RESEARCH PAPER

Phenotypic Profiling and Characterization of Extracellular Polymeric Substances (Eps) From A Novel *Priestia Megaterium* Strain

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Received: 10/08/2026 **Accepted: 20/08/2026** **Published: 07/09/2026**

ABSTRACT

Biofilms are structured microbial communities enclosed within an extracellular polymeric substance (EPS) matrix that enhances microbial survival and offers diverse biotechnological applications. The present study aimed to isolate, identify, and characterize a biofilm-forming bacterium from the biofilm developed on boiled rice water and to evaluate the functional properties of its EPS. Biofilm samples were serially diluted and cultured on nutrient agar, yielding five morphologically distinct bacterial isolates. Among these, one isolate exhibited strong biofilm-forming ability and was selected for further characterization. Morphological and biochemical analyses identified the isolate as a Gram-positive, short rod-shaped bacterium. Molecular identification through 16S rRNA gene sequencing confirmed the isolate as *Priestia megaterium*. EPS extracted from biofilms formed on glass beads was characterized using Fourier Transform Infrared (FTIR) spectroscopy, revealing the presence of alcohol, amine, carbonyl, aldehyde, nitrile, and aromatic functional groups. Biochemical analysis demonstrated that the EPS was rich in carbohydrates and proteins. Functional evaluation showed strong dose-dependent antioxidant activity, achieving 76.25% free radical scavenging at 500 µL concentration. Moderate antibacterial activity (3–9 mm inhibition zones) and mild antifungal activity against *Aspergillus* species were also observed. Notably, the isolate initiated biofilm formation within 10 minutes of surface attachment, indicating rapid colonization ability. These findings demonstrate that *P. megaterium* isolated from boiled rice water produces a bioactive EPS with significant antioxidant potential and moderate antimicrobial properties, highlighting its promise for applications in biomedical, pharmaceutical, food, agricultural, and environmental biotechnology. This study emphasizes the potential of naturally occurring microorganisms from traditional substrates as sustainable sources of valuable bioactive compounds.

Keywords: Biofilm, Extracellular Polymeric Substance (EPS), *Priestia megaterium*, Boiled Rice Water, Antioxidant Activity, Antibacterial Activity, FTIR, Biotechnology.

How to cite this article: S. Murugan*1 S. Devi bala2. Phenotypic Profiling and Characterization of Extracellular Polymeric Substances (Eps) From A Novel Priestia Megaterium Strain. Int J Drug Deliv Technol. 2026;16(80s):1713-1721. DOI: 10.25258/ijddt.16.80s.212

.INTRODUCTION

Rice (Oryza sativa) is a fundamental dietary staple for over half of the world's population, serving as a vital source of carbohydrates and essential nutrients. In the conventional cooking method, rice experiences thermal hydrolysis, which leads to the release and dispersion of soluble starches into the cooking water. This results in a significant amount of nutrient-rich wastewater, commonly known as rice starch water. Upon cooling, this byproduct develops a thick, milky, and gelatinous film on the surface, abundant in organic matter such as free sugars, amino acids, vitamins, and minerals. Traditionally, it has been employed in ethnomedicine, skincare, and food processing as a thickening agent. Additionally, this starchy matrix creates an ideal habitat rich in carbon and nitrogen that actively promotes microbial growth. Depending on various external environmental factors, including temperature, pH, and duration of exposure, this medium can support both beneficial fermentative organisms and potentially harmful pathogenic microbes.

In environments that are both aqueous and rich in nutrients, over 99% of naturally occurring bacteria shift from a free-swimming, planktonic state to a sessile lifestyle. This shift promotes the development of microbial biofilms, which are characterized as highly organized, surface-associated communities of microorganisms that are embedded within a self-generated matrix of extracellular polymeric substances (EPS). The EPS matrix, which is mainly made up of lipopolysaccharides, proteins, nucleic acids, and lipids, functions as both a mechanical and biochemical barrier. This structural intricacy provides biofilm-associated bacteria with

increased phenotypic resistance to environmental stressors, host immune responses, and standard antimicrobial agents when compared to their planktonic counterparts. The process of biofilm development is a highly regulated, multi-stage morphogenetic process that is influenced by mechanical attachment, genetic transcriptomic changes, and intercellular signaling pathways collectively referred to as Quorum Sensing.

Although biofilm phenotypes have been recognized since the groundbreaking microscopic studies conducted by Antoni van Leeuwenhoek and Louis Pasteur, their contemporary significance extends into essential industrial, environmental, and medical fields. In marine and industrial environments, bacterial biofilms modify the physicochemical characteristics of submerged surfaces, leading to macrofouling and severe biocorrosion, which considerably increase maintenance expenses. In contrast, within medical settings, these organized microbial communities are significant etiological factors in persistent, chronic infections and contamination of medical devices by foreign bodies. Considering that geographical, seasonal, and substrate-specific differences greatly influence the composition, growth rate, and structural density of the EPS matrix, it is imperative to assess biofilm dynamics on organic byproducts for effective biotechnological management and waste treatment. As a result, comprehending how specialized bacterial strains colonize and utilize starch-rich agricultural residues offers crucial insights into microbial ecology and pathogen virulence. To explore this issue, the current study intends to investigate the molecular and phenotypic traits of biofilm-forming bacteria that utilize the organic starchy layer generated as a byproduct of boiled rice.

