RESEARCH PAPER

Optimization and characterization of Exopolysaccharide produced by Micrococcus endophyticus DMW6 and its biological potential

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ABSTRACT
Endophytic bacteria produce exopolysaccharides (EPS), which have drawn a lot of interest because of their various industrial uses and bioactivities. In this study, we concentrated on the biological potential, molecular identification, optimization, and characterization of EPS generated by Micrococcus endophyticus DMW6, which was isolated from seawater. Production conditions were optimized by evaluating the effects of carbon and nitrogen sources, pH, temperature, and Nacl concentration, resulting in enhanced EPS yield. The purified EPS was characterised by GCMS and it identified several bioactive sugar derivatives in the EPS.Finally the cytotoxic effect of EPS was studied against A549 cells by MTT, AOEB, DAP and comet assay and the treatment with the EPS significantly reduced the viability of A549 cells, suggesting its promising role as a natural anticancer agent. Overall, the study establishes the EPS from M. endophyticus DMW6 as a promising bioactive molecule, with future research needed to evaluate its mechanism of action, large-scale production, and industrial or therapeutic applications.
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INTRODUCTION
Since the ocean covers more than 70% of the planet's surface, a vast array of marine species provide a plentiful supply of natural products, and marine organisms are becoming recognized as a source of novel bioactive substances. (Lindequist, 2016) Of the 1212 different kinds of aquatic microorganisms, roughly half of all organic matter on Earth is produced by marine microbes.(Field C.B et al.,1998).Exopolysaccharides are important among them. These polymers are a component of the dissolved organic carbon (DOC) present in marine environments and aid in a variety of processes, such as particle formation, sedimentation, and the cycling of dissolved metals. (Ding,Y.X. et.al 2008,Verdugo.P. 2012). Many living things, including microbes, plants, and algae, excrete EPSs, which are biopolymers. These high molecular weight carbohydrate polymers are an essential component of the extracellular polymers that adhere to microorganisms in the marine environment. (Patel VH. et.al., 2022). Based on their distribution, bacterial exopolysaccharides are divided into two types: free slime polysaccharides, which are firmly attached to the cell surface, and capsular polysaccharides, which are fully or partially released into the extracellular environment. Bacteria rely on EPS for a variety of essential processes, including cell adhesion and growth, protection against unfavourable environmental circumstances, symbiotic relationships with plants, avoiding desiccation, nutrition compartmentation, and antibiotic resistance. (Jyoti.k et al., 2024). Numerous marine bacterial species have been reported to manufacture extracellular polymers (EPSs).These include cyanobacteria, mesophilic bacteria, and some extremophiles (thermophiles and psychrophiles). They have been discovered in numerous samples of salt lakes, hydrothermal vents, marine hot springs, soil, sea ice, seawater, and 33 marine salterns.
They can be found throughout the ocean. (Singh et al., 2011; Mohamed et al., 2019). The structure of microbial marine exopolysaccharides is varied and complex. (Wang et al., 2020) The biological importance of these polymers, which

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include a significant amount of the dissolved organic carbon in oceans and serve as survival strategies in hostile conditions including polar seas, deep-sea vents, and hypersaline habitats, is frequently highlighted by the fact that marine bacteria create EPS. (Poli A et al.,2010). Temperature, pH, nutrient availability, microbial diversity, and the selection of specific bacterial strains are among the optimization parameters used in EPS synthesis. (Jyoti K et al.,2024). Bacterial EPS can change their molecules and give them new, beneficial features because it contains a variety of functional groups, such as hydroxyl, carboxyl, carbonyl, acetate, etc. (Aditya T., et al., 2022). Bacteria from several genera that produce EPS, including *Vibrio*, *Pseudoalteromonas*, *Alteromonas*, *Halomonas*, *Marinobacter*, *Bacillus*, *Pseudomonas*, *Rhodococcus*, *Kocuria*, *Enterobacter*, and *Shewanella*, have been discovered in seawater, sediments, hydrothermal vents, sea ice, and hypersaline ponds.(K.Sahli and A. Djekoune, 2022). Additionally, a variety of industries, including the petroleum, food, pharmaceutical, and cosmetics sectors, can employ marine EPS due to its improved thickening, stabilizing, gelling, and emulsifying qualities. (Casillo et al., 2018; Roca et al., 2016). Marine EPS provides a variety of biotechnological uses, including anti-aging compounds for the cosmetics industry, foaming agents for wastewater treatment, and thickening agents. (Al- Nahas et al., 2011). Marine bacterial EPSs are attractive targets for the pharmaceutical and biomedical industries because of their antifreeze, antioxidant, anticancer, anti-inflammatory, immunological, and antibacterial qualities. They are polymers of carbohydrates that have unique biological properties and complex structures. (Parkar, D.et.al., 2017). One potential application of the maritime environment is the isolation of novel microorganisms (actinomycetes, bacteria, and fungus) that are powerful producers of a range of bioactive natural chemicals. (Bramhachari et al., 2007; Park et al., 2019). Microbes, in particular, are ubiquitous in the oceans and have effectively adapted to challenging conditions (such as such as salinity, low temperature, high or low pH and osmotic pressure).(Qi, M et.al., 2025). The need for EPS produced by bacteria, especially marine-derived bacteria, has increased recently due to the variations between terrestrial and marine settings as well as the manner in which bacteria adapt and develop to thrive. To date, only a few number of EPS-producing bacterial taxa isolated from severe marine environments have been commercialized. (Catalão M et.al., 2023). Hence the present study focuses on the identification of *Micrococcus endophyticus DMW6*, optimization and characterization of its exopolysaccharide, and evaluation of its cytotoxic activity.

