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Optimization And Characterization Of Exopolysaccharides Produced By Bacillus Mycoides Isolated From Soil

S. Dhivya[1*], Dr. M. Mekala[2], Dr. R. Ragunathan[3]
[1]Research Scholar, Department of Microbiology, Sri Ramakrishna College of Arts and Science for Women, Coimbatore, Tamil Nadu, India.
[2]Director and Supervisor, Micro Biotek lab, Coimbatore, Tamil Nadu, India.
[3]Director and Supervisor, Centre for Bioscience and Nanoscience Research, Coimbatore, Tamil Nadu, India.

Abstract
The production of exopolysaccharide (EPS) by bacteria that live in soil has a significant biotechnology application with a wide range of uses in environmental remediation, food industry, and medicine. The current work focuses on isolating and thoroughly characterizing EPS-producing Bacillus mycoides isolated from soil with the aim of optimizing growing conditions for enhanced EPS production. Initially, soil samples were collected, and bacterial strains were isolated and screened for EPS production. This screening was performed using morphological, biochemical profiling, and molecular identification methods, a very effective EPS generator among the isolates was recognized and verified as Bacillus mycoides. Using a one-variable-at-a-time (OVAT) approach, several physicochemical parameters, such as pH, temperature, carbon and nitrogen sources, and incubation duration, were adjusted to increase EPS yield as an optimized medium for enhanced EPS production. A variety of analytical methods, such as Gas Chromatography–Mass Spectrometry (GC-MS), Thin Layer Chromatography (TLC), Fourier Transform Infrared Spectroscopy (FTIR), and UV–Visible Spectroscopy, were utilized to investigate the EPS that was extracted from the optimized culture. These techniques identified possible bioactive components, defined the composition of monosaccharides, and verified the existence of particular functional groups of exopolysaccharides. Overall, the study demonstrates that EPS derived from Bacillus mycoides exhibits significant promise for industrial and biomedical applications. Its enhanced yield, structural integrity, and antimicrobial potential highlight its suitability for the formulation of environmentally friendly and sustainable microbial polysaccharide-based products.
Keywords *: Exopolysaccharides, Bacillus mycoides, optimization, Antibacterial*

INTRODUCTION

Microbial exopolysaccharides, are high-molecular-weight polymers that are released into the extracellular matrix as a result of the growth and metabolism of a wide variety of microorganisms. These microorganisms include bacteria, fungus, and certain types of algae. Because they promote surface adherence, offer defense against environmental stressors, and are crucial for the production of biofilms, these naturally occurring biopolymers are vital for microbial survival. Because of their structural diversity and numerous uses in industries like pharmaceuticals, food, cosmetics, agriculture, and environmental remediation, microbial EPS have attracted increasing attention in recent years. Unlike polysaccharides derived from plants or algae, microbial EPS can be synthesized under controlled fermentation conditions, offering an environmentally sustainable and scalable alternative to synthetic polymers. (Almalki, 2020).

This research focuses on the isolation of a high-yield EPS-producing strain of Bacillus mycoides from soil, followed by the optimization of its culture conditions to enhance EPS production. The purified EPS will then undergo detailed physicochemical characterization through techniques such as UV–Vis spectroscopy, FTIR, TLC, and GC-MS. Subsequently, the antimicrobial potential of the EPS will be evaluated against common bacterial pathogens. Within the wide array of EPS-producing microorganisms, species from the genus Bacillus have garnered particular attention. This is due to their robust metabolic profiles, non-pathogenic nature, and capacity to produce significant amounts of EPS under diverse environmental conditions. Bacillus mycoides,

a Gram-positive, rod-shaped bacterium commonly found in soil, is recognized for its rapid growth and its ability to secrete surface-active polysaccharides. While extensive research has been conducted on EPS production in Bacillus subtilis, Bacillus licheniformis, and Bacillus cereus, relatively few studies have explored Bacillus mycoides. especially with respect to optimizing production parameters and characterizing the chemical and functional aspects of the EPS it generates. Optimizing physiochemical parameters is essential for maximizing EPS production and lowering associated costs for potential industrial applications. Conventional techniques like the one-variable-at-a-time (OVAT) approach help elucidate the effect of individual factors, while more advanced statistical methods, such as Response Surface Methodology (RSM), allow for interactive analysis and more efficient optimization.

Following production, Spectroscopic analyses such as UV–Visible and FTIR spectroscopy provide insights into the functional groups present and the overall purity of the sample. UV–Vis absorbance can help detect the presence of protein contaminants or associations with aromatic molecules, whereas FTIR spectroscopy identifies characteristic peaks associated with hydroxyl, carboxyl, and glycosidic linkages. Following acid hydrolysis, the monosaccharide composition is ascertained by Thin Layer Chromatography (TLC), which provides details on the constituent sugars, including glucose, mannose, and galactose. The sensitivity of gas chromatography–mass spectrometry (GC-MS) makes it possible to precisely identify chemical components and minute structural variations, both of which are essential for evaluating possible bioactivities and industrial applications.

The biological activity of microbial EPS is one of its most advantageous characteristics. Numerous EPS have immunomodulatory, antibacterial, anti-inflammatory, and antioxidant properties, which make them attractive options for application in the pharmaceutical and medical industries. This work contributes to the fundamental understanding of microbial EPS biosynthesis and supports the development of cost-effective, sustainable bioproducts with relevance to pharmaceutical and industrial applications and also offer promising avenues for innovation in healthcare, agriculture, and environmental management. (Bajpai et al., 2016).