MATERIALS AND METHODS

1: Sample Preparation and Microbial Isolation

1.1 Sample Collection and Processing

The hybrid Ponni rice variety (Myang Ebos 6080/2 and Taichung 65) was sourced from a local commercial supplier in Thoothukudi, Tamil Nadu, India. To create the starch matrix, a 250 g sample of the rice was placed into a 1000 mL beaker, immersed in 500 mL of distilled water, and subjected to thermal hydrolysis at a temperature of 100°C for a duration of 30 minutes. After the boiling process, the liquid portion was separated from the solid grains using a sterile cotton cloth. The collected rice water was then incubated in a sterile beaker under static conditions at room temperature for 10 minutes, which facilitated the formation of a distinct gelatinous starchy layer on the surface.

1.2 Isolation and Screening of Biofilm-Forming Bacteria

To isolate bacteria, a 1 mL sample of the newly developed surface starchy layer was homogenized in 99 mL of sterile distilled water and subjected to serial dilutions up to a factor of 10^{-7}. Individual dilutions were then spread-plated onto nutrient agar matrices and incubated at 37°C for 24 hours to facilitate colony growth. Based on macro-morphological differences, five distinct colony types were isolated and subsequently sub-cultured into sterile nutrient broth for 24 hours at 37°C to prepare inoculum for screening.

The biofilm-forming ability of the isolates was assessed qualitatively using a tube staining assay. Luria-Bertani (LB) broth (1 mL) was inoculated with 100 µL of the overnight bacterial culture and incubated statically at 37°C for 72 hours. After incubation, the liquid media was decanted, and the tubes were rinsed with Phosphate Buffered Saline (PBS, pH 7.3) to eliminate free-floating planktonic cells. The adherent biomass was air-dried and stained with 0.1% (w/v) crystal violet reagent. Excess background stain was removed through washes with deionized water. The presence of a visible, chemically stained macro-film along the bottom and internal walls of the tube indicated positive biofilm production. The confirmed biofilm-producing strain was then purified on nutrient agar plates using the quadrant streaking technique and incubated at 37°C for 24 hours.

2: Phenotypic and Biochemical Characterization

2.1 Morphological Identification via Gram Staining

The structural classification of the isolated biofilm-forming strain was initially evaluated using standard Gram staining techniques. A thin bacterial smear was prepared on a clean glass slide, air-dried, and heat-fixed over a burner flame. The fixed specimen was stained with crystal violet for 60 seconds, rinsed with water, and exposed to Gram's iodine mordant for an additional 60 seconds. Decolorization was performed by gently rocking the slide with 95% alcohol for 20 seconds until the primary stain stopped bleeding, followed immediately by an aqueous wash. The smear was counterstained with safranin for 20 seconds, rinsed, blot-dried, and examined under a compound light microscope to determine Gram reaction and cellular morphology.

2.2 Biochemical Phenotyping (IMViC Tests)

To assess the metabolic profiles of the bacterial population, a standardized IMViC test suite was performed: Indole Test: The isolate was inoculated into peptone water broth and incubated at 37°C for 24 hours. After incubation, Kovac's reagent was added to identify tryptophan degradation and the subsequent production of indole.Methyl Red (MR) Test: The culture was transferred into MR-VP broth and incubated at 37°C for 24 hours. Methyl red

indicator dye was introduced to evaluate whether the organism could produce and maintain stable organic acid end-products through glucose fermentation, remaining below a critical pH threshold of 4.4. Voges-Proskauer (VP) Test: After a 24-hour incubation in MR-VP broth at 37°C, the culture was treated with 0.5 mL of alpha-naphthol and 0.3 mL of potassium hydroxide (KOH) to determine if glucose was converted into acetoin. Citrate Utilization Test: The isolate was streaked onto Simmon's Citrate Agar slants and incubated at 37°C for 24–48 hours to evaluate its ability to produce citrate-permease and utilize citrate as its sole carbon and energy source.

3: Molecular Identification and Genetic Sequencing

3.1 Genomic DNA Extraction and Electrophoresis

Total genomic DNA was extracted from pure bacterial cultures using the NucleoSpin® Tissue Kit (Macherey-Nagel) in accordance with the manufacturer's instructions. An aliquot of the liquid culture was lysed by adding 180 µL of T1 buffer and 25 µL of Proteinase K, and this mixture was incubated in a water bath set at 56°C until complete cellular lysis occurred. To eliminate RNA contamination, 5 µL of RNase A (100 mg/ml) was added and allowed to react for 5 minutes at room temperature. The solution was then treated with 200 µL of B3 buffer at 70°C for 10 minutes, followed by the addition of 210 µL of absolute ethanol, and subsequently loaded onto a NucleoSpin® Tissue column. After centrifugation at 11,000 × g for 1 minute, the silica membrane was washed in succession with 500 µL of BW buffer and 600 µL of B5 buffer. The purified genomic DNA was eluted using 50 µL of BE buffer. The structural integrity and yield of the DNA were assessed through 0.8% agarose gel electrophoresis in 0.5X Tris-Borate-EDTA (TBE) buffer containing 0.5 µg/mL ethidium bromide, which was run at 75 V and visualized using a UV transilluminator (Genei/Bio-Rad).

