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Isolation and Characterization of Bacillus Subtilis BSP1 from Soil: Antimicrobial Activity and Optimization of Fermentation Conditions

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Abstract: This study focused on the isolation, characterization, and evaluation of the antimicrobial and antioxidant activities of a crude extract from *Bacillus subtilis* isolated from rhizosphere soil. Through biochemical and physiological assessments, followed by whole genome sequencing, the isolate was confirmed as *Bacillus subtilis* BSP1. We examined the antimicrobial activity of *B. subtilis* BSP1 metabolites against various pathogenic bacteria and fungi. To enhance its antibacterial efficacy, we optimized the fermentation medium to maximize the secretion of antibacterial agents. Our findings demonstrated that the crude extract exhibited notable antimicrobial properties against various pathogenic bacterial and fungal isolates. The antioxidant test revealed a dose-dependent increase in the extract's DPPH scavenging activity and reducing power, with an impressive 98.9% DPPH scavenging activity at 30 mg/mL. Importantly, safety assessments indicated a lack of hemolytic activity on human red blood cells, with only 1.3% hemolysis at 100 mg/mL, suggesting its potential suitability for practical applications. In summary, *Bacillus subtilis* BSP1, isolated from soil, appears to be a promising candidate for antibiotic production. Its significant antimicrobial and antioxidant properties, combined with its safety profile, highlight its potential applications in medicine, agriculture, and biotechnology.

Keywords: *Bacillus subtilis* isolate; identification; fermentation; medium optimization; antimicrobial activity; antioxidant

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1. Introduction

The growing issue of bacterial resistance to antibiotics leads to thousands of deaths annually. This challenge arises from the bacterial development of resistance to widely used antibiotics, such as Vancomycin [1]. In 2014, the World Health Organization (WHO) declared the increase in multidrug-resistant bacterial infections as a major global health challenge [1]. The main causes of the global antibiotic resistance problem are the excessive use of antibiotics, their improper prescribing practices, and their widespread application [2].

Multidrug-resistant bacteria (MDR), such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Enterobacteriaceae*, pose significant challenges in hospitals due to their role in severe infections like pneumonia and bloodstream infections [3]. The issue of multidrug resistance impacts both Gram-positive bacteria, such as *Staphlylococcus aureus*, *Streptococcus pneumoniae*, *Enterococcus faecium*, and *Enterococcus faecalis*, as well as Gram-negative bacteria, including *Escherichia coli* and *Acinetobacter baumanii* [4]. This rise in antibacterial resistance is a serious risk to health and to available medications, affecting cancer treatment, surgical procedures, and transplants [4]. To address this issue, various alternative treatments have been investigated. However, the use of natural products derived from plants is limited by the variability in their chemical composition and biological activities. These variations can be influenced by the geographical area, the time of collection, and the specific plant origin [5]. In addition, a limitation is that some plant metabolites may exhibit varying levels of effectiveness against different strains within the same genus [5]. Therefore, employing antagonistic microorganisms that produce substances to inhibit pathogens has emerged as one of the most effective solutions. A promising strategy involves using the antimicrobial peptides (AMPs) produced by bacteria classified as Generally Recognized as Safe (GRAS). AMPs are particularly appealing because they show minimal risk of resistance development compared to traditional antibiotics. Moreover, AMPs are considered safer as they decompose into amino acids, unlike other treatments that may generate potentially toxic metabolites [6]. AMPs show minimal resistance development and primarily eliminate microbes by forming pores in their membranes, making it difficult for these organisms to develop resistance. AMPs are considered as a vital class of new antibacterial, antifungal, and antiviral agents. They hold promise for treating infectious diseases, parasitic infections, and potentially even cancer and HIV infections [7].

Bacillus species, which are Gram-positive, rod-shaped, motile, and spore-forming bacteria, are commonly found in various environments, particularly in soil. Their ability to survive in extreme conditions is largely due to their capability to produce endospores through simple and rapid developmental processes [8]. Biological control agents (BCAs) are organisms that can suppress or inhibit the growth of animal or plant pathogens. The use of *Bacillus* as BCA has also been shown to reduce agricultural costs by decreasing the reliance on fertilizers and pesticides [8]. The designation of *Bacillus subtilis* as GRAS positions it as an excellent option for biological control against pathogenic microorganisms [5]. The *Bacillus* genus is known for its production of a diverse array of AMPs, positioning it as a promising candidate in the exploration of new inhibitory compounds [9]. It is estimated that at least 4–5% of the genetic material in any strain belonging to the *B. subtilis* group is involved in the synthesis of antimicrobial compounds (AMCs), which are mainly AMPs [10]. During the production of a biological product, the formation of active metabolites is significantly influenced by the characteristics of the microbial strain and the conditions of the growth medium. Several factors such as the composition of the medium, pH, temperature, and duration of cultivation affect the antibiotic capabilities of microorganisms [11]. Several studies have demonstrated that glycerol serves as an appropriate and cost-effective substrate for the biosynthesis of antimicrobial compounds by *Bacillus subtilis* [5]. The identification of the ideal composition of the growth medium and the optimization of the process conditions for *B. subtilis* cultivation are crucial for maximizing the production of the targeted antimicrobial compounds [5].

On the other hand, the generation of reactive oxygen species (ROS) can lead to oxidative harm to biological macromolecules. Oxidative stress arises from an imbalance between the production of ROS—such as superoxide anions, hydroxyl radicals, nitric oxide, and lipid radicals—and the body's antioxidant defenses, which include enzymes, proteins, and vitamins. This imbalance can be due to an excessive production of ROS or a deficiency in the antioxidant system, leading to ROS accumulation and the initiation of oxidative stress. This condition can damage cells and tissues [12]. This damage can contribute to several health issues such as atherosclerosis, heart disease, aging, and inflammatory conditions. Moreover, an overproduction of ROS has been linked to the development of cancer [13].

