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BIOFILM PRODUCTION AND ANTIBACTERIAL POTENTIAL OF *BACILLUS SUBTILIS* ISOLATED FROM SOIL

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ABSTRACT

Bacillus subtilis (*B. subtilis*) is a ubiquitous soil bacterium recognized for its probiotic potential, biofilm-forming capacity, and promising bacterium for agricultural biocontrol applications. We investigated growth characteristics, biofilm formation, and antibacterial profile of *B. subtilis* isolated from diverse soil environments. Ten soil samples from each site including animal hospital site, fruit plant rhizosphere and laboratory surroundings were collected. The soil samples were cultured on nutrient agar, Luria-Bertani agar and rabbit blood agar. Identification was confirmed via Gram's staining, spore staining and motility assays, revealing Gram-positive, rod-shaped, spore-forming, motile bacilli. Phenotypic characterization was performed using soil (0.7% agar), semi-solid (0.6% agar), and liquid (0.4% agar) media to assess colony morphology, motility and biofilm formation. Biofilm formation was quantitatively evaluated using a microtiter plate assay with crystal violet staining, demonstrating significantly higher mean absorbance values (0.37) compared to controls (0.086), indicative of robust biofilm production across isolates. Antibacterial activity was assessed using the spread plate method against *Staphylococcus aureus*, *Salmonella typhi*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, and *Escherichia coli.* Notably, selective inhibition was observed against *E. coli,* with no detectable activity against other tested pathogens. Spearman's rank correlation analysis revealed a weak association (0.434-0.789) between biofilm formation and antibacterial activity.

***Keywords*:** Biofilm, Soil bacterium, *B. subtilis*, Antimicrobial resistance.

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1. INTRODUCTION

Bacillus subtilis is a Gram-positive, motile, ubiquitous, and spore-forming bacterium; commonly found in a wide range of environments, including soil, plants, and digestive systems of animals, and has been a focus of scientific research for more than a century (Su et al., 2020; Zeng et al., 2025). Its ecological adaptability and well-characterized genetics have made it a model organism for microbiology and biotechnology. *B. subtilis*'s strong physiological adaptations, such as endospore formation, biofilm development, and the synthesis of antimicrobial compounds, are responsible for its capacity to endure in harsh environments like high salinity and nutrient limitation (Akinsemolu et al., 2024). According to recent genomic and metabolomic studies, environmental *B. subtilis* strains harbor highly conserved biosynthetic gene clusters that produce lipopeptides and polyketides, thereby greatly enhancing their ecological competitiveness and antimicrobial efficacy (Theatre et al., 2021; Qiao et al., 2024). In agricultural contexts, *B. subtilis* enhances plant growth and soil health by improving nutrient availability through nitrogen fixation and phosphorus solubilization, producing enzymes such as proteases and amylases, and suppressing soil-borne pathogens like *Fusarium spp.* and *Rhizoctonia solani*. Additionally, its probiotic effects support animal health by improving gut microbiota, digestion, and immunity, highlighting its role in sustainable agriculture and reducing reliance on chemical fertilizers and pesticides (Mukhopadhyay et al., 2023; Ren et al., 2025). Additionally, it has been demonstrated that rhizosphere-associated *B. subtilis* strains induce systemic resistance (ISR) in plants, thereby improving resistance to a variety of phytopathogens by boosting defense signaling pathways mediated by ethylene and jasmonic acid (Arnaouteli et al., 2021).

The main adaptive feature of *B. subtilis* is its ability to form structured biofilms, which facilitate root

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colonization and enhance persistence in the rhizosphere and protect bacterial cells from environmental stress such as salinity stress and drought (Ongena & Jacques, 2021; Çam et al., 2023). Plant resilience is enhanced by biofilms, which offer a stable niche that sustains microbial activity and fortifies plant-microbe interactions. Complex quorum sensing networks and extracellular matrix elements like exopolysaccharides and amyloid fibers control the biofilm architecture in *B. subtilis.* These elements work together to improve surface adhesion, nutrient retention and resistance to environmental stressors (Vlamakis et al., 2013).

In addition to its agricultural importance, *B. subtilis* exhibits significant antimicrobial activity against a broad spectrum of human and plant pathogens. This activity is largely mediated by the production of diverse antimicrobial compounds *i.e.* surfactin, iturin and fengycin, which disrupt pathogen cell membranes and inhibit their growth (Adjei et al., 2025). *B. subtilis* is a promising candidate for alternative antimicrobial strategies because of the synergistic action of these lipopeptides, which has also been linked to strong antibiofilm activity against multidrug-resistant pathogens (Lilge et al., 2022; Santos-Lima et al., 2023). These properties highlight its potential in therapeutic applications, mainly in addressing the global challenge of antimicrobial resistance (Shinde et al., 2025).