MATERIALS AND METHOD

Collection of water sample:

The sample of seawater was collected from a coastal region of Tuticorin, South India, and stored in an ice box until it was transported to the lab in a sterile plastic container.

Isolation of marine organism:

After completely mixing the marine water sample, 1 mL of the mixture was aseptically transferred into 9 mL of sterile physiological saline (0.85% NaCl) to obtain a 10^{-1} dilution. Serial dilutions were prepared up to 10^{-6} using the same diluent under aseptic conditions (Nwosu et al., 2019).

Plating and Incubation

Aliquots (0.1 mL) from suitable dilutions were spread evenly onto Zobell Marine Agar (ZMA) plates using a sterile glass spreader. The plates were kept in an incubator at 28–30 °C for 24–72 hrs in an inverted position. Marine agar was used to ensure proper salt concentration and nutrient composition suitable for marine bacterial growth (Dash et al., 2013).

Selection and Purification of Isolates

Following incubation, plates were examined for bacterial growth. Colonies differing in size, shape, color, elevation, margin, and surface texture were selected. Individual colonies were picked and streaked repeatedly on fresh marine agar plates to obtain pure cultures. (Cappuccino & Sherman, 2014).

Screening for EPS production

For exopolysaccharide (EPS) production, screening of isolated colonies was done using media composed of (g/L): glucose 20, $CaCO_3$ 0.1, NH_4NO_3 0.8, K_2HPO_4 0.6, KH_2PO_4 0.05, $MnSO_4 \cdot 4H_2O$ 0.1 and yeast extract 1.0 (Kim et al., 1998). Isolates showing mucoidal growth on marine agar media were further screened for EPS production.

Morphological and biochemical identification

The characteristics of the bacterial isolates were determined by their colony morphology, including parameters such as shape, size, color, elevation, and surface texture. Subsequently, a series of biochemical assays, including catalase, oxidase, and carbohydrate fermentation tests, were conducted to evaluate their metabolic properties.

Molecular identification of *Micrococcus endophyticus* DMW6

The EPS produced bacterial isolate was identified by 16S rDNA sequencing. The GeneJET Genomic DNA Isolation Kit (Thermo Fisher Scientific) was used to extract genomic DNA in accordance with the manufacturer's instructions. Universal primers 27F were used to amplify the 16S rDNA gene (5' AGAGTTTGATCMTGGCTCAG 3') and 1492R (5' TACGGYTACCTTGTTACGACTT 3') under the following PCR conditions: initial denaturation at 95°C for 4 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, with a final extension at 72°C for 10 min.

Agarose gel electrophoresis was used to confirm the PCR result and purified prior to sequencing. Sequencing was carried out by Eurofins Genomics, India Pvt. Ltd. (Bangalore, India). The sequences that were obtained were examined using the BLASTn program against GenBank to determine sequence similarity, submitted to NCBI GenBank for accession, and a phylogenetic tree was developed using the neighbor-joining method in MEGA7 software to infer evolutionary relationships.

Isolation and purification of EPS

The method of acetone precipitation has been altered to separate EPS (Sudhamani et al., 2004). To extract cells, Bacterial cultures were centrifuged for seven minutes at 9000 rpm. After adding two volumes of cold acetone to the supernatant, it was kept in incubator for overnight at 4 °C. After centrifuging the precipitate for 15 minutes at 4 °C at 12,000 rpm, it was collected for further examination.

GC–MS Analysis of EPS

The exopolysaccharide (5mg) from *Micrococcus endophyticus DMW6* was hydrolysed using 2 M sulfuric acid at 105°C for 10 hrs. Following cooling, the hydrolysate was neutralized, and any precipitates were removed by centrifugation. The resulting supernatant was filtered and lyophilized. Derivatization of the dried hydrolysate was done using 1:1 mixture of pyridine and BSTFA at 80°C for 16 hrs. A portion of the derivatized sample was injected into a GC-MS system fitted with an HP5MS column (30 m × 0.25 mm × 0.25 µm). At first, the column temperature was maintained at 100°C, ramped to 260°C, and maintained at 260°C, with helium as the carrier gas at a flow rate of 1 ml/min. The identification of monosaccharides was done by comparing the obtained spectra with the NIST library.

Optimization of EPS production

The consequences of various carbon (glucose, lactose, trehalose, mannitol, maltose, fructose and galactose), incubation temperatures (10, 15, 20, 25,30,35, and 40°C), pH values (4.0,5.0,6.0, 7.0, 8.0 and 9.0) were screened. Further, the effect of sodium chloride concentration (0.5,1.0,1.5,2.0,2.5,3.0,3.5,4.0,4.5 and 5.) on EPS production was also examined (Farag et al., 2020; Fourati-Ben Fguira et al., 2005).

Cytotoxic Assessment of EPS from *Micrococcus endophyticus* DMW6

Cell Line and Culture Conditions

The human lung adenocarcinoma cell line A549 was used to assess the cytotoxic ability of EPS from *M. endophyticus* DMW6. The cells were kept at 37 °C in a humidified 5% CO2 environment in Dulbecco's Modified Eagle's 111 Medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics (penicillin 100 U/mL, streptomycin 100 µg/mL).

