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Antimicrobial and plant growth-promoting activity of *Bacillus subtilis* isolated from mangrove soil

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Abstract *Bacillus subtilis* strain was isolated from mangrove soil and evaluated for its biocontrol and plant growth-promoting potential. The strain exhibited key traits including indole-3-acetic acid (IAA) production, biofilm formation, and tolerance to high salinity (up to 10% NaCl). It showed broad-spectrum antimicrobial activity, inhibiting the growth of both fungal and bacterial pathogens. Antifungal activity was demonstrated using well-diffusion and dual-overlay assays, effectively suppressing *Aspergillus niger*, *Rhizopus arrhizus*, and *Mucor mucedo*. Antibacterial effects were observed against *Escherichia coli*, *Bacillus albus*, and *Xanthomonas* sp. The cell-free supernatant of the isolate significantly inhibited fungal spore germination, as confirmed by turbidity assays. LC-MS analysis of the supernatant revealed the presence of antimicrobial compounds, including palmitic acid derivatives, fatty acid amides, and pyridine-based molecules. Bio-priming wheat seeds with this *Bacillus* sp. led to enhanced seed germination and increased shoot and root lengths, especially under saline soil conditions. These findings highlight the potential of this mangrove-derived *B. subtilis* strain as a promising bioinoculant for sustainable agriculture, particularly in salt-affected environments.

Keywords: *Bacillus subtilis*, Biocontrol agent, Antibacterial, Antifungal, Plant-growth-promoting bacteria (PGPB)

Introduction

Global annual production of vital food crops in 2020 included 761 million metric tons of wheat (Guarin *et al*., 2022), while rice production was 755 million metric tons in 2019 (Bin Rahman and Zhang, 2023). The loss of these crops due to pests and pathogens was estimated to be approximately 21.5% in wheat, and 30% in rice (Savary *et al*., 2019). In order to prevent crop loss due to pathogenic fungi and pests, most farmers use a variety of fungicides and pesticides. As of 2019, 2 million tonnes of pesticides, out of which 17.5% are fungicides, were used to mitigate crop loss due to pathogenic fungi and pests (Sharma *et al*., 2019).

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The extensive use of fungicides and pesticides has an adverse effect on the food chain and the environment. Pesticides can harm beneficial insects, birds, fish, and other non-target organisms, disrupting ecosystems and reducing biodiversity, e.g., beetles are major predators of slugs that feed on seedlings but the pesticides ingested by slugs are sufficient to kill beetles. Neurotoxicity in fish has also been demonstrated due to the direct action of these chemicals on the activity of acetylcholinesterase (AChE) in fish. The presence of pesticides and fungicides in agricultural run-off contaminates soil, water bodies, and non-target vegetation. Moreover, these pesticides and fungicides do not decompose readily, and there is bioconcentration and bioaccumulation of these chemical compounds (Aktar *et al*., 2009).

Pesticides are not only harmful to the environment and the ecosystem but also to humans that come in contact with it (US EPA, 2015). Farmers come directly in contact with the pesticides and depending on the duration of exposure to the pesticide, also suffer from acute pesticide poisoning which presents as skin, eye and mouth irritation, vomiting, and respiratory distress (Boedeker *et al*., 2020).

In order to overcome the adverse effects of the extensive use of pesticides and protect the environment, it is crucial to adopt sustainable agricultural practices. These practices are designed to maintain soil fertility and enhance the quality of crops while preserving the environment. A feasible alternative to chemical pesticides is the use of plant-growth-promoting (PGP) microbes with biocontrol potential.

Plant growth promoting microorganisms have been isolated from various soils including mangrove soils (Bashan and Holguin, 2002). Mangroves exist in saline environments and PGP microorganisms isolated from mangrove rhizosphere are halotolerant and these can probably support plant growth in saline soils. Halotolerant plant-growth-promoting microorganisms isolated from mangrove soil include *Arthrobacter* spp., *Pseudomonas* spp., and *Bacillus* spp. Some of the microorganisms found in mangroves have also been reported to have biocontrol potential (Pallavi *et al*., 2023).

Bacillus subtilis is a common soil isolate known to promote plant growth. *Bacillus subtilis* enhances the growth of various plants like *Prosopis strombulifera* (Argentine screwbean), *Solanum lycopersicum* (tomato), and *Triticum aestivum* (wheat). Synergistic interactions between *B. subtilis* and root nodule bacteria have exhibited a positive effect on plant growth (Elbagory *et al*., 2022). It can also act as a biocontrol agent that can actively induce systemic resistance (ISR) in many plant crops which leads to increased resistance of the crops to various diseases and decreases the number of phytopathogenic infections (Hashem *et al*., 2019). The present study focused on the antimicrobial activity

and role of *Bacillus subtilis* DQ005496 isolated from mangrove soil in promoting plant growth.

Materials and methods

Isolation and identification of organisms from mangrove soil

A mangrove soil sample was collected from Navi Mumbai, Maharashtra (19.0762° N, 72.9881° E). Various bacterial strains were isolated and tested for growth inhibition against different fungal and bacterial species. Among them, one bacterial strain exhibited both antifungal and antibacterial activity. The organism was identified using biochemical tests and Sanger sequencing of its 16S rRNA gene.

The bacterial 16S rRNA gene was amplified using universal primers 27F (5'-CCA GAG TTT GAT CMT GGC TCA G-3') and 1492R (5'-TAC GGY TAC CTT GTT ACG ACT T-3') (Weisburg *et al*. 1991). The amplified PCR product was purified and sequenced using the BDT v3.1 cycle sequencing kit and an ABI 3500XL Genetic Analyzer. Additional internal primers were used for a near-full length sequence.

For phylogenetic analysis, up to 10 closest-neighbour sequences from different taxa among the top 1000 hits with the highest similarity were retrieved and aligned using MUSCLE aligner (Edgar, 2004). The alignment was manually inspected and used to create a consensus phylogram using a neighbour-joining algorithm with 1000 iterations in MEGA11 software (Tamura *et al*., 2021).

Determination of antimicrobial activity

A dual-overlay assay was performed to ascertain the inhibitory effect of *Bacillus subtilis* against various fungal and bacterial cultures, as described by Zhao *et al*. (2022). For the assay, two 4.0 cm long lines of bacterial culture were streaked on Nutrient agar and incubated for 48 h. Sabouraud agar medium and Nutrient agar medium were used for making the second layer with the test fungal spores (0.1mL,10^6 spores/mL) or bacterial culture (0.1mL of 0.1 OD_{600nm}) respectively. The plates were incubated at 28°C±2 and observed after 48h to confirm the presence of a zone of clearance around *Bacillus subtilis*.

Cell free supernatant preparation for turbidity assay, well-diffusion assay and fluorescence microscopy was done. To obtain the cell-free supernatant (CFS) for these assays, nutrient broth of half the strength was prepared and inoculated with *B. subtilis* for 48 h at 28°C. The CFS was acquired by centrifugation at

10,000g for 15 min (Islam *et al.,* 2022), followed by passing through a 0.22µm filter.