Given the heterogeneity of soil environments, *B. subtilis* isolates exhibit considerable strain-level variability in growth characteristics, biofilm formation capacity, and antibacterial potential. Understanding this variability is essential for identifying efficient strains with enhanced functional traits for targeted applications. According to recent comparative research, strain- specific variations in secondary metabolite clusters and regulatory genes have a major impact on biocontrol effectiveness and ecological fitness (Put et al., 2024; Zeng et al., 2025). Therefore, the present study aims to characterize the growth, biofilm production, and antibacterial activity of *B. subtilis* isolated from varied soil ecosystems, with a view toward their potential utilization in sustainable agriculture and biomedical applications.

2. MATERIALS AND METHODS

2.1. Sample Collection

Soil samples were collected from five distinct locations, i.e., animal hospital soil, fruit plant soil, roadside soil, sewage water-infested soil, and laboratory soil, with two samples per site (n=10). All samples were collected aseptically using sterile spatulas and containers and transferred to the Biosafety Laboratory at the University of Agriculture, Faisalabad, Pakistan, for in vitro isolation and characterization of *B. subtilis*. All handling was performed under sterile conditions to prevent contamination.

2.2. Isolation and Culturing of Samples

Samples were homogenized in sterile distilled water and streaked onto nutrient agar. Plates were incubated at 37°C for 24-48 hours. Colonies with characteristic morphology were selected and sub-cultured on Luria- Bertani agar and blood agar for confirmation. Media preparation and sterilization followed standard protocols (Chen et al., 2024; Golnari et al., 2024).

2.3. Identification of *B. subtilis*

Isolates were identified on basis of colony morphology, Gram staining, motility and spore formation. Routine biochemical tests *i.e.* Catalase, Methyl red, Voges Proskauer, Indole, Glucose acid production and Maltose fermentation tests were performed to support identification, following standard microbiological protocols (Shoaib et al., 2020).

2.4. Growth and Stress Tolerance

Growth kinetics were evaluated in nutrient broth at 37°C. Salt tolerance was assessed by inoculating isolates onto nutrient agar supplemented with increasing NaCl concentrations (up to 10%) and incubating at 37°C. Growth patterns were compared with reference strains to differentiate *B. subtilis* from related species (Madigan et al., 2021).

2.5. Phenotypic Characterization and Biofilm Formation

Isolates were examined for growth on solid (0.7% agar), semi-solid (0.6% agar), and liquid (0.4%) agar media to evaluate colony morphology, motility and biofilm formation under varied conditions. Biofilm-forming ability was quantified using a 96-well microtiter plate assay. Overnight cultures were standardized (OD630= 0.2), incubated at 37°C for 24 h, stained with 0.1% crystal violet, and absorbance was measured at 550 nm. Assays were performed in hexaplicate and repeated thrice (Stepanović et al., 2007).

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2.6. Antibacterial Activity

Antibacterial activity was tested against *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella typhi*, and *Salmonella typhimurium* using the spread plate method. Indicator bacteria were isolated from various sources and cultured on selective media (e.g., MacConkey agar for *E. coli*, Staph1-10 for *S. aureus*). *B. subtilis* was grown in nutrient broth for 24–48 hours, and sterile filter paper pieces were soaked in the broth for 2 hours. Nutrient agar plates were swabbed with indicator bacteria, and *B. subtilis*-soaked filter papers were placed on top, incubated at 37°C for 24–48 hours. Clear zones of inhibition were recorded (Cappuccino & Welsh, 2017), with selective activity observed against *E. coli*.

2.7. Statistical Analysis

All experiments were performed in triplicate. Spearman's rank correlation was used (Wisniewski & Brannan, 2024) to assess the relationship between biofilm formation and antibacterial activity. Percentage positivity was calculated as:

$$\text{Percentage Positivity} = (\text{Number of Positive Samples}/\text{Total Samples}) \times 100$$

3. RESULTS

After being successfully isolated from soil samples, *B. subtilis* was phenotypically characterized and its ability to form biofilms and its antibacterial activity were assessed. The isolates showed morphological and physiological characteristics consistent with *B. subtilis* and demonstrated measurable biofilm formation and antimicrobial potential.

1.1. Isolation and Morphological Identification of *B. subtilis* from Soil

A total of ten soil samples were collected from different sources (Table 1) for the isolation of *B. subtilis*. Bacterial identification was performed through the observation of culture characteristics on nutrient agar. The *B. subtilis* isolates produced medium sized, round, opaque, flat-drying, gray-white colonies (Fig. 1).

