Study of Exopolysaccharide produced by a marine bacterium for development of biopolymer films

[1]Anuradha S. Pendse*, [2]Shaikh Zaveria Shamimuddin and [3]Neel Tikekar

[1*]Associate Professor, [2]Student, [3]Assistant Professor
Department of Microbiology, Wilson College, Mumbai 400007, Maharashtra, India
*Corresponding author email address: dranuradha.pendse@wilsoncollege.edu

Abstract: With the advent and growing popularity of fast foods and comfort foods, the volume of petrochemical based plastics has increased significantly in the packaging industries. However, the limited recyclability and non-biodegradable nature of these plastics has raised serious environmental concerns. Although these synthetic polymers offer excellent mechanical and thermal properties, the associated harmful environmental effects advocate the need for sustainable alternatives such as microbial derived biopolymers. The present study aimed at isolation of exopolysaccharide (EPS) producing bacteria from various sources for development of biopolymer films. The highest yield of EPS among 15 isolates screened in this study was obtained from a marine bacterium, identified as *Vibrio tritonius* based on molecular methods. Under optimised conditions of inoculum size (0.5 OD and 7ml volume), media (pH 7.5; supplemented with 4% galatose, 1% ammonium sulphate and 1% tryptone) and incubation conditions (under shaker for 48h), *V. tritonius* yielded 0.54g% EPS. Following purification, a 53.4% reduction in EPS weight was observed indicating successful removal of proteins and salts. The purified EPS was 0.46mm thick, smooth, with 34.62% moisture content, flexible and translucent (transparency of 1.64). It was completely soluble within 6 h in water. The observed properties of biopolymer film were comparable to kefiran, fucopol and Pseudomonas derived biopolymers. Overall, the findings of this study highlight the potential of obtained EPS as an eco-friendly alternative to petrochemical based plastics.

IndexTerms **- marine bacteria, exopolysaccharide, biopolymer film, eco-friendly, sustainable**

I. INTRODUCTION

Bacteria possess a unique ability to adapt to the environmental conditions prevalent in a wide range of ecological niches, including extreme habitats with fluctuating pH and temperatures (Marzban and Tesei, 2025). Among the many specialized cellular structures and adaptive mechanisms used by bacteria to overcome desiccation, osmotic changes and other environmental stressors, is the extracellular polymeric substance (EPS) or exopolysaccharides (Dogsa et al., 2005). It is a structural component composed of carbohydrate chains that forms a hydrated matrix surrounding the cell (Kaur and Dey, 2023). EPS production is also essential for the development and stability of biofilms, where it acts as promoters of cellular cohesion to facilitate surface attachment. In fact, the capsular EPS forms represent the earliest stage of biofilm formation (Dave et al., 2020).

EPS have been isolated from various microorganisms, including extremophiles, halophiles, psychrophiles, thermophiles, and alkaliphiles. Interestingly, the physicochemical properties of EPS produced are unique to the distinct habitats of the bacteria producing those (Donot et al., 2012). For example, deep-sea vent bacteria are known for producing EPS with high metal-binding capacities. The poly-anionic characteristics of these biopolymers in marine settings allow for strong interactions with cations and trace metals. These properties are attributed to the presence of uronic acids, which are present in concentrations up to 50% of the total polysaccharide composition (Qi et al., 2022). EPS from psychrophiles functions as a cryoprotectant. In hypersaline environments, EPS stabilizes microbial communities by maintaining hydration and ion balance (Qi et al., 2022; Saha et al., 2020). Presently, the structurally diverse and functionally versatile microbial EPS are in high demand for various biotechnological applications. Besides development of biopolymer films for packaging, many bacterial EPS has shown bioactive properties such as antioxidant, immune-modulatory and therapeutic activities. They have also shown immense potential in development of biomaterials such as hydrogels, sponges and biodegradable films (Shukla et al., 2019; Álvarez et al., 2021). Overall, the biodegradable and non-toxic nature of bacterial EPS has significantly raised their demand as a promising alternative to synthetic polymers.

Typically, EPS occurs in two primary forms known as capsular and slime EPS. The capsular EPS remains closely associated with the cell surface, while the slime EPS is secreted into the surrounding environment. These forms differ slightly in composition but have a common function associated with microbial adhesion, colonization, and stress tolerance (Salimi and Farrokh, 2023). EPS synthesis in bacteria is achieved through specific biosynthetic pathways, including the Wzx/Wzy-dependent pathway, ABC transporter–dependent pathway, and synthase-dependent pathways. Based on the specific pathway followed, the resulting polymer is either a homo-polysaccharide or hetero-polysaccharide. The monomeric composition, branching pattern, and molecular weight of the biopolymer also depend on the pathway followed by the bacterial strain (De Vuyst and Degeest, 1999; Prakash Devesh et al., 2017). Additionally, many studies have suggested that cultivation parameters such as carbon availability, pH, temperature, and incubation time greatly influence EPS yield and characteristics (Mouro et al., 2024; Ali et al., 2019; Alves et al., 2011; Bibi et al., 2021; Prakash Shyam et al., 2021).