While synthetic antioxidants such as butylated hydroxyanisole and butylated hydroxytoluene have been widely used to prevent lipid oxidation, concerns about their safety have emerged, including potential liver damage and carcinogenic effects [14]. As a result, there has been an increased interest in discovering safer and natural antioxidants from biological resources as substitutes for synthetic ones [14]. *Bacillus* species, when employed as probiotics, can produce antioxidant enzymes such as superoxide dismutase (SOD) and glutathione, which efficiently neutralize free radicals [15].

The aim of this study was to isolate, identify, and characterize a potent antimicrobial-producing strain of *B. subtilis* from Lebanese soil. The research focused on optimizing fermentation conditions to enhance the production of antibacterial compounds by the isolated *B. subtilis* BSP1 strain. Additionally, the biocontrol efficacy of *B. subtilis* BSP1 against various pathogenic bacteria and fungi was evaluated. Furthermore, the study explored the antioxidant potential of *B. subtilis* BSP1 and performed a hemolysis analysis to assess its safety profile.

2. Materials and Methods

2.1. Isolation and Screening of Bacillus Species

Soil samples were collected from the rhizosphere of ten flower cultivation areas within Beirut Arab University's Garden in Debbieh, Lebanon, recognized as a rich source of *Bacillus* species. Using a sterile inoculating spoon, the soil samples were obtained from each location at a depth of 10–15 cm. The method used for *Bacillus* isolation was described by Gajbhiye et al. [16] with minor modifications that included adding the fungicide fluconazole to selectively favor the spore-forming bacteria while inhibiting fungal growth. Then, the soil samples collected from the ten locations were blended in sterile bags to ensure a homogenous soil mixture. To enrich the spores from the soil, 1 g of the soil mixture was mixed with 9 mL of distilled water and heated in a water bath at 80 °C for 10 min. Subsequently, 100 µL of the suspension was spread onto nutrient agar (NA) plates supplemented with fluconazole to inhibit fungal growth. These plates were incubated at 37 °C for 40 h. After 40 h of incubation, individual colonies were selected and transferred to the freshly prepared NA plates. The NA used in the study consists of the following (g/L): peptone, 5; beef extract, 3; sodium chloride, 5; and agar, 15. Finally, the characteristics of the isolated bacterial colonies were identified based on their color, shape, and Gram staining, and through a series of biochemical tests including the catalase test, Voges/Proskaver (VP) test, and mannitol fermentation test.

2.2. Molecular Identification of the Isolates

Further characterization of the bacterial isolates utilized Matrix-Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) technology (BioMérieux, Craponne, France). For more precise identification, whole genome sequencing (WGS) was performed. Genomic DNA was extracted using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, Irvine, CA, USA). This DNA served as a template for the Polymerase Chain Reaction (PCR) amplification of the target DNA regions using universal primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGCTACCTTGTTACGACTT-3′). The resulting nucleotide sequences were directly compared against the known sequences in the Genbank database.

2.3. Test-Microorganisms

The antimicrobial properties of the isolated *B. subtilis* crude extract were evaluated against a diverse range of microorganisms. The tested microorganisms include two Gram-positive bacteria (*Micrococcus luteus* and *Bacillus cereus*), two Gram-negative bacteria (*Citrobacter freundii* and *Stenotrophomonas maltophilia*), three types of filamentous fungi (*Aspergillus flavus*, *Aspergillus niger*, and *Rhizopus arrhizus*), and one yeast (*Candida albicans*). The bacterial isolates were sourced from the Specialized Microbiology Laboratory at Beirut Arab University, Debbieh, where they were isolated by Mezher et al. [17]. *Bacillus cereus*,

specifically, was isolated from the soil on the university campus between June and July. The filamentous fungi and yeast were previously detected and isolated from nuts found in the Lebanese market [18]. This selection of microorganisms aimed to evaluate the antibacterial and antifungal effectiveness of the *B. subtilis* strain isolated.

For preservation, the bacterial isolates were re-cultured on the NA slopes in test tubes and incubated at 37 °C for 24 h. Meanwhile, the fungi and yeast were maintained on Potato Dextrose Agar (PDA) slopes in test tubes, incubated at 28 °C for 48 h, and then stored at 4 °C. To ensure their viability, the bacterial isolates were transferred to fresh NA media, while the fungi and yeast were transferred to new PDA plates every two months [19]. The PDA media used for the fungi and yeast typically consists of the following (g/L): peptone, 5; HM peptone B, 1.5; yeast extract, 1.5; sodium chloride, 5; and agar, 15.

2.4. Production of B. subtilis Crude Extract

The fermentation procedure was conducted using a method outlined by Pathak and Keharia et al. [20]. Single colonies of the isolated *Bacillus subtilis* were introduced into a 1000 mL flask containing synthetic media which served as the fermentation medium to produce secondary metabolites. The composition of the fermentation media was as follows (g/L): glycerol, 10.00; L-glutamic acid, 5.00; $KH_2PO_4 \cdot 2H_2O$, 1.00; $K_2HPO_4 \cdot 2H_2O$, 1.00; $MgSO_4 \cdot 7H_2O$, 0.20; $CaCl_2 \cdot 2H_2O$, 0.02; $FeSO_4 \cdot 2H_2O$, 0.05; and NaCl, 0.01. The initial optical density (OD) at 600 nm of the bacterial culture was adjusted to 0.05 before its incubation at 30 °C for 96 h on an orbital shaker set at 150 rpm. After the incubation, the *B. subtilis* fermentation broth was centrifuged at 6000× *g* for 20 min using a centrifuge (C2006, Centurion, Aberdeen, UK). Then, the resulting pellet was dissolved in butan-1-ol. The crude extract was concentrated using a rotary evaporator (BUCHI Rotavapor r-461, Flawil, Switzerland). A 250 mL flask was used for the evaporation process, which was conducted under a vacuum of 20–30 mbar for a duration of 2–4 h. The concentrated crude extract was stored in a pre-weighed glass for further analysis.

