IJARSCT
International Journal of Advanced Research in Science, Communication and Technology
International Open-Access, Double-Blind, Peer-Reviewed, Refereed, Multidisciplinary Online Journal
ISSN: 2581-9429
Volume 6, Issue 7, April 2026
Impact Factor: 8.2

Antibacterial and Antifungal Efficacy of Exopolysaccharides Produced by *Bacillus subtilis*

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Abstract: *The increasing resistance of pathogenic microorganisms to commonly used antibiotics has created an urgent need for alternative antimicrobial agents. In the present study, exopolysaccharides (EPS) produced by Bacillus subtilis were extracted and evaluated for their antimicrobial potential against selected pathogenic microorganisms. The bacterial isolate was identified based on morphological, biochemical, and 16S rRNA gene sequencing methods. EPS was produced in liquid culture, purified by solvent precipitation, and characterized using FTIR, HPLC, and SEM analyses. The antimicrobial activity of the purified EPS was tested against Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, and Candida albicans. The EPS showed significant antibacterial activity against both Gram-positive and Gram-negative bacteria, with maximum inhibition observed against E. coli and S. aureus. Moderate antifungal activity was observed against C. albicans, while no activity was detected against Aspergillus niger. Minimum inhibitory concentration (MIC) studies indicated a concentration-dependent inhibition of microbial growth. Cytotoxicity and haemolysis assays demonstrated that the EPS was non-toxic and biocompatible at effective concentrations. Overall, the results of this study suggest that EPS produced by Bacillus subtilis has promising antimicrobial properties and may be explored further as a natural and safe alternative to synthetic antimicrobial agents.*

Keywords: Exopolysaccharides (EPS), Antimicrobial activity, Physicochemical characterization, FTIR, HPLC, Biocompatibility

I. INTRODUCTION

The global rise in antimicrobial resistance (AMR) has emerged as one of the most critical threats to public health, significantly limiting the effectiveness of conventional antibiotics against infectious diseases. Pathogenic bacteria such as Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, and Klebsiella pneumoniae, along with opportunistic fungi like Candida albicans, have developed resistance to multiple classes of antimicrobial agents, leading to prolonged illnesses, increased healthcare costs, and higher mortality rates (Ventola, 2015; WHO, 2020). This alarming trend necessitates the exploration of novel, safe, and sustainable antimicrobial alternatives.

Microbial exopolysaccharides (EPS) have attracted considerable scientific interest due to their structural diversity and wide range of biological activities, including antimicrobial, antibiofilm, antioxidant, immunomodulatory, and cytoprotective properties (Freitas et al., 2011; Caggianiello et al., 2016). EPS are high-molecular-weight biopolymers secreted extracellularly by microorganisms and play a vital role in cell protection, adhesion, biofilm formation, and environmental adaptation. Importantly, microbial EPS are biodegradable, non-toxic, and biocompatible, making them promising candidates for biomedical, pharmaceutical, and food applications (Patel et al., 2012).

Among EPS-producing microorganisms, Bacillus subtilis is a well-characterized, Gram-positive, rod-shaped bacterium widely distributed in soil and environmental ecosystems. It is recognized as a Generally Recognized As Safe (GRAS) organism and is extensively used in industrial biotechnology due to its ability to synthesize a variety of bioactive

DOI: 10.48175/IJARSCT-33032

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metabolites, including enzymes, lipopeptides, pigments, and extracellular polysaccharides (Schallmey et al., 2004; Earl et al., 2008). EPS produced by B. subtilis have been reported to exhibit significant antimicrobial activity by disrupting microbial cell membranes, inhibiting biofilm formation, and interfering with essential cellular processes of pathogenic microorganisms (Wang et al., 2019; Liu et al., 2021).

The antimicrobial potential of B. subtilis-derived EPS against both Gram-positive and Gram-negative pathogens has gained attention as an alternative strategy to combat drug-resistant infections. Studies have demonstrated that these EPS can effectively inhibit pathogenic bacteria such as S. aureus and E. coli, as well as opportunistic fungi, highlighting their broad-spectrum antimicrobial capability (Kumar et al., 2020; Xu et al., 2022). Additionally, evaluating the safety profile of EPS through cytotoxicity and haemolysis assays is crucial to establish their suitability for therapeutic and biomedical applications.

In this context, the present study investigates the efficacy of purified exopolysaccharides produced by Bacillus subtilis against clinically significant pathogenic microorganisms, with a focus on antimicrobial activity, minimum inhibitory concentration (MIC), and biocompatibility. The findings of this study aim to contribute to the development of microbial EPS as a promising natural antimicrobial agent and provide a scientific basis for their potential application in pharmaceutical and healthcare industries.