Well diffusion assay was performed as described below with CFS as well as the bacterial culture (Kerr, 1999). CFS was concentrated and the volume reduced to 40mL from 100mL using a rotary vacuum evaporator (40^{o}C). To determine antifungal activity the test cultures used were *Aspergillus niger*, *Rhizopus arrhizus*, and *Mucor mucedo*; to determine antibacterial activity the test cultures used were *E. coli*, *Bacillus albus*, and *Xanthomonas oryzae*. These cultures were obtained from Microbial Type Culture Collection and Gene Bank (MTCC). The test fungal spore suspension of 0.1mL (10^8 spores/mL) was surface spread on Sabouraud agar plates and bacterial cultures (0.1mL of 0.1 OD_{600nm}) were spread on Nutrient agar medium. Wells with a diameter of 6mm were cut through and 20µL of the bacterial culture, cell-free extract, concentrated cell-free extract, and saline (as control) were added to the wells. The plates were then incubated for up to 48 h to observe the zone of clearance.

Turbidity assay studied sporulation inhibition of different fungal spores in the presence of various concentrations of CFS (Somashekaraiah *et al*., 2019). To each well of the 96-well microtiter plate 10µL of fungal spore suspension along with 100µL of double-strength Sabouraud broth and 100µL of bacterial CFS was added to give 10^8 spores/mL of the test fungus.

The turbidity assay was also used to determine the inhibitory effect of the CFS against various bacteria. Each well contained varied concentrations of the CFS diluted with nutrient broth (100 µL) and 10µL of 0.1 OD_{600nm} of bacterial culture. The 96-well microtiter plates were incubated at 37°C for 24 h with orbital shaking for 10 seconds, and the absorbance at 600nm was measured every 30 min in case of bacteria and every 20 minutes for fungi using BioTek, EPOCH2 Plate Reader. The data was analysed using Gen 5 Data Analysis Software Version 2.07.17.

A fluorescence microscopic study was carried out to visualize the effects of CFS on fungal spores and bacteria. The experiment was carried out in cavity slides. Actively growing cultures of *Bacillus albus*, actively growing mycelia of *Rhizopus arrhizus* and *Aspergillus* spp. were used along with the CFS of *Bacillus subtilis* (48 h) and propidium iodide (PI). To the slides, 100µL of the bacterial or fungal cultures were added along with 1µL of 5µM of propidium iodide (PI) stain. The slides were observed at 0 h and then incubated for 2 h in a water bath in order to prevent the slides from drying out. The slides were then observed after 2 h under both white light as well as fluorescent light using the Leica M205 FCA fluorescence stereo microscopes.

Plant growth-promoting activity

IAA Production: For the quantitative determination of indole-3-acetic acid (IAA) production, nutrient broth with 0.1% tryptophan was used. The IAA production was estimated, by the Salkowski's reagent, after incubation for five days under shaking conditions (Sarker and Al-Rashid, 2013). The absorbance at 530nm was measured using BioTek, EPOCH2 Plate Reader. The data was analyzed using Gen 5 Data Analysis Software Version 2.07.17.

The IAA production was quantified in the presence of different salt concentrations as well. The medium used was nutrient broth with 0.1% tryptophan and salt (NaCl) at different concentrations (2-10%). The incubation was at 28°C for 48 h at 100 rpm (Khan *et al*., 2021). The quantification was done using the method described above.

Phosphate solubilization: *Bacillus subtilis* strain was spot inoculated on National Botanical Research Institute's phosphate growth medium (NBRIP) agar plates to check its ability to solubilize phosphate (Amri *et al*., 2023). The culture plate was incubated at 28ºC for 7 days. Isolates that showed a clear zone around the colonies were considered to be phosphate solubilizers.

Siderophore production: For the detection of siderophore production by the isolates, the culture suspension (0.2 OD_{600nm}) was spot inoculated on Chrome azurol S (CAS) agar plate and incubated at room temperature for 48-72 h. Production of siderophores was indicated by a change in the colour of the medium from blue to orange/yellow surrounding the bacterial growth (Louden *et al*., 2011).

Nitrogen fixation: To detect nitrogen fixers, the isolates were streaked on Ashby's mannitol agar plates and incubated at room temperature for 5 days. The isolates that showed visible growth on Ashby's agar plate were considered to be nitrogen fixers (Jiménez *et al*., 2011).

Potassium solubilization: For the detection of potassium solubilizers, 10µL of the culture suspension (0.2 OD_{600nm}) was spot inoculated on sterile Alexandrov's agar plate. The plate was incubated at room temperature for 4 days and checked for clear zones around the colonies (Boubekri *et al*., 2021).

Biofilm formation: *B. subtilis* was grown in nutrient broth for 18 h and the density was adjusted to 0.1 OD_{600nm}. Then, 20µL of culture was added to 200µL of Trypticase soy broth and incubated for 3 days in 96-well microtiter plates. The cells were fed with fresh media every 24 h. After 3 days, the wells were washed twice and incubated with 0.1% crystal violet for 30 min. Residual/non-reacted crystal violet was washed. Then, 200µL of 95% ethanol was added to each well for 30 min (Metzler, 2016). The absorbance was measured at 540nm using

BioTek, EPOCH2 Plate Reader. The data was analysed using Gen 5 Data Analysis Software Version 2.07.17.

Bacterial salt tolerance: The salt tolerance of *B. subtilis* was determined by inoculating 1% of the culture suspension (0.2 OD_{600nm}) in nutrient broth containing different concentrations of NaCl (2%, 4%, 6%, 8%, 10%, 12%). The tubes were incubated at room temperature for 5 days and the OD was checked spectrophotometrically at 600nm. The cultures showing growth at high salt concentrations were considered to be salt-tolerant (Panigrahi *et al*., 2020).

Germination assay and pot assay: Wheat seeds were surface sterilized by immersion in 0.1% $HgCl_2$ for 5 mins. The seeds were then washed 5 times with sterile distilled water to get rid of any traces of $HgCl_2$ and were coated with either saline (control) or bacterial suspension (*B. subtilis*, 0.1 OD_{600nm}) in a flask under shaking conditions for 60 mins to enable bacterial seed coating. Following this, the seeds were placed in a sterile petri dish containing a dry Whatmann filter paper and allowed to dry in laminar air flow for 2 h. Then, fungal spores (*Rhizopus arrhizus* 10^8 spores/mL) and *Bacillus albus* (0.1 OD_{600nm}) suspension were sprayed on some of the pre-primed or unprimed (control) seeds respectively. At least 30 different seeds were kept in six different combinations for seed germination assay (control-without any bacteria; seeds with *B. subtilis* only, seeds with spores of *Rhizopus arrhizus* only; seeds with *Bacillus albus* only; seeds with *B. subtilis* DQ005496 and spores of *Rhizopus arrhizus*; seeds with *B. subtilis* DQ005496 and *Bacillus albus*) for 5 days in a sterile Petri plate. The germination rates were checked and the experiment was repeated thrice.

After 5 days, the germinated seeds were used for pot assay (Khan *et al*., 2022). Wheat seeds, coated with bacterial isolate (*Bacillus subtilis*), were transferred to pots containing sterile soil. The pots were divided into two sets - set 1 (no NaCl added to soil) and set 2 (exposed to salt stress i.e., 320 mM NaCl). Both the sets of plants were watered regularly with tap water. Plants obtained from uncoated seeds were kept as control. After 15 days, the plants were removed and their shoot length, root length and fresh weight were measured.