2. MATERIALS AND METHODS

2.1 Collection of samples

Samples of soil were meticulously gathered from different cotton-growing locations and placed in sterile plastic containers, where they were stored at room temperature until further analysis. (Hereher et al., 2018)

2.2. Screening of Bacterial Strains Using Agar Media (Crowded Plate Technique)

After being inoculated into 9 mL of saline solution, the soil samples were diluted in a series of steps up to a temperature of 10 degrees Celsius. MRS (Man, Rogosa, and Sharpe) agar plates were treated with 0.1 milliliters of each dilution when it was applied to them. For 48 hours, the plates were incubated at 37°C. By streaking over newly made agar media, isolates with noticeable, mucoid colony shape were chosen and further purified. Following purification, the colonies were stored for further use at 4°C. (Angelin and Kavitha, 2020)

2.3. Screening of EPS Producing Bacterial Strains Using Quadrant Streaking Method & Its Biomass Calculation:

Colonies of interest were isolated using the crowded plate method. Chaudhari et al. reported that the plates were incubated at 37°C for 24 hours (2017a). The ability of the selected bacterial strains to produce extracellular polymeric substances (EPS) was evaluated by analyzing the biomass. The biomass was computed using the following formula after the entire weight was recorded: (Paulo et al., 2012)

Cell mass = weight of the tube with pellet - weight of the empty tube.

2.4. Morphological and Biochemical Characterisation of Bacterial Isolate:

Following the guidelines provided in Bergey's Manual of Determinative Bacteriology, the high exopolysaccharide (EPS)-producing bacterial strains designated as B1 was carefully examined. For biochemical characterization, the purified isolates were subjected to several tests, including the Voges-Proskauer test, methyl red reaction, indole production, catalase activity, carbohydrate fermentation analysis, and oxidase activity assessment. (Jenny et al., 2018)

2.5. Sequencing of Exopolysaccharides-Producing Bacteria:

Procedure for DNA Amplification and Gel Extraction

Standard phenol-chloroform extraction and ethanol precipitation were used to obtain genomic DNA from the selected EPS-producing bacterial isolate B1. The DNA's content and purity were evaluated using a NanoDrop spectrophotometer and agarose gel electrophoresis (Ergene et al., 2018)

In order to amplify the 16S rRNA gene, the universal primers known as 16S Forward Primer (5'-AGAGTTTGATCMTGGCTCAG-3') and 16S Reverse Primer (5'-TACGGYTACCTTGTTACGACTT-3') were utilized in a heat cycler known as an Eppendorf Mastercycler. For the 25 µL PCR reactions, the following components were included: 1 µL of forward and reverse primers with a concentration of 10 µM, 1 µL of template DNA, 9.5 µL of nuclease-free water, and 12.5 µL of 2X master mix consisting of Taq polymerase, dNTPs, $MgCl_2$, and reaction buffer. (Jurášková et al., 2022)

Under UV transillumination, the 1500 bp amplicon was removed from the gel using a sterile scalpel. Following the manufacturer's instructions, the PCR product was extracted using the QIAquick Gel Extraction Kit (Qiagen). Prior to sequencing, the DNA was again measured after being eluted in 30 µL of elution buffer. Initial denaturation at 95°C for 5 minutes, 35 cycles of denaturation for 30 seconds at 95°C, annealing at 55°C for 30 seconds, and final extension for 10 minutes were the PCR amplification settings. (Caggianiello et al., 2016)

2.6. Phylogenetic Relationships:

The isolates' 16S rDNA sequences were examined using NCBI's Basic Local Alignment Search Tool (BLAST) to find closely similar sequences for DNA amplification and sequence identification. MEGA program used neighbor-joining (NJ) to determine evolutionary relationships. The gene sequencing data were submitted for an accession number. (Miri et al., 2021)

2.7. Optimization of Exopolysaccharides Producing Strains:

The effects of various components of the growth medium on microbial exopolysaccharide synthesis were systematically investigated using highly efficient bacterial strains. To visually represent the outcomes, standardized graphical illustrations were generated. Key physiological and nutritional factors examined included inoculum size, pH, temperature, and different carbon and nitrogen sources, all aimed at optimizing exopolysaccharide production by the isolates during the static growth phase. (Moghannem et al., 2018)

2.8. Production of Exopolysaccharides with Optimized Medium:

The optimal growth medium consisted of 1.0 g/L of sodium chloride, 0.6 g/L of yeast extract powder, 3.0 g/L of KHPO3, 3.0 g/L of both KHPO3 and KHPO3, 1.0 g/L of sodium chloride, and 1.0 g/L of beef extract. It was decided to elevate the pH to 7.0 in order to stimulate the bacterial strains to produce a greater amount of EPS. While the medium was being compressed at a rate of 15 pounds per square inch, it was sterilized for fifteen minutes at 121 degrees Celsius. Immediately following the sterilizing process, the mixture was put into an incubator and rapidly stirred for thirty to sixty minutes. After that, it was left in the incubator for the entire night. (Malick et al., 2017)

