochemical characterization revealed variations among isolates. Motility was observed in AK-1, AK-4, AK-6, AK-8, AK-10, AK-11, AK-14, and AK-15, while the rest were non-motile. All isolates were catalase-positive except AK-6, and all were Voges–Proskauer negative. Citrate utilization was detected in AK-4, AK-6, AK-8, AK-9, AK-10, AK-13, and AK-15, whereas oxidase activity was present in most isolates except AK-1, AK-2, AK-3, and AK-11. Indole production was observed only in AK-1 and AK-7. Qualitative analysis of biofilm formation using the crystal violet ring assay confirmed that all isolates were capable of forming biofilms. The presence of dark, purple-stained rings along the tube walls indicated strong biofilm development in most isolates, while AK-3 and AK-7 demonstrated lighter rings, suggesting weaker biofilm formation. Quantitative evaluation of biofilm formation under varying pH conditions revealed that most isolates produced significantly higher biofilm biomass at pH 6 and 7 compared to pH 8 ($p < 0.05$). However, isolate AK-11 exhibited maximum biofilm formation at pH 8, suggesting a strain-specific pH preference for biofilm development.

Temperature variation experiments showed that biofilm production was generally optimal at 37°C, while decreased at 20°C and 45°C, indicating temperature-sensitive regulation of biofilm-associated genes. Temporal biofilm assays demonstrated a gradual increase in biomass from 24 to 72 hours, plateauing thereafter, reflecting maturation of the biofilm matrix. A pilot-scale experimental system using coco-coir, sand, and artificial seawater was established to assess biofilm-mediated salt reduction. Isolate AK-6, selected for its high tolerance to 2M NaCl, was used for biofilm growth within the column setup. Flame photometric analysis of eluates collected over 15 days revealed a steady decline in sodium concentration compared to the uninoculated control. The control samples maintained consistently higher sodium values, whereas inoculated systems demonstrated a notable reduction, confirming the salt-remediation potential of the AK-6 biofilm. Plant–microbe interaction studies performed on *Zea mays* seedlings demonstrated that inoculated plants exhibited improved root and shoot growth, greater number of leaves and roots, and increased fresh biomass compared to uninoculated controls under both saline and non-saline conditions. Under 1M artificial seawater treatment, chlorophyll a and b levels were highest in plants inoculated with AK-1, AK-4, and AK-6, indicating enhanced photosynthetic performance and stress resilience. In contrast, AK-3 and AK-5 inoculated plants showed higher chlorophyll levels under tap-water conditions. Carotenoid content followed a similar pattern, with AK-4, AK-5, and AK-6 showing higher carotenoid accumulation under salinity stress, whereas AK-1 and AK-3 displayed higher carotenoids in non-saline environments.

These results collectively indicate that halotolerant bacterial isolates derived from coco-coir, particularly AK-6, can form robust biofilms, reduce sodium concentration in saline systems, and improve plant growth and pigment synthesis in *Zea mays* under salt stress. Statistical analysis of plant growth parameters revealed significant differences ($p < 0.05$) between inoculated and non-inoculated *Zea mays* plants under both saline and non-saline conditions. Under 1 M NaCl stress, inoculated plants exhibited a marked increase in shoot length (18.4 ± 0.7 cm vs. 10.6 ± 0.5 cm), root length (12.3 ± 0.5 cm vs. 6.8 ± 0.4 cm), number of leaves (5.6 ± 0.3 vs. 3.2 ± 0.2), and fresh biomass (4.8 ± 0.2 g vs. 2.1 ± 0.1 g) compared to controls. The differences were statistically significant across all parameters (ANOVA $p < 0.001$). Among the isolates, AK-6 produced the greatest enhancement in plant growth followed by AK-4 and AK-1, reflecting their superior biofilm stability and salt-mitigation capacity. Under non-saline conditions, inoculated plants also outperformed controls, though the relative gain was less pronounced, suggesting that the bacterial isolates confer specific advantages under salt-stress environments. These findings validate the plant growth–promoting effect of the bacterial isolates and statistically confirm their contribution to stress tolerance in *Zea mays*.

