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# *In Vitro* Antioxidant and Anti Biofilm Potential of Bacterial Exopolysaccharide from Halophiles: Separation and Chemical Characterization

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**Abstract:** The halophilic salt pan region in Tuticorin, Tamil Nadu, India, was sampled for its sedimentary soil. The samples were then serially diluted and placed on Zobell marine agar to determine which bacterial strain was most successful in producing Exopolysaccharide (EPS). From halophilic samples, seven distinct bacterial strains were identified and given the names VSM1 through VSM7. The isolate's 16S rRNA gene sequencing and further phylogenetic analysis identified the bacterial strain as *Halobacillus trueperi* VSM4, which is listed in the GenBank (Accession no. MK829848). Purification and characterization of the EPS were achieved by means of FT-IR and and HPLC, investigations. The analysis revealed that its chemical makeup was made up of 73.15 ± 0.11% carbohydrates, 22.18 ± 0.17% sulphate, and 7.27% 9.15 ± 0.26% uronic acid. Exopolysaccharide was examined for its antimicrobial efficacy against a number of clinical infections, and the zones it produced were documented. The results of the antimicrobial and antibiofilm experiment indicate that *Staphylococci* sp., the clinical pathogen, was more vulnerable to the bacterial polysaccharide. Ascorbic acid was used as the benchmark while the exopolysaccharide's overall antioxidant activity was examined. Exopolysaccharide's optimum activity was estimated and determined to be 91.16 ± 0.19 per cent. Exopolysaccharide has a reducing power of 78.13 ± 0.4% when compared to reference compound. Gallic acid was used as the standard in the polysaccharide hydrogen peroxide scavenging experiment. The ideal EPS activity was 83.27 ± 0.71%.

**Keywords:** Exopolysaccharide, Total antioxidant capacity, FTIR, GCMS, Microbial strain, *Halobacillus trueperi*

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## Introduction

Microorganisms are the chief components on Earth that maintain structural integrity. In particular, marine territory represents a wide array of diversified microorganisms that perform fundamental activities for the aquatic ecosystem. The primary production is basically dependent upon the presence of microorganisms on Earth, and among these, the bacterial Exopolysaccharide (EPS) occupies a prominent position. EPS is one of the significant biopolymers that are essential to making up the dissolved organic carbon (DOC) in aquatic ecosystems and contribute to several processes, such as particle formation, sedimentation, and recycling of dissolved metals. In addition to that, extremophiles are one of the most significant microorganisms for therapeutic applications due to their biopotential. A variety of extremophilic microorganisms have been isolated from various geographic locations for industrial requirements possessing thermal stability, low pH, or very high saline concentrations (Merino *et al.*, 2019; Chambi *et al.*, 2021).

The presence of saline lakes and soils is the chief producer of halophilic microorganisms capable of surviving in salt-rich environments (Barghin *et al.*, 2014). Exopolysaccharides (EPS), lectins, and polyhydroxyalkanoates are just a few of the biopolymers that halophilic bacteria produce to protect themselves from potentially dangerous environmental elements. In order to defend themselves from potentially dangerous environmental elements, the halophiles secrete EPS and other biopolymers which may adhere to different surfaces and form a protective layer. It is possible to utilize EPS as an additive to enhance the rheological and textural properties of food products because of its structure and unique properties. Antioxidants, immunomodulators, antiviral, anti-inflammatory, and anticancer medicines are some of their potential uses in pharmaceutical and biological applications. Medical professionals are quite interested in EPS, and halophilic EPS in particular, because of the regulatory influence they have on certain tumour cells (Shankar *et al.*, 2021).

Microorganisms are capable of synthesizing EPS, which has various biotechnological applications, especially in the paper, food, and textile industries. Furthermore, it is also used in the cosmetic and pharmaceutical industries according to its biopotential. Mechanical strength and emulsifying activity of bacterial exopolysaccharides are the chief characteristic features of their choice for industrial applications. Because of its renewable activity, microbial EPS has been a significant alternative to petroleum in the industry for the last few decades (Shahnavaz *et al.*, 2015; Maheswari *et al.*, 2019). EPS production might be enhanced by adjusting environmental parameters including pH, temperature, and fertilizer supply. There have been recent efforts to identify EPS-producing bacterial strains from a variety of sources, including saltwater and soil (Pawar *et al.*, 2013) and soil polluted with crude petroleum oil (Razack *et al.*, 2013). The present study was concentrated on *in vitro* antioxidant and anti-biofilm potential of bacterial exopolysaccharides from halophiles and separation and chemical characterization of these exopolysaccharides.