Fruit infection assay

First, uniform fruits without physical injuries were selected. Then, they were immersed in a 2% sodium hypochlorite solution for 2 minutes, rinsed with sterile tap water, and air-dried for approximately 2 h in a clean plate inside a biosafety cabinet. After drying, the fruits were wounded with a sterile needle 3 times to a depth of approximately 2 mm in the middle of the fruit. *Rhizopus arrhizus* was used for infection in tomatoes and potatoes; whereas *Aspergillus*

spp was used for tomatoes only. For inoculation, the spore suspension (10^8 spores/mL) was swabbed to each wound site, while sterile saline solution served as the control. Then after an hour, bacterial suspension (*Bacillus subtillis* 10^6cfu/mL) was sprayed on some of the fruits previously inoculated with fungal spores. Inoculated fruits were then placed in a sealed container with high humidity (about 95%) at 25°C. Disease progression was monitored by measuring lesion diameters after 3 days for tomatoes and 10 days for potatoes.

Liquid Chromatography-Mass Spectrometry

To extract the potential antimicrobial compounds from the CFS, ethyl acetate was added to the CFS. The mixture was incubated on a rocker at room temperature for 18 h. The tubes containing the mixture were allowed to stand for a few hours, enabling the separation of the aqueous and organic phases. The ethyl acetate layer containing the extracted compounds was utilized as the ethyl acetate extract for further analysis (Shoaib *et al*., 2020).

The compounds present in the extracts were determined using Online Hyphenated Reversed-Phase Liquid Chromatography-Mass Spectrometry (O-HRLCMS). The Q-Exactive Plus Biopharma, Thermo Scientific, was used for the same, with Hypersil GOLD 150 x 2.1 MM, 1.9 microns (Thermo Scientific) column. The mobile phase consisted of 0.1% formic acid in Milli-Q water and acetonitrile for the optimal separation and elution of analytes. The analytes were identified utilizing the Orbitrap mass analyzer through full-scan mode mass spectrometry. The data acquisition software employed was Thermo Scientific Xcalibur, Version 4.2.28.14, while data processing was carried out using Compound Discoverer 3.2 SP1.

Statistical analysis

The data were subjected to a one-way analysis of variance (ANOVA) with a Tukey's post hoc test to evaluate the efficiency of biopriming on various plant-growth parameters (germination, shoot length, root length, number of leaves and roots). The differences between the treatment means were tested at a significance level of 0.05. The experiments were performed in triplicates in two sets, and the standard deviation was determined.

Results

Isolation and characterization of bacterial isolates from mangrove soil

In order to obtain antibiotic-producing organisms from mangrove soil samples, crowded plate technique was used. Different strains were isolated and checked for their antifungal and antibacterial activity. Among several strains, one *Bacillus* sp. exhibited anti-fungal as well as anti-bacterial properties. It was determined as Gram-positive, spore-forming rods, with the capacity to tolerate salt (NaCl) up to 10%. Based on biochemical tests and 16SrDNA sequence the isolated organism (*Bacillus* spp. or JPP), was identified as *Bacillus subtilis* (DQ005496) (supplementary Figure 1).

Antimicrobial properties of Bacillus spp. with well-diffusion, dual -overlay assay and turbidity assay

The biocontrol capacity of *Bacillus subtilis* (DQ005496) was evaluated in the present study by co-culture techniques against a variety of species, with a focus on *Rhizopus arrhizus* and *Bacillus albus*. *Bacillus subtilis* (DQ005496) exhibited remarkable antimicrobial activity, demonstrating its potential as a natural agent against a wide range of fungi and bacteria. The antimicrobial activity of *Bacillus subtilis* DQ005496 was determined using techniques such as well-diffusion, dual-overlay and turbidity assay. The diffusion of the antimicrobial substance creates zones of inhibition, depicted as clear areas around the well/streaks. Larger zones indicated greater antimicrobial potency (Figure 1).

Turbidity assay was performed to determine the antifungal potential of bacterial cell-free supernatant. Instead of fungal cultures, spore suspension was added to the wells of a 96-well plate with or without the bacterial CFS. The absorbance of the wells increases only if the spores germinate and produce fungal mycelium. In the case of fungal spores from *Rhizopus* spp. and *Aspergillus* spp., there was a significant change in optical density (OD) after 12 h between the test and control. As for bacteria, a notable change between the test and control was observed after 7 h (Figure 2).