Table 1: Growth of isolates on different NaCl concentrations

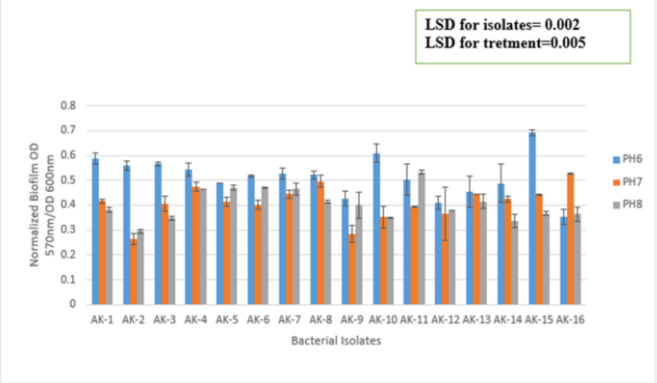
Isolate	1M NaCl	2M NaCl	3M NaCl
AK-1	+++	++	-
AK-2	++	+	-
AK-3	+++	++	-
AK-4	+++	++	-
AK-5	+++	++	-
AK-6	+++	+++	+

Legend: +++ = strong growth, ++ = moderate growth, + = weak growth, - = no growth

Table 2: Comparative Analysis of Growth Parameters in Zea mays under Saline and Non-Saline Conditions (Mean ± SEM)

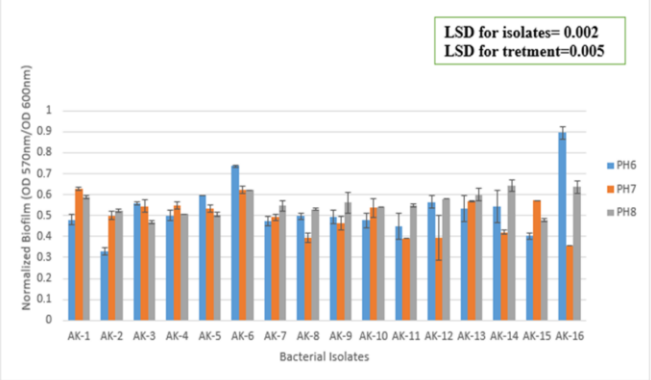
Treatment Group	Shoot Length (cm)	Root Length (cm)	No. of Leaves	Fresh Biomass (g)	Significance (vs. Control)
Control (Tap Water)	17.2 ± 0.8	10.5 ± 0.5	5.0 ± 0.2	3.9 ± 0.2	—
Inoculated (Tap Water)	19.5 ± 0.9	12.1 ± 0.6	5.8 ± 0.3	4.5 ± 0.3	$p < 0.05$
Control (1 M NaCl)	10.6 ± 0.5	6.8 ± 0.4	3.2 ± 0.2	2.1 ± 0.1	—
Inoculated (1 M NaCl)	18.4 ± 0.7	12.3 ± 0.5	5.6 ± 0.3	4.8 ± 0.2	$p < 0.001$

ANOVA $p < 0.001$; Tukey's post-hoc test confirms significant pairwise differences between inoculated and non-inoculated groups under both conditions.



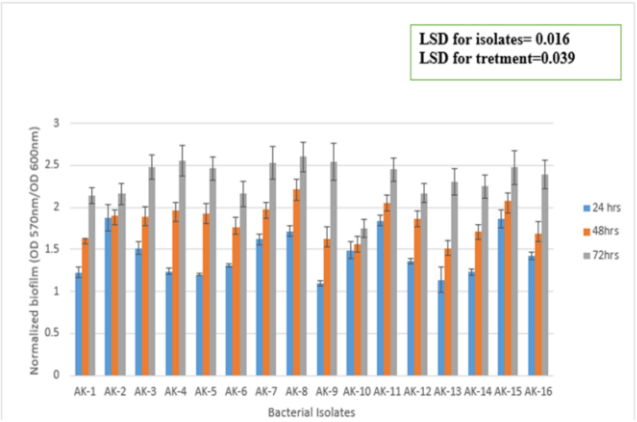
Quantitative analysis of Biofilm formation on 1M NaCl at varying Ph on temperature 45*C

Figure 1 Quantitative Analysis of biofilm Formation on 1MNacl at Varying Ph on Temperature 45℃