2.5. Optimization of Fermentation Media Conditions

The various fermentation media compositions were tested to optimize the production efficiency of antibacterial peptides by *B. subtilis*. A one-factor-at-a-time approach was employed to analyze the influence of the various environmental and media factors, including temperature, incubation time, and different carbon and nitrogen sources. This method involved modifying one independent variable while maintaining the others at constant levels. The following tested sources of carbon were glycerol, glucose, and mannitol. L-glutamic acid, peptone, and sodium nitrite were examined as nitrogen sources. Additionally, different temperatures (30 °C, 37 °C, and 42 °C) and incubation times (48 h, 96 h, and 128 h) were also investigated. The optimized conditions of each factor were then combined and examined to detect the maximum antibacterial activity.

2.6. Antibacterial Susceptibility Analysis

The antibacterial activity of the crude extract of *Bacillus subtilis* was assessed using the agar disk-diffusion method against the four bacteria under study. A volume of 100 µL from a freshly prepared bacterial suspension (0.5 McFarland) was uniformly spread on a Mueller/Hinton agar (MHA) plate. Filter paper disks (6 mm) were soaked with 100 µL of different concentrations of the bacterial extract (2.5, 5, and 10 mg/mL). These saturated disks were then positioned on the surface of an MHA plate and incubated at 37 °C for 24 h. Distilled water and doxycycline (250 µg/mL) were used as control and reference antibiotics, respectively [21]. After the incubation, the size of the inhibition zone around the paper disk was determined. A positive result was defined as a zone with a diameter > 7 mm [22].

2.7. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The assay was conducted in circular 96-well microtiter plates. Initially, 10 µL of the freshly prepared bacterial suspension was combined with 90 µL of Mueller/Hinton broth (MHB) to achieve an inoculum concentration of 5×10^5 CFU/mL [21]. The composition of MHB is as follows (g/L): beef infusion form, 300; casein acid hydrolysate, 17.5; and starch, 1.5. Subsequently, 11 dilutions of the bacterial crude extract (ranging from 0.001 to 10 mg/mL) were carried out from the initial stock concentration of 80 mg/mL. A total of 100 µL of each dilution of the bacterial crude extract was added to each well to have a final volume of 200 µL in each well. Three replicates of each dilution were used. A row was designated for the reference antibacterial drug, doxycycline. In addition, a row was designated for the positive control, containing MHB and bacterial suspension, while another row served as the negative control with only MHB. The plates were incubated at 37 °C for 24 h. After the incubation, the optical density was measured at 595 nm using an ELISA microplate reader [23]. The minimum inhibitory concentration (MIC) was determined as the lowest extract concentration showing no visible growth. The MIC value was identified by the first clear well, which indicates a complete inhibition of bacterial growth [24].

To determine the Minimum Bactericidal Concentration (MBC), 10 µL from each clear well was transferred onto MHA plates, followed by incubation at 37 °C for 24 h [25]. The MBC was identified as the lowest concentration where three or fewer colonies were observed, representing a 99.9% kill rate of the tested inoculum [24].

2.8. Antifungal Susceptibility Analysis

The initial assessment of the antifungal efficacy of the crude extract involved using the disk-diffusion method outlined by Mnif et al. [26]. To evaluate its antagonistic effect on the hyphal growth of the four studied fungi, a Petri plate containing 10 mL of PDA was prepared with the studied fungus at the center. Different concentrations of the crude extract (3.75, 7.5, and 15 mg/mL, respectively) were added, and the plates were incubated at 25 °C for 5 days. Control plates were treated with distilled water only. The radial expansion was recorded every day, and the crude extract's effect in inhibiting mycelial growth was quantified as a percentage compared to the expansion observed on the control medium. The mycelial growth inhibition was calculated according to the provided formula [26]:

$$\text{Mycelial growth inhibition percentage (MGI\%)} = \frac{d_c - d_t}{d_c} \times 100$$

where d_c and d_t represent the diameters of mycelial growth in the control and treated plates, respectively.

2.9. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC)

The MIC of the crude extract was established using a double dilution method conducted in sterile 96-well microtiter plates. Each well contained 100 µL of potato dextrose broth. In total, 1.5×10^3 CFU/mL of the target fungus was inoculated into each well after dilution [21]. The fungicide Amphotericin B at a concentration of 100 µg/mL served as the positive control, while a medium without any inoculation was utilized as the negative control. The plates were then incubated at 30 °C for 48 h, and the MIC values for each fraction were determined [20]. The MIC is described as the smallest concentration that completely inhibits visible fungal growth [26].

To determine the MFC, 10 µL from each well was extracted and inoculated onto fresh PDA media, then incubated for 72 h at 28 °C. The absence of mycelial growth in the new media was considered indicative of a fungicidal effect [27]. The MFC was defined as the minimum concentration that killed at least 99.9% of the tested inoculum [28].

2.10. DPPH Radical Scavenging Assay

The antioxidant activity of the bacterial peptides was evaluated by 1,1,diphenyl1-2 picrylhydrazyl (DPPH) free radical scavenging assay according to the method described by Giri et al. [29]. Five concentrations of the crude extract were prepared and ascorbic acid was taken as a reference positive control (5, 10, 15, 20, and 30 mg/mL). For the DPPH preparation, a concentration of 0.2 mM was dissolved in methanol and kept in the dark. Then, 1 mL of the prepared DPPH was mixed with 1 mL of each sample then the tubes were left in the dark for 30 min. For the control, it was prepared by mixing 1 mL of DPPH with 1 mL of methanol instead of the tested samples. The blank sample contained distilled water. After incubation, the absorbance of each solution was recorded at 517 nm using a spectrophotometer. The results were expressed as mean values. The percentage of DPPH scavenging efficacy was determined using the equation below [30]:

$$\text{Percentage of DPPH scavenging activity } (\%) = \frac{A_c - A_s}{A_c} \times 100$$

A_c = absorbance of the control and A_s = absorbance of the sample

The values for the half-maximal inhibitory concentration (IC_{50}) were calculated based on the obtained results. The IC_{50} represents the concentration of the crude extract necessary to neutralize 50% of the DPPH free radicals in comparison to the control that lacks a sample [30].