2.9 Extraction and purification of Exopolysaccharides:

Exopolysaccharides (EPS) were extracted using an alcoholic extraction method. Absolute ethanol was mixed with the EPS culture media in a 1:1 ratio. The resulting mixture was stored at 4°C overnight to reduce the solubility of the EPS, promoting its precipitation. The mixture was then centrifuged at 10,000 rpm for 5 minutes to collect the precipitate. The pellet was washed with deionized water and dried. The yield of EPS after extraction was calculated using the designated formula.

$$\text{EPS yield } (\text{mgL}^{-1}) = \frac{\text{Dry weight of EPS (g)}}{\text{Volume of production media (L)}}$$

The precipitate of crude extracellular polymeric substances (EPS) was reconstituted in deionized water as part of the purification process. Following that, the EPS solution was incubated for 40 minutes at 37°C with intermittent stirring after 0.5% trichloroacetic acid (TCA) was added. After the incubation, the mixture was centrifuged for five minutes at 10,000 rpm to remove any remaining proteins from the sample. (Berekaa, 2014)

2.10 Purification efficiency Using carbohydrate estimation:

A test tube was filled with two milliliters of the substance. Five milliliters of sulfuric acid were then added after 0.05 milliliters of 80% phenol. The acid was poured rapidly, making sure that the stream was aimed at the liquid's surface rather than the test tube walls for the best mixing. The tubes were let to stand for ten minutes before being shook. Before absorbance measurements were made, they were then kept in a water bath that was kept between 25°C and 30°C for ten to twenty more minutes. The absorbance of the characteristic yellow-orange color was recorded using a UV spectrophotometer at 490 nm. A standard calibration curve for glucose was developed within the concentration range of 10 - 150 mg/L, and the total carbohydrates in each liter of extract were calculated in milligrams to report the results. (Moghannem et al., 2018)

The purification efficiency of EPS was calculated employing the specified formula;

$$\text{Purification efficiency } = \frac{\text{Total carbohydrate}}{\text{Yield of Extraction}} \times 100$$

2.11 Characterisation of Exopolysaccharides:

2.11.1 UV – Visible spectrophotometry analysis utilizing the phenol-sulfuric acid method:

For the purpose of determining the total amount of carbohydrates, the Anthrone method was utilized. An aliquot of the pellet containing 0.5 milliliters was mixed with the crude extracellular polymeric substances (EPS) that were extracted from the production medium. Following this, 2.5 milliliters of anthrone reagent was added. Following the completion of the components' thorough mixing, the mixture was next heated to 90 degrees Celsius in a water bath.

Throughout the entire process of heating, the absorbance at 620 nm was monitored in order to determine the amount of carbohydrates present. (Saadat et al., 2019)

2.11.2 Fourier Transform Infrared Spectroscopy Analysis:

To create pellets, potassium bromide (KBr) was used to finely grind the EPS. A Shimadzu FTIR spectrophotometer was used to record the FTIR spectra in the 400–4000 cm^{-1} wavelength range. A total of 32 scans were conducted, and the average spectrum was calculated after obtaining the individual spectra at a resolution of 2 cm^{-1}. (Sethi et al., 2019)

2.11.3 TLC Analysis

Using thin-layer chromatography (TLC), a qualitative analysis was performed to determine the composition of monosaccharides that were present in the exopolysaccharides (EPS). For the preparation of the hydrolyzed EPS sample, 10 mg of purified EPS was subjected to treatment with 2 M trifluoroacetic acid (TFA) at 121 degrees Celsius for a period of two hours. This was followed by the neutralization and concentration of the hydrolysate. As the stationary phase, a silica gel 60 F254 TLC plate was utilized in the experiment. The plate contained approximately 5 microliters of the sample as well as monosaccharide standards, which included glucose, galactose, mannose, and other similar substances. The mobile phase was a solvent system that was composed of butanol, acetic acid, and water in the proportions of 2:1:1 (volume/volume/volume). After producing the chromatogram, the plate was allowed to dry and then visualized by spraying it with an aniline-diphenylamine reagent and then heating it at a low temperature for ten minutes at a temperature of one

hundred degrees Celsius. A comparison was made between the Rf values of the sample spots and those of the standard sugars in order to arrive at an identification. (Zhou et al., 2012)

2.11.4 GCMS Analysis

GC-MS was used to determine the monosaccharide content of isolated exopolysaccharides (EPS) after acid hydrolysis and derivatization. About 10 mg of pure EPS were hydrolyzed for two hours at 121°C using 2 M TFA. The hydrolysate was reduced with sodium borohydride, acetylated with acetic anhydride, and dried under nitrogen to produce alditol acetates. Ethyl acetate was used to extract, filter, and concentrate the derivatized components. An Agilent 7890A GC system with a 5975C mass selective detector and a DB-5MS capillary column was used to analyze the samples. The temperature rose from 80°C to 280°C over ten minutes at a pace of 10°C per minute. A 1 µL injection volume of helium was utilized as the carrier gas in split mode. The NIST Mass Spectral Library was used to identify chemicals based on retention durations and compare them with standards, revealing the EPS's monosaccharide profile and bioactive components. (Singh et al., 2011)