MTT Assay

The MTT test was used to evaluate cell viability following EPS treatment. Before being administered with different EPS concentrations, A549 cells were seeded and given time to adhere. As controls, untreated cells were used. To enable formazan production, MTT reagent was applied after incubation. The relative vitality of the cells was evaluated by measuring absorbance at 570 nm after the dissolution of formazan crystals in dimethyl sulfoxide. (Mosmann, 1983).

Acridine Orange/Ethidium Bromide (AO/EB) Dual Staining

Necrotic and apoptotic morphological alterations were assessed by AO/EB staining. Both control and treated cells were stained using AO/EB dye and viewed under a fluorescence microscope to assess nuclear morphology and membrane integrity. (Kasibhatla et al., 2006).

DAPI Staining

Both EPS-treated and control cells were fixed, washed, and stained with DAPI to further assess nuclear changes; chromatin condensation and fragmentation were observed under a fluorescence microscope to detect apoptosis. (Baskić et al., 2006).

Comet Assay

DNA damage in EPS-treated cells was evaluated using the alkaline comet test. Alkaline electrophoresis was performed on the cells after they were embedded in agarose, lysed to extract proteins, and labelled with a fluorescent dye that binds to DNA. Comet tail development was used to measure DNA damage under a fluorescence microscope. (Singh et al., 1988).

RESULTS AND DISCUSSION

Isolation of Marine bacteria

Marine water collected from the coastal region of Tuticorin, South India, yielded cultivable heterotrophic bacteria when plated on Zobell Marine Agar. Growth of bacteria was noted after incubation at 28–30 °C for 24–72 h, confirming the existence of viable marine microorganisms in the sampled coastal environment. Due its appropriate salinity and nutritional content, marine agar has been extensively used to isolate bacteria from the shore and open ocean. The formation of distinct colonies on this medium indicates that the selected medium successfully encouraged the development of bacteria that were adapted to salt. (Dash et al., 2013). Similar qualitative findings of bacterial proliferation from samples taken in the ocean have been reported in earlier studies on coastal microbial diversity.

Screening for Exopolysaccharide (EPS) Production

Purified isolates were examined for the production of exopolysaccharides using a glucose-enriched EPS screening medium. During incubation, some isolates had a slimy, mucoidal colony form, which is considered an early indication of EPS generation. The observed colony morphology is aligned with previous discoveries that the production of polysaccharides causes EPS-producing marine bacteria to have mucoid or slimy features. (Sutherland, 2001; Flemming & Wingender, 2010). Similar qualitative screening methods for EPS-producing marine bacteria have been effectively employed by earlier researchers, showing the importance of mucoidal colony morphology as an early selection criterion. (Nwosu et al., 2019).

Morphological and biochemical identification

Identification of the isolate was achieved by studying their morphological characters and biochemical features, as recorded in Table 1 and 2 & (Fig : 1) These are compatible with the previously described traits of Micrococcus species isolated from maritime settings (Walif et al., 2019; Martins et al., 2016).

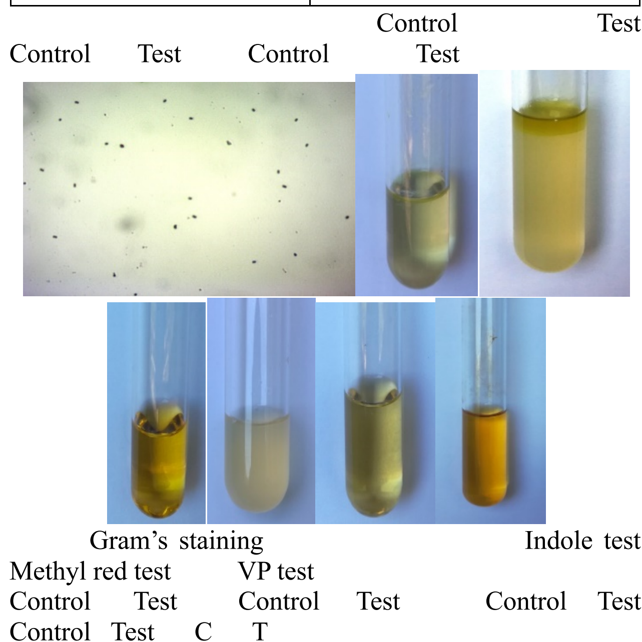
Table 1: Observation of morphological characteristics of *Micrococcus endophytics* DMW6

Morphological features		Cultural features	
Size	Small (1–2 mm)	Gram's staining	Gram-positive
Shape	Circular	Shape	Cocci (tetrads/clusters)
Elevation	Convex	Capsule staining	– (non-capsulated)
Texture	Smooth, butyrous	Spore staining	– (non-sporing)
Colour	Yellow to creamy	Motility	Non-motile
Margin	Entire (smooth)	Flagella	Absent