## Materials and Methods

### *Isolation and screening of potential isolate*

In order to test for EPS-producing bacteria using Zobell marine agar medium, a sample of halophilic silt soil was obtained. The formation of mucus around the bacterial strain was considered to be the EPS producer and it was selected for further experimental analysis.

### *Molecular identification of candidate strain by 16S rRNA gene sequencing analysis*

Using 25 cc of LB broth and a temperature of

35 °C, the tested bacteria was cultured. After 5 min of spinning at 5,000 rpm, the pellets were extracted from the mixture. Then added 400 µl of sucrose TE buffer (Tris EDTA) to the pellet for supplementation. In order to create the reaction mixture, 1 µl of 0.5 M EDTA (pH 8.0), 60 µl of SDS, and 3 µl of proteinase-K (20 mg/ml) were added. The mixture was then heated to 55°C for incubation. Concurrently, it was spun for 3 min at 7,000 rpm to separate the particles and liquids. The filtrate underwent two discrete isolation methods. The first used a phenol:chloroform (1:10) mixture, and the second involved a chloroform:isoamylalcohol (24:1) mixture. Polymerase chain reaction amplification was performed after bacterial DNA was collected using primers. We used the PCR Clean-up Kit (Millipore) to remove any contaminants from the final results. Following the addition of primers, the PCR product was sequenced using the ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits. The results obtained were sent to the National Centre for Biotechnology Information (NCBI) for their sequence deposit database.

### *Characterization of exopolysaccharide*

### *Estimation of carbohydrate, sulphate and uronic acid content of exopolysaccharides*

Determination of total carbohydrate content was performed by phenol-sulfuric acid method of DuBois *et al*. (1956). Lloyd *et al*. (1961) method was used for estimation of the extract's sulphate content by using barium chloride gelatin. The method of Bitter and Muir (1962) was used to investigate the measurement of uronic acid, with glucuronolactone serving as a reference.

### *FTIR analysis of EPS*

To make a 3 mm diameter salt disc, one part extract was combined with 90 parts dried potassium bromide (KBr) separately using a Perkin-Elmer FT-IR spectrometer. The mixture was then compacted. Absorption measurements were taken from these discs using an infrared spectrophotometer, which ranges from 400 to 4000 $cm^{-1}$.

### *HPLC analysis of EPS*

A refractive index detector was used to verify the separated components, and a C18 column (LC-10VP Shimadzu) was run at 1.0 ml/min at 20 °C to evaluate any bacterial EPS. After incubating the sample at 30°C for 60 min, 1.25 ml of 72% sulphuric acid was used to hydrolyse the potential bacterial EPS. After putting the mixture into the water bath, which contained 13.5 ml of distilled water, it was left to soak for 4 h. After the liquid cooled, 3.1 ml of 32% NaOH were added to it. The hydrolyzed potential bacterial EPS and its methanol derivatives were tested for sugar content using high-performance liquid chromatography (Mahendran *et al*., 2013).

### *Biological activities of exopolysaccharide*

### *Antimicrobial activity of exopolysaccharide*

*Klebsiella* sp., *Staphylococcus* sp., *Escherichia coli, Pseudomonas* sp., (4 bacteria) *Aspergillus niger*, and *Candida* sp. (2 fungi) were the human clinical pathogens chosen for this investigation, and the EPS's antimicrobial activity was examined against them.

The antimicrobial activity of EPS was determined against clinical pathogens by using the swab method. By using a well cutter, two holes were made on the Muller-Hinton agar plate. One well containing 10% distilled water was loaded into the respective label, and another well containing the sample was loaded. The results were documented based on the formation of an inhibition zone for each well and expressed in millimeters (Maheswari AND Karthiga, 2016).

### *Evaluation of antibiofilm effect of EPS*

### *Ring Biofilm Assay*

The effect of EPS on ring biofilm formed at air-liquid interface was studied by growing MRSE in glass (hydrophilic) test tubes. Glass test tubes holding 2 ml of TSB and 1% inoculum (~1 × 106 CFU/ml) was supplemented with increasing concentrations of EPS (2 to 10 mg/ml). The tubes then incubated for 24 h in shaking condition (160 rpm) at 37°C. Biofilm formed on the glass tubes

was stained with CV (0.4% w/v) as mentioned above to visualize the difference developed upon EPS treatment and photographed (Sparjan *et al*., 2024).

### *In vitro antioxidant activities of EPS*

#### *Determination of total antioxidant capacity (TAC)*

We started by making a reagent solution in a 3.0 ml volume that included 0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate. After mixing the sample with this solution, it was incubated for 90 min in a water bath set at 95 $^{o}$C. Prieto *et al.* (1999) used ascorbic acid as the standard to assess the absorbance at 695 nm after 15 min.