2.12. Antibacterial activity:

The sample's antibacterial activity against food-borne pathogenic microbes, such as E. coli, S. aureus, K. pneumoniae, and S. typhi, was evaluated using the Kirby-Bauer well diffusion method. 39 grams of Mueller-Hinton agar medium were combined with 1000 milliliters of distilled water to create the medium. Then, 70 microliters of the food-borne pathogens were evenly distributed across the agar surface. Wells, each 5 mm in diameter, were created using a stainless-steel cork borer, and each well was labeled accordingly. Each well was filled with 70 microliters of the bacterial strains, which were prepared under optimized growth conditions and purified using trichloroacetic acid (TCA). The agar plates were incubated at 37°C for 24 hours. To evaluate antibacterial activity, the widths of the zones of inhibition surrounding the bacterial strains were measured in millimeters. A positive control was established by placing a Cefazolin 30 mg antibiotic disc on the agar, and distilled water was used as the negative control. (Mohamed et al., 2018)

3. RESULTS AND DISCUSSION:

3.1 Screening of Bacterial Strains that Produce EPS and Calculation of their Biomass:

Using serial dilutions, various bacterial strains were examined through the crowded plate technique. Bacterial colony growth was observed on MRS agar medium at a controlled temperature of 37°C for 48 hours. Colonies formed using the crowded plate technique were analyzed for their ability to produce exopolysaccharides (EPS) via the quadrant streaking method. Among the isolates, ten distinct strains were evaluated for their biomass to assess EPS-producing bacterial growth. In the biomass assessment, Strain B1 exhibited the highest biomass yield of 0.269 g/m, surpassing all other bacterial strains evaluated. The results are summarized in Table 1. (Mu'minah et al., 2015)

Table 1 Biomass details of bacterial strains

Sample	Biomass (g/m)	Sample	Biomass(g/m)
B1	0.269	B6	0.103
B2	0.203	B7	0.132
B3	0.027	B8	0.126
B4	0.174	B9	0.11
B5	0.186	B10	0.112

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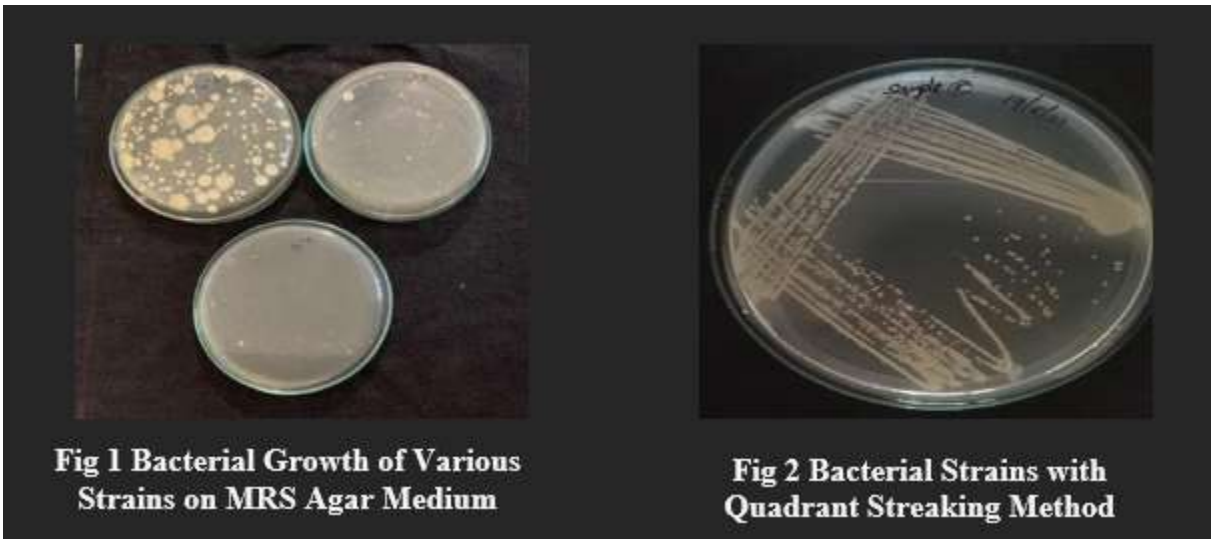

Fig 1 Bacterial Growth of Various Strains on MRS Agar Medium

Fig 2 Bacterial Strains with Quadrant Streaking Method

3.2 Morphological and Biochemical characterization of bacterial Isolate:

Morphological features of specific strains were carefully documented for the isolates cultured on Nutrient agar medium. The identified bacterial strain B1 was characterized as bacilli in morphology and exhibited Gram-positive properties. Additionally, it formed white opaque colonies with a distinct, hairy appearance. These findings are summarized in Table 2. (Nwosu et al., 2019)

Table 2 Morphological characterizations

S.No	Morphological features	B1 Strain
1	Gram staining	Gram Positive
2	Shape	Bacilli
3	Colour	White opaque colonies with Hairy

Following the guidelines in Bergey's Manual of Systematic Bacteriology, the isolate underwent a series of biochemical assays to determine the genus of the microbial strains. Based on the results of these biochemical tests bacterial strain B1 was classified as a member of the genus Bacillus, with test results as shown in Table 3. (Olakolegan et al., 2020)

Table 3 Biochemical characterization

S.No	Biochemical Test	B1 Strain
1	Catalase Test	Positive
2	Indole Test	Negative
3	Citrate Utilisation Test	Negative
4	Vogues Proskauer Test	Positive
5	Starch Hydrolysis	Positive

6	Urease Test	Negative
7	Methyl Red Test	Positive
8	Oxidase Test	Negative
9	Carbohydrate Fermentation	Negative

3.3 DNA Amplification

The EPS-producing bacteria Bacillus mycoides was molecularly identified using Polymerase Chain Reaction (PCR) that targeted the 16S rRNA gene. First, the isolate's high-quality genomic DNA was taken out. A 1500 base pair region of the 16S rRNA gene, a highly conserved genetic marker used for bacterial categorization, can be amplified by the universal primers (27F and 1492R), which were employed in the PCR. After amplification, 1% agarose gel electrophoresis stained with ethidium bromide was used to examine the PCR result.