Materials and Methods

Isolation and Identification of Bacillus subtilis

The bacterial isolate used in this study was obtained through standard microbiological techniques. Morphological characterization was performed based on colony appearance on nutrient agar, including size, shape, color, and elevation. Gram staining was carried out to determine cellular morphology and Gram reaction. Biochemical characterization was conducted using standard biochemical tests such as indole, methyl red, Voges–Proskauer, citrate utilization, catalase, oxidase, urease, starch hydrolysis, hydrogen sulfide production, and carbohydrate fermentation tests.

Molecular Identification by 16S rRNA Gene Sequencing

Genomic DNA was extracted using the HiPurA Bacterial DNA Purification Spin Column Kit (HiMedia, India). The quality of extracted DNA was confirmed by 1% agarose gel electrophoresis. PCR amplification of the 16S rRNA gene (~1500 bp) was performed using primers BS168F and BS168R. PCR products were purified and sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit and analyzed on an Applied Biosystems 3500xL Genetic Analyzer. The obtained sequences were edited and analyzed using BLAST against the NCBI database for species confirmation.

Production and Purification of Exopolysaccharide (EPS)

The confirmed B. subtilis isolate was cultured in YEPD medium under optimized growth conditions. After incubation, the culture broth was centrifuged to remove bacterial cells. EPS present in the cell-free supernatant was precipitated using cold acetone (3:1 v/v) and incubated at 4 °C. The precipitated EPS was collected, washed with ethanol, and dried for further analysis.

Characterization of EPS and Bacterial Metabolites

Fourier Transform Infrared (FTIR) spectroscopy was used to identify functional groups present in the EPS and bacterial biomass. High-Performance Liquid Chromatography (HPLC) was performed to analyze the metabolite profile of the bacterial extract. Scanning Electron Microscopy (SEM) was used to study surface morphology and EPS-associated biofilm structures.

Antimicrobial Activity Assay

The antimicrobial activity of purified EPS was evaluated against pathogenic microorganisms including Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Candida albicans, and Aspergillus niger. The zone of inhibition was measured in millimeters to assess antimicrobial efficacy.

Minimum Inhibitory Concentration (MIC) Assay

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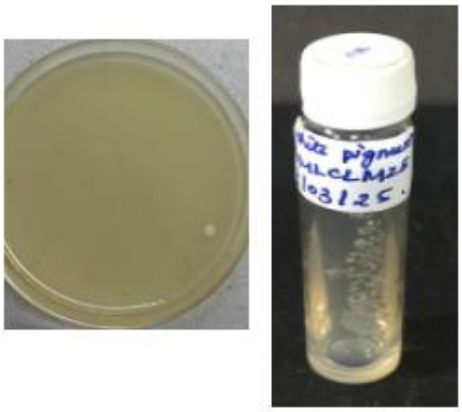
MIC was determined using a microbroth dilution method in ELISA plates. Serial dilutions of EPS were tested against pathogenic strains, and microbial growth inhibition was assessed spectrophotometrically. MIC was defined as the lowest EPS concentration resulting in ⩾50% growth inhibition.

Cytotoxicity and Hemolysis Assays

Cytotoxicity was evaluated using the MTT assay on cultured cell lines obtained from NCCS, Pune. Cell viability was measured spectrophotometrically at 570 nm. Hemolytic activity was assessed using human red blood cells (RBCs), and hemoglobin release was measured at 540 nm to determine EPS biocompatibility.

II. RESULTS AND DISCUSSION

Morphological, Biochemical, and Molecular Characterization



The isolated bacterium exhibited small, round, convex, white colonies and appeared as Gram-positive, rod-shaped cells under microscopic observation.

Biochemical test	Results
Indole test	negative
Methyl red	negative
Vp	positive
Citrate	positive
Catalase	positve
Oxidase	positive
Urease	negative
Starch hydrolysis	negative
Hydrogen sulfide	negative
Carbohydrate	fermentation
Glucose	positive
Fructose	positive
Lactose	positive