#### *Determination of reducing power*

4 ml of the reaction mixture was supplemented with a 1% w/v potassium ferricyanide solution in phosphate buffer (0.2 M, pH 6.6) and then incubated at 50°C for 20 min. To stop the reaction, a 10% (w/v) TCA solution was prepared by mixing distilled water with 0.1% ferric chloride (w/v). At 700 nm, the absorbance was determined, as stated by Yamaguchi *et al*. (1998).

#### *Hydrogen peroxide scavenging assay*

Gulcin *et al*. (2004) used a hydrogen peroxide test to evaluate the exopolysaccharide's capacity to scavenge free radicals. 10 ml of hydrogen peroxide was dissolved in 0.1 M of phosphate buffer saline at a pH of 7.4. To measure the absorbance at 230 nm in the UV spectrophotometer, 2 ml of hydrogen peroxide solution and 1 ml of the extract were combined after 10 min of incubation at 37°C.

#### *DPPH radical scavenging assay*

In methanol, a DPPH solution was prepared at a concentration of 0.1 mM. Afterwards, 1 ml of this solution was combined with 3 ml of sample at 50 µg, 100 µg, 250 µg, 500 µg, 750 µg, and 1000 µg. After 10 min, the absorbance at 517 nm was recorded. To get the scavenging activity %, we employed following formula (Blois, 1958):

$$\text{Percentage of Scavenging} = ((A_o - A_1) / A_o) \times 100$$

Where $A_o$ is absorbance of control and $A_1$ is absorbance of sample turbidity factor.

## Results

### *Screening of exopolysaccharide producing bacteria*

Samples of soil and sediment were taken from the salt pan-halophilic area of Tuticorin. To screen for bacterial strains that efficiently produce EPS, the samples were diluted in a serial fashion and then placed on Zobell marine agar. Isolated from halophilic materials are seven distinct bacterial strains, identified as VSM1 to VSM7. Table 1 shows the proportion of total carbohydrates found in the exopolysaccharides, which were calculated using the phenol-sulfuric acid technique using glucose as a reference. The strain with the highest EPS production was chosen, VSM4, from among them.

### *Morphological identification of candidate bacterial isolate*

The Gram's staining was done for the isolate. As per the microscopical observation, Gram positive and rod shape were observed.

### *Molecular identification of bacteria*

Phylogenetic analysis and sequencing of the 16S rRNA gene of the isolate confirmed that the bacterial strain was *Halobacillus trueperi* VSM4, which is stored at the GenBank (Accession No. MK829848).

### *Analysis of biochemical composition*

We evaluated the total carbohydrates, proteins, sulphate, and uronic acid in the exopoly-saccharides. The phenol-sulphuric acid technique was used to quantify the total carbohydrate content in the exopolysaccharides, with glucose serving as a reference. The result showed a percentage of total carbohydrate of 73.15 ± 0.11%. Figure 1 shows that the exopolysaccharides had a sulphate content of 22.18 ± 0.17% and a uronic acid value of 9.15 ± 0.26%.

### *FT-IR spectrophotometer analysis*

Isolated polysaccharide FT-IR spectra were confirmed using a Perkin-Elmer FT-IR system (Fig. 2). In the C=O medium (carbonyls), signals at 1677.95 and 1557.41 $cm^{-1}$ demonstrated the

Table 1: Identification of efficient EPS producing bacteria

| S. No. | Colonies | Exopolysaccharide production (%) |
|---|---|---|
| 1 | VSM1 colony | 13.0 ± 0.40 |
| 2 | VSM2 colony | 38.6± 0.14 |
| 3 | VSM 3 colony | 31.0± 0.36 |
| 4 | VSM 4 colony | 57.1 ± 0.29 |
| 5 | VSM 5 colony | 47.0± 0.32 |
| 6 | VSM 6 colony | 37.6 ± 0.57 |
| 7 | VSM 7 colony | 52.9 ± 0.53 |

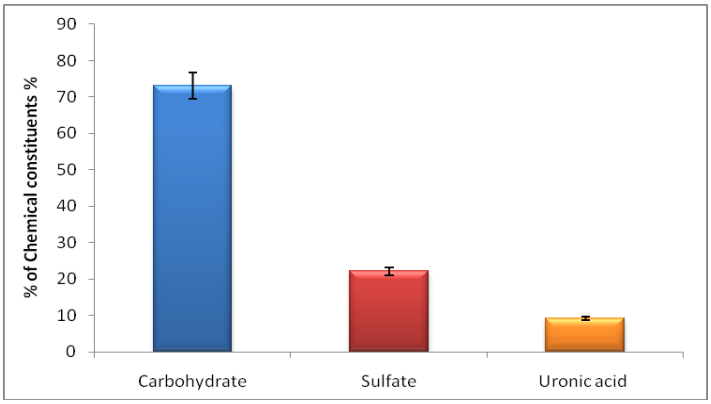



Fig. 1: Analysis of biochemical composition.