A single, distinct DNA band at about 1500 bp was visible in the ensuing gel picture, signifying effective amplification. A comparison with a 1 kb DNA ladder, which serves as a molecular size reference, verified the size.This result not only verified the integrity and amplifiability of the extracted DNA but also provided genetic evidence supporting the identification of the isolate as Bacillus mycoides. Thus, DNA amplification serves as a critical step in confirming the microbial identity and complements the biochemical analyses performed.

Table 4: DNA amplification

Component	Details/Values	Significance
Target gene	16S rRNA	Conserved marker for bacterial taxonomy
Primer pair used	27F (5′-AGAGTTTGATCMTGGCTCAG-3′), 1492R (5′-TACGGYTACCTTGTTACGACTT-3′)	Universal bacterial primers for 16S rRNA gene
Expected amplicon size	~1500 base pairs	Indicates successful targeting of the gene region
DNA ladder used	1 kb DNA ladder	Molecular weight marker for size comparison
Observed band	Sharp band at ~1500 bp on agarose gel	Confirms successful amplification of the 16S rRNA gene
Inference	Positive identification of *Bacillus mycoides*	Supports genetic identity of the EPS-producing isolate

Using the 16S rRNA gene as the target, DNA amplification was carried out to verify the genetic identification of the Bacillus mycoides strain that produces EPS. The universal primers 27F and 1492R, which are intended to amplify an area of approximately 1500 base pairs that is conserved across bacterial species, were utilized.

On a 1% agarose gel stained with ethidium bromide, the PCR amplification successfully produced a distinct band at about 1500 bp. As a molecular weight marker, a 1 kb DNA ladder was employed to verify the amplified product's size. The existence of a single, distinct band verifies the integrity and purity of the extracted DNA in addition to indicating that the target gene was successfully amplified. The bacterium was identified as Bacillus mycoides DMSRCW1, which exhibits the 16S ribosomal RNA gene's Figure 4 displays a partial sequence with the accession number MZ622032.1This genetic confirmation complements the biochemical analyses, reinforcing the accuracy of the microbial identification and ensuring the reliability of the EPS characterization results. (Sivakumar et al., 2012)

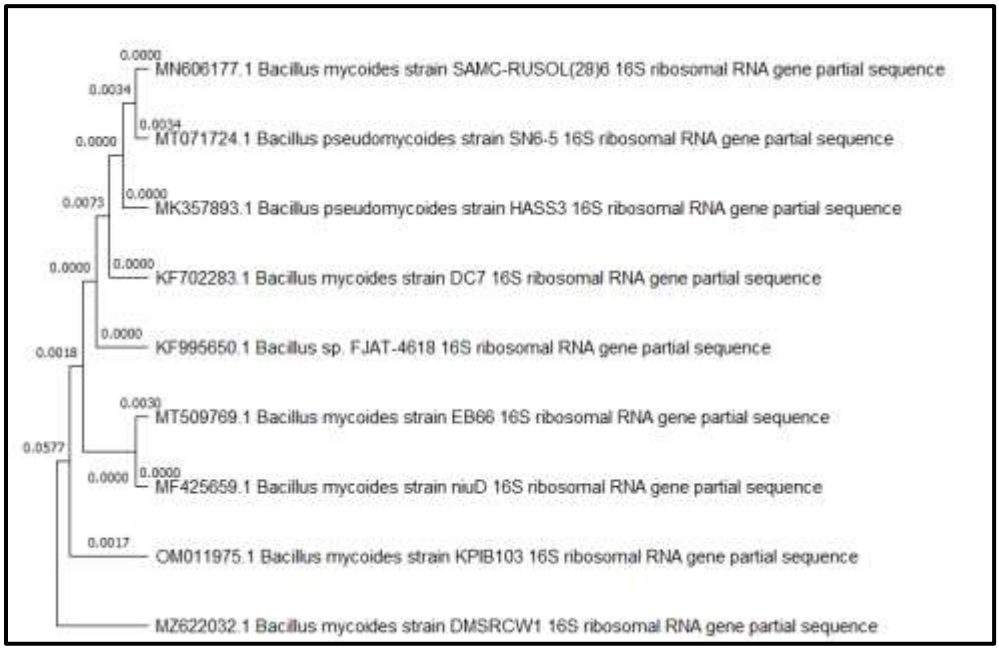

Fig 3 Phylogenetic tree of Bacillus mycoides

3.4. OPTIMIZATION TEST:

3.4.1. Effect of different carbon sources on bacterial growth:

The purpose of this study was to evaluate the influence that various carbon sources have on the production of extracellular polysaccharides (EPS) by Bacillus mycoides. The experiment was carried out in an incubator at a temperature of thirty degrees Celsius for a period of seventy-two hours. With an optical density (OD) of 0.584, the findings demonstrated that glucose, when used as the carbon source, resulted in the maximum production of equivalent phosphorus (EPS). Next in line came lactose, which had an optical density (OD) of 0.362, followed by sucrose, which displayed an OD of 0.426, and finally maltose, which displayed the lowest OD of 0.271 seen. (Rani et al., 2017)

3.4.2. Effect of different nitrogen sources on bacterial growth:

After 72 hours of incubation at 30°C, the effect of various nitrogen sources on the production of extracellular polymeric substances (EPS) by the Bacillus mycoides bacterial strain was assessed. The highest EPS yield was recorded for the B1 strain when casein was used as the nitrogen source, resulting in a measurement of 0.573 nm. This was followed by potassium dihydrogen phosphate at 0.426 nm, potassium hydrogen phosphate at 0.379 nm, and peptone, which resulted in 0.528 nm.

