

MOLECULAR CHARACTERISATION AND FATTY ACID PROFILING OF MARINE BACTERIA *SHEWANELLA* ALGAE

Submitted in partial fulfillment of the requirements for the award of a
Bachelor of Science degree in Biotechnology

By
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INSTITUTE OF SCIENCE AND TECHNOLOGY

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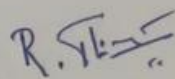
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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **REVANTH SAI.G (40770044) B.Sc Biotechnology**, who carried out the project entitled **"MOLECULAR CHARACTERISATION AND FATTY ACID PROFILING OF MARINE BACTERIA SHEWANELLA ALGAE"** under the supervision of Dr. R. THIRUGNANASAMBANDAM, Col. Dr. Jeppiaar Research Park, Sathyabama Institute of Science and Technology, Chennai from November 2022 to April 2023.


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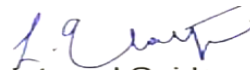

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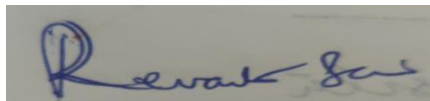
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DECLARATION

I, **REVANTH SAI.G (40770044)** hereby declare that the Project Report entitled “**MOLECULAR CHARACTERISATION AND FATTY ACIDPROFILING OF MARINE BACTERIA SHEWANELLA ALGAE**” done by me under the guidance of **Dr. P. PRAKASH** at Department of Biotechnology, Sathyabama Institute of Science and Technology, Chennai and **Dr. R. THIRUGNANASAMBANDAM** at Col. Dr. Jeppiaar Research Park, Centre for Ocean Research is submitted in partial fulfillment of the requirements for the awardof Bachelor of Science degree in Biotechnology.

DATE: 12/5/23

PLACE : Chennai
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A handwritten signature in blue ink, appearing to read 'Revanth Sai.G', is written on a light-colored rectangular background.

SIGNATURE OF THE CANDIDATE

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ABSTRACT

Shewanella algae are marine bacteria widely distributed in various marine environments. This bacterium has received much attention due to its ability to produce various bioactive compounds and its potential role in bioremediation. In this study, we aimed to molecularly characterize *Shewanella* algal strains isolated from the location in Marakkanam (12.1899°N, 79.9249°E), Chennai, Tamil Nadu, India, and analyze their fatty acid composition. The presence of unsaturated fatty acids suggests that *Shewanella algae* can adapt to different environmental conditions and have potential applications in biofuel production. Taken together, These findings help us understand the diversity and potential of marine bacteria in various biotechnological applications. Further studies are needed to examine the bioactive compounds produced by these strains and their bioremediation potential.

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LIST OF ABBREVIATIONS

A	Adenine
Bp	Base Pair
C	Cytosine
DNA	Deoxyribonucleic acid
EPS	Exo polymeric substances
Kbp	Kilo Base Pairs
LB	Luria-Bertani
Mbp	Mega base pairs
NaCl	Sodium chloride
PBS	Phosphate-Buffered Saline
Rpm	Rotation per minute
RT	Room temperature
T	Thymine
TSB	Tryptic Soy Broth
µg	Microgram
mL	Milliliter
µL	Microliter

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CHAPTER: 1

1.1 INTRODUCTION

1. SHEWANELLA

Shewanella is a distinct genus of the *Shewanellaceae* family that includes non-fermentative Gram-negative, motile rods with positive oxidase and catalase reactions. This genus' species are widely spread in nature, particularly in aquatic habitats such as seawater and freshwater, as well as in marine organisms. The bacterium genus *Shewanella* contains a diverse range of facultative anaerobes found in both marine and freshwater settings (Satomi M *et al.*, 2014). *Shewanella* species have a wide metabolic profile and have been isolated from harsh settings with high pressure, temperature, pH, and salinity. These organisms have been studied for their involvement in bioremediation and as prospective bacteria for microbial fuel cell development. However, numerous *Shewanella* species are known to be harmful to humans, animals, and marine life. The isolation of these organisms as disease-causing agents is increasing, particularly for *Shewanella algae* and isolated strains are increasingly carrying novel antibiotic-resistance genes that can spread to other Enterobacteriaceae members. Here we review what is known about infections caused by *S. algae* and highlight its role as an emerging human pathogen. Furthermore, they have been reported in clinical settings and are considered an emerging source of human infections, with *Shewanella algae* and *Shewanella putrefaciens* being responsible for most of these infections (Janda JM *et al.*, 2014).

2. HISTORY AND TAXONOMY

Shewanella putrefaciens was the first species found in this genus. Derby and colleagues isolated it from spoiled butter in 1931 and identified it as a member of the *Achromobacter* genus. This species' classification and naming have changed several times since then. Because of its rod shape, motility, and non-fermentative metabolism, Long and Hammer categorized this species as *Pseudomonas putrefaciens* in 1941. Shewan *et al.* (1960) classified it as belonging to the *Pseudomonas* genus. This species was reassigned to the genus *Alteromonas* due to its low (G + C) content in comparison to other pseudomonads (43-53% vs. 58-

72%). MacDonell and Colwell identified a new genus *Shewanella* in 1985 based on a study of 5S rDNA gene sequences. This species was transferred from the *Alteromonas* genus (Levin RE *et al.*, 1972). MacDonell and Colwell designated a new genus *Shewanella* after Dr. James Shewan for his contributions to fisheries microbiology in 1985, based on a comparison of 5S rDNA gene sequences (5). At the time, *S. putrefaciens*, *Shewanella benthica*, and *Shewanella haredai* were the only species identified in this genus and were categorised as members of the Vibrionaceae family (which had common phenotypic features) (Horikoshi K *et al.*, 1998).

The introduction of 16S rRNA sequence analysis resulted in the formation of the *Alteromonadaceae* family, and the genus *Shewanella* was one of its members. Ivanova *et al.* classified this massive family into eight families in 2004, including the Shewanellaceae family, of which *Shewanella* is the sole genus (Christen R *et al.*, 2004). Within the genus *Shewanella*, there are currently 63 recognized species, 49 of which have been discovered since the turn of the century. At the genetic level, *Shewanella* species are exceedingly varied. In one study, eleven *Shewanella* genomes from six different species had only 54% sequence identity in the core genome (McMeekin T *et al.*, 2005).

For many years, 16S rDNA gene sequencing was the 'gold standard' method for describing bacterial phylogeny, but low substitution values between closely related species in the *Shewanella* genus, as well as mounting evidence that the 16S rDNA gene within the *Shewanella* species is undergoing concerted evolution, suggest that this target is unsuitable for determining phylogeny (Dikow RB *et al.*, 2011; MacGregor BJ *et al.*, 1999). Housekeeping genes, such as *gyrB* and *ropB*, have also been employed as phylogenetic targets to distinguish between closely related *Shewanella* species, and are thought to be more accurate than 16S rDNA.