2.11. Hemolysis Activity of B. subtilis

The hemolytic potential of the crude extract of *B. subtilis* on human blood cells was assessed following a referenced procedure [31]. The human red blood cells were type O^+ and collected from a laboratory, sourced from normal patients, and preserved with EDTA to prevent coagulation. The blood cells were separated from whole blood by centrifuging at room temperature for 10 min at 2500 rpm. These red blood cells were then washed three times with a 10 mM sodium phosphate buffer (pH 7.0) in 150 mM NaCl phosphate-buffered saline (PBS). The resulting sediment was resuspended in PBS to form a 5% (*v*/*v*) red blood cell/PBS mixture. Various concentrations of the crude extract (from 0.5 to 100 mg/mL) in the same buffer were then introduced, and the mixture was incubated for 1 h at 37 °C. After the incubation, the solutions were centrifuged at room temperature for 10 min at 2500 rpm. The optical density (OD) of the supernatant was measured at 540 nm. Positive and negative controls were implemented using SDS and phosphate-buffered saline (PBS), respectively. The percentage of hemolysis was calculated according to the following formula [25]:

$$\text{Hemolysis percentage } (\%) = \frac{OD_{(crude\ extract\ treated\ sample)} - OD_{(buffer\ treated\ sample)}}{OD_{(SDS\ treated\ sample)} - OD_{(buffer\ treated\ sample)}} \times 100$$

2.12. Statistical Analysis

All the experiments were conducted at least 3 times. The values were presented as the mean ± standard error of the mean (SEM). The statistical significance was determined between each factor and the control using the *t*-test. The calculated *p*-values were interpreted as follows: $p > 0.05$ was not considered significant (ns). Image J 1.53e (National Institute of Health, Bethesda, MD, USA) was used to measure the zone of inhibition obtained from the disk-diffusion assay. Origin 2021 (OriginLab Corporation, Northampton, MA, USA) was used for the analysis and graphs.

3. Results

3.1. Isolation and Identification of Bacterial Isolates

Nine distinct bacterial colonies were isolated from the Petri dishes to establish pure cultures. During the preliminary characterization, the Gram-stained isolates underwent microscopic observation, revealing that seven of them belonged to the *Bacillus* genus, namely

B01, B02, B03, B04, B05, B06, and B07. These isolates were characterized as Gram-positive and displayed a rod-like shape. Subsequently, a second screening involved biochemical tests, including the catalase test, Voges/Proskaver (VP) test, and mannitol fermentation test. The results obtained are listed in Table 1.

Table 1. Biochemical characteristics of *Bacillus* species isolates.

Biochemical Tests	Bacterial Colonies						
	BC01	BC02	BC03	BC04	BC05	BC06	BC07
Gram staining	+	+	+	+	+	+	+
Catalase test	+	+	+	+	+	+	+
VP test	+	+	+	+	+	+	+
Mannitol fermentation	+	+	−	−	−	+	+

BC01–07: species of *Bacillus* isolates; positive (+); negative (–).

3.2. Molecular Identification and Characterization of Bacterial Isolates

Based on the MALDI-TOF results, the isolates BC01, BC02, BC06, and BC07 discovered in the current study were identified as *Bacillus subtilis* species, while the isolates BC03, BC04, and BC05 were classified as *Bacillus cereus* species. Consequently, BC01, identified as *Bacillus subtilis,* was selected for further in vitro study. To confirm these identifications, molecular analysis WGS was conducted for the isolates BC01 and BC03. Upon the comparative analysis of the sequences of the newly isolated strains with those in the GenBank database, the bacterial strains were identified as *Bacillus subtilis* BSP1 for the BC01 isolate, with accession number SAMN41887931, and *Bacillus cereus* strain, with accession number SAMN39716293 for the BC03 isolate.

3.3. Optimization of Fermentation Media Conditions

The findings, presented in Figure 1, revealed that glycerol exhibited the most significant effect on the antibacterial activity of *B. subtilis* among the tested carbon sources, with an inhibition zone of 20 mm. Glucose also displayed antibacterial activity, albeit to a lesser extent compared to glycerol. In contrast, when mannitol was used as the sole carbon source, no observable antibacterial effect was detected. Similarly, L-glutamic acid and peptone demonstrated notable inhibitory effects with a zone diameter of 22 mm among the nitrogen sources. Among the incubation temperature and incubation time, 42 °C and 128 h, respectively, were found to be more effective. Furthermore, the combination of the optimized conditions demonstrated a larger zone of inhibition, which measured 32 mm.

3.4. Disk-Diffusion Assay for Evaluating Antibacterial Activity of B. subtilis BSP1

In this study, the four examined bacteria displayed significant susceptibility to the antibacterial effects of the *B. subtilis* crude extract. A positive result was defined as a zone with a diameter > 7 mm [22]. The results of the diameter of the zones of inhibition exhibited by the crude extract with three concentrations (2.5, 5, and 10 mg/mL) against *B. cereus*, *M. luteus*, *C. freundii*, and *S. maltophilia* are represented in Table 2. All the results were statistically significant with a p-value < 0.05. The recorded zones for the crude extract ranged between 7.8 mm and 21.6 mm.

3.5. Determination of MIC and MBC

Following the investigation of the antibacterial activity of the crude extract through the disk-diffusion assay, the broth microdilution method was employed to determine the MIC and MBC of the crude extract against the examined bacteria [32]. The MIC values, MBC values, and the MBC/MIC ratios of the crude extract are presented in Table 3. The findings regarding the MIC and MBC of the crude extract from *B. subtilis* indicated that the crude extract exhibited the lowest MIC (0.312–0.624 mg/mL) and MBC (0.624–2.5 mg/mL) values against *M. luteus* and *B. cereus*, respectively. However, Gram-negative bacteria (*C. freundii, S. maltophilia*) exhibited higher MIC values equal to 2.5 mg/mL. The MBC values were comparatively higher, ranging from 2.5 to 5 mg/mL against *C. freundii* and *S. maltophilia*, respectively.