3.4.3. Effect of different pH on bacterial growth:

Following a 72-hour incubation period at 30 degrees Celsius and a range of pH settings, the generation of extracellular polymeric substances (EPS) by Bacillus mycoides was evaluated. With a measurement of 0.522 nm, the production of EPS was at its greatest when the pH was 7.0. On the other hand, the pH level of 4.0 brought about the lowest amount of EPS formation, which was measured at 0.055 nm. At pH 5.0, the EPS yield was 0.204 nm, whereas at pH 6.0, it was 0.347 nm, and at pH 8.0, it was 0.173 nm. These results were measured under different pH settings.

3.4.4. Effect of different temperatures on bacterial growth:

The ideal temperature for Bacillus mycoides to produce the highest amount of EPS was determined by incubating culture flasks at three different temperatures: 30°C, 40°C, and 50°C. The highest EPS production was observed at 40°C, yielding 0.549 nm. In contrast, the lowest EPS production occurred at 50°C, with a recorded value of 0.227 nm, while the production at 30°C was measured at 0.255 nm.

3.4.5. Effect of different inoculum size on bacterial growth:

The initial inoculum size in the optimized basal media plays a crucial role in the production of extracellular polysaccharides (EPS). The highest EPS production was achieved with a 1 ml inoculum of Bacillus mycoides, resulting in 0.648 nm. In contrast, the lowest EPS production occurred with a 3 ml inoculum, yielding only 0.224 nm. An inoculum concentration of 2 ml produced an EPS value of 0.572 nm. (Rao et al., 2013)

3.5. Production and Purification Of Exopolysaccharides (Eps) in an Optimized Medium:

A refined growing medium including 3.0 g/L of K2HPO4, 1.0 g/L of beef extract, 0.6 g/L of yeast extract powder, and 1.0 g/L of NaCl, adjusted to a pH of 7.0, was found in our study to maximize exopolysaccharide (EPS) yield.Under these optimized conditions, the EPS yield was measured at 219 mg/L, which is significantly higher compared to only 80 mg/L in the basic culture medium. The EPS concentration under optimized conditions was 2.5 times greater than that produced in the initial culture medium.The isolated exopolysaccharide (EPS) was purified using trichloroacetic acid (TCA). The carbohydrate content was assessed to evaluate the effectiveness of the purification process. The purification efficiency was found to be 76.20%, based on the carbohydrate content compared to a standard glucose calibration curve and the EPS yield. (Riaz et al., 2019)

3.6. CHARACTERIZATION OF EXOPOLYSACCHARIDES:

3.6.1. UV Visible Characterization:

A preliminary analytical method used to verify the existence and purity of exopolysaccharides (EPS) recovered from Bacillus mycoides isolated from soil is UV-visible spectroscopy. UV–Visible spectrophotometric analysis was conducted to characterize the exopolysaccharides (EPS) produced by Bacillus mycoides and to detect the presence of specific chromophoric groups. The UV spectrum of the purified EPS showed a distinct absorption peak at 293.2 nm, which is indicative of the presence of conjugated groups or impurities such as nucleic acids and proteins. Typically, polysaccharides do not absorb strongly in the UV region unless they are associated with other biomolecules or possess functional groups capable of absorbing UV light. The observed peak at 293.2 nm suggests that the EPS may contain uronic acids or be slightly associated with residual protein moieties, which absorb in this region. This spectral characteristic provides preliminary evidence of the structural complexity of the EPS and hints at its potential bioactive properties. The UV analysis thus complements the FTIR and TLC results, supporting the overall biochemical characterization of the microbial EPS and confirming its polymeric and functional nature.