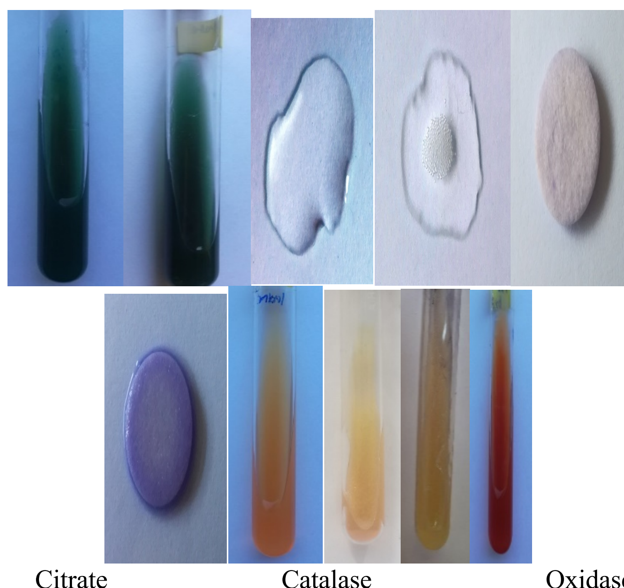
Table 2 : Observation of biochemical characteristics of *Micrococcus endophytics* DMW6

Biochemical test	
Indole	–
Methyl Red (MR)	–
Voges–Proskauer (VP)	–
Citrate Utilization	–
Catalase	+
Oxidase	+
Urease	–
Nitrate Reduction	–
Gelatin Hydrolysis	–
TSI Reaction	Alkaline/No change (K/NC)
H_2S Production	–
Gas Production	–

Control Test
Control Test Control Test



Gram's staining Indole test
Methyl red test VP test
Control Test Control Test Control Test
Control Test C T



Citrate Catalase Oxidase
Urease TSI

Fig :1 Morphological and Biochemical identification

Molecular identification of *Micrococcus endophyticus* DMW6

16S rRNA gene sequencing was used to identify the targeted isolate. The 16S rRNA gene was successfully amplified by PCR using the universal primers 27F and 1492R, which produced an amplicon of about 1.5kb.Sequence analysis using the BLASTn program revealed close similarity with *Micrococcus endophyticus,* and the isolate was designated as *Micrococcus endophyticus DMW6.* The sequence was uploaded to the GenBank database of the NCBI and an accession number (OQ772211) was obtained. Phylogenetic analysis using the neighbor-joining method in MEGA7 software showed that the isolate clustered with reference *M. endophyticus* strains, confirming its taxonomic affiliation. (Fig 2) *M. endophyticus* DMW6's identification offers a molecular foundation for additional research on its EPS-producing potential. According to (Nisha P. et al., 2023), the study highlights the importance of connecting molecular identification with exopolysaccharide production capabilities in Micrococcus species by describing EPS production in a strain identified by 16S rRNA sequencing, illustrating EPS characterization and potential functional properties. 16S rRNA gene sequencing demonstrated the isolate's identification as *Micrococcus endophyticus* DMW6, which is compatible with the initial species description by Chen et al. (2009), who classified *M. endophyticus* as a novel taxon using a polyphasic strategy that combines sequence data and chemotaxonomic markers.

OQ772211.1 *Micrococcus endophyticus* strain DMW6 16S ribosomal RNA gene, partial sequence

>GAGTAACCTGCCCTTGACTCTGGGATAAGCCCGG
GAAACTGGGTCTAATACCGGATAGGAACGTCCACC
GCATGGTGGTTGTTGGAAAGATTTATCGGTCATGG
ATGGACTCGCGGCCTATCAGCTTGTTGGTGAGGTA
ATGGCTCACCAAGGCGACGACGGGTAGCCGGCCT
GAGAGGGTGACCCGCACACTGGACTGAGAACGAT

CAGACTCTACGGAGCAGCAGTGGGGATATGCACA
TGGCGAAAGCTGATGCAGCGACGCCGCGTGAGGG
ATGACGGCCTTTCGGGTTGTAAACCTCTTTCAGTA
GGGAAGAAGCGAAAGTGACGGTACCTGCAGAAG
AAGCACCGGCTAACTACGTGCCAGCAGCCGCGGT
AATACGTAGGGTGCGAGCGTTATCCGGAATTATTG
GGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCT
GTCGTGAAAGTCCGGGGCTTAACCCCGGATCTGC
GGTGGGTACGGGCAGACTAGAGTGCAGTAGGGGA
GACTGGAATTCCTGGTGTAGCGGTGGAATGCGCA
GATATCAGGAGGAACACCGATGGCGAAGGCAGGT
CTCTGGGCTGTAACTGACGCTGAGGAGCGAAAGC
ATGGGGAGCGAACAGGATTAGATACCCTGGTAGTC
CATGCCGTAAACGTTGGGCACTAGGTGTGGGGAC
CATTCCACGGTTTCCGCGCCGCAGCTAACGCATTA
AGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTA
AAACTCAAAGGAATTGACGGGGGCCCGCACAAGC
GGCGGAGCATGCGGATTAATTCGATGCAACGCGA
AGAACCTTACCAAGGCTTGACATGTTCTCGATCGC
CGTAGAGATACGGTTTCCCCTTTGGGGCGGGATCA
CAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTG
AGATGTTGGGTTAAGTCCCGCAACGAGCGCAACC
CTCGTTCCATGTTGCCAGCACGTAATGGTGGGGAC
TCATGGGAGACTGCCGGGGTCAACTCGGAGGAAG
GTGGGGACGACGTCAAATCATCATGCCCCTTATGT
CTTGGGCTTCACGCATGCTACAATGGCCGGTACAA
TGGGTTGCGATACTGTGAGGTGGAGCTAATCCCAA
AAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAAC
TCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCA
GATCAGCAACGCTGCGGTGAATACGTTCCCGGGC
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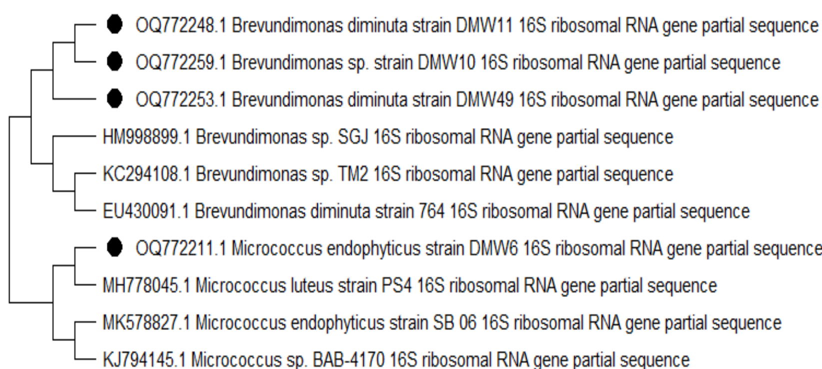