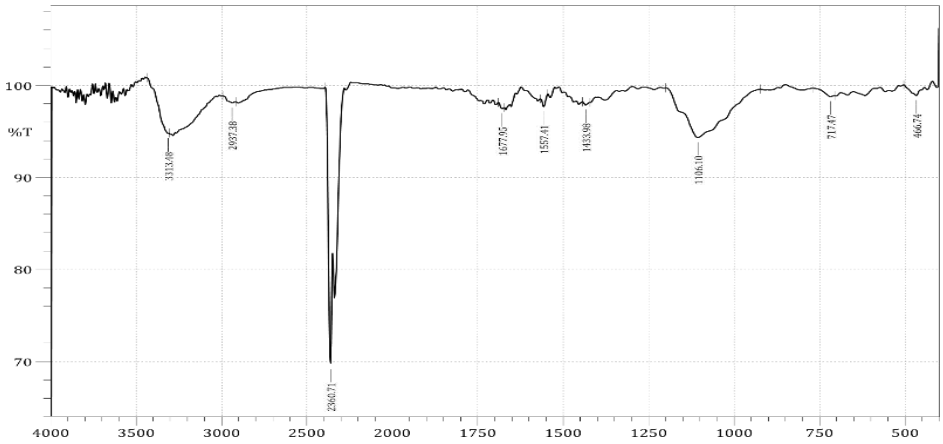



Fig. 2: FT- IR spectrum of polysaccharides.

stretching vibration. In the case of aromatic and aliphatic amine C-N stretch bending, respectively, the bands at 1433.98 $cm^{-1}$ and 1106.1 $cm^{-1}$ flatten out. The stretch bending of alkyl halides (C-Cl) was identified by the signal at 717.47 $cm^{-1}$. The week signals at 466.74 $cm^{-1}$ suggest the formation of rings.

### *HPLC analysis of the exopolysaccharide*

The peaks seen in our crude EPS extract after running it through the C18 column confirm the presence of neutral sugars. Figure 3 shows the results of comparing the acquired peaks to the standard retention time of carbohydrates, which was determined to be 2.120 (fructose).

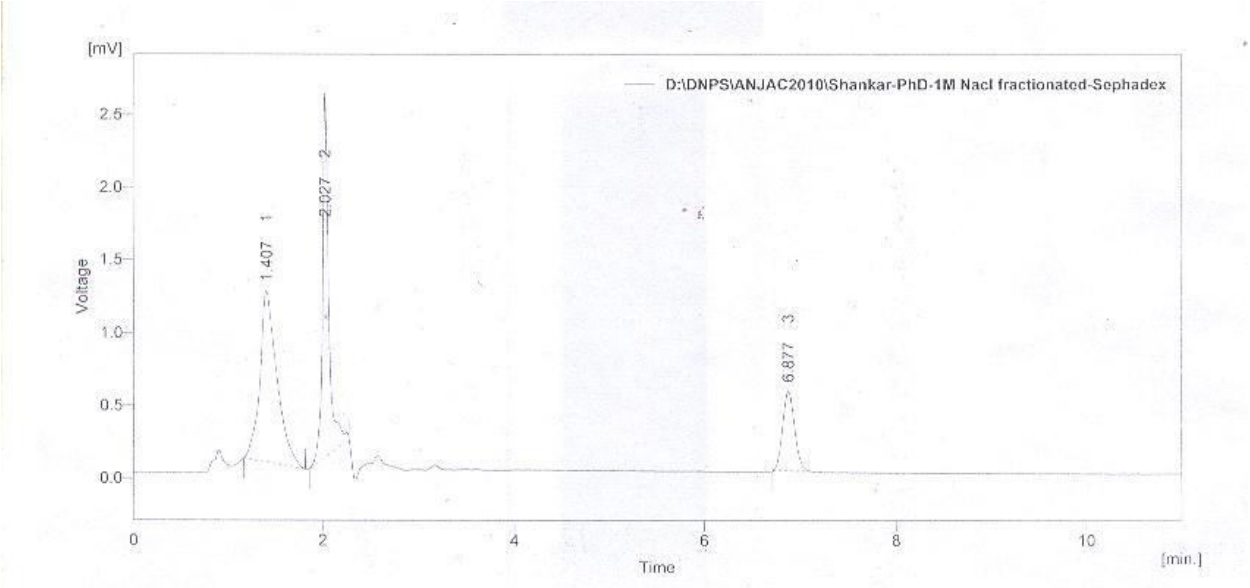



Fig. 3: HPLC analysis of the exopolysaccharide.