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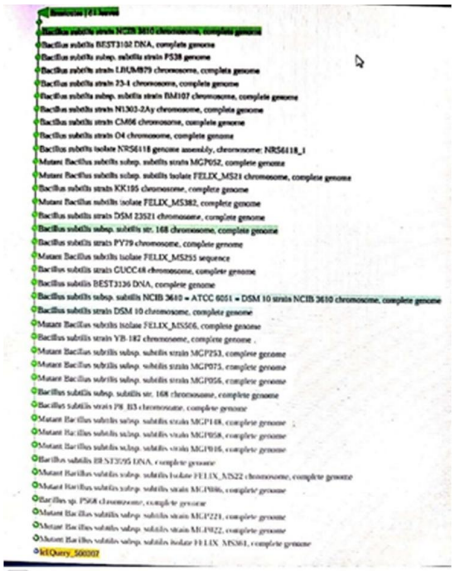
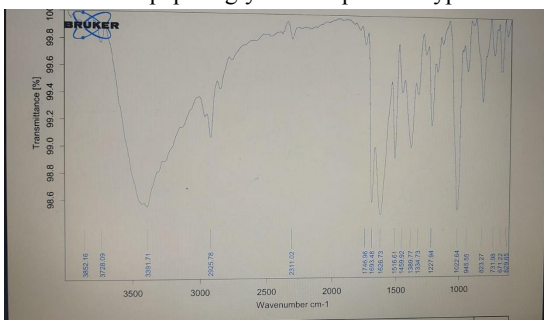


Biochemical test results, including positive catalase, oxidase, Voges Proskauer, citrate utilization, and carbohydrate fermentation, were consistent with characteristics of Bacillus subtilis.

Molecular identification through 16S rRNA gene sequencing further confirmed the isolate as B. subtilis, validating its taxonomic identity.

FTIR and HPLC Analysis

FTIR analysis revealed characteristic absorption bands corresponding to hydroxyl, amide, ester, and polysaccharide functional groups, confirming the presence of proteins, lipids, and carbohydrate-rich EPS. Strong bands in the 1227–1022 cm^{-1} region indicated polysaccharide and peptidoglycan components typical of Gram-positive bacteria.



W1 Sample

The FTIR spectrum of Bacillus subtilis (Sample code: W1) displays characteristic absorption bands corresponding to the major biochemical constituents of the bacterial cell, including proteins, lipids, polysaccharides, and cell wall components. The broad band observed in the region of 3390–3300 cm^{-1} is attributed to O–H and N–H stretching vibrations, indicating the presence of hydroxyl groups and amide functionalities associated with proteins and polysaccharides in the bacterial cell envelope. The absorption peak around 2925 cm^{-1} corresponds to aliphatic C–H stretching vibrations of $–CH_2$ and $–CH_3$ groups, confirming the presence of membrane lipids and fatty acid chains, while the distinct peak at 1746 cm^{-1} is assigned to C=O stretching of ester functional groups characteristic of phospholipids in the bacterial membrane.

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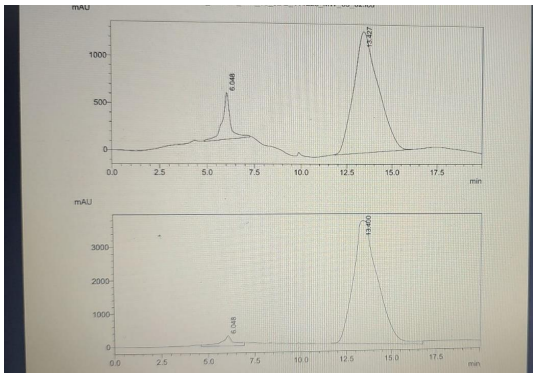
Prominent bands in the regions of 1639–1626 cm^{-1} (amide I) and 1516 cm^{-1} (amide II) indicate peptide bond vibrations, confirming the abundance of proteinaceous components. Additionally, bands observed between 1450 and 1380 cm^{-1} are associated with C–H bending and symmetric stretching of carboxylate (COO^-) groups, reflecting amino acid residues and organic acids. Strong absorption bands in the range of 1227–1022 cm^{-1} correspond to C–O–C and C–O stretching vibrations, characteristic of polysaccharides, peptidoglycan, and teichoic acids, which are key structural components of Gram-positive bacteria, while peaks below 950 cm^{-1} are attributed to phosphate and sugar ring vibrations, supporting the presence of nucleic acids and carbohydrate moieties. Overall, the FTIR spectral profile of Bacillus subtilis (W1) confirms the presence of proteins, lipids, carbohydrates, and phosphate-containing compounds, validating the typical biochemical and structural features of Gram-positive bacterial cells.

HPLC



W1 Sample

The HPLC chromatographic profile of Bacillus subtilis (Sample code: W1), recorded using PDA detection at 400 nm, exhibits a well-resolved pattern of peaks, indicating the presence of multiple UV-absorbing biomolecules in the sample extract. A distinct peak observed at a retention time of 6.048 min with a peak area of 14,728,097 and an area percentage of 10.655% suggests the presence of a moderately abundant compound, likely associated with secondary metabolites, peptides, or small aromatic biomolecules produced by B. subtilis. In addition, a dominant and sharp peak appearing at approximately 13.4 min indicates a major component with higher hydrophobicity and stronger interaction with the stationary phase; the sharpness and symmetry of this peak reflect efficient separation, good column performance, and high compound purity. Such late-eluting peaks are commonly attributed to lipopeptides or other non-polar bioactive secondary metabolites characteristic of Bacillus subtilis. The stable baseline and clear peak resolution throughout the chromatographic run demonstrate minimal interference and good analytical reproducibility, confirming the metabolic diversity of B. subtilis and the suitability of the HPLC method for bacterial metabolite profiling.