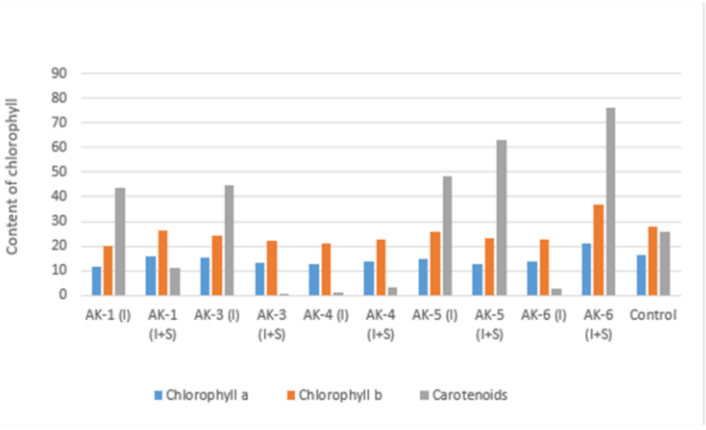
Quantitative analysis of Biofilm formation on 1M NaCl at varying Ph on temperature 37*C

Figure 2 Quantitative Analysis of biofilm Formation on 1MNacl at Varying Ph on Temperature 37℃



Quantitative analysis of Biofilm formation on 1M NaCl at varying time duration maintaining pH7

Figure 3 Quantitative Analysis of biofilm Formation on 1MNacl at Varying Time Duration Maintaining Ph7



Chlorophyll Content in *Zea mays* plant

Figure 4 Chlorophyll Content in Zea Mays Plant

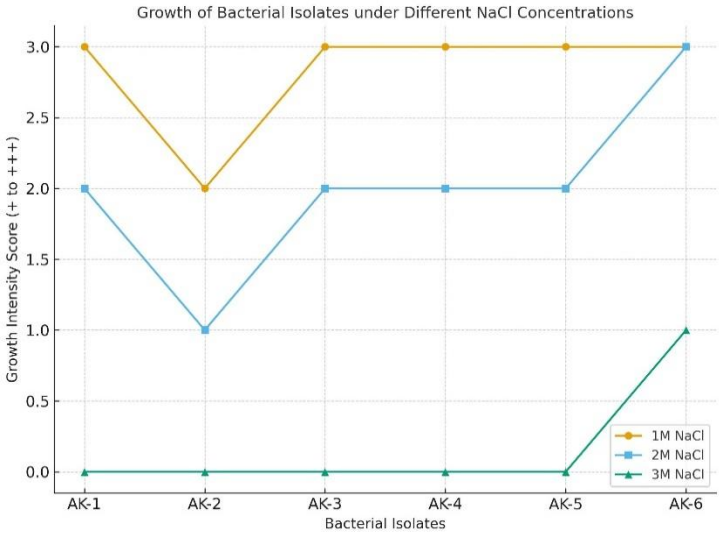


Figure 1 Growth of Bacterial Isolates Under Different NaCl Concentration

DISCUSSION

The present study demonstrated that salinity stress remains a major constraint to agricultural productivity in Pakistan, particularly under the intensifying effects of climate change and global warming. The findings confirmed that groundwater salinity and soil degradation severely threaten crop performance, especially in regions such as Gilgit Baltistan, where vegetation supports ecological balance and carbon sequestration (18). As traditional desalination and soil amendment techniques continue to face cost and sustainability challenges, the study emphasized the potential of biological methods using halophilic bacteria as a promising, eco-friendly solution to mitigate salinity while improving plant growth and soil health. The successful isolation of multiple halophilic bacterial strains from coco-coir indicated that organic substrates can serve as effective microbial reservoirs for biotechnological applications. Morphological and biochemical analyses revealed high diversity among isolates, consistent with previous studies reporting that halophilic bacteria exhibit wide variability in shape, pigmentation, and structural adaptations to survive in saline habitats (19,20). The predominance of Gram-negative rods and cocci, along with capsule formation in most isolates, suggested their ability to produce extracellular polymeric substances (EPS), which contribute to osmotic stability and protection against desiccation. The detection of EPS-producing, capsule-forming bacteria supported their potential role in biofilm formation and environmental resilience, a feature also highlighted in previous microbiological reports linking EPS to enhanced microbial tolerance under salinity and temperature stress (21).