3.2 16S rRNA Amplification and Sanger Sequencing

The Polymerase Chain Reaction (PCR) amplification of the specific 16S rRNA gene was carried out using a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems). The reaction mix totaled 10 µL, which included 5 µL of 2X Phire Master Mix, 4 µL of distilled water, 1 µL of template DNA, and 0.25 µL of each specialized forward (16S-RS-F: 5'-CAGGCCTAACACATGCAAGTC-3') and reverse (16S-RS-R: 5'-GGGCGGWGTGTACAAGGC-3') primers. The amplification protocol consisted of an initial denaturation step at 98°C for 1 minute, followed by 40 cycles comprising denaturation at 98°C for 5 seconds, primer annealing at 60°C for 10 seconds, extension at 72°C for 15 seconds, and a final extension at 72°C for 2 minutes. The resulting amplicons were resolved on 1.2% agarose gels alongside a 2-log DNA ladder standard (NEB).

The PCR Mix (10 µL) was composed of:

- 2X Phire Master Mix : 5.00 µL
- Distilled Water : 4.00 µL
- Template DNA : 1.00 µL
- Forward Primer : 0.25 µL
- Reverse Primer : 0.25 µL

To eliminate unincorporated primers and dNTPs, 5 µL of the PCR product was mixed with 0.5 µL of ExoSAP-IT enzyme mix, incubated at 37°C for 15 minutes, and subsequently denatured at 85°C for 5 minutes. Cycle sequencing was conducted using the BigDye Terminator v3.1 Kit, employing a specialized reaction matrix consisting of 6.6 µL distilled water, 1.9 µL of 5X sequencing buffer, 0.3 µL of forward primer, 0.3 µL of reverse primer, 0.2 µL of sequencing mix, and 1 µL of treated template, over 30 thermal cycles. Following sequencing, cleanup was performed through precipitation with 3M sodium acetate, EDTA, and absolute ethanol. The resulting pellets were washed with 70% ethanol, air-dried, resuspended in 10 µL of highly deionized formamide (HiDi), and analyzed on an ABI 3500 DNA Analyzer using the Sanger method. The raw chromatograms were assessed with Sequence Scanner Software v1 and aligned using Geneious Pro v5.1.

4: Extraction and Functional Bio-Assays of EPS

4.1 Extraction of Extracellular Polymeric Substances (EPS)

To obtain the EPS matrix, the selected bacterial isolate was cultivated overnight in LB broth and subsequently diluted 1:100 into 20 mL of fresh LB broth within a flask containing sterile 2 mm diameter glass beads. The system was incubated at 37°C for a duration of 72 hours to promote biofilm development on the surfaces of the beads. Planktonic cells were eliminated by decanting the media and washing the beads twice with PBS. The beads were then vortexed to dislodge the biofilm, and the resulting suspension was centrifuged at 15,000 rpm for 20 minutes at 4°C. The pellet was resuspended in 30 mL of cold 0.2 M sulfuric acid (pH 1.1) and mechanically homogenized with a glass tissue homogenizer. The mixture was stirred intermittently at 4°C for 3 hours and centrifuged at 15,000 rpm for 20 minutes to separate the total EPS from the supernatant fraction. The chemical functional groups present in the EPS were analyzed using Fourier-Transform Infrared (FTIR) spectroscopy.

4.2 Biochemical and Functional Quantification Assays

- **Carbohydrate and Protein Estimation:** The total carbohydrate content present in the extracted EPS was quantified using the

Anthrone method at a wavelength of 620 nm, employing ice-cold anthrone reagent alongside glucose standards. The total protein content was assessed at 595 nm through the Bradford assay method, utilizing bovine serum albumin (BSA) standards.

- **Antioxidant Activity:** The total antioxidant capacity of the EPS was evaluated at various concentrations (0.1–0.5 mL) through the phosphomolybdenum method. This involved a reaction mixture containing sulfuric acid, sodium phosphate, and ammonium molybdate, which was incubated at 95°C for 90 minutes. The absorbance was measured at 570 nm against an ascorbic acid positive control, and the results were calculated using standard percentage inhibition equations.
- **Antagonistic and Antimicrobial Screening:** Antagonistic interactions among isolates were assessed by performing cross-streaking tests with test organisms against a central perpendicular streak of the target isolate on nutrient agar. Pathogenicity was screened based on hemolytic activity observed on blood agar slants. The antimicrobial efficacy of the pure EPS extract was evaluated using the agar well diffusion method; Mueller-Hinton Agar (MHA) was utilized for bacterial testing, while Rose Bengal Agar was used for fungi. Isolate swabs were evenly spread, 8 mm diameter wells were created, filled with the EPS extract, and incubated (24 hours for bacteria and 48 hours for fungi) to measure the clear zone of inhibition in millimeters.

RESULTS

1. Sample Preparation and Bacterial Isolation

Incubation of boiled rice water at room temperature for 10 minutes resulted in the formation of a distinct surface layer (Fig.1). Bacterial isolation from this surface layer was performed via serial dilution up to 10^{-7} followed by spread plating on nutrient agar. Following 24 hours of incubation, multiple morphologically distinct bacterial colonies emerged. Five distinct colonies were selected for further study based on their specific macroscopic features: Colony 1: Yellow, circular, smooth surface. Colony 2: White, circular, convex surface. Colony 3: Pale yellow, irregular, mucoid surface. Colony 4: Yellowish-white, circular, rough surface. Colony 5: White, irregular, rough surface.