Fig : 2 Phylogenetic analysis of Micrococcus endophyticus strain DMW6

Optimization of EPS production

Our results demonstrated that the amount of exopolysaccharide (EPS) generated by *Micrococcus endophyticus DMW6* increased with temperature, pH, NaCl concentration, and carbon source selection. The isolate exhibited maximum EPS production at pH 7.0 and 35 °C (Fig. 3A &B), and 3.5% NaCl (Fig. 3C), suggesting that these conditions promote metabolic activity and polymer synthesis. Trehalose supported the largest EPS yield among the several carbon sources studied (glucose, lactose, trehalose, mannitol, maltose, fructose, and galactose),(Fig 3D) indicating that trehalose may be converted more effectively into EPS precursors under the tested conditions. The observed increase in EPS yield with incubation time, temperature, pH, NaCl level, and carbon source is in accordance with other discoveries where optimization of cultural conditions significantly enhanced EPS production in diverse bacteria. For instance, Tandel and Minocheherhomji (2025) discovered that *Bacillus licheniformis* KP062r's EPS output was significantly increased by modifying temperature, pH, and carbon supply; sucrose and neutral pH favored polymer synthesis. Research on the probiotic *Lactobacillus rhamnosus* additionally revealed that specific variables (pH, temperature, and carbon supply) greatly increased the generation of EPS (Polak Berecka, M.,et., 2014)