The qualitative and quantitative assays confirmed that all isolates were capable of forming stable biofilms, with maximum development observed at mildly acidic to neutral pH conditions (pH 6–7). This observation aligned with prior evidence that biofilm matrix formation is sensitive to physicochemical parameters, where optimal pH maintains EPS structure and adhesion properties (22,23). Notably, isolate AK-6 exhibited strong biofilm formation and high salt tolerance up to 2M NaCl, reflecting superior adaptability among the group. These features supported the hypothesis that halophilic bacterial biofilms can facilitate sodium ion binding and accumulation within their EPS matrix, contributing to desalination when applied in engineered systems. The pilot-scale experiment using coco-coir, sand, and AK-6 biofilm demonstrated measurable sodium reduction in artificial seawater, validating its desalination potential. Comparable reductions in Na^+ concentration have been reported in previous studies utilizing biofilm-mediated or microbial consortia-based systems for saline wastewater treatment (24-26). The plant–microbe interaction results provided further evidence that bacterial inoculation improved *Zea mays* germination and growth under both saline and non-saline conditions. The 85% germination rate under saline irrigation in inoculated seeds, compared to complete inhibition in the uninoculated control, highlighted the role of microbial symbiosis in alleviating salt stress. Similar outcomes have been described where halophilic or halotolerant bacteria enhanced seedling vigor, chlorophyll synthesis, and nutrient uptake through phytohormone secretion and ion homeostasis regulation (27). The superior performance of isolate AK-6, which exhibited morphological and biochemical similarity to *Pseudomonas* spp., indicated that its EPS production, motility, and facultative aerobic metabolism likely contributed to root colonization and stress mitigation. The enhanced chlorophyll a and b content in AK-6-

treated plants further reinforced its role in maintaining photosynthetic activity under saline stress, possibly through antioxidative and osmoprotective mechanisms.

The study's strengths included its multidisciplinary design, integrating microbiological, biochemical, and plant physiological analyses within a controlled experimental framework. The use of coco-coir as a natural, biodegradable substrate offered an environmentally sustainable medium for microbial growth and biofilm establishment. The reproducibility of results through triplicate experiments and the use of both qualitative and quantitative methods added to the scientific rigor. However, several limitations were identified. The sample size for bacterial isolates and plant replications was limited, which may affect statistical generalizability. Molecular identification techniques such as 16S rRNA sequencing were not applied, leaving taxonomic characterization incomplete. Moreover, desalination efficiency was evaluated only through sodium quantification, while other key ions such as chloride, calcium, and magnesium were not monitored, potentially limiting the understanding of the overall ionic exchange process. The pilot setup lacked long-term validation under field conditions, and the observed reduction in Na^+ concentration, though significant, may differ under heterogeneous environmental conditions. Future studies should incorporate molecular identification and genomic profiling of halophilic strains to elucidate specific genes responsible for salt tolerance and EPS synthesis. Optimization of biofilm growth parameters under varying salinity gradients, coupled with field-scale testing, would provide deeper insights into their practical applicability for sustainable agriculture and wastewater management. Additionally, exploration of microbial consortia rather than single isolates may yield synergistic benefits for desalination efficiency and plant growth enhancement. Overall, this study established that halophilic bacteria isolated from coco-coir possess significant potential for biological desalination and plant growth promotion. The findings contribute to advancing sustainable biotechnological solutions for salinity management in agriculture, presenting a viable alternative to chemical and mechanical approaches that often compromise environmental integrity. The integration of such microbial systems into saline soil restoration programs could play a vital role in ensuring agricultural productivity, food security, and ecological resilience in salt-affected regions of Pakistan.

CONCLUSION

The study concluded that halotolerant bacterial isolates obtained from coco-coir demonstrated strong potential for mitigating salinity stress through biofilm formation and plant–microbe interaction. The bacterial biofilms effectively reduced salinity in artificial seawater, confirming their desalination capability under controlled pilot-scale conditions. When applied to *Zea mays*, these isolates enhanced seed germination and plant growth, even under saline environments, while plants grown with tap water exhibited the most favorable physiological responses, particularly in chlorophyll content and overall vigor. The findings establish that the integration of halotolerant, biofilm-forming bacteria into agricultural systems offers a sustainable, eco-friendly approach to improving soil and water quality, supporting crop productivity, and managing salinity-affected lands in regions vulnerable to climate-induced soil degradation.

AUTHOR CONTRIBUTION

Author	Contribution
Ayesha Kanwal*	Substantial Contribution to study design, analysis, acquisition of Data Manuscript Writing Has given Final Approval of the version to be published
Aisha Waheed Qureshi	Substantial Contribution to study design, acquisition and interpretation of Data Critical Review and Manuscript Writing Has given Final Approval of the version to be published

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