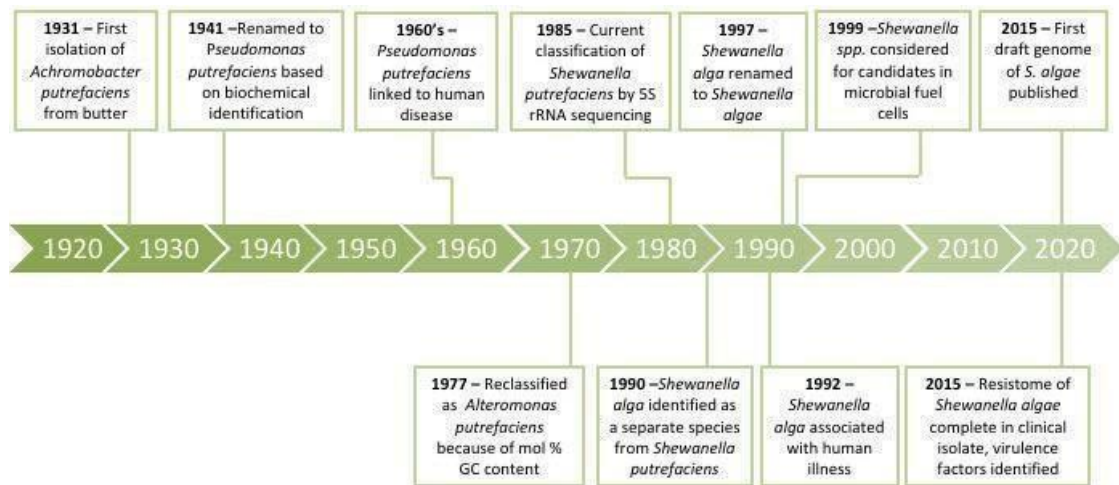


Figure 1.1 Overview of *Shewanella* species

1.3 HABITAT AND ECOLOGY

Shewanella species are found everywhere in nature; nevertheless, most of these bacteria appear to have originated in the sea. They can be retrieved from sea creatures, macroalgae, marine sediments, organic detritus, deep sea, iced fish, and coastal locations. They can be found in salt marshes, soil, freshwater, and natural energy reserves, among other places. As previously stated, *S. putrefaciens* was the first species in this genus to be identified as causing food deterioration. It was first isolated from rancid butter and has since been discovered as a food spoiler in a variety of foods, including poultry, beef, and shellfish. It rarely causes salted food products to deteriorate. *Shewanella* species thrive in marine fish that are high in trimethylamine oxide (TMAO).

The fishy odor produced by these species after decomposing marine fish is caused by the conversion of TMAO to TMO (trimethylamine). These microbes have also been isolated in the clinical setting; they have been identified from both patients and hospital effluent (wastewater). *Shewanella alga* has been isolated from a variety of locations including sediments from New Hampshire's Great Bay, an oil field in Canada, oil brine, seawater, marsh sediments in the United States, muddy sediments from Arcachon Bay, and sea sediment from eastern China.

S. alga has been isolated from several taxa, including fish, echinoderms, mammals such as sea lions, wild birds, and poultry.

However, *S. alga* has most typically been identified from human clinical material as an infective disease-causing bacterium in the literature (Yousfi K *et al.*, 2016).

1.4 BIOLOGICAL CHARACTERISATION

Shewanella species are found all over the world in freshwater and marine locations including intertidal and benthic zones, sediments, terrestrial ecosystems, and oilfield wastelands (Holt HM *et al.*, 2005; Nealson KHaS *et al.*, 2006). Because of their physiological diversity, they can be found in a wide range of settings around the world, where temperature, salinity, and pH can vary considerably depending on the season (Bozal N, Montes MJ, *et al.*, 2002). Antarctic coastal areas, Amazonian shelf muds, and the orbital cavity of St. Marcian's mummified bones are examples of extreme environments where they have been isolated (Venkateswaran K *et al.*, 1998). *Shewanella* species are known to exist as free-living bacteria in marine environments and sediments, in commensal relationships with marine life (Ivanova EP *et al.*, 2003), in mutualistic relationships with some organisms such as the *Loligo paelei* squid (Leonardo MR *et al.*, 1999), and in parasitic relationships with humans and marine life, where they are known to cause disease (Barbieri E *et al.*, 2001; Janda JM *et al.*, 2012).

Shewanella species also have sophisticated sensory and regulatory systems that allow them to exist at low temperatures (less than 4 C), high salt concentrations, and a wide variety of barometric pressures (Kato C *et al.*, 2001; Kan J *et al.*, 2011). This is owing to *Shewanella* species' capacity to metabolise a wide range of terminal electron acceptors, including heavy metals and radio nucleotides (such as iron, magnesium, and chromium), as well as environmental contaminants such as arsenic and uranium (Heidelberg JF *et al.*, 2002). *Shewanella* species are thus the most respiratory diverse creatures known to date.

Furthermore, the metabolic properties of *Shewanella* species have generated interest in employing them for bioremediation and sustainable energy production. These features may be the key to *Shewanella* species' ecological success, allowing them to exploit specific niches in severe habitats that change in temperature, pH, and salinity periodically and are unfriendly to other organisms. This could also explain the genus's high genotypic and phenotypic diversity.

5. CLINICAL ASPECTS

Skin and soft tissue infections, sepsis, hepatobiliary disease, and otitis media are all clinical manifestations of *Shewanella* species, particularly *S. algae* and *S. putrefaciens*, and are like conditions caused by marine halophilic bacteria such as *Vibrio* species and *Aeromonas* species (Myung DS *et al.*, 2009). Otitis media (29.2%), cellulitis (abscesses, ulcers, wound infections; 24.9%), gastroenteritis (17.6%), septicaemia (11.6%), and bacteraemia (4.3%) were the most common infectious presentations associated with *S. algae*, accounting for 199 of the 239 cases. When clinical symptoms of *Shewanella* species are compared in general, they share similar traits. Of the 227 patients who presented with a *S. algae* infection, 145 had previously reported hepatobiliary illness, cancer, diabetes mellitus, and neutropenia. While the majority of these are still linked to *S. algae* infection (hepatobiliary disease (1.8%), malignancy (3.0%), and diabetes (5.6%), there are more prominent comorbidities such as previous ear disease (28.8%), abdominal problems (renal/liver; 13.7%), hypertension (6.0%), broken skin (4.7%), and a compromised immune system (1.3%).

6. PATHOGENICITY OF *SHEWANELLASPP.*

Before the early 1990s, *S. putrefaciens*' species identification was synonymous with the clinical link between the genus *Shewanella* and human infections. However, in 1992 (Nozue, H *et al.*, 1992), Nozue *et al.* characterised 46 strains previously classified as "*S. putrefaciens*" and discovered that 33 of these isolates (72%) were *S. algae* (formerly "*S. alga*") based on the conventional biochemical properties consistent with the latter species designation and the mol% G+C content. The first report of *S. algae*-producing bacteremia in two people with lower leg ulcers was published shortly after this investigation (Domínguez, H *et al.*, 1996). Since that publication, it has been conclusively demonstrated that the vast majority (>90%) of strains linked to human illnesses that were identified using complete conventional biochemical panels or 16S rRNA gene sequencing to the species level are *S. algae*. This finding was corroborated by Janda and Abbott (Janda, J.M and S.L. Abbott *et al.*, 2012) in a retrospective analysis of 35 instances of *Shewanella* infection described in the literature, where 24 of 26 isolates identified by one or both methods (conventional and 16S rRNA) ultimately identified *S. algae*. It is yet unknown what

disease *S. putrefaciens* sensu stricto truly causes or how frequently it is recovered from clinical samples compared to the more prevalent *S. algae* based on these cumulative data and other investigations. Recent molecular studies indicate that the number of *Shewanella* species that cause human sickness is increasing, even though *S. algae* rather than *S. putrefaciens* currently appears to be the leading human pathogen in the genus. Using 16S rRNA gene sequencing, it was determined that a presumed case of *Vibrio vulnificus* necrotizing fasciitis and sepsis in Japan was really caused by *Shewanella haliotis* (Tadera, K *et al.*, 2010).