Table 2: Antimicrobial activity of the EPS was tested against certain clinical pathogens

| S. No. | Test organisms | Control (Water) | Exopolysaccharide concentration (mg) | | | | |
|---|---|---|---|---|---|---|---|
| | | | 0.2 | 0.4 | 0.6 | 0.8 | 1 |
| | Bacterial sp. | Zone of inhibition ( mm ) | | | | | |
| 1 | *Klebsiella* sp. | _ | 6 | 8 | 11 | 15 | 18 |
| 2 | *Staphylococci* sp. | _ | 12 | 15 | 18 | 21 | 23 |
| 3 | *Escherichia* sp. | _ | 7 | 9 | 11 | 12 | 13 |
| 4 | *Pseudomonas* sp. | _ | 10 | 12 | 13 | 15 | 17 |
| | Fungi sp | | | | | | |
| 5 | *Aspergillus niger* | _ | 7 | 9 | 13 | 15 | 16 |
| 6 | *Candida* sp. | _ | 8 | 10 | 11 | 13 | 15 |

## *Biological activities of exopolysaccharides*

### *Antimicrobial Activity*

### *Antimicrobial activity of exopolysaccharide*

Antimicrobial activity of the exopolysaccharide was tested against certain clinical pathogens, and their zones were tabulated. The antimicrobial assay shows that the clinical pathogen *Staphylococci* sp. was more susceptible to the bacterial polysaccharide, and it showed the highest zone of 35 mm, as shown in Table 2 and Figure 4. Other clinical isolates showed a low zone, indicating resistance to the exopolysaccharide.

### *Ring Biofilm Assay*

Qualitative assay also known as Ring Biofilm Assay was a tube method which is used for detecting biofilm development. In response to different

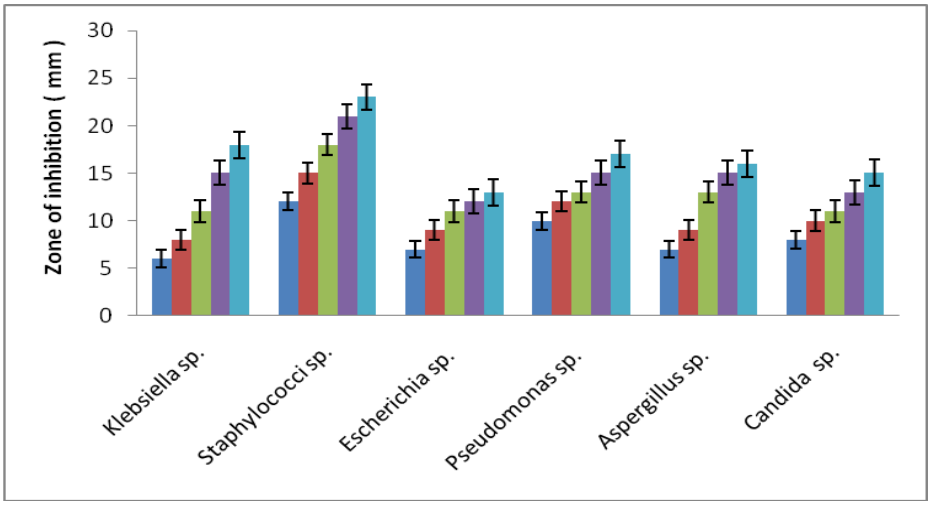



Fig. 4: Antimicrobial activity of the EPS was tested against certain clinical pathogens.