Given the increasing demand for sustainable biomaterials, microbial EPS have gained significant attention for their ability to form biopolymer films that can be used in the packaging industries (Shukla et al., 2019). Recent advances in biomaterial research have reported successful film formation from microbial EPS such as kefiran, fucopol and Pseudomonas derived polymers. These biopolymers are biodegradable and flexible, and show potential applications in food, agricultural and biomedical industries (Rimada and Abraham, 2003; Ferreira et al., 2014).

The present study was carried out to screen EPS producing bacteria from various environmental sources, followed by optimization, extraction, purification and partial characterization of the extracted EPS.

II. RESEARCH METHODOLOGY

2.1 Sample Collection

A total of 10 samples were collected in this study. Soil samples were collected from Wilson college garden and mangrove region. Water samples were collected from sea, wells and rivers in Mumbai city. Other samples included spoiled grains and vegetables. All samples were collected in sterile zip-lock bags or sterile flasks and stored at 4°C until further processing for isolation of EPS producing bacteria.

2.2 Enrichment and Isolation

To enrich the microbial populations, 1g of each soil sample was suspended in 10mL of sterile phosphate-buffered saline (PBS). From this suspension, 1 mL was inoculated into 50mL of sterile nutrient broth (NB) supplemented with 1% (w/v) glucose and incubated at Room Temperature (RT; ~28ºC) on a shaker (150 rpm) for 24h. Similarly, 1mL suspensions prepared from spoiled grain or vegetable samples were inoculated into NB containing 1% glucose and incubated under same conditions. Water samples were centrifuged at 5000 rpm for 20min, and 1mL of the supernatant was inoculated into 50mL of sterile seawater broth (SWB) containing 1% glucose. The flasks were incubated on shaker for 24h. Post-enrichment, all cultures were streaked onto nutrient agar (NA) and seawater agar (SWA) plates supplemented with 1% glucose and incubated at RT for 24h. Colonies exhibiting mucoid, shiny morphology were selected as potential EPS producers and maintained as pure cultures on respective slants.

2.3 Detection of EPS using microscopic and qualitative methods

2.3.1 Gram staining

Before detection of EPS, the gram nature of the isolates was assessed using standard gram staining technique and confirmed with KOH test. For the later, a loopful of bacterial culture was mixed with one drop of 3% KOH solution on a clean slide for 10–15 s. Formation of a viscous, gel-like thread indicated a Gram-negative bacterium, while the absence of string formation indicated a Gram-positive bacterium (Buck, 1982).

2.3.2 Capsule staining

To confirm presence of capsules, the Maneval's staining method was carried out. A loopful of bacterial culture was mixed with a drop of 1% Congo red and spread on to a clean, dry, grease free slide and spread into a thin film. After air-drying (without heat fixation), the smear was flooded with Maneval's stain for 1–2 min. Excess stain was removed, and the slide was air-dried and examined microscopically. The capsule appeared as a clear halo surrounding the stained bacterial cell (Maneval, 1941).

2.3.3 EPS assay

To qualitatively detect EPS production, the Congo Red Agar (CRA) assay was employed. The isolates were spot inoculated on CRA plates containing 0.08 g% Congo red dye and 1% glucose, and incubated at RT for 24 h. After incubation, the colony colour was assessed using a six-point colour intensity scale; where deep black, black and almost black were considered positive for EPS production, and Bordeaux, deep red, and red indicated negative results (Ferreira et al., 2015; Ignatova & Ivanova, 2017).

2.4 Extraction of EPS by Solvent Precipitation

For EPS extraction, the optical density (OD) of all isolates was adjusted to 0.5 at 540nm. From this bacterial suspension, a 1mL inoculum was transferred into 50 mL of sterile broth and incubated under shaking conditions for 24h. After incubation, 0.5% (v/v) formalin was added and the culture was kept at 4°C for 1h to stabilize the EPS. The culture broth was centrifuged at 5500 rpm for 20min, and the supernatant was collected carefully. To precipitate EPS, three volumes of chilled 95% ethanol were added to the supernatant, and the mixture was incubated at RT for 30min. The resulting precipitate was recovered by centrifugation at 3000 rpm for 15min, and residual ethanol was allowed to evaporate. A small volume of acetone was added to wash the EPS pellet. The weight of the obtained EPS was noted in a pre-weighed aluminum foil boat. The EPS was then dried completely to remove moisture and weighed again to determine the EPS yield (Dave et al., 2020; Gaikwad et al., 2022; Prakash Shyam et al., 2021; Yuksekdag et al., 2008).