Recent reports of a second *S. haliotis* infection in a 52-year-old Thai woman who had handled fresh seafood and developed necrotizing fasciitis and sepsis have been made (Poovorawan, K *et al.*, 2013). Liu and coworkers also noted that *S. haliotis* was the root cause of 5 out of 9 cases (or 56%) of *Shewanella* bacteremia (Liu, P-Y *et al.*, 2013). Three of these five individuals had monomicrobial *S. haliotis* bacteremias and underlying hepatobiliary illness at the time of their presentations. *Shewanella xiamenensis*, a fourth *Shewanella* species, was linked to a health care-associated peripancreatic infection in 2011 where *Shewanellae* were found alongside *Staphylococcus epidermidis* in drainage and surgical samples. Finally, it has been determined that several clinical isolates are members of the *Shewanella oneidensis* species (Zong, Z *et al.*, 2011). Sadly, there was no information on these isolates, therefore the origins and potential clinical implications of the species are still unclear (Venkateswaran K *et al.*, 1999).

1.7 ANTIBIOTIC RESISTANCE IN SHEWANELLA ALGAE

Although *Shewanella* is typically thought of as an environmental genus, numerous human infections have been reported in recent years. *Shewanella* infections are treated with surgery, drainage, and antibiotic therapy, and the most frequently prescribed antibiotics are lactams, aminoglycosides, and quinolones, which are frequently used in antimicrobial chemotherapy. The majority of this genus's members are sensitive to carbapenems, lactamase inhibitor combinations, aminoglycosides, chloramphenicol, erythromycin, aztreonam, and quinolones, as well as third- and fourth-generation cephalosporins (Satomi M *et al.*, 2014; Janda JM, Abbott SL, *et al.*, 2014). *Shewanella* species strains can differ in their susceptibility (Bruun B *et al.*, 2005).

Shewanella algae and the *S. putrefaciens* species that are most frequently linked to infections in humans are typically susceptible to aminoglycosides, carbapenems, erythromycin, and quinolones but resistant to penicillin. Their level of sensitivity to ampicillin and cephalosporins varies. This genus is becoming increasingly resistant to antibiotics. There have been reports of resistance to colistin, a last-resort antibiotic in human medicine. According to reports, *S. algae* isolates were resistant to colistin while *S. putrefaciens* isolates were vulnerable. In addition to strong resistance to heavy metals, (Holt HM *et al.*, 2004) *Shewanella putrefaciens* strains isolated from Korean shellfish also exhibited high resistance to cefalotin, gentamicin, erythromycin, vancomycin, ampicillin, rifampicin, and streptomycin (Kang CH *et al.*, 2016).

1.8 SHEWANELLA AN EMERGING PATHOGEN

According to descriptions, *Shewanella* species are uncommon, opportunistic, or secondary human pathogens acquired in the community that are frequently linked to skin and soft tissue illnesses. Most sickness reports in the literature read like case studies, and *Shewanella* species have not been thoroughly researched as pathogens. Most case studies were published after 2000 due to advancements in clinical diagnostic technology that made it possible to identify the genus (Sáez-Nieto JA *et al.*, 2016). While *S. putrefaciens* and *S. algae* make up more than 85% of *Shewanella* infections known to have occurred, *S. halotis* and *S. xiamenesis* have also been linked to human illness (Tadera K *et al.*, 2014; Tena D *et al.*, 2016).

Additionally, *S. oneidenesis* has been isolated from clinical samples (Poovorawan K *et al.*, 2013), although its function in pathogenesis is yet unclear. When the National Communicable Disease Centre identified an unidentified CD Group Ib strain with oxidase positivity and H₂S production (which is consistent with the phenotypic classification of *P. putrefaciens*) as the cause of 22 different infections, King reported the first case of *Shewanella* infection in humans. The pathogen was not identified as *P. putrefaciens*, as it is now known to be *S. putrefaciens*, until 1970. Soon after, case investigations documenting septicaemia and infected leg ulcers brought on by *P. putrefaciens* (*S. putrefaciens*) were presented. The fact that some *Shewanella* species were potentially harmful to humans was first recognised in

these publications. When *shewanella algae* was identified from two individuals who had bacteraemia connected to lower leg ulcers, it was the first time that *shewanella algae* was described as a human pathogen (Zong Z *et al.*, 2011; Moser DP *et al.*, 1999).

1.9 PREVENTION

Avoiding aquatic conditions with open wounds or trauma to the body, or covering the wound with waterproof bandages before entering the water, are strategies used to avoid infection by *S. algal*. To prevent *S. algae* from entering the gastrointestinal tract, it is recommended that all food (especially seafood) be thoroughly prepared before eating and that significant amounts of water from marine or freshwater environments be avoided. Finally, scrub down any marine-related equipment (e.g., fishing hooks and diving equipment) to ensure that no surviving *S. algae* remain as a reservoir of infection.

CHAPTER - 2

2. LITERATURE SURVEY

1. MORPHOLOGICAL, PHYSIOLOGICAL, AND BIOCHEMICAL CHARACTERISTICS

There is no uniform biochemical or physiological profile in the genus of *Shewanella* (Lemaire *et al.*, 2020). As marine microorganisms, *Shewanella* spp. were originally isolated with marine agar 2216E, forming round, smooth, medium-sized, and raised colonies (Fang *et al.* 2019). On nutrient agar, the colonies are light brown, round and raised with intact margins. Selective media are also used for the initial isolation of *Shewanella* strains, such as thiosulphate citrate bile salts (TCBS) sucrose agar, MacConkey agar, and blood plates. Colonies on TCBS are medium-sized, smooth, raised, rounded, with colourless edges and black centres. On MacConkey agar, 1–2mm yellow-brown colonies can be seen, while on sheep's blood agar, some *Shewanella* species such as *S. algae* and *S. putrefaciens* can produce conspicuous β -haemolysis, and some strains can also form mucoid colonies. As researchers studied that β -haemolysis was detected in all *S. algae* strains but only in a couple of *S. putrefaciens* isolates (Khashe and Janda 1998). Derby and Hammer described the following general biochemical features of *Shewanella* spp.: Gram-negative, indole-negative, and gelatinase-, sucrose-, and maltose-positive (Ha and Bw *et al.*, 1931). This genus is positive for catalase and can reduce nitrate (NO_3^-) to nitrite (NO_2^-). Members of this genus are aerobic or facultative anaerobic, and some species can ferment D-glucose and N-acetylglucosamine to produce acid, usually without gas. Most of the *Shewanella* strains also present H_2S -producing, urea-, arginine hydrolase-, lysine decarboxylase-negative phenotypes. This article summarizes physiological and biochemical properties of seven common *Shewanella* spp. (Holt *et al.*, 2005).