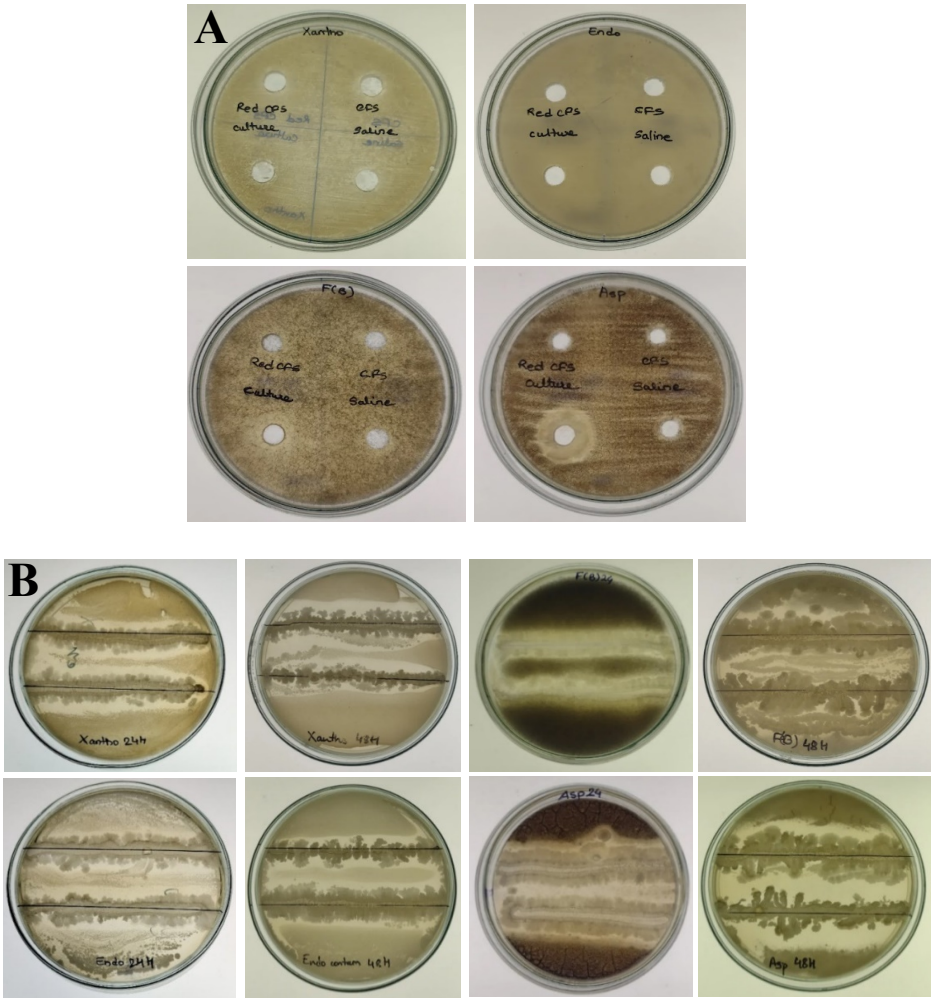


Figure 1. Antimicrobial activity of *B. subtilis* DQ005496 against plant pathogens (A) Well-Diffusion Assay: Representative image of a well-diffusion assay to assess antimicrobial activity. Wells were bored and impregnated with a test substance (culture suspension 0.1 OD_{600n} of *B. subtilis* DQ005496 or cell-free supernatant 25µL) on an agar plate swabbed with target microorganisms. (B) Dual Overlay Assay: Representative image of a dual overlay assay to assess antimicrobial activity. Parallel streaks of *Bacillus subtilis* DQ005496 2 cm apart were overlayed with plant pathogens (10^8/mL fungal spores or 0.1 OD_{600nm} of the target bacterial suspension) after 24 h. (A) and (B) A zone of clearance was observed after 48 h due to the production of antimicrobial substances.

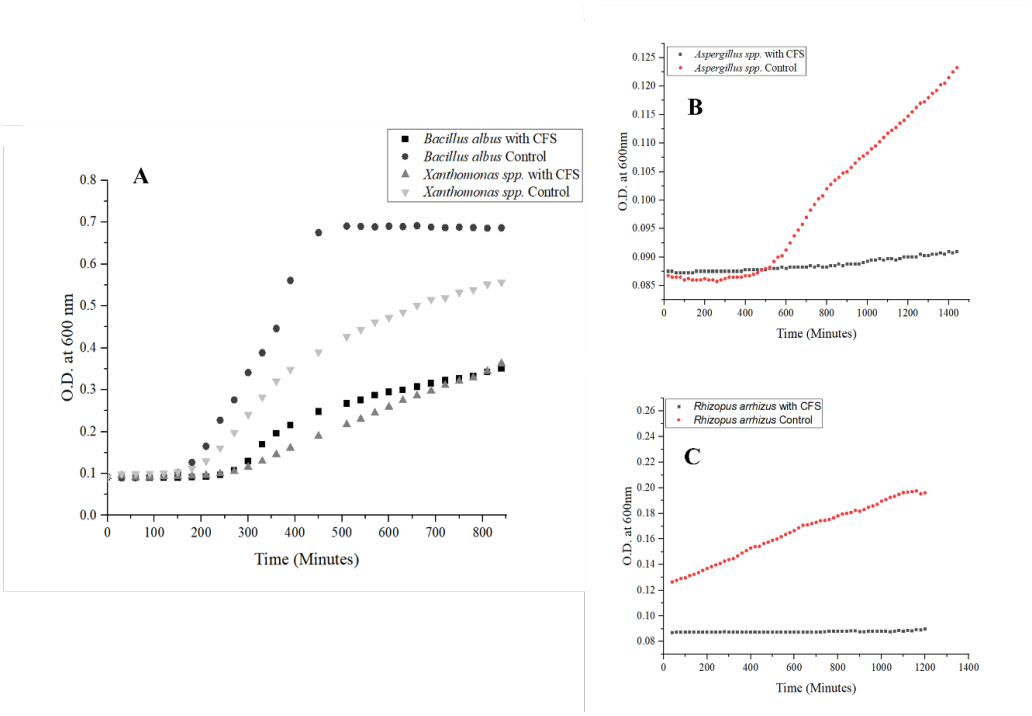


Figure 2. Graphical representation of turbidity Assay for bacteria (A) and fungi [(B) *Aspergillus niger*, (C) *Rhizopus arrhizus*]. 96-well microtiter plates were loaded with 100µL of cell-free supernatant and target organism (20µL of bacterial culture suspension 0.1 OD_{600nm} or 20µL of fungal spores at a concentration of 10^8/mL). Growth was monitored by measuring the absorbance at 600nm. (A) Bacteria: a notable change in the inhibitory effect against bacterial strains was observed after 7 h between the test and control. (B, C) Fungi: a significant change in the inhibitory effect against fungi was observed after 12 h. Data is representative of sets of triplicates.

Staining of bacterial and fungal spores with Propidium Iodide

Propidium iodide (PI) stain is a fluorescent dye commonly employed to assess cell viability, serving as a valuable tool for distinguishing live cells from those with compromised cell coverings. *Rhizopus arrhizus* and *Bacillus albus* cell suspensions were kept for 4 h with the concentrated *Bacillus subtilis* cell-free supernatant along with propidium iodide. A clear increase of red fluorescence was observed in both the cultures after 2 h. This experiment indicates the degradation of the fungal spore, mycelial cell wall and bacterial cell wall due to the cell-free supernatant of *B. subtilis* (Figure 3).

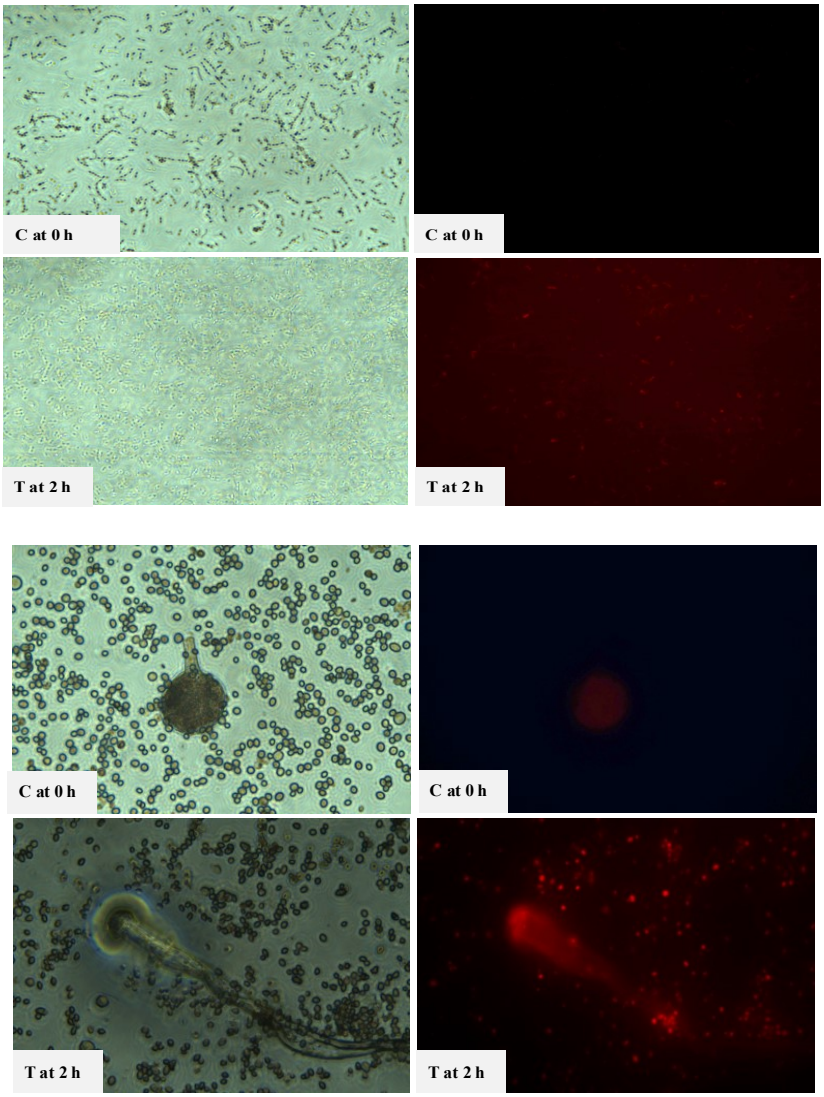


Figure 3. Representative micrograph illustrating propidium iodide (PI) staining for cellular viability assessment. A) *Bacillus albus* and B) *Rhizopus arrhizus* were treated with cell-free supernatant of *B. subtilis* DQ005496 for 2 h. Dead or damaged cells with compromised cell wall, uptake PI, resulting in red fluorescence upon binding to DNA. Live cells with intact membranes exclude PI and remain unstained.