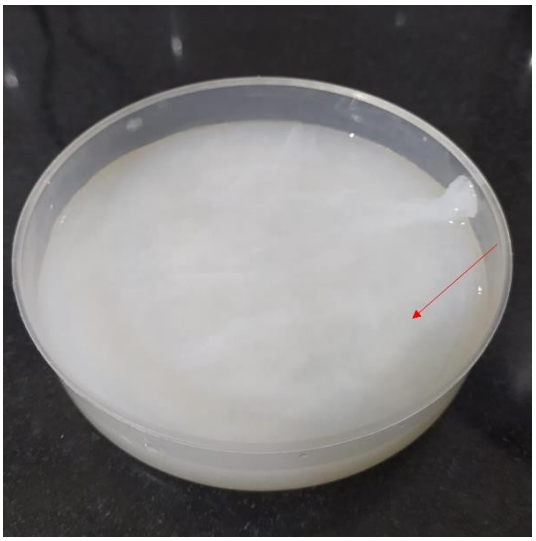


Fig.1: Boiled rice water sample

2. Screening for Biofilm-Forming Strains

Primary screening using the qualitative glass tube assay revealed that all five isolated strains were positive for biofilm formation. Among these, one specific strain demonstrated exceptionally strong biofilm production and was prioritized for downstream analysis. This phenotype indicates an efficient production of extracellular polymeric substances (EPS), which are critical for stable structural architecture and surface adherence. The strain displayed rapid surface sensing and attachment capabilities, initiating observable biofilm formation within 10 minutes of exposure to a solid substrate (Fig.2).

Fig.2: stained tube showing biofilm attachment

3. Morphological and Phenotypic Characterization

The selected strong biofilm-producing colony was successfully purified via the quadrant streaking method on nutrient agar (Fig. 3). Gram staining and subsequent microscopic examination identified the organism as a Gram-positive, short rod-shaped bacterium. Cellular arrangement occurred predominantly in pairs as well as in short and long chains (Fig. 4). The retention of the crystal violet stain confirms a characteristic thick peptidoglycan cell wall layer common to rod-shaped genera like *Lactobacillus* and *Bacillus*.

Fig. 3:Quadrant streak plate of biofilm forming bacteria Fig. 4: Microscopic views of Gram Stain of the isolate

4. Biochemical Profiling

The isolated strain underwent standard biochemical testing to determine its metabolic characteristics: Indole Test: Negative; addition of Kovac's reagent produced no color change, confirming the absence of the tryptophanase enzyme.

Methyl Red (MR) Test: Positive; the medium turned red, demonstrating mixed acid fermentation where glucose is metabolized into stable acidic end products. Voges-Proskauer (VP) Test: Negative; no color change occurred after adding alpha-naphthol and KOH, indicating that the strain does not synthesize acetoin during glucose fermentation.Citrate Utilization Test: Positive; the Simmons citrate agar shifted from green to blue, confirming the organism's capacity to utilize citrate as a sole carbon source under aerobic conditions.This specific pattern (Indole-, MR+, VP-, Citrate+) highlights metabolic versatility and matches profiles common to species within the *Bacillus* group.

5. Molecular Identification and Phylogenetic Analysis

Genomic DNA was isolated utilizing a commercial tissue kit, and its quality was verified via agarose gel electrophoresis under UV light. Polymerase Chain Reaction (PCR) amplification was conducted using 16S rRNA primers (Forward: CAGGCCTAACACATGCAAGTC; Reverse: GGGCGGWGTGTACAAGGC). The resulting PCR products were resolved on a 1.2% agarose gel containing ethidium bromide. Sequence analysis and alignment using BLAST tool against the NCBI non-redundant nucleotide database yielded a 95% identity match (1055/1111 bp) with a 3% gap fraction across a 1077 base pair alignment length (Fig.6). The alignment generated a score of 1705 bits and an E-value of 0.0 (Fig.5). Phylogenetic tree construction and graphic summaries established strong homology, confirming the identity of the bacterial isolate as *Priestia megaterium* (submitted to GenBank under accession number FRWBB08 *Priestia megaterium* PV413214)(Fig.7).