2.5 Molecular Identification

For molecular identification of the bacterial isolate, a 24 h old culture was outsourced to Hi-Gx360® Solutions, Hi-media Laboratories Pvt Ltd, Thane, for 16S rRNA sequencing. The bacterial DNA was amplified and sequenced using BDT v3.1 chemistry on an Applied Biosystems 3500XL Genetic Analyzer. Amplification was performed using universal primers, and the resulting PCR product was subjected to Sanger sequencing. The obtained consensus sequence was analyzed using the Basic Local Alignment Search (BLASTn) tool against the NCBI type strain database to identify closely related bacterial species. Key alignment parameters such as bit score, alignment length, query coverage (qcovs) and percentage identity (pident) were evaluated to ensure accurate classification. A phylogenetic tree was constructed to visualize the evolutionary relationship of the potential isolate with closely related bacterial species. The tree was generated using the Neighbor-Joining (NJ) algorithm, and bootstrap analysis was performed with 1,000 replications to assess the statistical robustness of each branch. Bootstrap values above 70% were considered reliable indicators of evolutionary support.

2.6 Detection of Carbohydrates in EPS

The presence of carbohydrates in the extracted EPS was confirmed by the Molisch test. A few drops of saturated α-naphthol solution were added to the EPS extract, followed by careful addition of concentrated H_2SO_4 along the test tube wall. The appearance of a

violet or purple ring at the interface indicated a positive result, confirming the presence of carbohydrates. A negative control containing distilled water showed no color change, validating the test reliability.

2.6.1 Phenol-sulfuric acid method

The total carbohydrate content in the extracted EPS was quantified using the phenol-sulfuric acid method (Masuko et al., 2005), using glucose as standard. This method is widely recognized for its simplicity, sensitivity, and ability to detect diverse carbohydrate types, including mono-, di-, oligo- and polysaccharides. In this assay, concentrated sulphuric acid hydrolyzes polysaccharides and oligosaccharides into monosaccharides. Pentoses (five-carbon sugars) are dehydrated to form furfural, whereas hexoses (six-carbon sugars) yield hydroxymethylfurfural. These compounds react with phenol to produce a yellow–gold chromophore, whose absorbance can be measured at 490 nm using a spectrophotometer (Gaikwad et al., 2022; Prakash Shyam et al., 2021).

2.6.2 Anthrone method

In addition, the Anthrone method (Layne, 1975) was also employed to quantify the total carbohydrate concentration in the EPS extract using a standard glucose curve. This colorimetric assay is known for its higher sensitivity for carbohydrate estimation (including mono-, oligo-, and polysaccharides). In the presence of concentrated sulphuric acid, carbohydrates are dehydrated to furfural derivatives, which react with Anthrone to form a blue–green complex measurable at 620 nm. For both assays, 20-100 µg/mL glucose concentrations were prepared to construct the standard curve and calculate the value of carbohydrates in the unknown sample.

2.7 Optimization of Physiochemical Parameters for Exopolysaccharide Production

Optimization of EPS production was performed by systematically varying one factor at a time. The physical and nutritional parameters included optical density (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, and 0.7), inoculum size (1ml to 7 ml), aeration (static and 150 rpm), incubation time (24 h to 120 h), pH (5.5, 6.5, 7.5, 8.5, and 9.5), carbon sources (1% glucose, sucrose, xylose, galactose, and mannitol at a concentration) and its concentration (1% to 10% with intervals of 2%), as well as organic (1% peptone, yeast extract, beef extract, and tryptone) and inorganic nitrogen (1% KNO_3, NH_4Cl, NH_4NO_3, $(NH_4)_2SO_4$, and $NaNO_3$) sources.

The effect of each variable was evaluated individually, and optimized conditions were sequentially incorporated into subsequent experiments. At the initiation of the experiment, SWB supplemented with 1% glucose was adjusted to pH 7.5 ± 0.2 and sterilized at 121°C for 20min. The media was inoculated with 1 mL of culture suspension (0.2 OD_{540nm}) and incubated under shaker conditions for 24h. After every optimization step, EPS was extracted using the solvent precipitation method, and the yield was analyzed (Ali et al., 2019; Alves et al., 2011; Bibi et al., 2021; Prakash Shyam et al., 2021; Patel Vishalkumar & Patel Dr. Falguni, 2023; Prasertsan et al., 2008; Gomaa & Yousef, 2020; Malick et al., 2017; Bramhachari P. V. et al., 2006; Bramhachari & Dubey, 2006; Krishnamurthy et al., 2020; Nwosu et al., 2019; Yuksekdag et al., 2008; Gangalla et al., 2021).

2.8 Purification of EPS

Partial purification of the extracted EPS was carried out using solvent precipitation method, followed by dialysis to remove salts proteins, and other impurities. A dialysis membrane (with pore size of 10 µm) was cut into ~10 cm sections and activated by immersion in 10g of EDTA dissolved in 1000mL of distilled water. After activation, the membrane was thoroughly rinsed with distilled water to eliminate residual EDTA. The partially purified EPS was re-dissolved in distilled water. For additional protein removal, the EPS was dissolved in 5% trichloroacetic acid (TCA) and transferred into the activated dialysis bag, which was sealed and submerged in distilled water (pH 7.0). Dialysis was performed for 48h, and the water was replaced after 24h. The pH of the dialysis water was monitored periodically to detect any changes resulting from the released impurities. After 48h, the dialyzed EPS solution was collected into a pre-weighed container and allowed to dry completely. The final EPS weight was recorded and compared with that of the partially purified sample (Dave et al., 2020; Gaikwad et al., 2022).