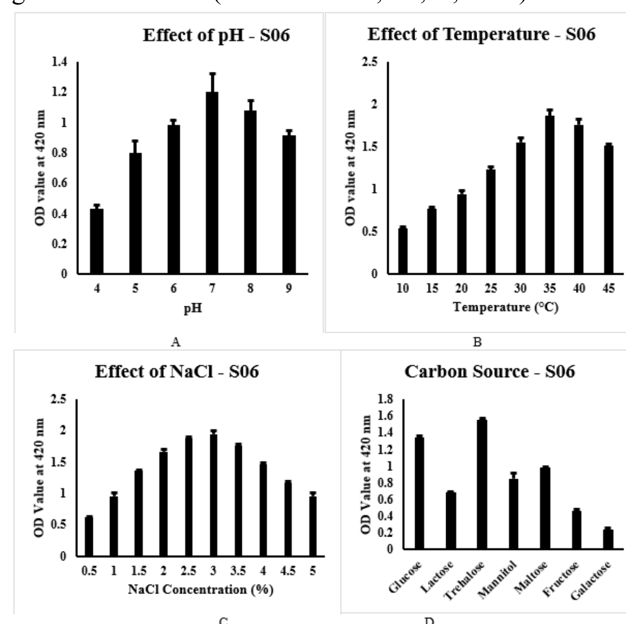


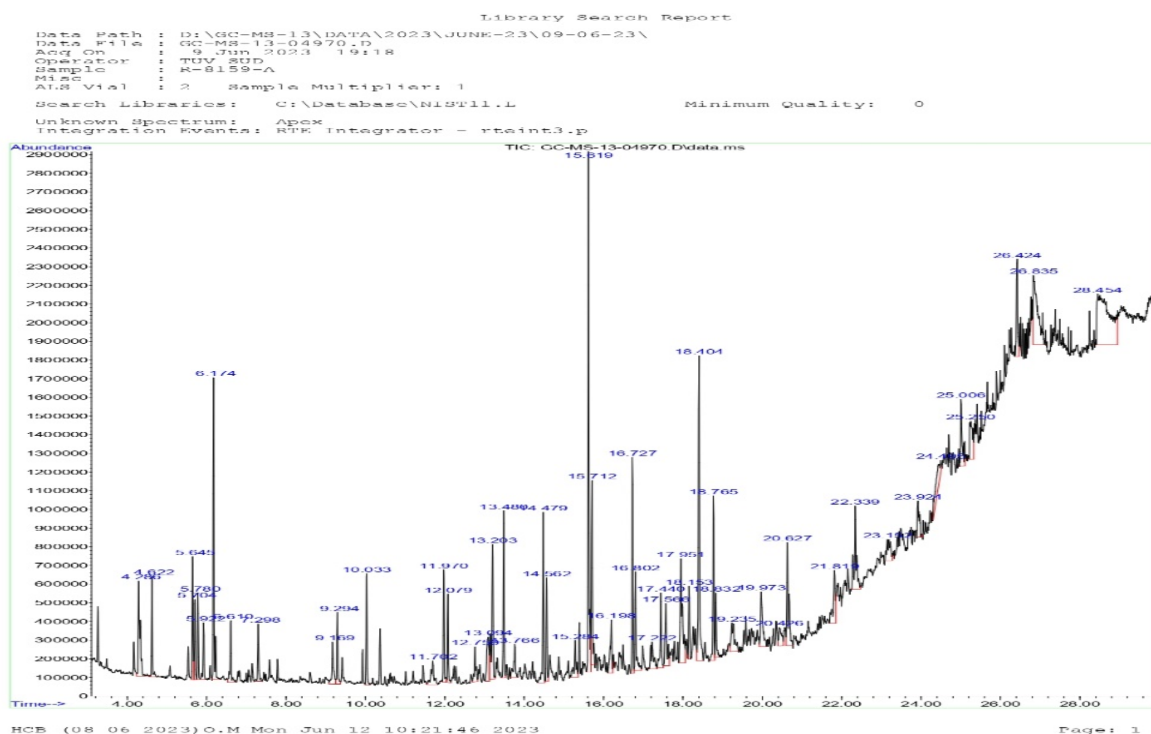
Fig 3 : Optimization of EPS production

GC–MS Analysis of EPS

GC-MS analysis was conducted to characterize the chemical makeup of the exopolysaccharide (EPS) produced by *Micrococcus endophyticus DMW6.* The chromatogram revealed multiple peaks across a broad retention time range, indicating the existence of a complicated mixture of chemical constituents in the EPS extract (Fig.4). Several peaks exhibited high intensity, suggesting the presence of dominant components, while numerous smaller peaks indicated minor constituents. The profile probably contains organic acids, sugar alcohols, and derivatives of carbohydrates, which are frequently reported in microbial EPS, even if the individual compounds could not be clearly identified using the NIST database. (Polak-Berecka et al., 2014; Ahmad et al., 2020; Nisha et al., 2023). The relative abundance and diversity of these constituents may contribute to the functional properties of the EPS, such as antioxidant, emulsifying, and biofilm-forming activities, as observed in EPS from other marine bacterial isolates. The GC-MS results are summarized in Table 3, which lists peak numbers, retention times, and relative intensities, providing an overview of the EPS chemical profile. According to (Sirin and Aslim, 2020), the MS detector used the NIST library to identify the majority of the peaks in the GC chromatogram as monosaccharide derivatives. The GC-MS data analysis revealed that the EI6-EPS, which represents the most intense region in the GC chromatogram, consists of at least five monosaccharides that were examined consecutively (time = 19–24min). The order of rhamnose > galactose > mannose > glucose > arabinose, with relative area% of 26.43, 25.07, 21.24, 14.42, and 12.84, respectively, was found by analyzing the similar area% because of the relative abundances of these moieties.

Table 3 : GC MS – Report table

Peak No.	RT (min)	Compound	Molecular Ion (m/z)	Library Match (%)	Relative Area (%)
1	4.286	p-Xylene	106	95	2.34
6	5.922	Benzene, 1-ethyl-3-methyl	5004383	90 - 95	0.78
26	15.712	Tetradecane	20529597	92–98%	3.18
35	18.404	1,2-Benzenedicarboxylic acid	32302919	92–97%	5.00



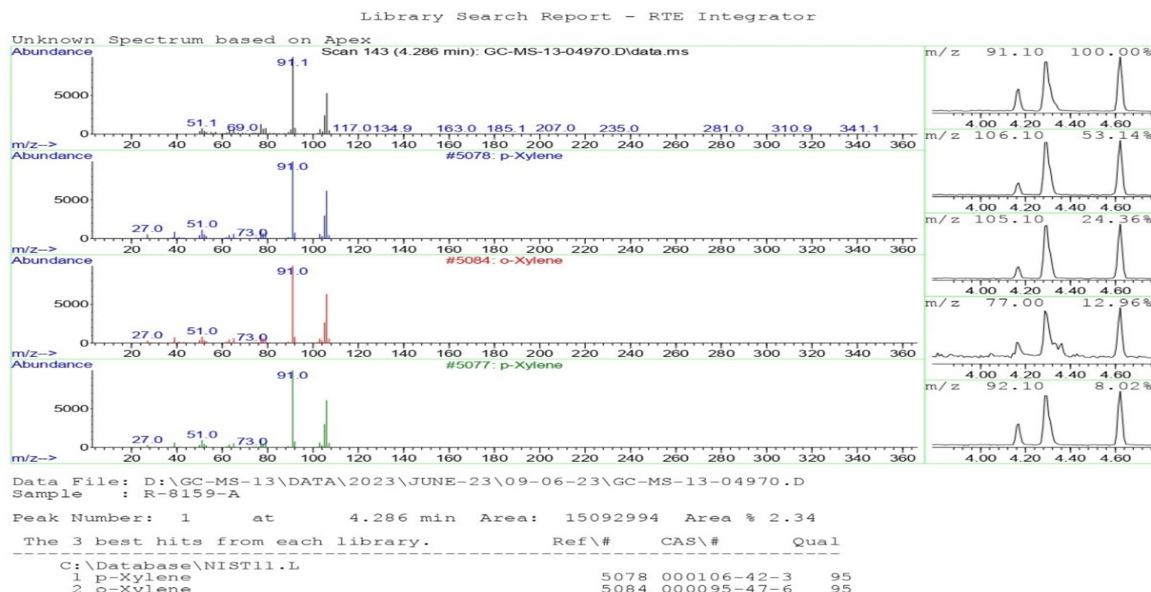
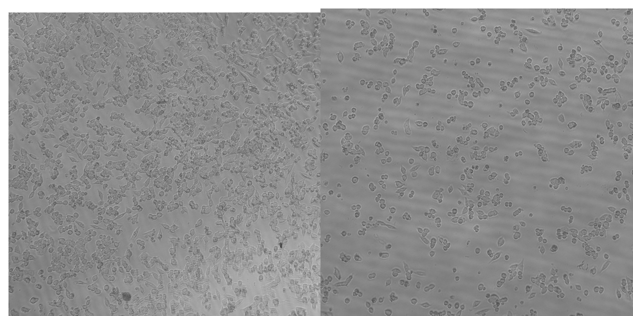