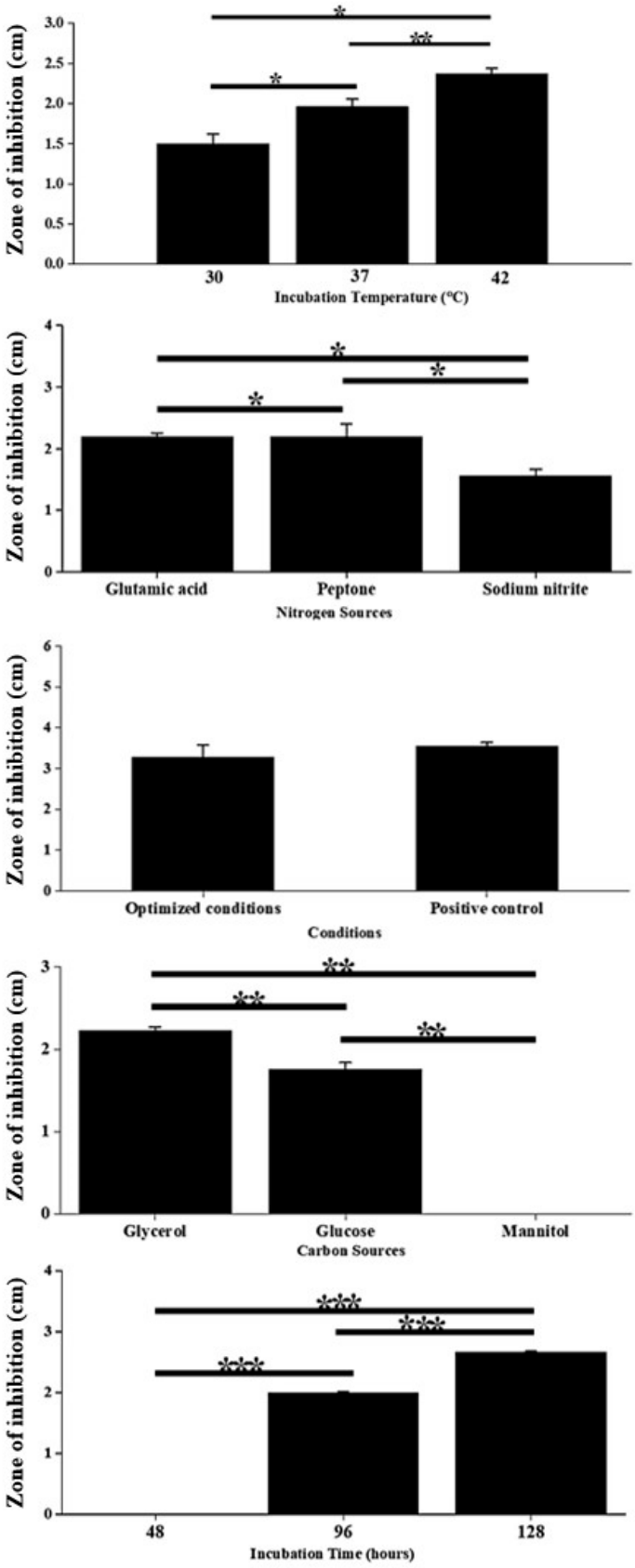

Figure 1. Effect of different carbon sources, nitrogen sources, incubation temperature, and incubation hours on the antibacterial activity of *B. subtilis* BSP1. The *p*-values were calculated: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Table 2. Zones of inhibition of the crude extract of *B. subtilis* BSP1 against the four bacterial isolates.

Microorganism	Zones of Inhibition (mm) *B. subtilis* Crude Extract (mg/mL)			Doxycycline (250 µg/mL)
	2.5	5	7.5	
B. cereus	8.8 ± 0.01	16.5 ± 0.08	17.4 ± 0.06	30.3 ± 0.03
p-value	2.30×10^{-4}	6.16×10^{-3}	1.33×10^{-3}	
significance	***	**	**	
C. freundii	10.0 ± 0.01	12.9 ± 0.03	13.5 ± 0.08	19.9 ± 0.05
p-value	2.79×10^{-3}	4.19×10^{-3}	4.36×10^{-2}	
significance	**	**	*	
M. luteus	16.2 ± 0.06	19.9 ± 0.05	21.6 ± 0.09	33.4 ± 0.05
p-value	1.74×10^{-3}	5.58×10^{-3}	2.64×10^{-3}	
significance	**	**	**	
S. maltophilia	7.8 ± 0.01	12.4 ± 0.02	15.3 ± 0.01	25.1 ± 0.01
p-value	3.05×10^{-4}	9.38×10^{-4}	7.60×10^{-4}	
significance	***	***	***	

Data are represented as the mean diameter of the zones of inhibition ± SEM (standard error of the mean). The *p*-values were calculated: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Table 3. MIC, MBC, and MBC/MIC ratios of the crude extract of *B. subtilis* BSP1 against the Gram-negative and Gram-positive bacteria.

Microorganisms	MIC (mg/mL)	MBC (mg/mL)	MBC/MIC
Gram-negative bacteria			
C. freundii	2.5	5	2.5
S. maltophilia	2.5	2.5	1
Gram-positive bacteria			
B. cereus	0.624	1.25	2
M. luteus	0.312	0.624	2

3.6. Disk-Diffusion Method for Evaluating Antifungal Activity

The initial demonstration of the antagonistic effects of the crude extract of *B. subtilis* against the various fungi was conducted using the disk-diffusion method. This activity was found to be dose-dependent and further confirmed through the radial growth method. The results indicated that the crude extract significantly impacted the radial growth of the fungi compared to the negative control, with a noticeable reduction in the fungal diameter correlating with increased concentrations. The inhibitory rates of the fungi ranged between 34.70% and 60.46%. The results obtained are shown in Table 4. The growth inhibition percentages were calculated at different doses, and the mean values showed statistical significance at *p*-values < 0.05.