Table 1: Number of samples collected for isolation of *B. subtilis* from soil

Places of sample collection	Numbers	Weight (gm/sample)	Sample (gm)	No. of isolates
Animal hospital	2	10	2	2
Fruit plant soil	2	10	2	2
Garbage water infested soil	2	10	2	2
Road side soil	2	10	2	2
Laboratory soil	2	10	2	1

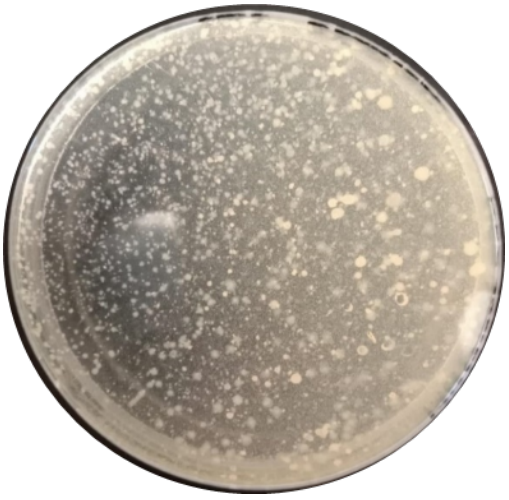

Fig. 1: Representative colonies of *B. subtilis* on nutrient agar. Colonies from soil-derived isolates (n=10) were medium sized, round, opaque, flat and gray-white in color, displaying consistent morphology across all samples.

1.2. Growth and Colony Characteristics of *B. subtilis* on Blood and LB Agar

Colonies previously confirmed as Gram positive bacilli, motile and spore forming on nutrient agar were further streaked on LB and rabbit blood agar. Plates were then incubated for 24-48 hours at 37°C. On blood agar, colonies appeared gray-white, round, flat, opaque, and exhibited complete hemolysis. However, on LB agar, colonies appeared as round, flat, gray-white, medium sized and opaque (Fig. 2).

1.3. Biochemical Characterization of *B. subtilis*

Biochemical characterization of *B. subtilis* was performed for confirmation. The results showed that the isolates were capable of metabolizing glucose with acid production, while no fermentation was observed for lactose or maltose. The Voges-Proskauer (VP) test was positive, indicating production of acetoin, whereas both the indole production and methyl red (MR) tests were negative as shown in Table 2.

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1.4. Phenotypic Adaptation of *B. subtilis* Across Solid, Semi Solid and Liquid Media

The phenotypic responses and growth patterns as shown in Fig. 3 of *B. subtilis* were evaluated across solid, semi-solid and liquid media. These conditions allowed assessment of changes in motility and biofilm formation as shown in Table 3.

Table 2: Biochemical profile of *B. subtilis* isolates recovered from soil samples (n=10)

Chemical tests	Observations	Results
Catalase test	Bubble and gas development	Positive
Methyl red test	No color change	Negative
Voges Proskauer test	Color change	Positive
Indole test	No ring formation and no color change	Negative
Glucose acid production	Gas formation	Positive
Maltose fermentation	No gas production	Negative
Growth in high salt concentration	Characteristic growth observed	Positive

Table 3: Phenotypic characterization of *B. subtilis* on solid, semi-solid and liquid mediums isolated from soil (n=10)

Parameters	Solid media	Semi solid media	Liquid medium
Agar consistency	0.7%	0.6%	0.4%
Motility	No motility	Slide motility	High motility
Biofilm	Thick biofilm	Slide biofilm	Thin biofilm
Observations	On solid media bacterial growth proliferate and colonies expanded both in vertical and horizontal directions	bacterial cells infiltration observed. on semi solid medium Collective migration of bacteria while proliferating and consuming nutrients	In liquid interface motile cells swim individually. *B. subtilis* restrict growing cells. Construct pellicle where Oxygen is high

Fig. 2: Representative picture of *B. subtilis* isolated from soil on Blood agar and LB agar (n=10).