Plant growth-promoting activity of Bacillus subtilis

The IAA production by *B. subtilis* was higher (28.74 µg/mL) after 6 days (Figure 4A). It was observed that 2% NaCl added to tryptophan containing

nutrient broth could enhance the IAA production to 44.78 µg/mL (Figure 4B). *B. subtilis* exhibited the capacity to form biofilms (supplementary Figure 2). Additional assays, such as phosphate solubilization, siderophore production, nitrogen fixation, and potassium solubilization, yielded negative results (data not shown) for this isolate.

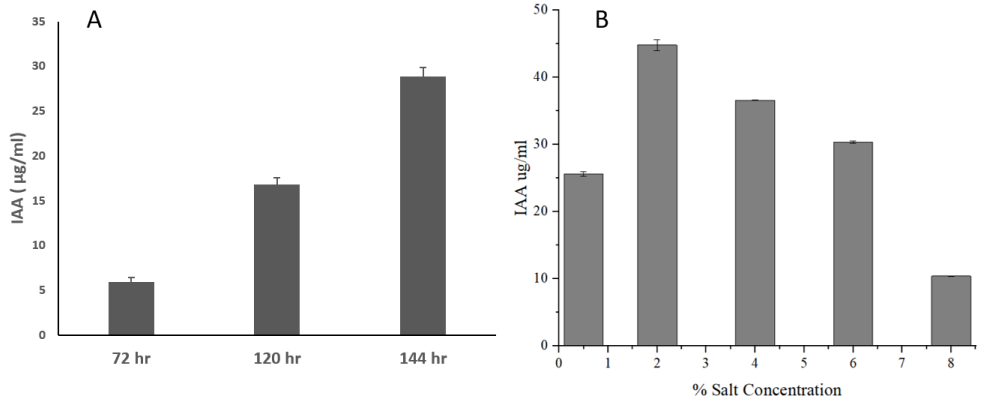


Figure 4. A) The indole-3-acetic acid (IAA) production by *B. subtilis* DQ005496 cultured in nutrient broth with 0.1% tryptophan for 144 h. IAA concentration was measured in µg/mL, and error bars represent standard deviations of 3 replicates. B) IAA production under varying salt concentrations: indole-3-acetic acid (IAA) production by *B. subtilis* DQ005496 cultured in nutrient broth with 0.1% tryptophan and salt (NaCl) at different concentrations (2-10%) for 48 h.

Among all the plant growth promotion assays, the germination assay was conducted first to validate the impact of *B. subtilis* on germination process. The germination rates were recorded five days after subjecting wheat seeds to biopriming with cultures. There was no significant difference among the germination of unprimed seeds and the *Bacillus subtilis* DQ005496 treated seeds which indicated that *Bacillus subtilis* DQ005496 was not inhibiting the natural germination rate. A reduced rate of germination was observed in wheat seeds primed with spores of *Rhizopus arrhizus* and *Bacillus albus* alone. Seeds treated exclusively with *Rhizopus arrhizus* exhibited almost no germination and displayed fungal growth. Notably, a significantly high germination rate was observed when the seeds were primed with *Bacillus subtilis* DQ005496 before the layering of fungal spores and *Bacillus albus* suspension. These findings strongly suggest that *Bacillus subtilis* DQ005496 acts as an effective biocontrol agent, inhibiting specific contaminants and enhancing wheat seeds' germination rate (Figure 5).

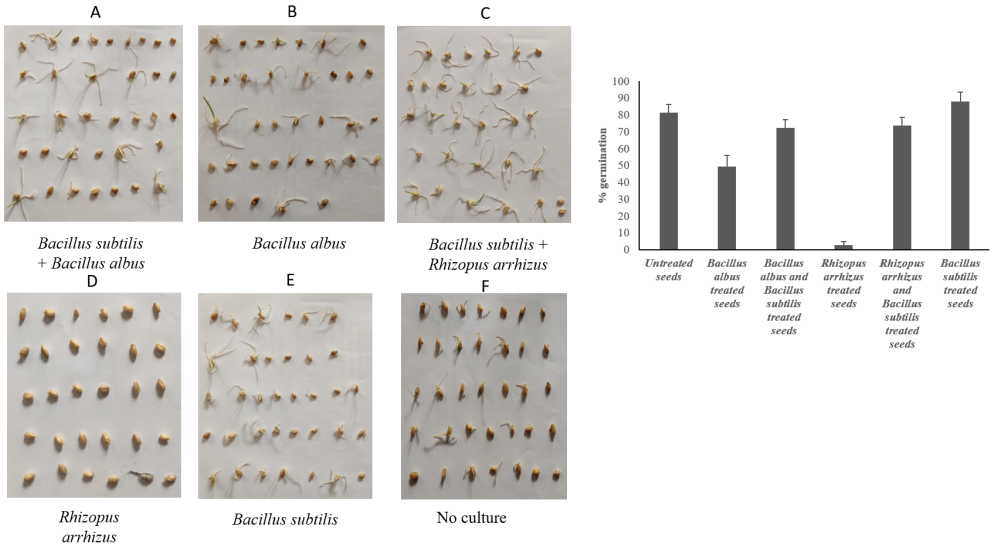


Figure 5. Germination assay: Sterilized wheat seeds were primed with either saline or bacterial suspension *B. subtilis* DQ005496 0.1 OD_{600nm}) with or without a sprayed layer of fungal spores of *Rhizopus arrhizus* (10^8 spores/mL) or *Bacillus albus* (0.1 OD_{600nm}). The seeds were kept for 5 days for germination. A to F are the representative pictures of the germination assay and the bar diagram represents the result of three such independent assays.

The effect of *B. subtilis* on plant growth promotion was carried out under salt stress conditions after biopriming the seeds and evaluating plant growth metrics, including root length, shoot length, leaf count, and root count. The plants with primed wheat seeds showed significantly increased shoot and root length in salt stress conditions after 15 days (Figure 6).