Fig.5: Isolated Bacteria Nucleotide sequencing chromatogram

>SR5151-27R_E08.ab1
ACCCTTCCGTTCCACCTTAGGCGGCTAGCT
CCTTACGGTTACTCCACCGACTTCGGGTGT
TACAAACTCTCGTGGTGTGACGGGCGGTG
TGTACAAGGCCCGGGAACGTATTCACCGC
GGCATGCTGATCCGCGATTACTAGCGATT
CCAGCTTCATGTAGGCGAGTTGCAGCCTA
CAATCCGAACTGAGAATGGTTTTATGGGA
TTGGCTTGACCTCGCGGTCTTGCAGCCCTT
TGTACCATCCATTGTAGCACGTGTGTAGC
CCAGGTCATAAGGGGCATGATGATTTGAC
GTCATCCCCACCTTCCTCCGGTTTGTCACC
GGCAGTCACCTTAGAGTGCCCAACTAAAT
GCTGGCAACTAAGATCAAGGGTTGCGCTC
GTTGCGGGACTTAACCCAACATCTCACGA
CACGAGCTGACGACAACCATGCACCACCT
GTCACTCTGTCCCCCGAAGGGGAACGCTC
TATCTCTAGAGTTGTCAGAGGATGTCAAG
ACCTGGTAAGGTTCTTCGCGTTGCTTCGA
ATTAAACCACATGCTCCACCGCTTGTGCG
GGCCCCCGTCAATTCCTTTGAGTTTCAGTC
TTGCGACCGTACTCCCCAGGCGGAGTGCT
TAATGCGTTAGCTGCAGCACTAAAGGGCG
GAAACCCTCTAACACTTAGCACTCATCGT
TTACGGCGTGGACTACCAGGGTATCTAAT
CCTGTTTGCTCCCCACGCTTTCGCGCCTCA
GCGTCAGTTACAGACCAAAAAGCCGCCCT
TCGCCCACTGGTGTTTCCTCCACATCTCTA
CGCATTTCACCGCTACACGTGGAATTTCC
GGCTTTTCTCTTCTGCACTCAAGGTTCCCC
AGATTCCCAATGACCCCTCCCACGGGTTG
GAGCCGGTGGGGCTTTTCACATCAGACTT
TAAGAAACCGCCTGGCGGCGGCGGCTTAA
CCGCCCAATAATTCCCGGATTACGCTGGC
CACCCTTACGTATTAACCGCGGCTGCTGG
CCACGGTAAGTAAGCCCGTGCTTTTCTGG
TAAGGTACGTCCAAGTACGGAGCCAGATA
CTCTCGGTACCTTGTTCCTTCCTACACAGA
GTTAGCGACCGAGCCTTCAATCACTTCAG
GCTGGGCGGATGTCTCTACGATGA

Fig.6 :Fasta format of Isolated Bacteria

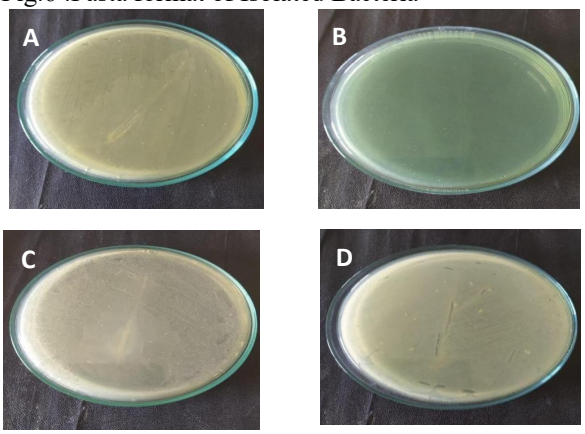


Fig.7: Phylogenetic tree

6. Co-culture Antagonistic and Hemolytic Activities

In vitro antagonistic assays showed no zones of inhibition among the five different bacterial strains isolated from the boiled rice water (Fig.8). The tested strains demonstrated mutual tolerance, suggesting a shared adaptation or commensal relationship within the nutrient-limited environment. Conversely, evaluation on blood agar plates revealed a distinct, clear, colorless zone surrounding the bacterial streak. This complete lysis of red blood cells confirms β hemolytic activity, indicating the secretion of diffusible hemolysins (Fig.9). This trait provides a competitive

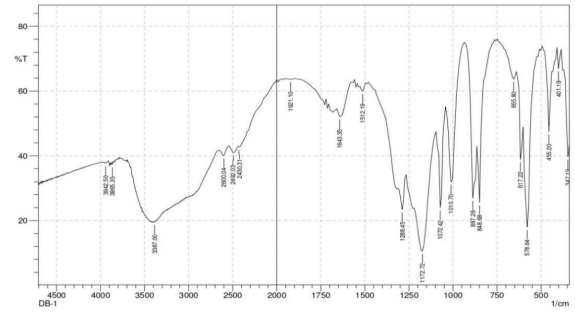
advantage in complex microbial niches and assists in nutrient acquisition.

Fig.8: Antagonist activity

Fig.9: Hemolytic Activity

7. Characterization of Extracellular Polymeric Substances (EPS)

Following biofilm cultivation on glass beads, the EPS matrix was extracted and analyzed using Fourier-Transform Infrared (FTIR) spectroscopy within the range of 400 to 4000 cm^{-1}. Key functional groups were identified through the following absorption peaks: 3942 & 3865 cm^{-1}: O-H stretch (alcohols/phenols) or N-H stretch (amines). 3387 cm^{-1}: Hydrogen-bonded N-H or O-H stretching vibrations. 2600 cm^{-1}: C-H stretching typical of aldehydes or carboxylic acids. 2492 & 2430 cm^{-1}: C≡N stretch (nitrile groups) and C=C stretch (alkynes). 1921 & 1643 cm^{-1}: C=O carbonyl stretching (aldehydes/ketones) and C=C alkene stretching. 1512 cm^{-1}: C-H bending or aromatic ring C=C stretching. 1288, 1172, & 1072 cm^{-1}: C-O stretching indicative of ethers, alcohols, or esters, alongside aliphatic C-N stretching. 1010, 887, 848, 655, & 617 cm^{-1}: Out-of-plane C-H bending vibrations associated with aromatic rings and alkyl groups. 578, 455, & 401 cm^{-1}: C-X stretching, organic skeletal bending, and potential metal-ligand stretching vibrations (Fig.10). Quantitative assays corroborated these structural data. The Anthrone method for carbohydrates yielded an optical density (OD) of 0.80 at 620 nm against a standard glucose blank of 0.77. The Bradford method for proteins yielded an OD of 0.52 at 595 nm against a bovine serum albumin standard of 0.29. These values establish that the carbohydrate fraction forms the primary structural backbone of the extracted EPS matrix, exceeding its total protein content.