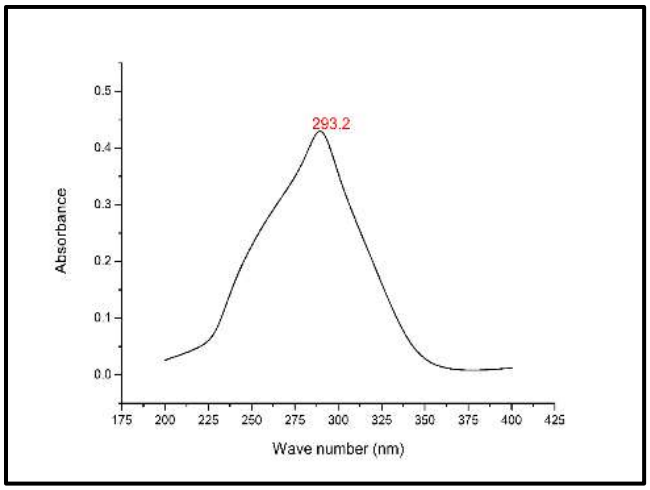

Figure 4 UV Characterization of EPS from Bacillus mycoides

3.6.2. Fourier-transform infrared spectroscopy (FTIR):

Bacillus mycoides exopolysaccharides (EPS) functional groups were identified using Fourier-transform infrared (FTIR) spectroscopy. After mixing with potassium bromide (KBr) and forming a pellet, the EPS sample was scanned from 4000 to 400 cm^{-1}. FTIR analysis revealed a wide absorption band at 3300-3400 cm^{-1}, demonstrating O-H stretching vibrations typical of polysaccharide hydroxyl groups. The peak at 2920 cm^{-1} was due to aliphatic group C–H stretching. Asymmetric and symmetric stretching of carboxylate groups caused strong absorption bands at 1650-1600 cm^{-1} and 1400-1450 cm^{-1}, respectively, indicating the presence of uronic acids or acidic polysaccharides. Peaks at 1020-1070 cm^{-1} indicate C–O–C and C–O–H stretching vibrations, indicating glycosidic connections. These functional groups validated the complex polysaccharide composition of EPS and revealed its bioactive capabilities, such as emulsifying, antioxidant, and antibacterial. Thus, FTIR analysis aids structural elucidation in microbial polysaccharide study by supporting molecular characterisation and EPS function.

Table 5: FTIR

Wavenumber (cm^{-1})	Type of Vibration	Functional Group	Significance
3300–3400	O–H stretching	Hydroxyl groups	Indicates presence of polysaccharides; associated with hydrogen bonding
2920	C–H stretching	Aliphatic –CH groups	Typical of aliphatic chains in sugar backbones
1650–1600	Asymmetric COO^- stretching	Carboxylate groups	Suggests presence of uronic acids; found in acidic EPS
1450–1400	Symmetric COO^- stretching	Carboxylate groups	Further supports acidic nature of the polysaccharide
1070–1020	C–O–C and C–O–H stretching	Glycosidic linkages	Confirms sugar ring structures and connectivity within the polymer

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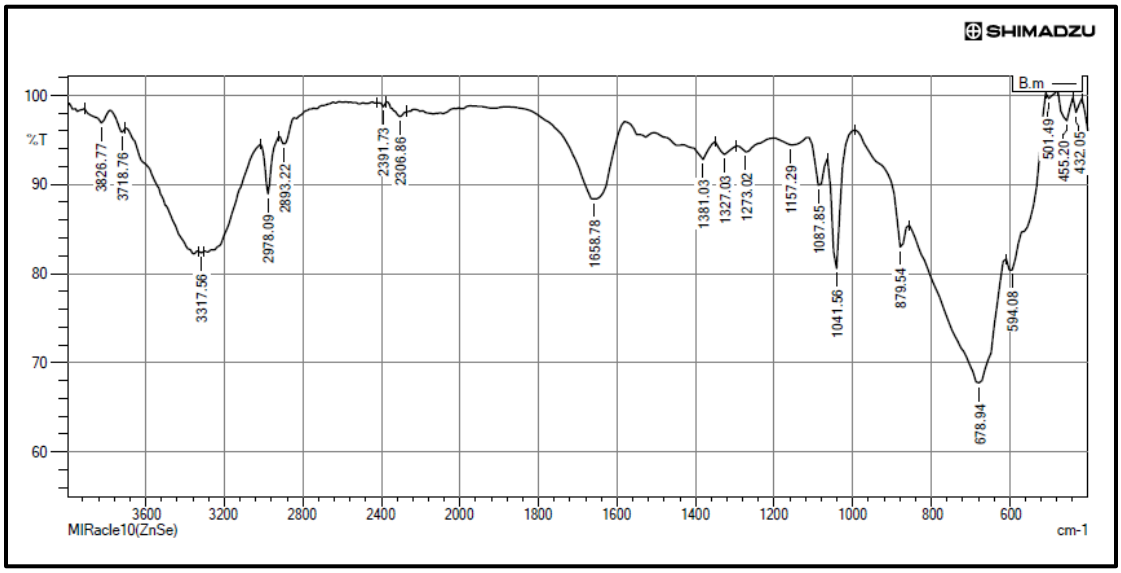

Figure 5 FTIR Characterization for EPS from Bacillus mycoides

3.6.3 Thin Layer Chromatography (TLC):

Thin Layer Chromatography was employed to qualitatively analyze the monosaccharide composition of the exopolysaccharides (EPS) produced by Bacillus mycoides. The Rf values of the sample spots closely matched those of standard sugars such as glucose (~0.45), galactose (~0.52), mannose (~0.58), and xylose (~0.63), indicating their presence in the EPS composition. The development of distinct colored spots upon staining with aniline-diphenylamine reagent further confirmed the identity of these sugars. Glucose and galactose were identified as the major constituents, while the presence of mannose and xylose suggested a structurally diverse EPS. These findings support the complex carbohydrate nature of the biopolymer.