2.9 Development of Biopolymer Film

A biopolymer film was developed using the extracted EPS. Briefly, 2% (w/v) of EPS was dissolved in 50 mL of distilled water and stirred continuously using a magnetic stirrer until complete dissolution. Once homogeneity was achieved, 35% (w/w) glycerol was added as a plasticizer, and the solution was stirred for an additional 15mins to ensure uniform mixing. The mixture was allowed to stand briefly to release entrapped air bubbles, then poured onto clean glass plates and left to dry at RT until a consistent film was formed (A. R. V. Ferreira et al., 2014; Ghasemlou et al., 2011).

2.10 Determination of Physical Properties of Films

Physical properties of the EPS such as thickness, moisture content, solubility and transparency were determined in this study. The thickness of the films was measured using a digimatic caliper (Mitutoyo No. 500-196-30, Japan) with an accuracy of 0.001 mm. Multiple measurements were taken and the average value was calculated to ensure precision (Ghasemlou et al., 2011). Moisture content was determined by measuring the weight loss of the films before and after drying. The difference in weight was used to calculate the moisture percentage (Ghasemlou et al., 2011). Film solubility in water was determined as the proportion of water-soluble dry matter released after immersion. Square samples (2 × 2 cm^2) were cut, weighed (initial dry weight), and immersed in 50mL of distilled water at RT with intermittent stirring for six hours (Ghasemlou et al., 2011). After immersion, the un-dissolved film fragments were collected, dried to constant weight, and reweighed (final dry weight). The percentage of total soluble matter (%TSM) was calculated using the equation:

$$\%TSM = \frac{Initial\ dry\ weight - Final\ dry\ weight}{\text{Initial dry weight}} X\ 100$$

Film transparency was evaluated by measuring light transmittance at 600 nm using a double-beam spectrophotometer (Systronics, Model 2203). Transparency (T) was calculated using the following equation (A. R. V. Ferreira et al., 2014):

$$T = -\log\frac{\text{T600}}{\text{x}}$$

Where T600 represents the transmittance at 600 nm and x is the film thickness in mm.

III. RESULTS AND DISUSSION

3.1 Isolation of EPS producers

A total of 15 EPS producing isolates were obtained in this study from various samples. Among these, 10 isolates were obtained from soil samples, 4 from water samples and 1 from food samples. Bacterial colonies exhibiting shiny, viscous and mucoid appearances were identified as potential EPS producers. Table 1 represents the qualitative and quantitative EPS production by isolates obtained in this study. Based on CRA method, Two isolates (W, R1) appeared very black, suggesting very high EPS production, four isolates (Z2, Z5, R, A) were black, indicating strong production and four isolates (S, Si, Z3, Z4) were almost black, suggesting moderate production. In contrast, four isolates (Z1, Tim, SwL, SwD) were red, indicating weak or no EPS production. Despite the red colonies on CRA plates, SwD demonstrated the highest EPS yield (0.2878 g%) followed by SwL (0.1548 g%) with the solvent precipitation method. This observation can be explained based on the fact that some bacterial species produce EPS with different chemical compositions, which may not effectively bind Congo Red in CRA medium, leading to appearance of red-colored colonies despite potential EPS synthesis (Ferreira et al., 2015; Ignatova-Ivanova, 2017).

Soil, water, and spoiled food samples are promising reservoirs of EPS producing bacteria. Soil microbes produce EPS to shield themselves from harsh conditions such as drought and resource scarcity (Costa et al., 2018). Spoiled food is abundant in lactic acid bacteria that synthesize EPS for metabolic stability and survival in acidic environment (Liang et al., 2024). The biofilm forming bacteria in river and well water samples are naturally enriched since EPS promotes adhesion and survival (Sooriyakumar et al., 2022). The marine bacteria primarily secrete EPS to withstand stress conditions like high salt concentrations (Kaur and Dey, 2023).

Media used in this study (NA and SWA with 1% glucose) supports robust bacterial growth and enhances EPS synthesis. NA provides essential organic nutrients for heterotrophic bacteria, in which glucose serves as a readily metabolizable carbon source promoting EPS. SWB is enriched with peptone and yeast extract, which maintains osmotic balance and provides essential nitrogen sources. In previous studies, isolation of EPS producing bacteria is reported using these media from soil and seawater (Shyam et al., 2021; Ifshani et al., 2018). Additionally, use of selective media such as glucose yeast extract medium and Thiosulfate citrate bile salts sucrose medium is reported for isolating *Enterobacter cloacae* and *Vibrio furnissii* respectively (Patel et al., 2023; Soundararajan et al., 2022). Similarly, alternative formulations such as minimal medium, Ruthenium red agar and Zobell marine medium have been employed for isolation of EPS producers (Yadav et al., 2024; Sørensen et al., 2022; Chaudhari et al., 2017; Bramhachari and Dubey, 2006). For extraction of EPS, ethanol precipitation is the most common method, while acetone and isopropanol are also used to influence molecular weight and purity. The choice of solvent and its ratio impacts precipitation efficiency, as higher solvent volumes improve recovery but may co-precipitate impurities. Centrifugation conditions (8000–12000 rpm for 10–30 min) also affect EPS separation, with excessive speeds potentially causing aggregation (Dave *et al*., 2020; Gaikwad *et al*., 2022; Prakash Shyam *et al*., 2021; Yuksekdag et al., 2008).