No.	Peak	Intensity	Corr. Intensity	Base (H)	Base (L)	Area	Corr. Area
1	347.19	39.706	9.454	370.33	339.47	9.037	0.708
2	401.19	66.983	6.271	408.91	385.76	3.571	0.429
3	455.2	47.44	23.909	486.06	416.62	13.737	3.411
4	578.64	18.001	36.879	601.79	509.21	31.814	11.074
5	617.22	38.869	16.849	640.37	601.79	11.132	1.653
6	655.8	63.641	3.813	748.38	640.37	16.626	0.475
7	848.68	25.603	22.35	864.11	763.81	22.916	2.892
8	887.26	26.975	26.63	933.55	864.11	22.95	5.693
9	1010.7	31.978	25.839	1033.85	941.26	26.216	7.964
10	1072.42	23.958	26.76	1095.57	1041.56	21.812	6.328
11	1172.72	10.628	30.375	1265.3	1103.28	103.744	39.227
12	1288.45	23.451	8.58	1311.59	1265.3	25.451	2.52
13	1512.19	59.949	1.884	1527.62	1481.33	9.792	0.238
14	1643.35	52.062	4.107	1658.78	1581.63	19.344	1.584
15	1921.1	63.645	0.179	1944.25	1897.95	9.057	0.032
16	2430.31	42.825	0.461	2438.02	2005.97	118.492	0.496
17	2492.03	40.934	1.957	2536.32	2438.02	37.93	1.062
18	2600.04	40.115	2.286	2654.05	2546.04	41.376	1.176
19	3387	19.574	5.692	3510.45	2661.77	487.469	52.089
20	3865.35	37.402	0.711	3873.06	3826.77	19.343	0.152
21	3942.5	37.668	0.499	3957.93	3919.35	16.238	0.111

Fig.10: Fourier-transform infrared (FTIR) spectroscopy

8. Functional Bioactivities of Extracted EPS

- **Antioxidant Capacity**: The EPS extract demonstrated significant phosphomolybdenum radical scavenging activity comparable to ascorbic acid. Free-radical neutralization increased proportionally with higher extract concentrations (Fig.11).

Fig.11: Antioxidant activity of EPS sample

- **Antibacterial Activity**: Agar well diffusion assays using the EPS extract at concentrations of µl, 75 µl, 100 µl, and 150 µl produced clear zones of inhibition against the co-isolated bacterial cultures from the fermented system. The inhibitory effect showed a direct concentration-dependent trend (Fig.12).

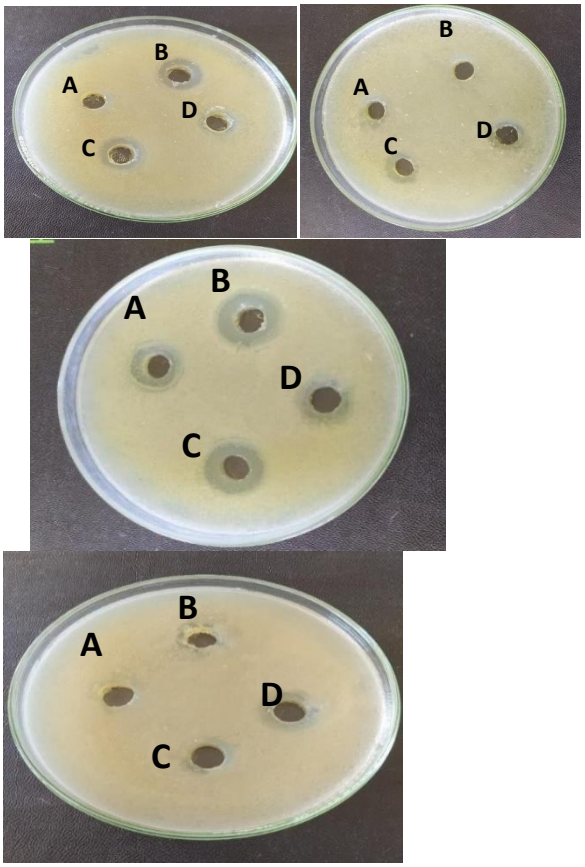


Fig.12: Antibacterial activity (A-50 µl, B-70 µl, C-100 µl, D-150µl)

- **Antifungal Activity**: The EPS extract displayed specific activity against the fungal pathogen *Aspergillus*. While lower concentrations (50 µl, 75 µl, and 100 µl did not produce noticeable inhibition, the maximum concentration of 150 µl generated a distinct 4 mm zone of inhibition, demonstrating a targeted threshold effect against fungal cell walls or spore germination (Fig.13).