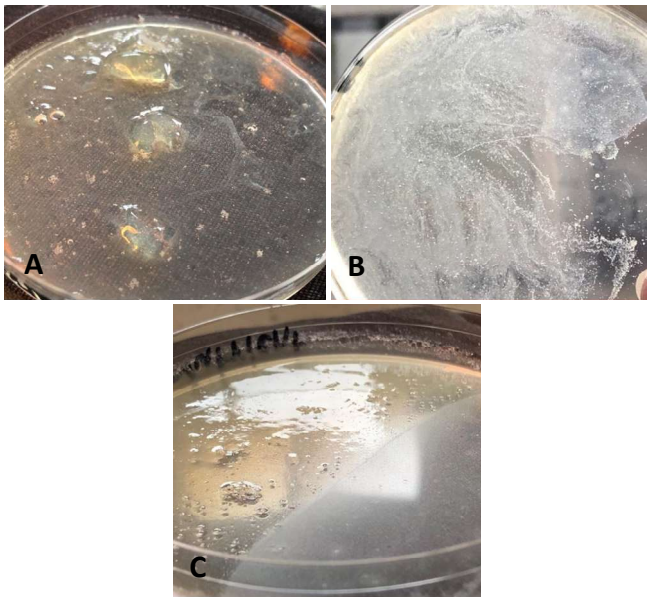

Fig. 3: Representative figures showing the growth and phenotypic behavior of *B. subtilis* on (A) solid media, (B) semi-solid media and (C) liquid media, including colony formation, collective motility with ring patterns and pellicle formation at the air-liquid interface.

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1.5. Assessment of Biofilm Production in *B. subtilis*

The biofilm-forming ability of the tested *B. subtilis* isolates was evaluated using the crystal violet assay. Based on the calculated OD values, the isolates exhibited varying degree of biofilm formation as shown in Fig. 4. Out of the total isolates, isolates 3,4 and 8 were categorized as weak biofilm producers. Isolates 1, 2, 7, 10, 11 and 12 demonstrated moderate biofilm-forming ability, while isolates 4, 6 and 9 were identified as strong biofilm producers. However, isolate 13 served as the negative control and showed no significant biofilm formation.

1.6. Antimicrobial Activity of *B. subtilis* against Pathogenic Microbes

The antibacterial activity of *B. subtilis* isolates was evaluated against five different pathogenic bacteria isolated from different sources. In result, zone of inhibition was only observed against *E. coli*, while no zone of inhibition was detected against *S. aureus*, *P. aeruginosa*, *S. typhi* and *S. typhimurium* depicted in Fig. 5. The antibacterial positivity rate was 20% (1/5). Overall, the isolates showed limited, narrow spectrum antibacterial activity despite universal biofilm formation.

4. DISCUSSION

The well-studied Gram positive, spore forming bacterium *B. subtilis* is found in soil and diverse environmental niches. Its physiological adaptability, which includes sporulation, motility, biofilm formation, and the synthesis of various bioactive secondary metabolites, is largely responsible for its ecological success (Zhang et al., 2025). Because of these characteristics, it is a valuable organism in biotechnology, agriculture, and biomedical applications, especially as a probiotic substitute for antibiotic growth promoters and a biocontrol agent (Ramírez-Pool et al., 2024; Gayithri et al., 2026). In the current study, *B. subtilis* was isolated from soil samples in the current investigation and identified using morphological, microscopic, and biochemical traits. The isolates' rod-shaped, Gram-positive morphology, motility, and spore formation were all in line with the species' typical taxonomic descriptions. Biochemical profiling (catalase positive, Voges-Proskauer positive, indole and methyl red negative) further verified their identity, while colony morphology on LB and blood agar displayed typical opaque, irregular growth patterns. These results align with the phenotypic traits of environmental Bacillus isolates that have been previously documented (Zeigler & Perkins, 2021).

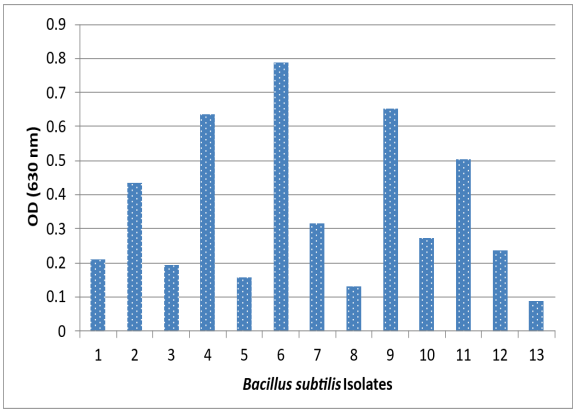

Fig. 4: Biofilm formation by *B. subtilis* isolates assessed using a microtiter plate assay. Cultures were adjusted to OD_{600}=0.2, stained with crystal violet, and absorbance measured at 550nm using a Thermo Multiskan ™ FC reader (n = 10).