Table 1: Qualitative and quantitative detection of EPS Producers

Isolates	Appearance on CRA plates	Quantified EPS (g%)
S	Almost Black	0.1238
Si	Almost Black	0.0401
Z1	Red	0.0040
Z2	Black	0.0056
Z3	Almost Black	0.0204
Z4	Almost Black	0.0244
Z5	Black	0.0336
Tim	Red	-
R	Black	0.0326
A	Black	0.0246
SwL	Red	0.1548
SwD	Red	0.2878
W	Very Black	0.0286
U	Red	0.0030
R1	Very Black	0.0348

3.2 Detection of Carbohydrates in EPS

The presence of carbohydrates in the extracted EPS sample from SwD was confirmed qualitatively using Molisch test. The carbohydrate content was determined to be 228 µg/mL with the phenol-sulphuric acid method and 312µg/ml with Anthrone method. This variation may be due to difference in the sensitivity and specificity of the two methods. The Anthrone method is often considered more sensitive to certain carbohydrate structures, while the phenol-sulphuric acid method provides a broader estimation of total sugars (Masuko et al., 2005; Layne, 1975). Compared to the previously reported carbohydrate content of *Vibrio harveyi* VB23 EPS (5.89 µg/mL; Bramhachari and Dubey, 2006), SwD showed considerably higher concentration.

3.3 Identification of potential isolate

The morphological, cultural and biochemical characteristics of SwD is represented in Fig. 1. The BLAST results revealed the closest match with *Vibrio tritonius* strain AM2 (NR_134830.1), showing 99.79% sequence identity with a bit score of 2628. Other closely related species included *Vibrio furnissii* (98.31% identity), *Vibrio fluvialis* (98.10% identity), *Allomonas enterica* (97.89% identity), and *Vibrio hepatarius* (97.81% identity). The phylogenetic tree is represented in Fig. 2. To provide an external reference for evolutionary comparison, *Paucibacter toxinivorans* (HG530249) was used as the out group to root the phylogenetic tree. The overall topology of the tree and the high bootstrap values (e.g., 89, 90, 46) indicated the reliability of the phylogenetic clustering.

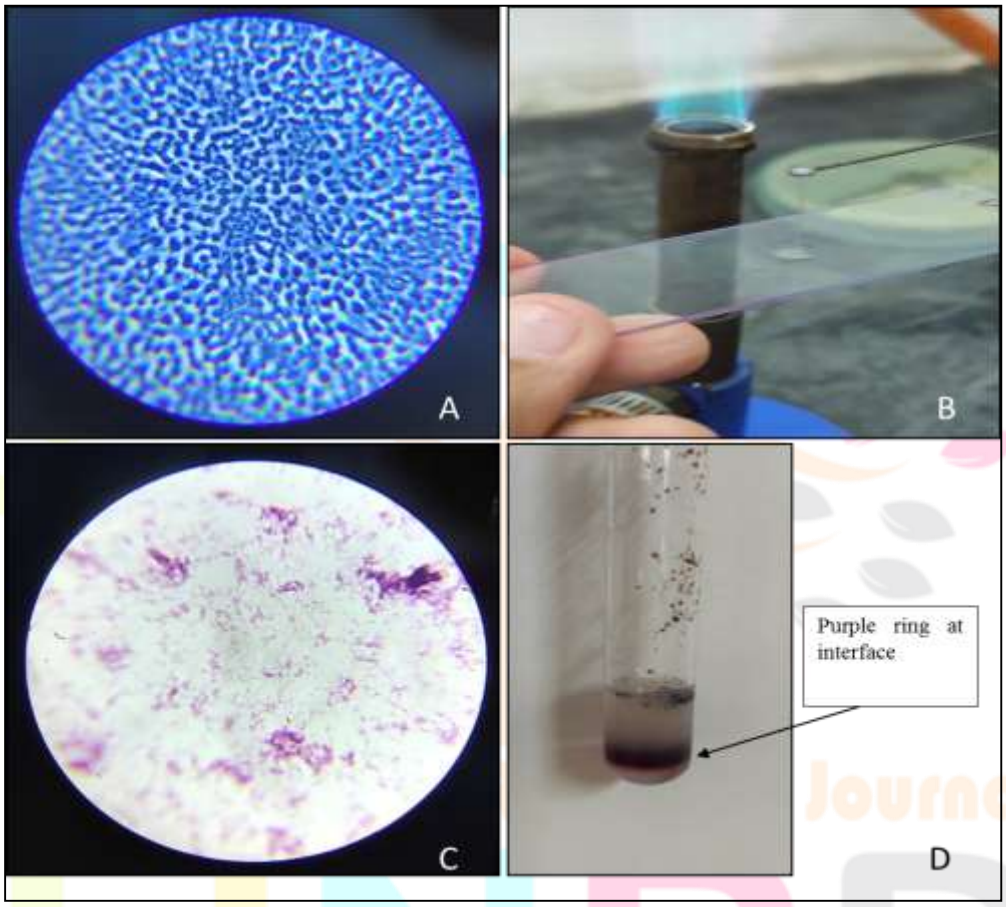

Fig 1: Characteristics of SwD isolate identified as *Vibrio tritonius* strain AM2