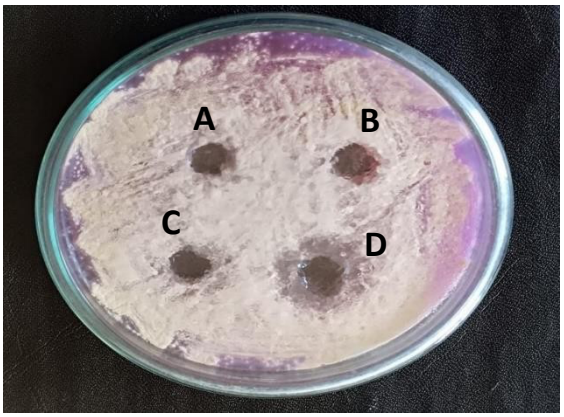


Fig.13: Antifungal activity (A-50 µl, B-70 µl, C-100 µl, D-150µl)

DISCUSSION

The spontaneous formation of a surface pellicle layer on boiled rice water indicates a dynamic migration of hydrophobic macromolecular fractions and lipids to the air-liquid interface. This boundary functions as a nutrient-rich, oxygen-abundant transient selection medium that facilitates a complex polymicrobial succession. While Rahman *et al.* (2021) isolated predominantly uniform whitish-yellow circular cocci from fermented cooked rice water, the identification of five phenotypically distinct colonies in this study underscores a significantly broader morphological spectrum influenced by variations in substrate composition and incubation conditions.

Screening through the foundational glass tube assay confirmed the baseline attachment capabilities of all five isolated strains, aligning with the screening success reported by Jain *et al*. (2013) and validating the efficacy of the assay. The priority isolate exhibited an accelerated attachment kinetic of 10 minutes, surpassing model organisms such as Escherichia coli (10–15 minutes) and demonstrating a performance comparable to Pseudomonas aeruginosa (5 minutes). This rapid surface colonization suggests an immediate upregulation of adhesion proteins or flagellar anchoring mechanisms, leading to the hyper-production of extracellular polymeric substances (EPS) to ensure spatial stability and enhance evolutionary fitness.Isolate Diagnostic & Bioactive Profile. Taxonomic Status: Gram-Positive Bacillus (Priestia megaterium). Metabolic Signature : Indole-negative, MR-positive, VP-negative, Citrate-positive. Matrix Architecture: Carbohydrate-dominant Extracellular Polymeric Backbone. Functional Capacity: Co-culture Tolerance, Beta-Hemolysis, Multi-Biocidal Actions.

Microscopic and staining evaluations identified the hyper-producing strain as a Gram-positive, chain-forming, short rod-shaped bacillus. Its thick peptidoglycan cell wall retains crystal violet dye, a characteristic taxonomic feature common to the Lactobacillus and Bacillus genera. The isolate's metabolic profile was delineated using the IMViC series (Indole-, MR+, VP-, Citrate+). The combination of a positive Methyl Red test and a negative Voges-Proskauer test confirm an exclusive reliance on mixed acid fermentation pathways. This metabolic pathway generates stable acidic end products that lower the microenvironmental pH, thereby outcompeting acid-sensitive strains. Additionally, its citrate-positive phenotype differentiates it from standard isolates described by Huligere *et al*. (2023), underscoring a metabolic versatility that enables the strain to thrive in carbon-limited environments by utilizing alternative organic substrates. Definitive taxonomic identification was achieved through targeting the 16S rRNA gene and PCR amplification. BLAST alignment confirmed a highly significant match of 1077 bp in length (95% identity, E-value: 0.0), positioning the strain within the Priestia megaterium clade (Accession: FRWBB08 Priestia megaterium PV413214).

Co-culture assays indicated the absence of inter-strain antagonism, suggesting long-term evolutionary adaptations. These adaptations facilitate the survival of cooperative, multispecies biofilms within competitive niches. This cooperative behavior contrasts with root-associated Bacillus strains, which actively produce biocidal metabolites against pathogens such as Magnaporthe oryzae. In contrast, the isolate exhibited pronounced β hemolysis on sheep blood agar. This active secretion of diffusible hemolysins aligns with behaviors observed in related species, such as Priestia entomophila. This behavior primarily serves as an ecological strategy to acquire bound iron and sustain dominance over neighboring microbial communities.

The structural mapping of the extracted exopolysaccharides (EPS) through Fourier Transform Infrared (FTIR) spectroscopy has revealed a highly intricate macromolecular assembly. The spectrum displays broad hydroxyl and amine stretching bands at 3942 and 3387 cm^{-1}, respectively, alongside sharp amide and aliphatic C-O modes at 1643, 1172, and 1072 cm^{-1}. This spectral profile substantiates the presence of an integrated network comprising carbohydrates, protein fractions, and distinctive functional groups such as aldehydes and nitriles. Colorimetric assays have further validated that the carbohydrate fraction constitutes the primary physical backbone of the matrix. This composition is consistent with the findings of Patel *et al.* (2018) while contradicting the protein-dominant models proposed by Jain *et al.* (2013). This suggests that *Bacillus megaterium* prioritizes carbohydrate assembly to create a highly hydrated, elastic matrix conducive to firm anchoring.