Fig : 4 GC MS – Chromatogram

Cytotoxic Assessment of EPS from *Micrococcus endophyticus* DMW6

MTT, AO/EB, DAPI, and comet tests were used to assess the cytotoxic impact of EPS on A549 cells. EPS-treated cells showed a dose-dependent decrease in cell viability in the MTT experiment when compared to untreated controls, suggesting reduced mitochondrial activity (Fig 5). While control cells showed consistent green fluorescence, EPS-treated cells showed apoptotic morphological abnormalities, such as chromatin condensation, nuclear disintegration, and changes in membrane permeability, according to AO/EB staining. (Fig 6) DAPI labelling supported the induction of apoptosis at the nuclear level by confirming nuclear condensation and disintegration in the cells that were treated (Fig 7). In contrast to control cells, which had unaffected nuclei, the comet assay revealed greater DNA movement in EPS-treated cells, which is suggestive of DNA strand breaks.(Fig 8).

MTT



A549 cells control **A549 cells treated**

Figure 5. Cytotoxic effect of marine bacterial exopolysaccharide on A549 cells assessed by MTT assay. Untreated control cells indicated greater formazan formation compared to EPS-treated cells.

The cytotoxic potential of EPS was evaluated using the MTT assay on A549 cells. EPS treatment resulted in a **dose-dependent decrease in cell viability,** with increased concentrations causing significant reduction compared to untreated controls. For example, Noufal, Sivaperumal, and Elumalai (2022) assessed the impact of EPS on MCF-7 Breast cancer cells in humans after extracting it from marine Actinobacteria (*Streptomyces* species). They displayed that the EPS suppressed about 50% of cell viability after 72 hours of exposure using the MTT assay, demonstrating selectivity as compared to non-cancer control cells and a dose-dependent cytotoxic action on cancer cells in vitro. Similarly, using the MTT assay, Bachay and Suker (2025) found that EPS produced from *Staphylococcus aureus* significantly inhibited the growth of MCF-7 cells. Their findings highlighted possible treatment selectivity by showing greater growth suppression in cancer cells relative to Vero normal cell lines

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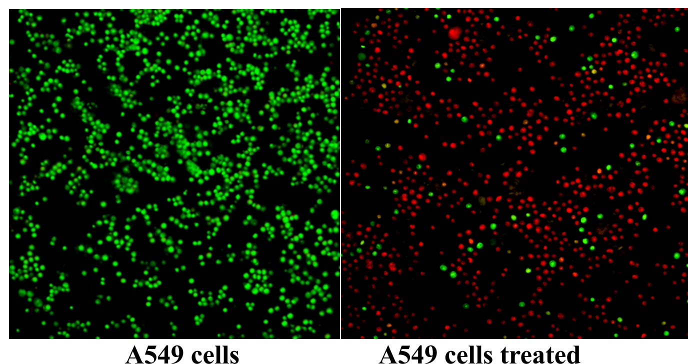
AO – EB Assay

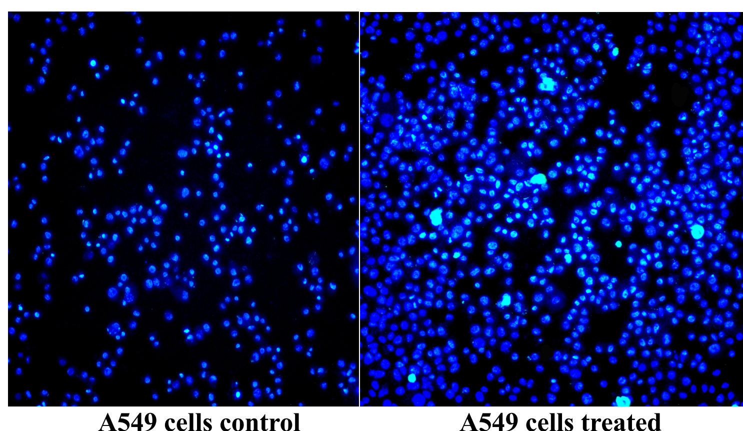


A549 cells **A549 cells treated**

Fig 6. AO/EB staining of A549 cells after EPS treatment showing apoptotic morphological changes compared to control cells