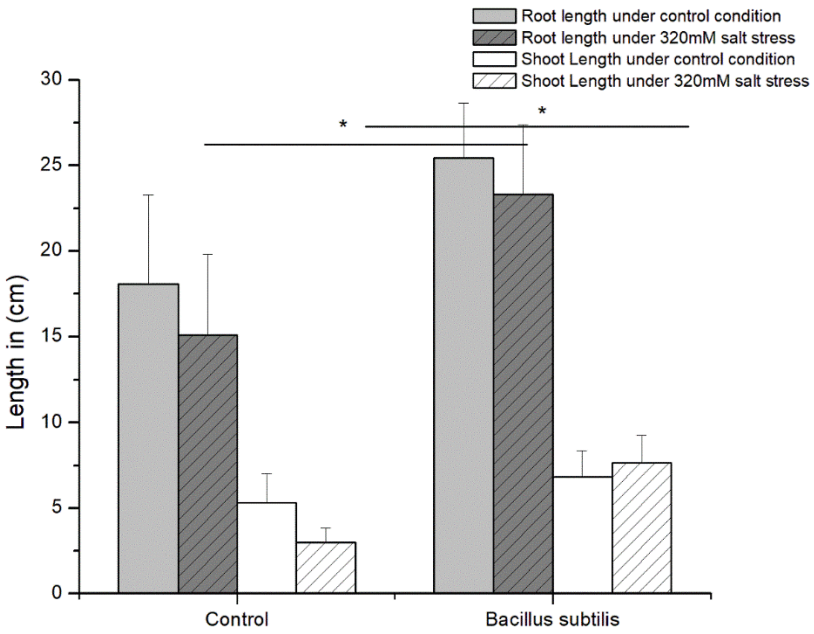


Figure 6. Pot assay evaluating the effects of *Bacillus subtilis* DQ005496 priming on sterile seeds subjected to two conditions: (1) No salt stress and (2) Salt stress of 320 mM NaCl. After a 15-day growth period, multiple parameters were measured, including shoot and root length, number of leaves (not shown), and root structure (not shown). Data are presented as mean values from 3 replicates, with error bars representing standard deviations. The statistical significance was determined using ANOVA followed by Tukey's post hoc test, *$p < 0.05$. There was a statistically significant increase in both shoot and root lengths in the test samples compared to the control samples under 320 mM NaCl salt stress.

Fruit infection assay

Rhizopus arrhizus is demonstrated its ability to infect both, tomatoes and potatoes. For a more comprehensive understanding of the biocontrol capabilities and impact of *Bacillus subtilis* DQ005496, tomatoes and potatoes were exposed to *Rhizopus arrhizus* and *Aspergillus* spp. Subsequently, they were treated with *Bacillus subtilis* cell suspension. The outcomes reveal that *Bacillus subtilis* possesses potential to avert infections and could extend the shelf life of economically significant crops, such as tomatoes and potatoes (Figure 7).

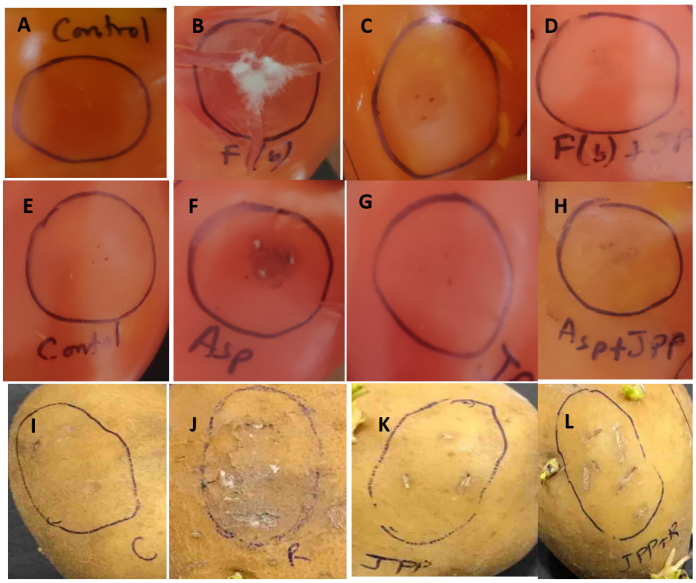


Figure 7. Representative pictures of fruit infection assay: Tomatoes and potatoes were first swabbed with 70% alcohol and then pricked with a sterile needle. The injured parts of the fruits were swabbed with fungal spores and/or sprayed with *Bacillus subtilis* suspension. Five fruits were kept per group. (A and E) Tomatoes were swabbed with saline only; (B and F) tomatoes swabbed with spore suspension of *Rhizopus arrhizus* (10^8/mL) and *Aspergillus* spp. (10^8/mL) respectively; (C and G) tomatoes pricked and sprayed with bacterial suspension (*Bacillus subtilis* 10^6cfu/mL); (D and H) tomatoes pricked and swabbed with spore suspension of *Rhizopus arrhizus* (10^8/mL) and *Aspergillus* spp. (10^8/mL) respectively. Then after an hour, bacterial suspension (*Bacillus subtilis* 10^6cfu/mL) was sprayed on them. In the next set of experiments, potatoes were taken. (I) Potatoes were swabbed with saline only; (J) potatoes swabbed with spore suspension of *Rhizopus arrhizus* (10^8/mL); (K) potatoes pricked and sprayed with bacterial suspension (*Bacillus subtillis* 10^6cfu/mL); (L) potatoes pricked and swabbed with spore suspension of *Rhizopus arrhizus* (10^8/mL). Then after an hour, bacterial suspension (*Bacillus subtillis* 10^6cfu/mL) was sprayed on them. The lesions were observed after 3 days for tomatoes and 10 days for potatoes.

Secondary metabolites in the cell-free supernatant of B. subtilis

The compounds with potential antimicrobial activity present in the extracts were determined using Online Hyphenated Reversed-Phase Liquid Chromatography-Mass Spectrometry (O-HRLCMS) (Figure 8). Several

compounds that have the potential to exhibit antimicrobial activity were detected. It included compounds with surfactant, antibacterial, antifungal activity (Table 1).