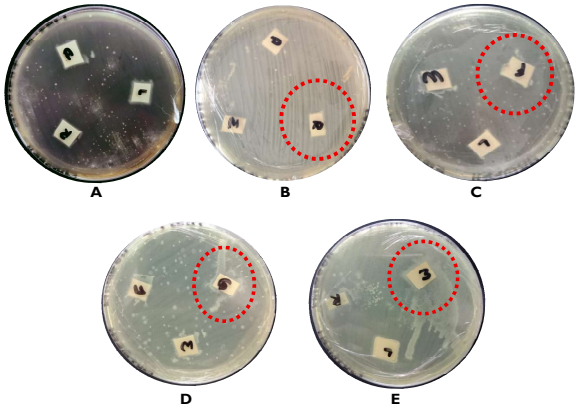

Fig. 5: Antibacterial activity of *B. subtilis* isolates against five pathogenic bacteria. (A) a zone of inhibition was observed only against *E. coli*. No zone of inhibition was detected against (B) *S. aureus* (C) *P. aeruginosa* (D) *S. typhi* (E) *S. typhimurium* respectively.

The isolates exhibited unique developmental behaviors on solid, semi-solid, and liquid media, demonstrating strong adaptability across various growth conditions. Interestingly, pellicle formation at the air–liquid interface was seen, which is indicative of *B. subtilis* developing structured biofilms in oxygen-rich environments. This species' highly coordinated multicellular process of biofilm formation is controlled by intricate genetic networks and environmental cues (Vlamakis et al., 2013). In soil ecosystems, these surface-associated communities promote long-term persistence and improve survival under stress (Chen et al., 2023).

The ability to form biofilms was demonstrated by every isolate in this investigation, suggesting that soil-derived *B. subtilis* populations share this trait. Extracellular polymeric substances (EPS), such as polysaccharides

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and structural proteins, which offer mechanical stability and environmental protection, are the main mediators of biofilm formation in *B. subtilis* (Vlamakis et al., 2013). Biofilm development in *B. subtilis* is tightly regulated by complex genetic networks involving Spo0A-dependent pathways, quorum sensing systems, and environmental signal integration, allowing the bacterium to adapt dynamically to soil and rhizosphere conditions (Wu et al., 2025). The ecological fitness of *B. subtilis* as a dominant member of soil microbial communities and its potential role in plant-microbe interactions and rhizosphere colonization are highlighted by the universal presence of biofilm formation among isolates (Sharma et al., 2026; Gayithri et al., 2026).

The current study found limited antibacterial activity despite strong biofilm-forming ability. Only *E. coli* was inhibited, while *S. aureus* showed no activity. This implies strain-dependent variability in the production of antimicrobial compounds, a phenomenon in *B. subtilis* populations that has been extensively studied. The production of antimicrobial metabolites such as surfactin, iturin, and fengycin is highly regulated and strongly influenced by environmental conditions and genetic background (Qin et al., 2025).

The calculated antibacterial positivity rate of 20% (1/5) further supports the limited antimicrobial spectrum observed under the experimental conditions. The absence of activity against *S. aureus* may be attributed to its clinical origin and potential resistance mechanisms, as pathogenic strains often exhibit increased tolerance to antimicrobial peptides and metabolic inhibitors (Flemming et al., 2016; Alam et al., 2025). This highlights how crucial target strain variability is when assessing environmental isolates' antimicrobial efficacy.

Crucially, the findings show a weak relationship between antibacterial activity and biofilm formation. Antibacterial activity depends on the biosynthesis of specialized secondary metabolites, whereas biofilm formation is primarily linked to environmental adaptation, surface colonization, and survival strategies. Strong biofilm producers do not always have high antimicrobial activity because these processes are independently regulated (Bais et al., 2021; Saiyam et al., 2024). Therefore, in *B. subtilis* isolates, biofilm formation should not be regarded as a direct predictor of antibacterial potential.

This study demonstrates that, in laboratory settings, *B. subtilis* is a highly adaptive, potent biofilm-forming bacterium with limited, strain-dependent antibacterial activity. Its antimicrobial potential is contingent and necessitates additional molecular research to maximize metabolite synthesis and regulatory comprehension.

5. CONCLUSION

In conclusion, soil-derived *B. subtilis* isolates demonstrated significant phenotypic diversity across various environments and a strong capacity to form biofilms. Only *E. coli* was susceptible to their restricted and selective antibacterial activity, with a weak correlation to biofilm formation. These results underline the need for additional molecular-level research while supporting its potential in biocontrol and agriculture.

Declarations

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Data Availability: All the data is available in the article.

Ethics Statement: This study did not require ethical review, as it did not involve human or animal subjects.

Author's Contributions: MIA conceptualized the study. NH performed laboratory work and analyzed the results. AA BM, BM, IA contributed to writing, data analysis, reviewing and proofreading the manuscript.

Generative AI Statements: The authors declare that no Gen AI/DeepSeek was used in the writing/creation of this manuscript.

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