The figure shows (a) Capsules stained with Manewals method, (b) KOH test showing string formation, (c) gram staining showing pink cells and (d) Purple ring in Molisch test confirming presence of carbohydrates

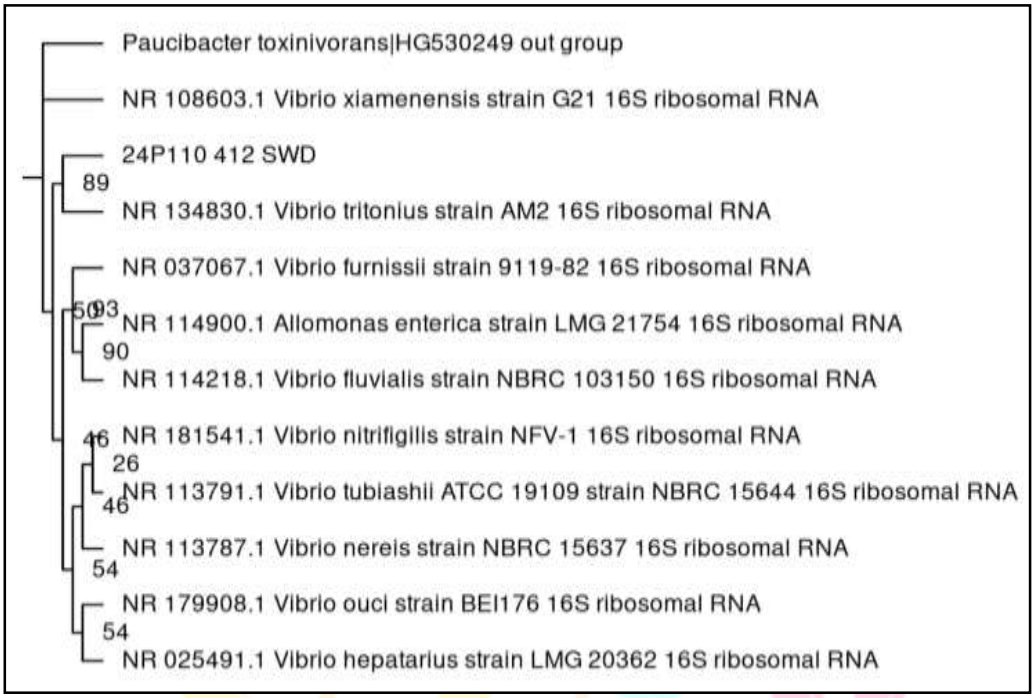

Fig 2: Phylogenetic analysis for evolutionary relationship

3.4 Optimization of EPS Production

Fig. 3 represents the optimum conditions for production of EPS by *Vibrio tritonius* AM2 isolated in this study. Maximum yield was obtained when 7ml culture of 0.5 O.D was inoculated in SWB (pH 7.5) media supplemented with 4% galactose, 1% ammonium sulphate and 1% tryptone, and incubated at RT for 48h under shaker conditions. Among other Vibrio sp. reported in literature, *V. harveyi* strain VB23 produced highest EPS yield during the late log phase in the mineral salts medium supplemented with 1.5% NaCl and 2% glucose (Bramhachari and Dubey, 2006). A marine isolate identified as *V. furnissii* VB0S3 produced the highest EPS yield in the mineral salts medium supplemented with 1.5% NaCl and 0.2% glucose (Bramhachari et al., 2007).

Comparing the above observations of NaCl requirement by Vibrio sp. isolated from different environments, it is evident that high salinity is not a necessity for survival of marine bacteria. Instead, it is likely that EPS helps to withstand the osmotic stress conditions in marine environment (Kaur and Dey, 2023). In fact, studies have suggested that Vibrio sp. are fragile and responds to salt stress through differential gene expression such as osmolarity response regulator gene (ompR), putative threonine efflux protein gene and Na+/H+ antiporter gene (Hu et al., 2022; Fu et al., 2014). The EPS production is also directly proportion to the growth of isolate during incubation. Hence, both incubation time and initial culture density plays an important role in maximizing EPS production. Higher microbial load enhances EPS synthesis, likely due to increased metabolic activity and efficient nutrient utilization. However, with higher incubation time or high initial culture density, nutrient depletion and oxygen limitation occurs along with the accumulation of metabolic byproducts, reducing EPS yield (Elmansy et al., 2023; Prakash Shyam et al., 2021). Similarly, the enhanced aeration in shaker conditions facilitates better oxygen diffusion, promotes bacterial respiration and EPS synthesis, whereas limited oxygen in static conditions restricts growth and EPS production (Biswas and Paul, 2017).