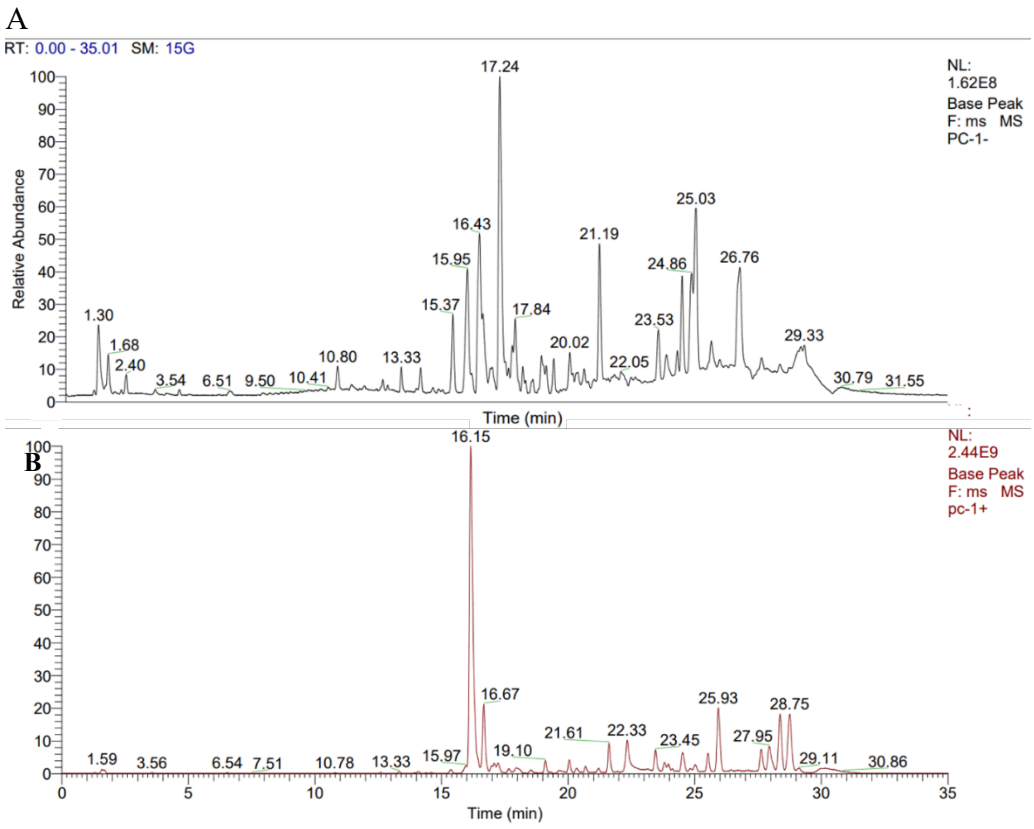
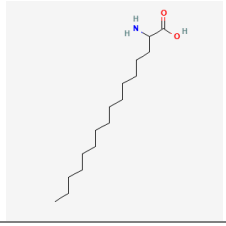
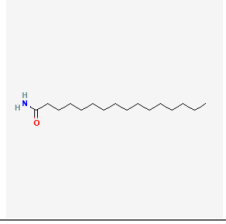
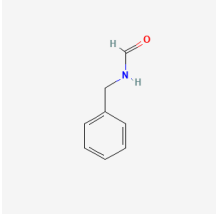
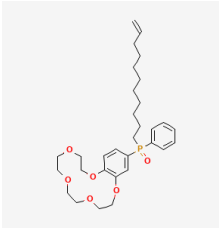
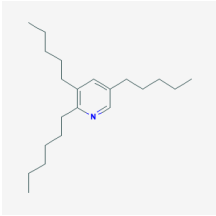
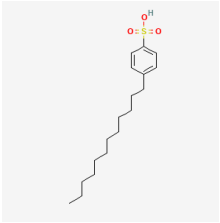
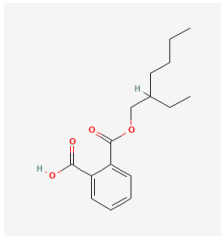
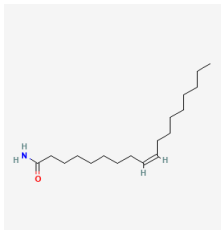
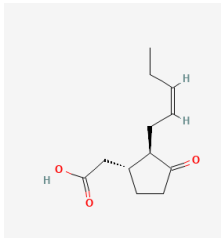


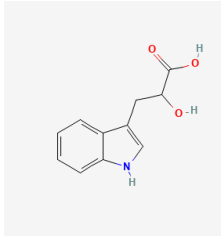
Figure 8. The ethyl acetate extract was prepared to extract the potential antimicrobial compounds from the CFS. This was then subjected to Online Hyphenated Reversed-Phase Liquid Chromatography-Mass Spectrometry (O-HRLCMS). The mobile phase consisted of 0.1% formic acid in Milli-Q water and acetonitrile for separation and elution of analytes. A) The mass spectrometric analysis for the positively charged principal components with the full-scan mode. B) The mass spectrometric analysis for the negatively charged principal components with the full-scan mode.

Table 1. LC-MS Analysis: compounds with antimicrobial activity

Sr. No.	Name	Structure	Calc. MW	RT [min]	Peak area %	Activity	Reference
1	2-Aminopalmitic acid		271.25	16.659	5.283962	Antibacterial and Antifungal	(Desbois and Smith, 2010)
2	Hexadecanamide		255.2552	25.969	3.542199	Antifungal	(Aryani *et al*., 2020)
3	N-Benzylformamide		135.0681	6.909	1.151009	Antibacterial and antifungal	(Shakhatreh *et al*., 2016)

4	2,3,5,6,8,9,11,12-Octahydro-1,4,7,10,13-benzopentaoxacyclopentadecin-15-yl(phenyl)10-undecenylphosphine oxide		544.2949	21.189	0.989664	Antibacterial and antifungal	(Şener Cemaloğlu *et al*., 2021)
5	2-Hexyl-3,5-dipentylpyridine		303.2914	19.093	0.835808	Antibacterial and antifungal	(Suyama *et al*., 1981)
6	4-Dodecylbenzenesulfonic acid		326.1912	20.013	0.22876	Antibacterial	(Wang *et al*., 2023)

7	Mono(2-ethylhexyl) phthalate (MEHP)		278.1508	20.773	0.092941	Antibacterial and antifungal	(Lotfy *et al*., 2018)
8	Oleamide		281.2708	24.505	0.039501	Antifungal	(Peng *et al*., 2023)
9	Jasmonic acid		210.125	17.021	0.020758	Plant growth, and defence	(Hashem *et al*., 2019)

10	Indole-3-lactic acid		205.073	11.343	0.013385	Antibacterial	(Zhou *et al*., 2022)

Discussion

Mangrove soils host diverse microorganisms and many of these halotolerant organisms are known to produce multiple secondary metabolites and promote plant growth. Plant growth-promoting bacteria enhance plant development either by stimulating the plant's own production of growth hormones or by synthesizing phytohormones that are readily accessible to the plant. Most species are known to increase indole acetic acid supply to plants (Olanrewaju *et al*., 2017).

Bacillus subtilis is commonly found in the rhizosphere and has exhibited plant growth promoting characteristics. *Bacillus subtilis* CW-S, reported to be a prebiotic organism increased the indole acetic acid levels, aiding significant growth in wheat and potato plants (Abuhena *et al*., 2022). *Bacillus* spp. exhibited plant growth promotion (PGP) traits such as nitrogen fixation and was found to have a positive impact on crops like potatoes, cucumber, and rice. Additionally, it was seen to enhance the growth of tomatoes by producing siderophores (Menendez *et al*., 2017). Increased IAA production by *Bacillus subtilis* NA2 at 72 h has been reported, but under salt stress decreased IAA levels were also observed (Gul *et al*., 2023). Many *Bacillus* spp. withstand high salt concentrations. *Bacillus subtilis* strain AS-4 showed a gradual increase in the optical density in the presence of 10% salt concentration (Satapute *et al*., 2012). *Bacillus subtilis* HG-15 was found to withstand high salinity of up to 30% (Ji *et al*., 2022), while *Bacillus subtilis* NA2 tolerated salinity up to 10% (Gul *et al*., 2023). *Bacillus subtilis* DQ005496 isolated in the present study produced the growth hormone IAA and also formed biofilm which may explain its ability to promote plant growth. Biofilm formation can enhance association with plant roots making the IAA available to plants. This isolate can grow in the presence of up to 10% NaCl and also produce IAA under saline conditions. This may facilitate alleviation of stress faced by plants in saline soils.