2. VIRULENCE FACTORS OF *SHEWANELLA ALGAE*

Pathogens must overcome many natural host defenses in order to bind to and infiltrate intestinal epithelial cells. Pathogen virulence factors include the ability

of pathogens to infiltrate human intestinal epithelial cells via virulence factors such as flagella, homoserine lactone signal molecules, gelatinases, proteases, and biofilm formation, all of which are required for the first stage of infectious illnesses(Dias *et al.*, 2019) Carla et al. studied *S. putrefaciens* attachment and internalization capabilities in post-confluent human colonic cancer (Caco-2) cells, which shared morphological and functional properties with normal small intestinal cells in vitro. The findings revealed that *S. putrefaciens* could bind to and internalize Caco-2 cells. Flagella, which enhance motility, adhesion, and biofilm formation, are found in species in the genus *Shewanella*. Mannose-sensitive haem agglutination (MSHA) and extracellular DNA (eDNA) both play critical roles in microbial adhesion(Gödeke, Binnenkade, and Thormann 2012)(Thormann *et al.*, 2004). β -haemolysins have been discovered to be involved in haemolytic activity, which is responsible for *S. algae* becoming more virulent (Khashe and Janda 1998). Moreover, *S. putrefaciens* was able to synthesize AHL molecules, which may coordinate the expression of numerous virulence factors, including the formation of biofilms(Dias et al. 2019). Chitinase, lipase, protease, elastin, and alkyl sulphate enzymes generated by some *Shewanella* species may also associate and harm host tissues, causing disruption and depletion of the mucus barrier. The genus *Shewanella* also features O-side chains or capsules with thicknesses ranging from 20 to 130 nm depending on the species (Martín-Rodríguez *et al.*, 2017). The polysaccharide polymers on *Shewanella*'s surface aid in its adherence to solid things and consequently infectivity(Korenevsky *et al.*, 2002). Many virulence-specific genes, including capsular polysaccharide biosynthesis, O antigen, and lasB (vibriolysin related gene), have been discovered through genome scanning(Leangapichart et al. 2021). The genes associated to type IV pili, which are controlled by external stimulation for direct movement, and the gene vasF, which is related to VAS T6SS, shed new light on *S. algae* pathogenicity. Virtually all *S. algae* strains possess the katA gene, whose product can breakdown hydrogen peroxide, protect cells from the harmful effects of hydrogen peroxide, and improve bacterial colonization in hosts. Furthermore, the discovery of genomic islands (GIs) containing a set of virulence genes and mobile elements suggested that GIs could aid in cross-species gene transfer and promote to the independent acquisition of virulence components in *S. algae*(Huang *et al.*, 2022).

3. BIOFILM FORMATION

1. BIOFILM

Biofilms are severe health concerns due to their multidrug resistance abilities, host defense, and other stresses (De La *et al.*, 2013). Therefore, it leads to chronic bacterial infections worldwide (Subhadra *et al.*, 2018). Bacterial biofilm is a multifaceted structure of communities with diverse bacterial colonies of cells in a group (Kostakioti *et al.*, 2013). Biofilm referred to the intricate three-dimensional (3-D) aggregation of bacteria attached to a surface and buried inflexibly in an Extracellular Polymeric Substance (EPS) matrix. Bacteria form biofilms in every substratum, and their associated infections in plants, animals, and humans (Lebeaux *et al.*, 2014). Besides, biofilms also play destructive roles in industrial pipelines corrosion (Lenhart *et al.*, 2014). Bacterial biofilms can attach to various materials such as metals, glass surfaces, plastic wares, tissues, and clinical devices. Bacterial communities also produce biofilm, especially on all medical implants, including vascular grafts, heart valves, intrauterine devices, pacemakers, prosthetic joints, catheters, sutures, and contact lenses to acute infections (Kannappan *et al.*, 2017). Bacteria inside the biofilm can also withstand harsh conditions and hold secreted polymers such as polysaccharides, extracellular DNA (e-DNA), proteins, and amyloidogenic proteins (Sharma *et al.*, 2019). The pathogenesis and persistence of bacterial pathogens are dependent on the formation and maintenance of intact biofilms (Xu *et al.*, 2000). Bacterial cells swathed in the biofilm are up to 1000-fold resistant to antibiotic agents. In this biofilm form, bacteria are more resistant to various antibacterial and chemical treatments. Biofilms offer the guard to the bacteria from pH, nutrients deficiency, and mechanical forces (Bryers *et al.*, 1993). Therefore, the biofilm matrix gives additional resistance to bacteria, leading to bad bug's infections like drug resistant bacteria. Novel biofilm inhibitors have been investigated from a various source in order to prevent biofilm formation and eliminate persistent biofilms. Although the research focused on identifying compounds able to target and inhibit this biofilm mode of bacterial growth is explicitly still inadequate. As a result, new therapeutic options are needed for controlling the biofilm associated infections. At present, the study of biofilm and its

strategies to eliminate without any resistant development is one of the utmost significant fields of research. Several reviews on biofilm inhibitors have already been reported, but our review mainly focuses on significant novel strategies to control biofilm mediated bacterial infections.

2. BIOFILM FORMATION IN BACTERIA

Generally, biofilm formation by bacterial pathogens on any substratum/layer involves five major stages (Kostakioti *et al.*, 2013). Attachment: at an initial stage, free-swimming planktonic cells reversibly attach to the biotic or abiotic surfaces through weak interactions such as acidbase, hydrophobic, Van der Waals, and electrostatic forces. Colonization: bacterial pathogens irreversibly attach to the surface through stronger interactions such as collagen-binding adhesive proteins, lipopolysaccharides, flagella, and pili. Proliferation: the multilayered bacterial cells are profoundly accumulated, and the enormous amounts of EPS are produced. Maturation: the attached multilayered bacterial cells grown into the matured biofilm with the typical 3D biofilm structure. Dispersion: after the complete development of biofilm, it is disassembled or dispersed using mechanical and active processes.

3. CHARACTERISTICS OF BIOFILM FORMATION

The formation of biofilm is a progressive process. Primarily, bacterial cells move onto a surface and adhere reversibly to the surface. In the second step, irreversible adherence occurs with the microcolonies expansion that produces an EPS matrix. Subsequently, the progress of the mature 3-D biofilm architecture emerges. Matured biofilms are more resistant to the host immune defenses and the action of antibacterial agents. During the dispersal of biofilm, the cells endure lysis and discharge from the biofilm community. Inside the host, bacteria produce biofilm on a biotic or an abiotic layer. The abiotic surface is typically coated with proteins or other biological molecules, forming a habituation film that changes cells adhesion. In biofilm formation, host cells can develop a fundamental part, and their components can be assimilated into the biofilm matrix (Lynch *et al.*, 2008).