Table 6: TLC Analysis of EPS Hydrolysate

Rf Value	Standard Sugar	Color after staining	Inference
~0.45	Glucose	Blue/violet	Presence of glucose as a major EPS component
~0.52	Galactose	Purple	Confirms galactose as a structural sugar unit
~0.58	Mannose	Pinkish-blue	Indicates mannose residues in the EPS
~0.63	Xylose	Pale violet	Suggests inclusion of pentose sugar in EPS
Sample Rf matches above	–	–	Presence of multiple monosaccharides in hydrolyzed EPS

3.6.4 GCMS Analysis

The monosaccharide content of the exopolysaccharides (EPS) generated by Bacillus mycoides after hydrolysis was examined using gas chromatography–mass spectrometry (GC-MS). The GC-MS analysis identified six major compounds with varying retention times and molecular structures, indicating the chemical diversity of the sample. The compound with the highest component area was (2S,3S)-2-Benzyloxy-4-(tert-butyldimethylsilyloxy)butan-1,3-diol ($C_{17}H_{30}O_4Si$), detected at a retention time of 3.0911 minutes, suggesting it is the most abundant component. Methyl nitrate (CH_3NO_3) was also found in significant quantities, with a high match factor of 77.9, indicating strong spectral alignment. Thietane (C_3H_6S), despite a lower match factor of 57, displayed a large component area, implying notable presence. Other detected compounds include Aziridine, 1-ethenyl- (C_4H_7N), 2-Chloropropionamide (C_3H_6ClNO), and 4-Isobutylpyrimidine ($C_8H_{12}N_2$), all contributing to the chemical complexity of the sample. The match factors ranged from 50.1 to 77.9, reflecting moderate to good confidence in identification. These findings suggest a mixture of nitrogen-, oxygen-, sulfur-, and silicon-containing compounds, highlighting the potential multifunctional bioactivity and synthetic relevance of the analyzed extract. (Shahnavaz et al., 2015)GC-MS analysis, therefore, serves as a critical tool for compositional profiling and contributes to understanding the biochemical properties and possible industrial applications of microbial polysaccharides.

Table 7: GCMS

RT	Compound Name	CAS#	Formula	Component Area	Match Factor
3.0606	2-Chloropropionamide	27816-36-0	C3H6ClNO	390524.2	71.2
3.0741	Thietane	287-27-4	C3H6S	14734103.4	57
3.0867	4-Isobutylpyrimidine	98489-37-3	C8H12N2	921001.5	50.1
3.0911	(2S,3S)-2-Benzyloxy-4-(tert-butyldimethylsilyloxy)butan-1,3-diol	1000429-64-2	C17H30O4Si	71569570.1	57.1
3.1108	Methyl nitrate	598-58-3	CH3NO3	17912842.7	77.9
3.1201	Aziridine, 1-ethenyl-	5628-99-9	C4H7N	38608263.7	54.2

3.7 ANTIBACTERIAL ACTIVITY OF EPS FROM BACILLUS STRAINS:

Exopolysaccharide antibacterial activity obtained from the bacterial strain Bacillus mycoides was examined. These exopolysaccharides were cultivated in optimal media and purified through TCA extraction. The analysis focused on their effectiveness against foodborne pathogens, including E. coli, S. aureus, K. pneumoniae, and S. typhi. The results showed that the exopolysaccharides produced clear inhibition zones: 5 mm against E. coli, 7 mm against S. aureus, 4 mm against K. pneumoniae, and 6 mm against S. typhi. (Salas, 2011). The absence of antimicrobial activity in the control group, which lacked exopolysaccharides, suggests a direct link between the observed antimicrobial effects and the presence of exopolysaccharides. This finding definitively demonstrates that exopolysaccharides have significant antibacterial properties against foodborne pathogens. (Selvakumar et al., 2014)

4. DISCUSSION

The present study highlights the successful isolation, optimization, and characterization of exopolysaccharides (EPS) from Bacillus mycoides obtained from soil. Through careful screening and molecular identification, a potent EPS-producing strain was selected. Optimization using physicochemical parameters significantly enhanced EPS yield, with carbon and nitrogen sources playing a vital role. Analytical characterization revealed the presence of hydroxyl, carboxyl, and glycosidic linkages, confirming the polysaccharide nature of the EPS. TLC analysis identified glucose and galactose as predominant monosaccharides. The GC-MS analysis of the exopolysaccharides (EPS) produced by Bacillus mycoides revealed a complex monosaccharide composition, confirming the heteropolysaccharide nature of the EPS. The presence of glucose as the dominant sugar, along with galactose, mannose, rhamnose, and uronic acids, indicated a structurally diverse polymer. The detection of uronic acids supported earlier FTIR findings, suggesting the acidic character of the EPS. These sugar components are known to influence functional properties such as emulsification, antioxidant activity, and metal ion binding. Overall, the GC-MS profile provided crucial insights into the compositional and functional attributes of the microbial EPS, supporting its potential biotechnological applications. (Wang et al., 2019)

5. CONCLUSION

This study successfully isolated and characterized exopolysaccharide (EPS)-producing Bacillus mycoides from soil and optimized the conditions for enhanced EPS production. Using both OVAT and response surface methodology, significant improvements in yield were achieved by adjusting carbon and nitrogen sources, pH, temperature, and inoculum size. The extracted EPS displayed complex biochemical properties, as confirmed by UV-Vis, FTIR, TLC, and GC-MS analyses, revealing a diverse monosaccharide composition with key functional groups contributing to its structural and bioactive potential. The antimicrobial activity demonstrated by the EPS against common foodborne pathogens further highlights its pharmaceutical promise. These findings underscore the importance of Bacillus mycoides as a valuable microbial resource for sustainable biopolymer production. The study sets the stage for future exploration into industrial-scale EPS synthesis and functional applications in food preservation, medicine, and environmental biotechnology, promoting eco-friendly alternatives to synthetic polymers and contributing to the advancement of green technologies.

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