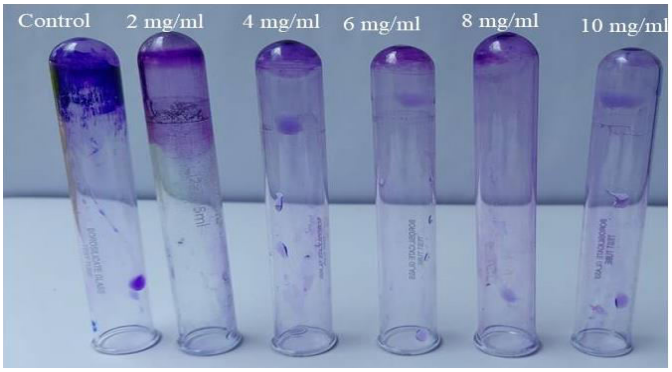
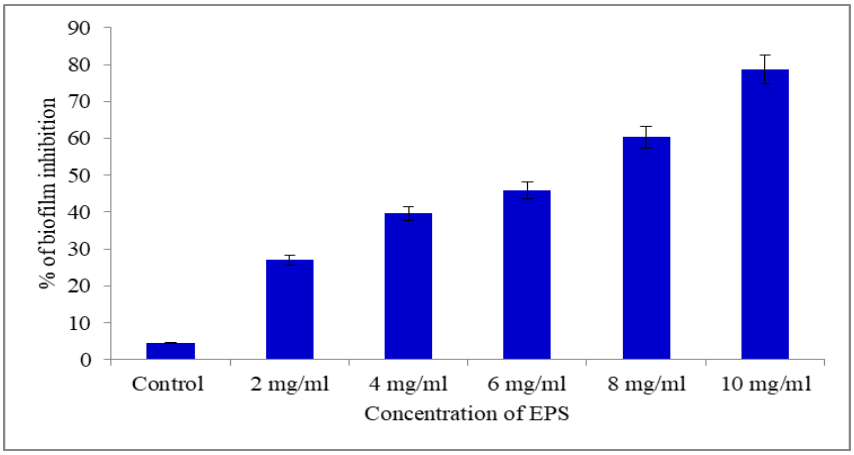



Fig. 5: Antibiofilm effect of EPS on *Staphylococci* sp by ring biofilm assay.




amounts of EPS the percentage inhibition of biofilms in *Staphylococci* sp was also studied. The Biofilm formation moderately reduced in the EPS different concentration such as 2, 4, 6, 8 and 10 mg/ml. The EPS 78.6±0.02% inhibition of biofilm formation by *Staphylococci* sp. Was highest at concentration tested (10 mg/ml) (Figs. 5, 6).

### *Antioxidant assays of exopolysaccharide*

#### *Total antioxidant capacity (TAC)*

The exopolysaccharide's total antioxidant activity was measured using ascorbic acid as a reference. As seen in Figure 7, the ideal exopolysaccharide activity was determined to be 91.16 ± 0.19%.

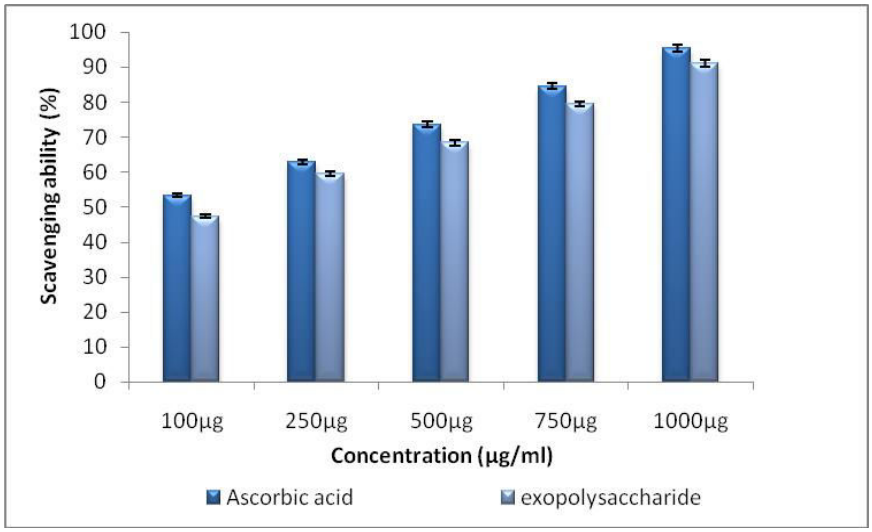



Fig. 7: Total antioxidant capacity of exopolysaccharide.