4. EPS EXTRACTION

1. EPS EXTRACTION FROM *S. ALGAE*

Extracellular polymeric substances (EPS) are a group of macromolecular compounds secreted outside the cell. These molecules create a slimy coating around cells, which acts as a protective barrier against some environmental stresses. EPS synthesis in *microalgae* takes place in the Golgi apparatus (Delattre *et al.*, 2016). Extracellular polymeric substances are characterized by a variety of chemical structures and a homopolymeric or heteropolymeric structure Paniagua- (Michel *et al.*, 2014). Microalgal EPS have a complex structure, which has evolved in response to diverse biotic and abiotic environmental conditions (Gaignard *et al.*, 2019). Depending on their properties and ability to bind to cells, several types of EPS have been reported in the literature. EPS that are weakly bound with cells and are secreted into the medium are referred to as soluble EPS (SL-EPS). In turn, bound EPS represent a type that is bound with cells (Naveed *et al.*, 2019). This fraction is divided into loosely bound EPS (LB-EPS) and tightly bound EPS (TB-EPS). LB-EPS and TB-EPS show a different composition and functionalities. LB-EPS show the highest Pb (II) adsorption abilities in comparison to TB-EPS and SB-EPS (soluble EPS) (Li *et al.*, 2021). The role of LB-EPS seems to be more important in response to adverse environmental changes than the role of other EPS fractions; hence, they serve a protection function in algal metabolism (Pan *et al.*, 2016). Both EPS fractions exert a different effect on the flocculating ability, as described. EPS may be involved in nutrient distribution, as the extracellular matrix contains some metabolites released by algal cells, e.g., nucleic acids, enzymes, lipopolysaccharides, and phospholipids. EPS components (proteins and humic substances) may be involved in redox processes as well. Suggest that proteins may be transferred from the inner layer (TB-EPS) to the outside of the cell (LB-EPS and SB-EPS). They also observed a decrease in protein content in TB-EPS in the death phase, whereas the protein content was comparable during the logarithmic growth phase in all EPS fractions (Wang *et al.*, 2020). However, the terminology of EPS is inconsistent; therefore, other terms for total EPS can be found in the literature, for example, exopolysaccharides, extracellular polysaccharides, extracellular

proteoglycan (EPG), exopolymeric substances, or extracellular polymers. Both fractions exhibit different properties and composition (Pierre *et al.*, 2019). However, claim that there are no significant differences. This review focuses on a released fraction referred to as EPS (Rossi and De *et al.* 2016). EPS are composed primarily of polysaccharides, proteins, lipids, and nucleic acids (Shi *et al.* 2017). Extracellular polymeric substances may occur as polysaccharides (with the predominance of sugar components), proteoglycans (containing proteins in sugar chains), or glycoproteins (with proteins as the major elements of the polymer) or they exhibit a different polymer type (Halaj *et al.*, 2019). EPS have high molecular weight, but there is also a fraction of low molecular weight polymeric substances (Villarcorte *et al.*, 2015). The weight of a polymer extracted from *Chlorella vulgaris* JSC-7 was $9.86 \times 10^3 \text{ g mol}^{-1}$ (Alam *et al.*, 2014). Whereas EPS from *Dictyosphaerium* strains contained a high molecular weight fraction (which was from $1 \times 10^6 \text{ g mol}^{-1}$ to, more than $2 \times 10^6 \text{ g mol}^{-1}$), as well as low molecular weight compounds in the range of 9000–56,000 g mol^{-1} (Halaj *et al.*, 2019). They are mostly negatively charged (hydrophobic) due to the presence of hydrolyzed functional groups. FTIR spectra show the presence of carboxyl, hydroxyl, and amino groups (Halaj *et al.*, 2019). Polysaccharides are one of the main components of EPS, accounting for 45–95% of EPS (Naveed *et al.*, 2019). The sugar units observed in microalgal EPS include glucose, galactose, mannose, arabinose, rhamnose, and fucose, with glucose and galactose as the most frequent

Monosaccharides can be modified by non-sugar residues such as pyruvate, sulfates, acyl groups, phosphates, O-acetyl-, and O-methyl groups (Badireddy *et al.*, 2010). EPS from *Porphyridium* sp. are mainly composed of xylose, glucose, and galactose. Protein accounts for 5.5% of the polymer. The negative charge of EPS is associated with the presence of negatively charged sulfate groups and glucuronic acids (Pignolet *et al.*, 2013). EPS from Chlorophyta is characterized by high contents of galactose (Gaignard *et al.*, 2019). Polysaccharides may form linear or branching structures with regular or irregular morphology related to the presence of various glycosidic bonds (Cybulska *et al.*, 2019). α -(1,3), β -(1,3), β -(1,4), and (2,1)- β are linkages observed in EPS. Most EPS are heteropolymers (Delattre *et al.* 2016). However, homopolysaccharides are observed in some cases, for example, the EPS synthesized by *Dunaliella tertioecelata* are (1→4)- α -D-glucans (Goo *et al.*

2013). Proteins are one of the main components of microalgal EPS. In terms of their functions, there are non-enzymatic structural proteins, which stabilize the EPS matrix (Xiao and Zheng *et al.*, 2016), and enzymatic proteins degrading exopolysaccharides, which can be a source of carbon and energy for cells (Flemming and Wingender *et al.*, 2010). As reported by the content of extracellular total protein per mg L⁻¹ of supernatant in different microalgae (green microalgae, diatoms, and red algae) is in the range from 0.5 mg L⁻¹ to 11 mg L⁻¹; when expressed as weight % of total EPS, it ranges from 0.5% to 16.9%. The analysis of experimental results of the protein content shown in literature reports reveals a large variation among Chlorophyta, which suggests that the protein content in EPS is not a species-specific trait. Analyses of Energies 2021, 14, 4007 4 of 23 the composition of *Chlorella vulgaris* EPS showed protein and carbohydrate contents of 25.6 mg L⁻¹ and 31.1 mg L⁻¹, respectively (Nguyen *et al.*, 2020). In turn, EPS synthesized by *Chlamydomonas reinhardtii*, i.e., another representative of Chlamydomonadales, contained 42.1% of proteins (Zhu *et al.* 2012). Investigations conducted on the effect of MgSO₄ on EPS production by *Heynigia riparia* (Chlorophyta) showed a protein level in EPS in the range from 15.27% to 24.19% (Liu and Miao *et al.* 2019). The EPS of Chlorophyta typically contains glycoproteins (Paulsen *et al.*, 1998).