Functional evaluation indicated a significant, concentration-dependent antioxidant capacity. This characteristic is attributed to the electron-donating hydroxyl and amine groups that can neutralize free radicals, which aligns with the antioxidant profiles documented by Tarannum *et al.* (2023). The cell-free EPS extract exhibited strong, dose-dependent antibacterial activity against co-isolated strains, reflecting the extensive protective properties observed in Lactobacillus matrices. Furthermore, the EPS demonstrated a distinct antifungal threshold at 150 µL, resulting in a 4 mm zone of inhibition against Aspergillus. This targeted control is derived from the embedded matrix proteins and glycolipids that compromise fungal cell wall integrity and inhibit spore germination. These overlapping bioactivities underscore the potential of P. megaterium EPS as a multi-targeted biomaterial for natural food preservation and protective bio-coatings.

REFERENCES

- Allonsius, C. N., et al. (2017). Intercellular mechanisms of purified exopolysaccharides against Candida biofilms and hyphal morphogenesis. *Journal of Applied Microbiology*.
- Christensen, G. D., et al. (1985). Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. *Journal of Clinical Microbiology*.
- Donlan, R. M., & Costerton, J. W. (2002). Biofilms: survival mechanisms of clinically relevant microorganisms. *Clinical Microbiology Reviews*.
- Dubois, M., et al. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*.
- Flemming, H. C., et al. (2016). Biofilms: an emergent form of bacterial life. *Nature Reviews Microbiology*.
- Flemming, H. C., & Wingender, J. (2010). The biofilm matrix. *Nature Reviews Microbiology*.
- Harwood, C. R., & Cutting, S. M. (1990). *Molecular Biological Methods for Bacillus*. John Wiley & Sons.
- Hibbing, M. E., et al. (2010). Bacterial competition: surviving and thriving in the microbial jungle. *Nature Reviews Microbiology*.
- Huligere, S. S., et al. (2023). Biochemical profiling of microflora isolated from traditional fermented food batters. *Journal of Food Science and Technology*.
- Isabelle, G., et al. (2010). Characterization of the strong diffusible hemolytic activity of *Pseudomonas entomophila*. *Environmental Microbiology*.
- Jain, A., et al. (2013). Screening of biofilm-forming bacteria from oral samples and characterization of their extracellular polymeric substances. *Journal of Oral Biology and Craniofacial Research*.
- Jeong, J., et al. (2017). Bactericidal and bacteriostatic activity of exopolysaccharides produced by *Lactobacillus kefiranofaciens*. *Carbohydrate Polymers*.
- MacFaddin, J. F. (2000). *Biochemical Tests for Identification of Medical Bacteria*. Lippincott Williams & Wilkins.
- Madigan, M. T., et al. (2018). *Brock Biology of Microorganisms* (15th ed.). Pearson.
- O'Toole, G. A., & Kolter, R. (1998). Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Molecular Microbiology*.
- Patel, A., et al. (2018). Chemical analysis and structural backbone validation of exopolysaccharides from environmental isolates. *International Journal of Biological Macromolecules*.
- Pratt, L. A., & Kolter, R. (1998). Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, pili, and antigen 43. *Molecular Microbiology*.
- Prescott, L. M., et al. (2002). *Microbiology* (5th ed.). McGraw-Hill Higher Education.
- Quang, N. H. (2022). In vitro antagonistic activity of rice root-associated bacteria against *Magnaporthe oryzae*. *Phytopathology*.
- Rahman, M. M., et al. (2021). Isolation and morphological characterization of bacteria from fermented cooked rice water. *Bangladesh Journal of Microbiology*.
- Rani, P., et al. (2017). FTIR spectral analysis of functional groups in bacterial exopolysaccharides. *Journal of Molecular Structure*.
- Rasamiravaka, T., et al. (2015). The impact of bacterial exopolysaccharides on fungal growth and morphogenesis. *Frontiers in Cellular and Infection Microbiology*.
- Salehizadeh, H., & Shojaosadati, S. A. (2001). Extracellular polymeric substances from *Bacillus* strains: production and characterization. *Biotechnology Letters*.

- Sharma, D., & Puranik, S. (2017). Inhibitory potential of bacterial exopolysaccharides against fungal pathogens. *Mycopathologia*.
- Suresh, K., et al. (2014). Antimicrobial properties of bioactive carotenoid metabolites from pigmented food bacteria. *Food Chemistry*.
- Sutherland, I. W. (2001). Biofilm exopolysaccharides: a shared and precious resource. *Trends in Microbiology*.
- Tannock, G. W. (2005). *Normal Microflora: An Introduction to Microbes Inhabiting the Human Body*. Springer Science & Business Media.
- Tarannum, S., et al. (2023). Antioxidant properties of bacterial exopolysaccharides: radical scavenging and reducing power validation assays. *Biomed Research International*.
- Trabelsi, I., et al. (2017). Antibacterial effect of exopolysaccharides produced by *Lactobacillus* spp. against foodborne pathogens. *International Journal of Food Microbiology*.
- Wingender, J., et al. (1999). *Microbial Extracellular Polymeric Substances: Characterization, Structure and Function*. Springer.
- Youssif, A. M., et al. (2020). Antioxidant potential and functional profiles of exopolysaccharides from marine *Halomonas* species. *Journal of Biotechnology*.