The isolate *Bacillus subtilis* DQ005496 increased the germination rate of wheat seeds and it did this even when the seeds were exposed to *Rhizopus arrhizus* or *Bacillus albus.* Root and shoot length were also significantly increased when *Bacillus subtilis* DQ005496 coated seeds were grown in saline conditions. Similar observations have been recorded by other investigators. *Bacillus subtilis* (pf4) increased germination rate by (96.5%) and root and shoot length in inoculated plants compared to un-inoculated plants (Hashem *et al*., 2019). An increase in root and shoot length was observed when maize seeds were inoculated with *Bacillus subtilis* strain HS5B5 (Song *et al*., 2023).

Plant growth and health is not just dependent on the presence of nutrients and growth hormones but also on its defence systems. Along with the inherent defence mechanisms of the plant, rhizosphere bacteria can play a crucial role by

producing antimicrobial substances that control growth of plant pathogens. *Bacillus subtilis* strain produces a variety of antimicrobial molecules that inhibit the growth of plant pathogens. *Bacillus subtilis* UTB1 has shown an inhibitory effect on *Aspergillus flavus* R5 found in pistachio nuts (Afsharmanesh *et al*., 2014). It has also shown inhibitory activity against *Aspergillus carbonarius* and *Aspergillus flavus* (Fira *et al*., 2018). Additionally, *B. subtilis* has also been active against *Xanthomonas campestris pv. campestris* strain (Monteiro *et al*., 2005). Other studies have also recorded the antifungal activity of *B. subtilis*. The supernatant obtained from *B. subtilis* FJ3 has shown antifungal activity against fungal pathogens like *Aspergillus* spp. and *Fusarium* spp. (Jan *et al*., 2023). The isolate in this study, *B. subtilis* DQ005496, has also demonstrated inhibitory activity against bacterial and fungal plant pathogens such as *Xanthomonas* spp., *Bacillus albus, Aspergillus* spp., and *Rhizopus arrhizus.* Studies have reported the role of several secondary metabolites of *Bacillus subtilis* that can act on the cell walls of other microorganisms (Su *et al*., 2020). This correlates with the data observed in this study with propidium iodide staining of *Bacillus albus* and *Rhizopus arrhizus*, where increased fluorescence indicated cell wall damage in presence of CFS. This cell wall damage may be due to one of the metabolites produced by *Bacillus subtilis* DQ005496.

The cell-free supernatant was analyzed with GCMS (data not shown) and LCMS. Several antibacterial, antifungal secondary metabolites were observed in the sample such as Quinic acid, Ethyl myristate, Hexadecanamide, Maltol, Mono-(2-ethylhexyl) phthalate (MEHP) etc. D-(-)-Quinic acid (retention time: 1.34 minutes) is known to inhibit the formation of biofilms, thereby preventing the aggregation and growth of various bacteria (Bai *et al*., 2019). Thyl myristate (retention time: 24.86 minutes) derived monomyristin shows promising antibacterial and antifungal properties against *Staphylococcus aureus, Aggregatibacter actinomycetemcomitans,* and *Candida albicans* (Jumina *et al*., 2018). Maltol (retention time: 33.25 minutes), is a chelating agent and has been identified as a potential antimicrobial agent in conjunction with small amounts of surfactants (Ziklo *et al*., 2021). Mono(2-ethylhexyl) phthalate (MEHP), various other phthalate derivatives are known to have antimicrobial activity. Mono-(2-ethylhexyl) phthalate (MEHP) is primarily known as a metabolite of di-(2-ethylhexyl) phthalate (DEHP) and has shown antibacterial activity against *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Salmonella typhimurium*, *Listeria monocytogenes*, *Escherichia coli*, and *Pseudomonas aeruginosa* (Lotfy *et al*., 2018). Additionally, CFS of *Bacillus subtilis* DQ005496 showed the presence of Indole-3-lactic acid (retention time: 11.34 minutes) and Jasmonic acid which are also crucial for plant growth, development, and defence mechanisms.

B. subtilis strain 10-4 and *B. subtilis* strain 26-D helped in prolonging the shelf life of potatoes against *Aspergillus* spp*., Phytophthora infestans,* and *Fusarium oxysporum* (Lastochkina *et al*., 2020). The present isolate, *B. subtilis* DQ005496, showed antifungal action, its ability to prolong shelf life of fruits and vegetables post-harvest was significant in the fruit infection assay. *B. subtilis* DQ005496 reduced the infection caused in tomatoes and potatoes on exposure to fungal plant pathogens like *Aspergillus niger* and *Rhizopus arrhizus.*

Therefore, *Bacillus subtilis* DQ005496 isolated from mangrove soil has the potential to function as an effective biocontrol agent and a plant growth-promoting bacterium specifically in salt stress conditions.

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Conflicts of interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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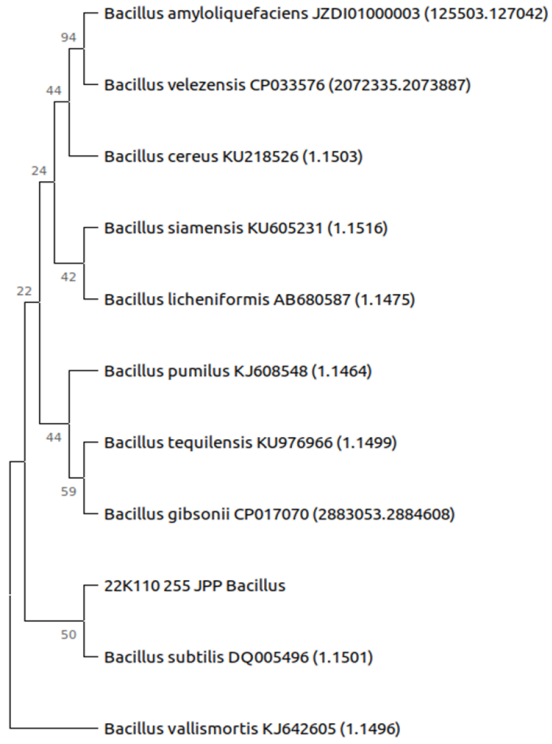
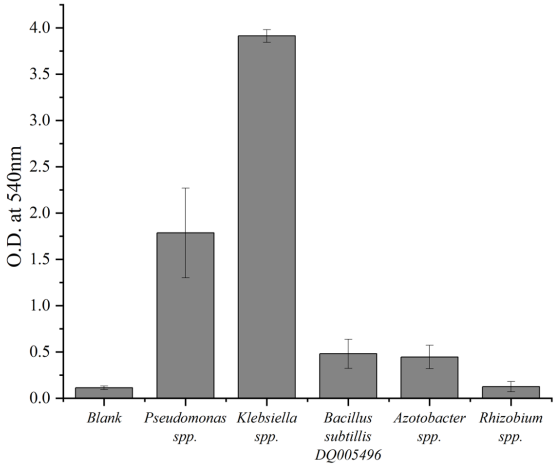


Supplementary Figure 1: Phylogenetic analysis



Supplementary Figure 2: Biofilm formation assay