2.5 FOURIER TRANSFORM INFRARED SPECTROSCOPY (FTIR)

FT-IR spectroscopy is a phenotypic approach used in chemistry to assess the chemical composition of various sample types. It is a quick, non-destructive, simple, low-cost, high-throughput analytical instrument that is based on the differential modes of vibration of different chemical bonds when subjected to an infrared beam (Griffiths and De Haseth, n.d.). It became possible because to the advancement of current interferometers and Fourier transform techniques (increased analysis time, repeatability, and sensitivity), as well as powerful hardware and software analytic tools. These and many subsequent studies paved the way for the use of this methodology for discrimination, classification, and identification of bacteria at various taxonomic levels (genera, species, serotype/serogroup, or even at the strain level), a topic that has been the subject of numerous review papers over the last decade (Wenning and Scherer *et al.*, 2013) (Naumann *et al.*, 2000) (Maquelin *et al.* 2002). Over the years, connections between phenotypic (FT-

IR) and genotypic features have been demonstrated blindly using a wide range of genotyping technologies with varying DNA targets and resolution(Rodrigues et al. 2015)(Campos et al. 2018). However, given that (i) many of these techniques do not link genomic evolution with phenotypic behaviour or key surface cell structures, and (ii) many studies have sample limitations, it is possible that some of the associations established are simply coincidences, or that the lack of correlation is not surprising(Beekes, Lasch, and Naumann 2007)(Leclère et al. 2014)(Jaya et al. 2012)(Jin, Zhang, and Martin et al., 2017).

2.6 GAS CHROMATOGRAPHY- MASS SPECTROMETRY

Because of their strong polarity and poor volatility, free FAs appear challenging to analyze by GC/MS without derivatization(Brondz et al., 2002). Derivatization of FAs into more volatile derivatives is required to improve detection sensitivity and separation, especially for samples containing trace quantities of FAs(Eder et al., 1995). The most common derivatives used in GC/MS analysis include methyl esters, pyrrolidide, picoliny esters, 4,4-dimethyloxazoline, and TMS. Without a doubt, the most significant disadvantage of GC/MS analysis is the difficulty of sample extraction involving derivatization. (Eder 1995) examined the methods for determining FAs by derivatization into methyl esters and proposed that sample introduction to GC be carefully planned to minimize discrimination between high-boiling and low-boiling samples. FAMEs also stated that polar stationary phases (e.g., CP Sil-88, SP-2340, and SP-2330) had low thermal stability and long retention time for FAMEs despite excellent isomer separation, whereas non-polar phases (e.g., CP-Sil 5CB, DB-5, and DB-1) had high thermal stability but poor selectivity. As a result, a column with median polarity (OV-225, DB-1701, and DB-225) can be chosen to strike a balance between thermal stability and separation.

AIM AND SCOPE

AIM

The aim of this work is the molecular characterization and analysis of the fatty acid composition of the marine bacterium *Shewanella algae* isolated from aquatic environment.

OBJECTIVES

For the isolation and identification of *Shewanella algae* strains using 16S rRNA gene sequencing. Performing a phylogenetic analysis to determine the relationship between *Shewanella algae* and other related bacterial species. Analysis of the fatty acid composition of *Shewanella algae* strains by gas chromatography-mass spectrometry (GC-MS) and identification of the major fatty acids profile.

SCOPE

The scope of this work includes the isolation and identification of *Shewanella algal* strains from the marine environment, molecular characterization of these strains using 16S rRNA gene sequencing, analysis of fatty acid composition using GC-MS, and potential biological Includes research for technology applications. This study focuses on strains of *Shewanella algae* isolated from the location in Marakkanam (12.1899°N, 79.9249°E), Chennai, Tamil Nadu, India. But can also be compared with strains from other marine environments. This paper does not cover the isolation or characterization of other marine bacterial species, nor the comprehensive characterization of bioactive compounds produced by *Shewanella algae*.

CHAPTER: 4

4. METHODS AND MATERIALS

1. MATERIALS

1. GLASSWARES

Petri plates, Test tubes, Conical flask, Absorbant and Nonabsorbent cotton, Beakers, Micro tips, Whatman Filter Paper, Funnel, Micropipette, Lighter, Spirit Lamp, Marker, Eppendorf tubes, cotton swab.

2. CHEMICALS

TSB agar (Tryptic soy broth + 3% of agar), LB Broth (Luria broth), Nutrient broth, NaCl, Sea water, 70% Ethanol, Distilled water, NaOH, Methanol, PBS Buffer, chloroform, n-hexane methanol Hydrochloric acid, Potassium hydroxide pellets

3. EQUIPMENT

Auto clave, weight balance, Centrifuge, Incubator, Hot air oven, Laminar air flow, Refrigerator, Water bath, Ph Meter, Shaker.

2. SAMPLE COLLECTION AND ISOLATION OF *SHEWANELLA* STRAIN

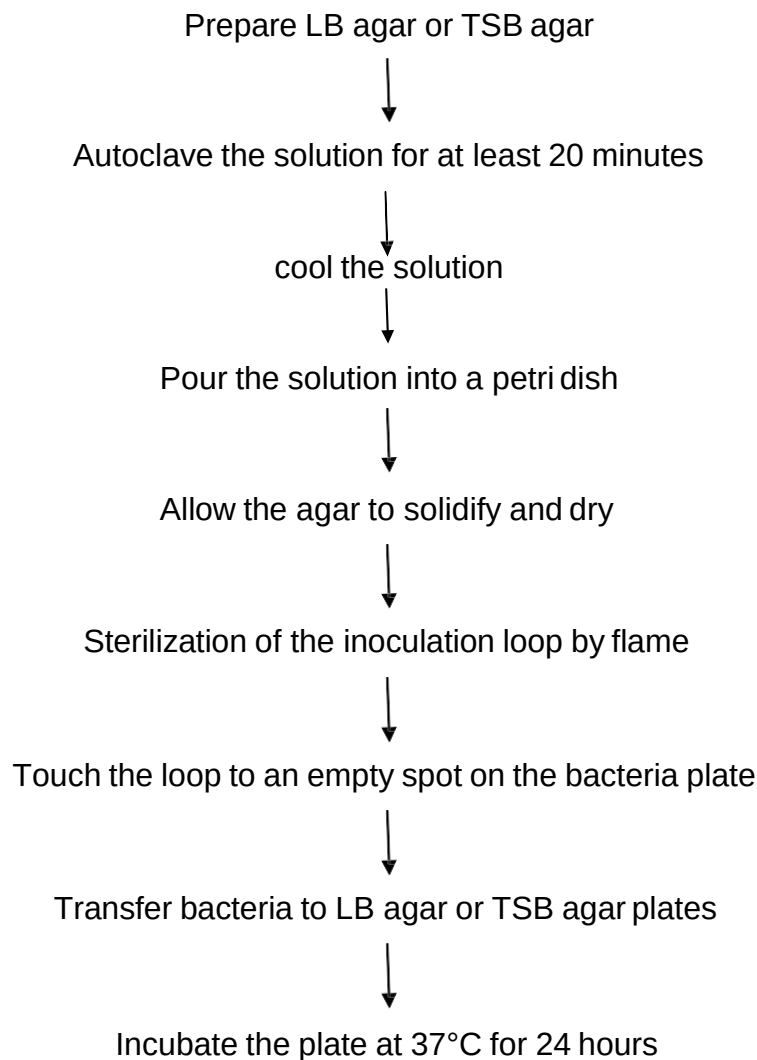
Samples of *Shewanella spp.* were collected from aquaculture ponds located in Marakannam (12.1899°N 79.9249°E), Chennai, Tamil Nadu, India. The tissues of the shrimp were homogenized by using TN buffer (0.02M Tris– HCl, pH 7.2, 0.4M NaCl) and inoculated in thiosulphate citrate bile sucrose (TCBS, Himedia) agar along with 2 % NaCl and incubated for 24h at 37°C for the development of colonies. The pure colonies were recognized by usual biochemical tests (Bauman, 1984) and stored for further experimental studies