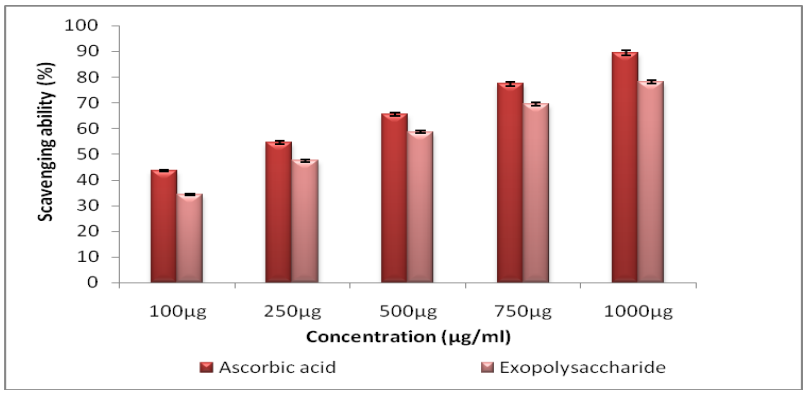



Fig. 8: Reducing power of exopolysaccharide.

*Reducing power*

Using ascorbic acid as a reference, Figure 8 shows the reducing power of exopolysaccharide. When compared to the reference chemical, the exopolysaccharide exhibited a reducing power of 78.13 ± 0.4%.

*Hydrogen peroxide scavenging assay*

Polysaccharide was tested in a hydrogen peroxide scavenging experiment with gallic acid which was used as a reference. The ideal EPS activity, as indicated in Figure 9, was 83.27 ± 0.71%.

*DPPH radical scavenging activity*

Polysaccharide was tested using the DPPH radical scavenging assay, with gallic acid used as the reference. In Figure 10, the polysaccharide's DPPH radical scavenging test exhibited a value of 61.09% ±0.13%.

## Discussion

Marine environment is the world's largest aquatic ecosystem, and marine bacteria account for more than 50% of all prokaryote biomass on Earth. These microbes are all capable of producing exopolysaccharides (EPS), which are extracellular biopolymers that make up a sizable portion of the dissolved organic carbon. Furthermore, exopolysaccharide production is frequently linked to biofilm formation, adhesion, surface colonization, and support for biochemical interactions between the bacterial strains and their surroundings (Casilo *et al*., 2018). Bacterial exopolysaccharide production has increased dramatically due to

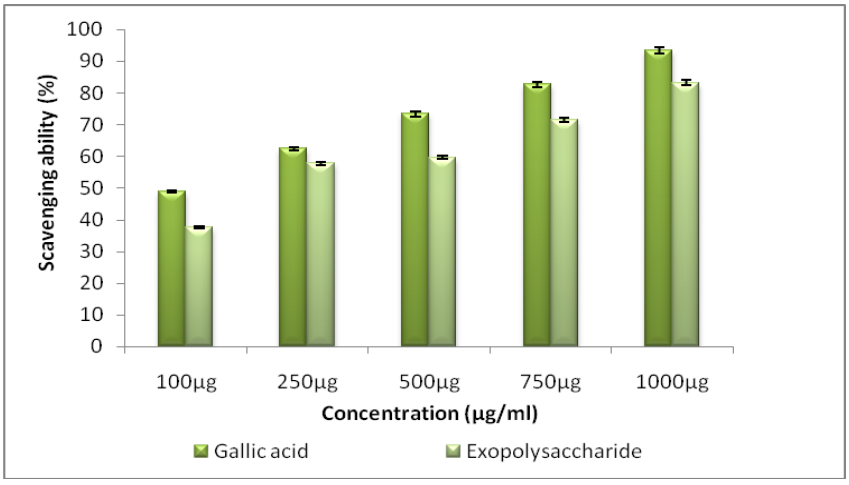



Fig. 9: Hydrogen peroxide scavenging assay exopolysaccharide.

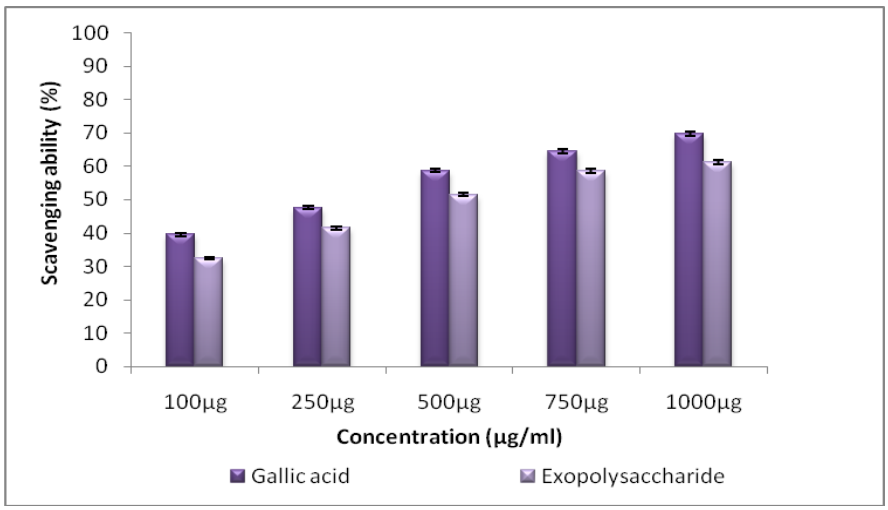



Fig. 10: DPPH radical scavenging assay of exopolysaccharide.

the need for natural biopolymers for various industrial applications (Sharma *et al*., 2017; Faten Hereher *et al.*, 2018).