AO-EB dual staining revealed apoptotic and necrotic changes in EPS-treated A549 cells. Control cells displayed a consistent green color, indicative of live cells. EPS-treated cells displayed increased orange/red fluorescence, suggesting early and late apoptosis as well as necrotic cells. Exopolysaccharide from Aphanizomenon flos-aquae was shown by Wang et al. (2021) to cause A431 epidermoid carcinoma cells to exhibit marked apoptotic features. The authors came to the conclusion that EPS treatment mostly triggered apoptosis rather than indiscriminate cytotoxicity, suggesting its anticancer potential. More recently, (Bachay and Suker 2024) evaluated the harmful impact of bacterial EPS on MCF-7 breast cancer cells and identified important apoptotic traits including membrane permeability and nuclear shrinkage using AO-EB labeling. The authors stressed the significance of integrating viability tests with fluorescence-based staining methods, emphasizing that AO–EB labeling offered crucial morphological data confirming biochemical apoptosis markers. As demonstrated by chromatin condensation, nuclear fragmentation, and changes in membrane permeability, AO/EB and DAPI labeling verified that the decrease in viability is linked to apoptosis (Kasibhatla et al., 2006; Baskić et al., 2006).

DAPI

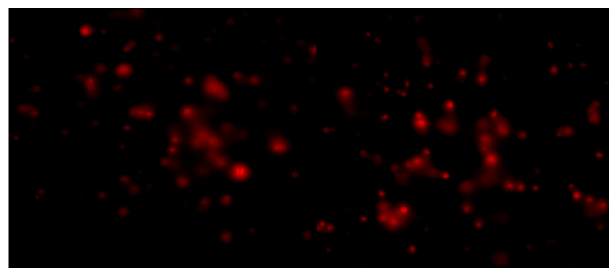


A549 cells control **A549 cells treated**

Fig 7: DAPI staining of A549 cells showing apoptotic nuclear morphological changes after EPS treatment compared with untreated control cells.

DAPI staining showed that EPS treatment induced nuclear morphological changes characteristic of apoptosis. and morphological alterations associated with apoptosis in nuclear fragmentation, and bright fluorescence, which intensified with increasing EPS concentration. These results corroborate the cytotoxic and pro-apoptotic effects of EPS on A549 cells EPS treatments can result in nuclear damage and apoptotic morphological changes in A549 cells, according to Toshkova-Yotova et al. (2024). The pro-apoptotic potential of EPS was confirmed in vitro by studies employing AO/EB and DAPI labelling, which showed chromatin condensation, nuclear fragmentation, and early/late apoptotic characteristics following exposure. This aligns with the typical hallmarks of apoptosis and confirms EPS's potential for use as an anticancer drug targeting cell death pathways.

Comet Assay



A549 cells control **A549 cells treated**

Fig : 8 Comet assay images showing increased DNA damage in EPS-treated A549 cells compared to control cells

The comet assay demonstrated that EPS treatment severely impacted DNA.in A549 cells. Cells exposed to EPS showed increased tail length and tail moment, indicating single-strand DNA breaks. DNA damage increased in a dose-dependent manner, confirming that EPS exerts genotoxic effects contributing to cell death. These findings are consistent with previous studies that found marine microbial exopolysaccharides to have pro-apoptotic and anticancer properties (Rajoka et al., 2020).Overall, the findings show that EPS from *M. endophyticus* DMW6 possesses significant cytotoxic and genotoxic potential, which may be explored for therapeutic applications.

CONCLUSION

Because of their unique polysaccharides and microbiologic al variety, marine environments are becoming the focus of more and more scientific inquir y. The optimization results of various factors showed that the isolate exhibited maximum EPS production at pH 7.0,35 °C and 3.5% NaCl and Trehalose supported the largest EPS yield among the several carbon sources studied (glucose, lactose, trehalose, mannitol, maltose, fructose, and galactose. The exopolysaccharide generated by *Micrococcus endophyticus DMW6* was subjected to GC-MS analysis, which provided a chemically varied profile that included a number of physiologically significant chemicals. These components suggest that the EPS matrix is structurally complicated. The functional and biological activities that are detected may be significantly influenced by these compositional characteristics. Overall, the GC-MS results validate this EPS's possible use in biotechnological and biological domains. The exopolysaccharide isolated from *Micrococcus endophyticus* demonstrated a strong cytotoxic effect against A549 lung carcinoma cells. MTT analysis confirmed a dose-dependent reduction in cell viability, while AO/EB and DAPI staining revealed characteristic apoptotic morphological changes. The comet assay further demonstrated DNA damage, supporting apoptosis-mediated cytotoxicity. All of these results suggest that the bacterial exopolysaccharide possesses promising anticancer potential against A549 cells. Despite the observed in-vitro efficacy, the precise molecular mechanisms and signaling pathways underlying the cytotoxic effect remain unclear. Future studies should focus on elucidating apoptosis-related gene and protein expression, oxidative stress involvement, and cell-cycle regulation. In vivo validation and toxicity profiling are also necessary to establish therapeutic relevance. Additionally, structural characterization and optimization of the exopolysaccharide may enhance its anticancer efficacy and selectivity.

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