3.7. Determination of MIC and MFC

The MIC values against the fungi were evaluated using the broth microdilution method. Significantly, these MIC values varied among the fungal isolates, indicating distinct sensitivities to the inhibitory effects of the substances. The MIC and MBC values of the crude extract against *C. albicans*, *A. flavus*, *A. niger*, and *R. arrhizus* are presented in Table 5. *C. albicans* exhibited the highest sensitivity, with the lowest MIC value recorded at 1.875 mg/mL, while *A. flavus* and *R. arrhizus* showed a relatively lower susceptibility with an MIC value of 3.75 mg/mL. *A. niger* demonstrated the highest resistance among the fungal isolates, presenting the highest MIC value of 7.5 mg/mL. The MFC value for the four tested fungal strains ranged between 3. 75 and 7.5 mg/mL.

Table 4. Mycelial growth inhibition of the crude extract of *B. subtilis* BSP1 against the four fungal isolates.

	Mean of Mycelial Growth Inhibition (%) *B. subtilis* Crude Extract (mg/mL)		
	3.75	**7.5**	**15**
A. flavus	43.8 ± 0.11%	46.39 ± 0.09%	50.13 ± 0.6%
p-value	3.05×10^{-3}	1.86×10^{-3}	6.86×10^{-4}
significance	**	**	***
A. niger	34.7 ± 0.13%	39.2 ± 0.08%	43.06 ± 0.11%
p-value	3.58×10^{-3}	1.24×10^{-3}	1.92×10^{-3}
significance	**	**	**
albicans	45.53 ± 0.15%	58.82 ± 0.17%	60.46 ± 0.13%
p-value	2.80×10^{-3}	2.09×10^{-3}	1.10×10^{-3}
significance	**	**	**
R. arrhizus	41.30 ± 0.08%	46.52 ± 0.09%	51.95 ± 0.07%
p-value	1.99×10^{-3}	1.94×10^{-3}	1×10^{-3}
significance	**	**	**

Data are represented as mean percentages of inhibition ± SEM%. *p*-values were calculated: ** $p < 0.01$ and *** $p < 0.001$.

Table 5. MIC, MFC, and MFC/MIC ratios of the crude extract of *B. subtilis* BSP1 against the fungi and yeast.

Microorganisms	**MIC (mg/mL)**	**MFC (mg/mL)**	**MFC/MIC**
Yeast			
C. albicans	1.875	3.75	2
Fungi			
A. flavus	3.75	3.75	1
A. niger	7.5	7.5	1
R. arrhizus	3.75	3.75	1

3.8. DPPH Scavenging Activity

The antioxidant activity of the crude extract was evaluated using the DPPH assay. The antioxidant activity of the crude extract was measured in the concentration range of 5–30 mg/mL. The results showed that the crude extract of *B. subtilis* has excellent antioxidant activity as compared with the standard ascorbic acid. As illustrated in Figure 2, the crude extract demonstrated potent antioxidant activity against DPPH in a concentration-dependent manner. The crude extract at a concentration of 5.0 mg/mL showed 73.5% DPPH scavenging activity. However, at the highest concentration (30 mg/mL), it exhibited 98.9% DPPH scavenging activity.

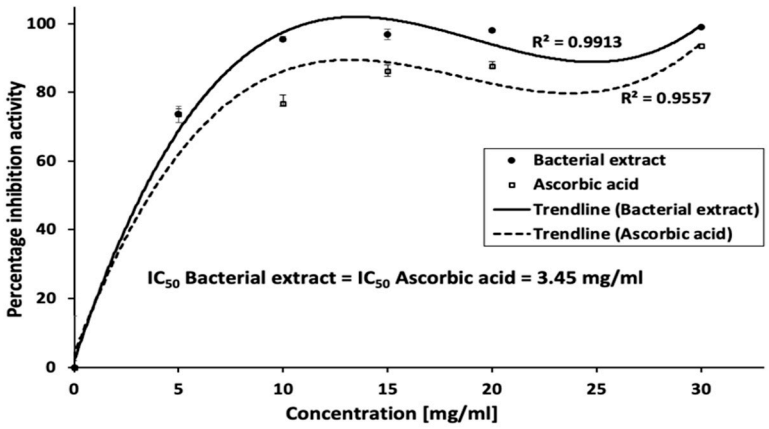

Figure 2. Antioxidant scavenging potential of the bacterial extract of *B. subtilis* BSP1 and ascorbic acid using the DPPH assay.

3.9. Hemolysis Activity of B. subtilis BSP1

The hemolytic activity of the crude extract was assessed across various concentrations. The results, as indicated in Table 6, revealed no noticeable hemolysis effects on the human erythrocytes. Even at the highest concentration tested (100 mg/mL), the crude extract showed minimal hemolytic activity on the human erythrocytes, with no noticeable hemolysis effects observed at the initial concentrations. Despite an increase in hemolytic activity to 1% at the highest concentration, this is significantly low compared to the 100% hemolytic activity exhibited by the positive control, indicating a negligible disruption of erythrocytes even at high concentrations.

Table 6. Hemolytic activity of the crude extract produced by *B. subtilis* BSP1.

Samples	Concentration	Percentage of Hemolytic Activity (%) ± SEM
SDS	1%	100
Crude extract of *B. subtilis*	0.5 mg/mL	0.45 ± 0.00019
	2.5 mg/mL	0.53 ± 0.00019
	5 mg/mL	0.6 ± 0.0002
	10 mg/mL	0.75 ± 0.00018
	100 mg/mL	1.3 ± 0.0021

4. Discussion

In the pursuit of novel and effective strategies to combat microbial infections, the isolation and characterization of unique bacterial strains capable of producing antimicrobial peptides have emerged as a promising avenue of research. The advancing challenge of antibiotic resistance indicates the urgency to explore alternative antimicrobial agents and natural sources, such as bacteria. The *B. subtilis* group strains have long been recognized for their potential to produce a diverse array of secondary metabolites, particularly AMCs [10]. This study focused on the isolation and identification of a *Bacillus subtilis* strain from a 10 cm deep rhizosphere with the potential to yield natural antimicrobial peptides. The *Bacillus* genus emerged as the predominant group among the bacteria antagonists present in the soil [33]. The isolates found in the current study were classified within the *Bacillus* genus and identified as *Bacillus subtilis* and *Bacillus cereus* species. The BC01 isolate was specifically identified as the *Bacillus subtilis* BSP1 strain.