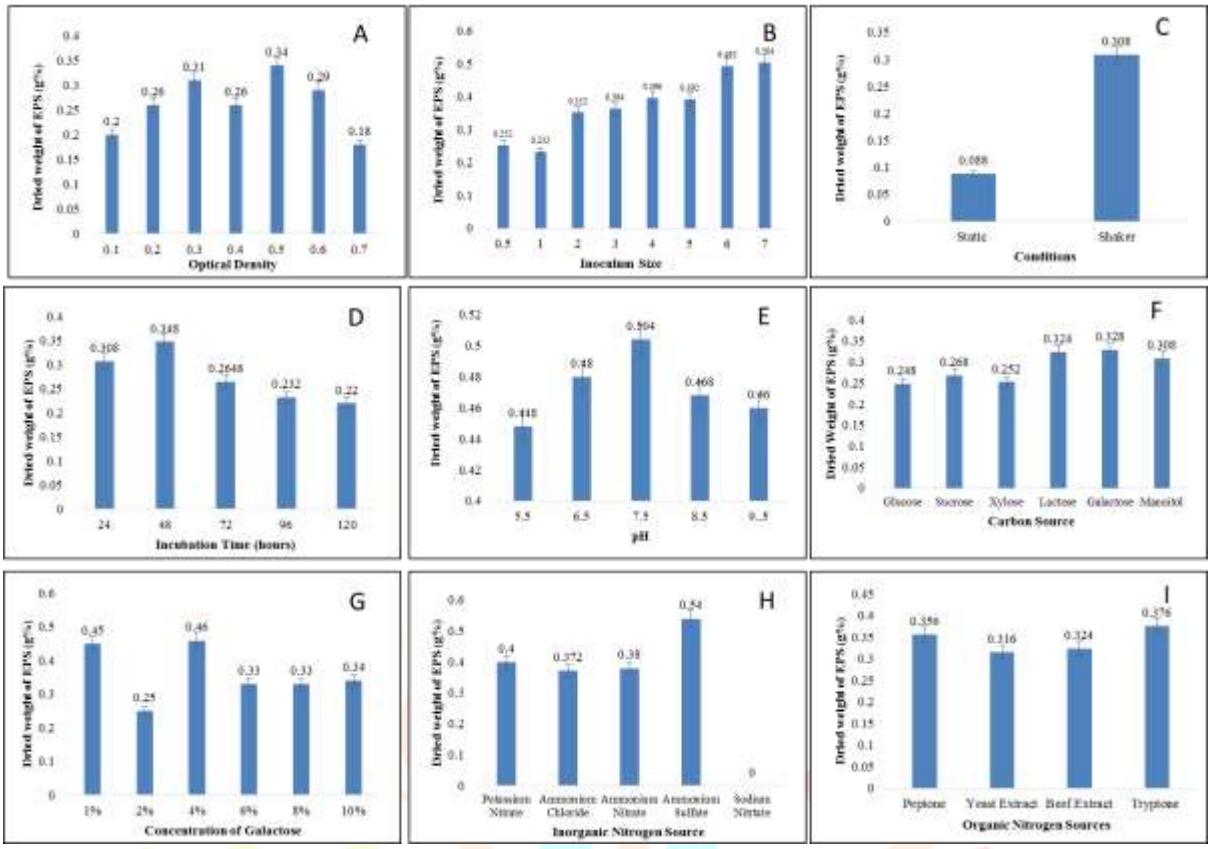

Fig 3: Optimization of EPS production by *Vibrio tritonius* strain AM2

Among other bacterial sp. the optimum nutrients for EPS production by *Virgibacillus salarius* BM02 were 4% sucrose and 0.75% peptone, and incubation period of 4.69 day (Gomaa & Yousef, 2020). *Enterobacter cloacae* WD7 produced highest yield of EPS in basal medium (pH 7.0) supplemented with 3% sucrose and 0.05% tryptone, and incubation conditions of 5 days at 30°C and 200rpm (Prasertsan et al., 2008). *Enterobacter cloacae* VHP-34, on the other hand, showed optimum EPS yield in presence of 10% glucose, 2% yeast extract, 2% peptone and 5% NaCl and gave a yield of 39.4 g/l which was 1.21 fold higher than the yield obtained in non-optimized media (Patel Vishalkumar & Patel Dr. Falguni, 2023). Among the Bacillus sp. the optimum conditions for EPS production by *Bacillus aerophilus* rk1 were pH 7.0, cultivation time 72 h, temperature 30°C, yeast extract and sucrose supplements (Gangalla et al. 2021) while *Bacillus cereus* KMS3-1 produced optimum yield at pH 7.0 in media containing 5g/L sucrose and 10 g/L yeast extract, in 120 h (Krishnamurthy et al., 2020).