HPLC profiling demonstrated well-resolved peaks, including a dominant late-eluting peak, suggesting the presence of hydrophobic bioactive metabolites such as lipopeptides or EPS-associated compounds. These findings indicate the metabolic diversity of B. subtilis and its ability to synthesize antimicrobial biomolecules.

SEM Analysis

SEM micrographs showed dense cellular aggregates embedded within an extracellular matrix, confirming active EPS secretion and biofilm-forming capability. The rough and compact surface morphology supports the protective and adhesive role of EPS, which is often associated with antimicrobial and antibiofilm activity.

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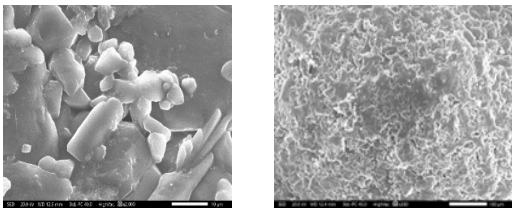
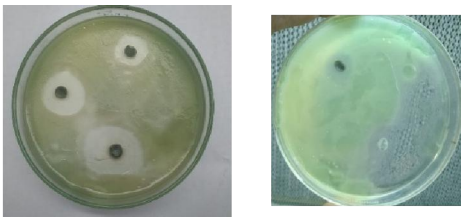
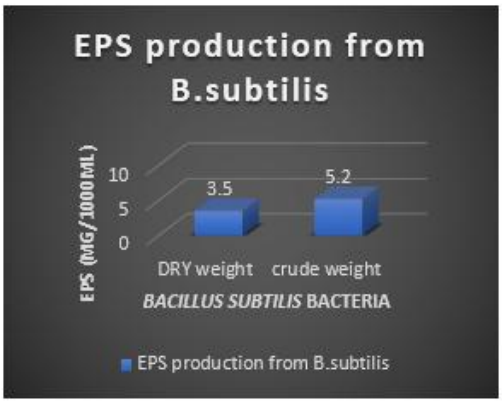


Antimicrobial Activity of EPS

Pathogenic Micro organisms	Inhibition zone of EPS (mm)
S. aureus	35
P. aeruginosa	27
E. coli	39
K. pneumonia	28
C. albicans	30
A. niger	NA



Purified EPS exhibited strong antimicrobial activity against all tested bacterial pathogens. The highest inhibition was observed against E. coli (39 mm) and S. aureus (35 mm), followed by K. pneumoniae and P. aeruginosa. Moderate antifungal activity was observed against Candida albicans, while no inhibition was detected against Aspergillus niger. These results suggest broad-spectrum antibacterial activity of B. subtilis EPS, particularly against Gram-positive and Gram-negative bacteria.



MIC and Growth Inhibition Analysis

MIC analysis revealed a concentration-dependent inhibition of pathogenic microorganisms. E. coli showed the highest susceptibility, with inhibition exceeding 50% at higher EPS concentrations. P. aeruginosa and C. albicans exhibited

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moderate sensitivity, while S. aureus showed gradual inhibition. These findings indicate that EPS interferes with microbial growth and metabolic activity, possibly through membrane disruption or nutrient limitation.

Cytotoxicity and Hemolysis Studies

MTT assay results demonstrated high cell viability at tested EPS concentrations, indicating low cytotoxicity. Hemolysis assays revealed minimal hemolytic activity at lower concentrations, confirming the biocompatible nature of EPS. These results suggest that B. subtilis-derived EPS is safe for potential biomedical applications.

III. CONCLUSION

In the present study, exopolysaccharides (EPS) produced by Bacillus subtilis were successfully extracted, characterized, and evaluated for their antimicrobial activity. The purified EPS showed effective antibacterial activity against both Gram-positive and Gram-negative pathogenic bacteria, with maximum inhibition observed against Escherichia coli and Staphylococcus aureus. Moderate antifungal activity was also observed against Candida albicans. MIC results confirmed that the antimicrobial effect of EPS increased with concentration.

Cytotoxicity and hemolysis studies indicated that the EPS exhibited low toxicity and good biocompatibility at effective concentrations. Overall, the findings suggest that EPS produced by Bacillus subtilis can serve as a promising natural antimicrobial agent. Further studies are required to understand its mechanism of action and to explore its potential applications in medical and pharmaceutical fields.

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