3. SUBCULTURING

Bacterial culture on agar plates is a common laboratory technique that involves following a series of steps to prepare a culture medium. First, it is important to note that 500ml of agar solution is required to make one bag of Petri dishes. To make the agar solution, 25 g of LB broth per 1000 mL or 30 g of TSB per 1000 mL were added to an appropriately sized beaker. Then water is added and stirred until

all the lumps are dissolved. Once done, 15 g of agar per liter is added to the beaker, but it will not dissolve. The mixture is then transferred to bottles and autoclaved for at least 20 minutes in the liquid cycle. After the liquid is autoclaved, it must be cooled before pouring it into the dish. The dishes must be tightly covered to avoid the formation of air bubbles during this process. Once the plates have been prepared, it is time to transfer the bacteria to the agar plate. Start by closing the valve at the bottom by twisting the collar, then touch the ring to an empty spot on the bacterial plaque. The bacteria can then be transferred to LB agar or TSB agar plates. Finally, the bacterial plates should be incubated at 37°C for 24 hours for bacterial growth. Following this procedure will provide a reliable method for culturing bacteria on agar plates.

Flowchart of Culturing Plate:



↓
Observe bacterial growth

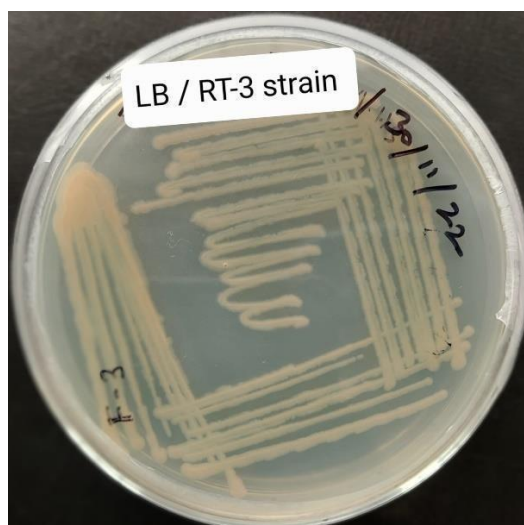


Figure 4.1: Subculturing of shewanella algae strain

4.4 DNA EXTRACTION

In this study, the protocol was used to extract DNA from overnight cell cultures. Biomass was collected by centrifugation at 6000 rpm for 5 minutes at 25° C. and the resulting pellet was mixed with 500 µl of extraction buffer. The mixture was centrifuged again at 7000 rpm for 10 minutes at 25° C. and the supernatant was discarded. Next, 500 µl of extraction buffer was added to the pellet and mixed well. The mixture was then incubated in a 65°C water bath for 1 hour before being centrifuged at 7850 rpm for 10 minutes at 25°C. Then transfer the supernatant to a new tube and add equal volumes of chloroform and ethanol were (24:1).

The mixture was centrifuged at 9500 rpm for 20 minutes at 25° C. and 600 µl of cold 100% ethanol was added to the aqueous phase. The tube was inverted several times and the mixture was kept at 4°C overnight. The resulting pellet was washed with cold 70% ethanol and the procedure were repeated with 90% ethanol. The pellet was dried and resuspended in TE-buffer containing ethylenediaminetetraacetic acid (Tris-EDTA).

DNA Extraction

- Collect biomass by centrifugation at 6000 rpm for 5 mins at 25°C
- Mix the resulting pellet with 500 µl of extraction buffer
- Centrifuge the mixture at 7000 rpm for 10 mins at 25°C and discard the supernatant
- Add 500 µl of extraction buffer into the pellet
- Mix the pellet well and incubate in a 65°C water bath for 1 hour
- Centrifuge the mixture at 7850 rpm for 10 mins at 25°
- Transfer the supernatant to a new tube and add equal volumes of chloroform and ethanol (24:1)
- Centrifuge the mixture at 9500 rpm for 20 mins at 25°C
- Add 600 µl of cold 100% ethanol to the aqueous phase
- Invert the tube several times and keep the mixture at 4°C overnight
- Wash the resulting pellet with cold 70% ethanol
- Repeat the procedure with 90% ethanol
- Dry the pellet and resuspend it in TE buffer containing ethylenediaminetetraacetic acid (Tris-EDTA).

5. BIOFILM FORMATION

The biofilm assay was carried out using the microtiter plate method with slight modifications. Firstly, the bacteria were incubated in a broth in test tubes. Then, 190 µl of medium and 10 µl of the bacterial sample were added to each well of the microtiter plate. The plate was incubated for 48 hours after the incubation. After the incubation period, the test tubes were washed with phosphate-buffered saline (PBS) buffer to remove non-adherent cells on the test tube wall. Then, 200 µl of 5% purple crystal was added to all tubes, and the tubes were incubated for one hour at room temperature. The purple crystal cells were then squeezed again, and washed with PBS buffer (200 µl). Ethanol (200 µl) was added to the tubes, and based on the visible color change, the samples were inserted into a multimode reader to obtain the OD value (500-600 nm).

Bacterial Biofilm Formation:

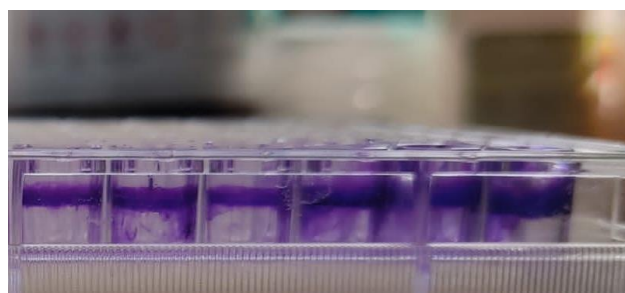
- Incubate bacteria in broth (test tubes)

- Add 190 μ l of medium + 10 μ l of bacterial sample to microtiter plate wells
- Incubate microtiter plate for 48 hours
- Wash test tubes with PBS buffer
- Add 200 μ l of 5% purple crystal to all tube
- Incubate for 1 hour at room temperature
- Squeeze purple crystal cells and wash with PBS buffer
- Add ethanol (200 μ l)
- Based on visible color change, insert samples into the multimode reader
- Measure OD value (500-600 nm).



(a)

Biofilm formation under different glucose concentration



(b)

Biofilm formation under different NaCl concentration

Figure 4.2: Biofilm formation in Shewanella algae

4.6 EPS EXTRACTION

In this study, extracellular macromolecules (EPS) were extracted by the modified ethanol precipitation method. A mature biofilm was grown on glass beads for 48 h at 37°C under static conditions. After 48 h, the consumed medium was discarded and the glass beads were washed with 1% PBS. The biofilm was disrupted by swirling the glass beads, and the disrupted biofilm sample was collected and harvested at 1000 rpm for 10 min at 4°C. To extract the EPS, the ethanol precipitation method was used, with some modifications. EPS is precipitated from the supernatant by mixing it with a pre-cooled volume of ethanol. The mixture was then incubated overnight at 4°C for 20 min. Then, EPS was purified through a 14 Kda cellulose dialysis membrane against deionized water at 4°C for 24 h. The dialyzed EPS solution was lyophilized and used for further characterization studies.