3.5 Purification and development of EPS

After optimization, 1.535g of EPS was extracted, which reduced to 0.716g on removal of proteins by TCA method and dialysis. The 53.4% reduction in mass suggests successful removal of impurities from the crude sample such as salts and proteins. The use of TCA in the early stages of extraction helps break down and remove unwanted protein contaminants and improve purification during dialysis. Some researchers also combine TCA with other precipitation agents, such as acetone or ammonium sulfate, to improve EPS purity (Dave et al., 2020; Gaikwad et al., 2022). The purified EPS appeared lighter in color and more homogeneous, signifying improved purity.

The EPS from *V. tritonius* successfully formed a 0.46 mm thin, smooth, translucent and flexible biopolymer film (with a slightly brownish tint) in 4 days (Fig. 4). The film had 34.62% moisture content, and was completely soluble in water within 6h (Fig. 5). The transparency index was calculated as 1.64, which is considered as moderate (translucent). In previous studies, successful formation of 0.058 to 0.067 mm thin kefiran-based films is reported using casting and solvent evaporation methods. The study further reported that addition of glycerol enhances flexibility of the films (Ghasemlou et al., 2011). A hydrophilic fucopol-based film also exhibited a brownish tint, as observed in this study (A. R. V. Ferreira et al., 2014). It also showed high permeability to water vapour, and maintained good barrier against oxygen and carbon dioxide. Moreover, these films showed good mechanical properties including high tensile strength, high elongation and low tension at break points, and low elastic modulus. EPS films derived from *Pseudomonas oleovorans* NRRL B-14682 were also reported to be transparent, flexible, and tough (Alves et al., 2011). These films were hydrophilic, but showed good stability in contact with liquid water, after auto-crosslinking reactions at low pH.

Fig 4: Biopolymer film produced by *Vibrio tritonius* strain AM2

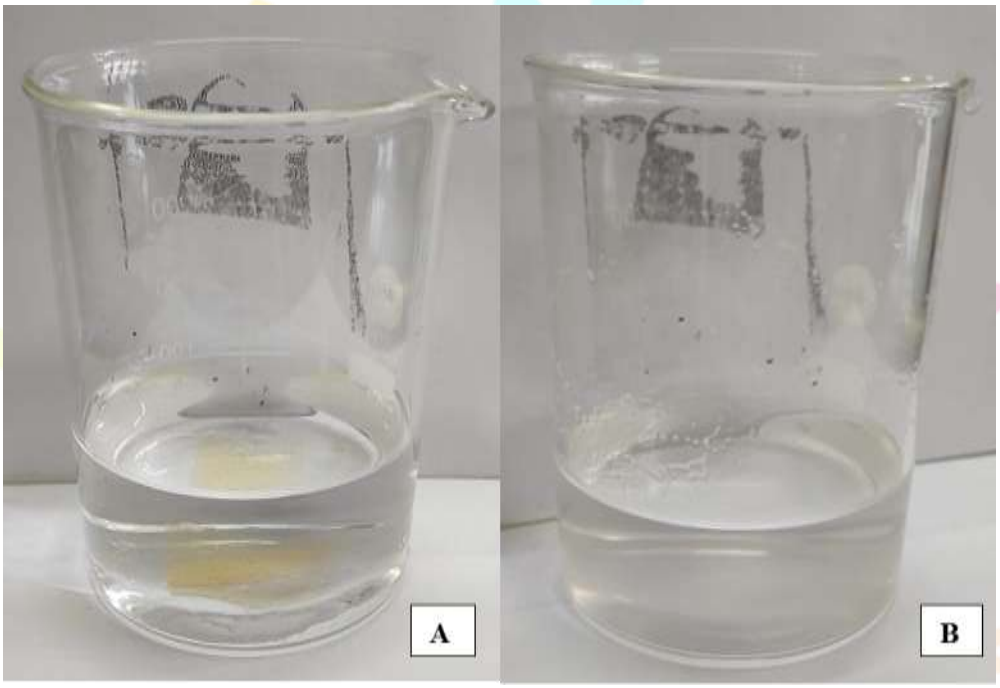

Fig 5: Solubility of biopolymer film at (a) 0 h and (b) in 6 h

Overall, the film exhibited good integrity and moderate transparency, suggesting its suitability for use as a natural, biodegradable polymer material for use in packaging, coating or biomedical applications.

IV. CONCLUSION

The present study demonstrates the potential of EPS producing bacteria to be a valuable resource for developing biodegradable films. For industrial, environmental and agricultural applications, more studies on improving mechanical properties of the formed biofilms is necessary to promote widespread use of such eco-friendly and sustainable microbial products on a larger scale. This study successfully developed a biofilm. However, it was translucent mainly due to the brown tint following the extraction and drying processes. Further studies on the same polymer but adjusted film thickness, polymer composition, and drying conditions can suitably improve transparency. Optimization of plasticizer concentration will further help in reducing molecular aggregation and improving film uniformity. These properties enhance optical properties of the biofilm; thereby increasing its potential applications.

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