In this study, synthetic media comprising essential minerals, carbon, and nitrogen sources were utilized as a fermentation media for the production of the secondary metabolites of *B. subtilis* BSP1. Although the majority of antibiotics operate independently of metal ions for their biological functions, some antibiotics, including bleomycin, streptonigrin, and bacitracin, require coordinated metal ions to preserve their correct structure and functionality. Significantly, the synthesis of bacitracin is inhibited by the metal chelator EDTA; however, this suppressive action can be reversed by adding an excess of Mn^{2+}, Co^{2+}, or Zn^{2+} to the culture [34].

The generation of antimicrobial secondary metabolites through microbial fermentation is strongly linked to the conditions within the culture [35]. Minor alterations in the medium composition or fermentation parameters can significantly influence both the quality and quantity of secondary metabolites, as well as the overall metabolic traits of microorganisms [36]. Certain *Bacillus* strains demonstrate efficacy against diverse microbial pathogens under the optimal medium composition [32]. The classical optimization approach, employing variations in carbon and nitrogen sources, incubation time, and temperature, significantly influenced the production of antibacterial peptides by *B. subtilis*. Different carbon sources were tested to identify the optimal one, and it was determined that the highest production of antibacterial peptides occurred when glycerol was used as the carbon source. This finding aligns with previous research where glycerol, employed as a carbon source, proved to be the most effective [34,37,38]. Although glucose is typi-

cally a favorable carbon source for bacterial growth, it retards the synthesis of numerous secondary metabolites [34,38]. In some microorganisms, the inhibitory impact of glucose has been linked to a decrease in pH due to the accumulation of organic acids. During the development of fermentation media for antibiotic production, it is frequently observed that polysaccharides or oligosaccharides outperform glucose as carbon sources [38].

Regarding the optimization of the incubation time, it was determined that 96 hours was identified as the optimal duration for antibiotic production. Previous studies investigating the influence of incubation time on secondary metabolite generation indicated that the optimized incubation time for the antibacterial activity of *B. subtilis* exceeded 48 h [34,39]. Although antibiotic formation usually occurs during logarithmic growth, and secondary metabolites may be produced throughout bacterial growth, they tend to predominate during the later stages of exponential growth and the stationary phase [34,40].

Three different nitrogen sources were employed to determine the optimal one, showing that the highest antibiotic production occurred when L-glutamic acid was used, while the lowest production was observed with sodium nitrite. These results were well corroborated by findings from other studies [34,41,42]. Glutamine and arginine are classified as the preferred nitrogen sources for *B. subtilis* [43]. The effectiveness of these nitrogen sources can be attributed, at least in part, to their ability to directly supply amino acids for the synthesis of antimicrobial metabolites, and/or they may serve as precursors for antibiotics [42]. Temperature plays a crucial role in regulating both the metabolism rate and the growth rate of microorganisms. The most effective inhibition against *M. luteus* was achieved at 42 °C. Wu et al. obtained a similar result while optimizing the conditions for the antibacterial effect of *B. subtilis*. It was demonstrated that the temperature at which bacteria exhibit the highest production of antimicrobial secondary metabolites may not align with the optimal temperature for their growth [35]. The optimized conditions, when combined, yielded zones of inhibition that were comparable to those of the positive control, as indicated by a *p*-value greater than 0.05, suggesting that the crude extract possesses an antibacterial effect similar to the positive control.

The *B. subtilis* BSP1 isolate exhibited strong antagonistic activity against various pathogenic microorganisms. Consistent with the findings of previous studies [13,25,32,44], our findings showed that the crude extract from *B. subtilis* BSP1 exhibited considerable antibacterial activity against various bacterial isolates, including both the Gram-positive and Gram-negative categories. The recorded zones for the crude extract ranged between 7.8 mm and 21.6 mm, indicating a proportional increase in the diameter of inhibition zones with a higher concentration of the extract. This observation suggests that the antibacterial activity exhibited by the *B. subtilis* extract is concentration-dependent [45]. However, it was observed that Gram-positive bacteria exhibited greater susceptibility compared to Gram-negative bacteria, showing notable growth-inhibitory effects against *M. luteus* (21.6 ± 0.09) and *B. cereus* (17.4 ± 0.06). These results were confirmed by the findings of Ramasubburayan et al. and Foldes et al. [13,46]. This variation in bacterial activity could be attributed to the differences in the cell wall composition between the two groups of bacteria, making Gram-positive bacteria more resistant to antibacterial substances [13]. Furthermore, numerous studies have pointed out that Gram-negative bacteria exhibit resistance to AMPs [47]. This resistance is characterized by the changes in the outer membrane's lipopolysaccharides (LPSs), alterations in the inner membrane's phospholipids, activation of efflux pumps, capture of AMPs by capsules or membrane vesicles, and proteolytic breakdown of AMPs [47].

MIC and MBC were utilized to assess the antibacterial effectiveness of the crude extract. The MIC (0.312 to 0.624 mg/mL) and MBC (0.624 to 1.25 mg/mL) values in *M. luteus* and *B. cereus* were lower than the MIC (2.5 mg/mL) and MBC (2.5 to 5 mg/mL) values in *C. freundii* and *S. maltophilia*. For the four bacterial strains examined in the research, the ratios of MIC to MBC are within the range of 1:4 (refer to Table 3). This suggests the compound's ability to function as a bactericidal agent, as any antibacterial agent with an MBC no more than four times the MIC is generally considered to be bactericidal [24].