In the present study, the potential bacterial strain's 16S rRNA gene was amplified by PCR using 16S rDNA universal primers. In our work, we sequenced both strands of the resulting PCR product. This follows in the same vein as the work of Mahendran *et al*. (2013), who used BLAST to determine a close relationship between an EPS-producing strain and *Lysinibacillus fusiformis* by analysing its 16S rDNA sequencing. The sulphate concentration of the EPS for *Halobacillus trueperi* was determined to be 27.12 ± 0.67% in the current investigation. The current study supports a previous report by Anita Iyer *et al*. (2005), which suggested that exopolysaccharide from *Enterobacter cloacae* had a sulphate concentration of 7%. Bramhachari *et al*. (2005) also found that *Vibrio furnissi* showed a 2.3% sulphate concentration in their EPS, whereas *Marinobacter* sp. showed a 2.7% sulphate content. Anitha Iyer *et al*. (2005) reported that the uronic concentration of bacterial exopolysaccharides from *Enterobacter cloacae* showed 9.23%, which lends credence to the current investigation.

Using a Perkin-Elmer FT-IR spectrometer, the pure polysaccharide FT-IR spectra was examined.

There was a contribution of C=O medium stretching vibration (carbonyls) at 1677.95 and 1557.41 $cm^{-1}$. The bend at 1433.98 $cm^{-1}$ for aromatic and 1106.1 $cm^{-1}$ for aliphatic amine C-N stretches is corresponding to the plane. Corpe *et al*. (1970) said that in order to determine whether the chemical has any unique functional groups, it is necessary to examine the FT-IR spectra. Consequently, a wide O-H stretching band at 3309 $cm^{-1}$ and a conspicuous C-H stretching band at 2895 $cm^{-1}$ were detected in the FTIR analysis of the polysaccharide of the marine isolate.

The peaks seen from the C18 column indicate that our crude EPS extract contains neutral sugars. At 2.120 (fructose), the retention period was shown to be distinct when compared to the typical length of carb retention. This research is similar to that of Shankar *et al*. (2021), who described the possible industrial applications of EPS generated from *Lactobacillus paracasei* and its carbohydrate and uronic acid composition.

In the present study, Qualitative assay also known as ring biofilm assay was a tube method which is used for detecting biofilm development. The EPS 78.6±0.02% inhibition of biofilm formation by *S. aureus* at highest concentration tested (10 mg/ml). Horn *et al*. (2013) reported that the impact of the EPS layer of *L. johnsonii* FI9785 on cell surface characteristics, biofilm formation and adhesion to tissues by making use of mutants where alterations in genes of an EPS cluster had led to differences in phenotype, EPS quantity and EPS composition.

This study found that exopolysaccharide had a total antioxidant activity of 91.16% ± 0.19%, a reducing power of 78.13% ± 0.4% compared to the standard compound, a hydrogen peroxide radical scavenging assay result of 83.27% ± 0.71%, and a DPPH radical scavenging assay result of 61.09% ± 0.13%. Similarly, Mahendran *et al*. (2013) discovered that *L. fusiformis* EPS extracts had the highest overall antioxidant capacity (80.13 ± 0.26%) in malt medium. On a weight-for-weight basis, we compared the radical scavenging capabilities of various polysaccharide fractions and evaluated their antiradical effectiveness against DPPH radicals. A study conducted by Abdel-Fattah *et al*. (2012) discovered that levan and two of its derivatives, SL1 and SL2, showed a significant ability to scavenge free radicals with DPPH. These results are consistent with the present study. Using scavenging tests with superoxide and 1, 1-diphenyl-2-picrylhydrazyl (DPPH), Chen *et al*. (2011) created a homogenous exopolysaccharide that exhibited high antioxidant activity *in vitro.*

## Conclusion

Based on the results of this investigation, it is clear that soil bacterial play a significant role in processing pharmacological activity. Because bacterial EPS may have a wide range of pharmacological effects, including antioxidant and antibiofilm properties, it has potential for use in a number of medicinal products.

## Conflict of Interest

The authors declare no conflicts of interest.

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