The *B. subtilis* BSP1 crude extract demonstrated antibacterial properties, a characteristic affirmed by numerous previous studies that have identified the presence of antibacterial molecules produced by the different isolates of *B. subtilis*. These compounds include bacteriocins [10,44], surfactins [29], bacillibactin [48], subtilosin A [48], mycosubtilin [27], and emycin [10].

Previous research [11,13,27,49] has demonstrated the antifungal properties of *B. subtilis*. The crude extract from *B. subtilis* BSP1 exhibited stronger inhibitory effects on growth, with a 60.46% mycelial growth inhibition observed against *C. albicans*, while showing weaker activity with an inhibition of 43.06% against *A. niger*, with a concentration of 15 mg/mL. There were significant differences ($p < 0.05$) observed in the fungal growth diameter of colonies, indicating a decrease compared to the wild type. Consistent with the present results, Todorova and Kozhuharova et al. and Ramasubburayan et al. [13,33] have reported that the crude extract of *B. subtilis* displayed low antifungal activity against *A. niger*. Some *Aspergillus* species like *A. niger* develop resistance to antifungal drugs. Moreover, the drug resistance seen in *Aspergillus* spp. is affected by the development of biofilms, offering temporary defense against antifungal medications and protecting the pathogen in adverse conditions. The extracellular matrix assists in the cohesion of hyphae, facilitating the creation of a biofilm structure that leads to reduced susceptibility to drugs [50]. The higher the concentration of the crude extract, the stronger the inhibitory impact on the fungi, and a gradual reduction in the diameter of fungi was noted. Our results corresponded with the results obtained by Mnif et al. and Liang et al. [26,51]. In this study, it was found that a notably higher concentration of the crude extract was necessary to demonstrate fungistatic (MIC) and fungicidal (MFC) effects on the pathogenic fungal strains in comparison to the bacterial isolates. Based on previous studies, an MFC/MIC ratio of $\leq$4 was considered fungicidal, while a ratio exceeding 4 was considered fungistatic [52,53]. Consistent with the previously mentioned statement, the MFC/MIC ratio observed for the crude extract against the majority of the tested fungal isolates was $\leq$4. This indicated that the crude extract exhibited significant fungicidal properties. Similar results were reported by Ramasubburayan et al. and Guillén-Navarro et al. [13,27].

The crude extract of *B. subtilis* BSP1 exhibited antifungal properties, as validated by numerous prior studies that have detected the existence of antifungal molecules produced by the various strains of *B. subtilis*. These metabolites include iturin A [54,55], fengycin [48,56], mycobacillin [10], diacetyl, and trans-2-octenoic acid [51].

Artificial antioxidants like butylated hydroxytoluene (BHT) and its variations are commonly employed in the pharmaceutical and food industries. Nevertheless, these substances come with adverse effects, raising public health concerns [29]. Therefore, the exploration of natural antioxidants holds significance for applications in both food and pharmaceuticals. In the current research, the antioxidant effectiveness of the raw extract was evaluated through the implementation of the DPPH assay. The DPPH radical scavenging assay is commonly employed to examine the capacity of compounds to function as the scavengers of free radicals or providers of hydrogen [57]. The results of the antioxidant assay showed that the crude extract from *B. subtilis* BSP1 exhibited a consistent increase in DPPH radical scavenging activity with rising extract concentrations. The highest observed activity was 98.97% at a concentration of 30 mg/mL. At this concentration, the *p*-value exceeded 0.05, indicating no significant difference. This implies that the crude extract's DPPH radical scavenging activity was comparable to that of the control (ascorbic acid). In fact, the observed changes in the extract's radical scavenging activity at various concentrations can be attributed to significant variations in the levels of biologically active metabolites [13]. Our results were in accordance with previous works that demonstrated that DPPH scavenging activity increased with the increasing concentration of *B. subtilis* extract [13,29,30,57]. The observed increase in the antioxidant activity in the crude extract of *B. subtilis* is primarily attributed to the synthesis of additional metabolites with antioxidant properties. This includes compounds such as phenolic and benzoic acids, as well as exopolysaccharides like levan, produced by the *Bacillus* genus [30]. This result was closely associated with

the IC_{50} values. The IC_{50} values for the crude extract and ascorbic acid were calculated, and they were found to be both equal to 3.45 mg/mL. This suggests that the crude extract demonstrated DPPH scavenging effectiveness similar to ascorbic acid. Smaller IC_{50} values signify more potent DPPH scavenging capability, whereas larger IC_{50} values indicate weaker scavenging ability [58].

Since antimicrobial compounds are applied in biomedical contexts, the evaluation of the degree of hemolysis in freshly collected human red blood cells was crucial. The hemolysis assay serves as a quick initial measure for toxicity evaluation. *B. subtilis* BSP1 exhibited no hemolytic activity, in which the hemolytic percentage at a concentration of 100 mg/mL was only 1.30%, with a dose-dependent increase in hemolytic activity, whereas the hemolysis obtained by SDS was considered 100% hemolysis. Similarly, various studies highlighting the use of *B. subtilis* strains, including the strains of URID 12.1 [25], IDC1101 [48], P223 [59], and LR1 [44] reported no hemolytic activities of the strains.

5. Conclusions

In summary, the findings of this study revealed that the crude extract obtained from *B. subtilis* BSP1, isolated from rhizosphere soil, exhibited strong antimicrobial properties against the pathogenic bacterial and fungal isolates tested. The results of the antioxidant assay indicated a dose-dependent increase in DPPH radical scavenging ability and reducing power. Additionally, the safety of the crude extract is affirmed, as it exhibited no hemolytic activity on human red blood cells, indicating its suitability for use. Therefore, it can serve as an environmentally friendly and promising bacterial strain capable of producing antibiotics for disease control in many fields. Future research should focus on the identification of the molecular structure of the active substances or metabolites generated by this strain and the investigation of its enzymatic activity to completely exploit its potential as an efficient biocontrol agent.

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