Overall, this protocol consists of several steps, including biofilm growth, EPS breakdown, and extraction, followed by EPS purification and lyophilization. These methods have been used to obtain EPS from mature biofilms grown on glass beads, which can be further characterized and investigated in future research.



Figure 4.3: EPS extract of *Shewanella algae* RT-3 strain

EPS extraction in Bacteria

- Grow mature biofilms on glass beads for 48 h under static conditions at 37°C.
- Discard the used medium and wash the glass beads with 1% PBS.
- Break the biofilm by swirling the glass beads.
- Collect and harvest disrupted biofilm samples at 1000 rpm for 10 min at 4°C.
- Precipitate EPS from the supernatant by mixing it with a pre-cooled volume of ethanol. Incubate the mixture overnight at 4°C for 20 minutes
- Purify EPS via membrane dialysis of 14 Kda cellulose with deionized water at 4 °C for 24h.
- Freeze-dry the dialyzed EPS solution.
- Use lyophilized EPS for further characterization.

4.7 ANTI-BACTERIAL SUSCEPTIBILITY TEST

To perform antibiotic susceptibility testing of a bacterial strain, cultures of this strain are prepared on an appropriate growth medium. Next, a sterile swab was used to transfer the bacterial culture to the Tryptic Soy Broth (TSB) plate. The antibiotic plates were then placed on the plate and incubated at 37°C for 24 h. After incubation, the zone of inhibition around each antibiotic plate was measured. This

zone of inhibition indicates that the area around the disc is not growing, indicating the effectiveness of the antibiotic.

By comparing the size of the zone of inhibition with interpretation criteria for specific antibiotics and bacteria, bacteria can be classified as susceptible, moderate, or resistant to antibiotics. The results are then recorded and can be used to guide antibiotic treatment of the infection. In general, the procedure involves several steps, including preparing the bacterial culture, inoculating the TSB agar plate, placing the antibiotic plate, incubating, measuring the zone of inhibition, interpreting the results, and recording the results.

8. FAME – (FATTY ACID METHYLESTER)

REAGENT PREPARATION

a) Saponification reagent:

NaOH + 15 ml of DH₂O

Add 15 ml of methanol to the above solution

Stir it until the pellets are dissolved in the solution

b) Methylation reagent:

6N of HCl 32.5 ml is added to 27.5 ml of Methanol

Stirred until it is mixed well

c) Extraction solvent:

n- Hexane

d) Washing solution:

30ml H₂O is added to 0.36g of NaOH pellets

Stir well until the pellets are dissolved

PROCEDURE

The following procedure was used to extract fatty acid methyl esters from overnight Quadrant plate cultures. First, the explants were harvested using screw-cap tubes. Next, 1.0 ml of saponification reagent was added to each culture and the mixture was vortexed for 5-10 s before placing in a boiling water bath (95-100°C) for 5 min. The tube was again vortexed and then kept in boiling water for an additional 25 min before being transferred to a cold-water bath for 30 min. Next, 2.0 mL of methylation reagent was added to each tube and shaken for 5–10 s before

placing it in an 80°C water bath for 10 min. The tube is removed from the water tank and placed in the cooling water tank. 1.25 ml of n-hexane was added to each tube and the tube was rotated for 10 min. The top layer of the solution is transferred to a new tube and the above procedure is repeated 3-4 times. Next, 3 ml of wash solution was added to the pooled or collected sample, and the tube was rotated for 5 min. In preparation for GC analysis, a syringe was used to transfer anhydrous sodium sulfate into the syringe. The needle is then withdrawn and the filter is securely attached to the syringe. Pour 3ml of n-hexane extract into the syringe and attach the plunger. The first drops of the filtered extract are removed before gently pressing the plunger to collect approximately 1 ml of the extract into the injection vial. The vial cap is then attached and the fatty acid methyl ester extract is now ready for GC analysis. To analyze the fatty acid composition, the vial was placed in a GC sampler and 1 ml of the extract was injected. GC analysis provides information about the type and number of fatty acids present in a sample, which is useful in many scientific applications.

FAME (Fatty Acids Methyl Esters) analysis:

- Harvest quadrant plate cultures using screw cap tubes.
- Add 1.0 mL of saponification reagent to each tube and vortex for 5–10 seconds.
- Place the tube in a boiling water bath (95-100°C) for 5 minutes, vortex, and place in boiling water for an additional 25 minutes.
- Transfer the tube to a cold-water bath for 30 min.
- Add 2.0 mL of methylation reagent to each tube and shake for 5-10 seconds. Place the tube in a water bath at 80°C for 10 minutes, remove the tube from the water bath, and place it in the cooling water tank.
- Add 1.25 mL of n-hexane to each tube and rotate for 10 min.
- Transfer the top layer of solution to a new tube and repeat the above steps 3-4 times.
- Add 3 mL of wash solution to pooled or collected samples and rotate for 5 min.

- Transfer anhydrous sodium sulfate to the syringe using the filter attached to the syringe.
- Pour 3 mL of the n-hexane extract into the syringe and attach the plunger.
- Collect about 1 mL of the extract in an injection bottle.
- Attach the vial cap and inject 1 mL of the extract into the GC autosampler to analyze the fatty acid composition.
- GC analysis is used to analyze the type and number of fatty acids present in the sample.

4.9 FTIR - (FOURIER TRANSFORM INFRARED SPECTROSCOPE)

To extract EPS, mature biofilms were grown on glass beads (2 mm diameter) under static conditions at 37 °C for 48 h. After 48 hours, spent material was discarded and biofilms were disrupted by vortexing the glass beads. Disrupted biofilm samples were collected and harvested at 7000 rpm for 10 min at 4 °C. To precipitate the EPS, the supernatant was mixed with 3 volumes of prechilled 100% ethanol and incubated overnight at 4 °C. Precipitated EPS was separated by centrifugation at 7000 rpm for 20 min at 4°C and purified through a dialysis membrane (cellulose) with a molecular weight cut-off of 14 kDa against deionized water for 24 hr at 4°C. The dialyzed EPS solution was lyophilized and used for FTIR characterization studies. The phenol-sulfuric acid method was used to measure EPS extracted from ethanol precipitation extraction and established methodology. To investigate the structural properties of pure EPS, FTIR spectroscopy was used in the spectral range of 4000–400 cm⁻¹ to determine the distribution functional group

CHAPTER-5

RESULTS AND DISCUSSION

5.1 ANTI-BACTERIAL SUSCEPTIBILITY TEST

Based on the results of the antibacterial susceptibility test, the *Shewanella algae* RT-3 strain that was isolated showed susceptibility to Amikacin, Ceftriaxone, Chloramphenicol, Doxycycline Hydrochloride, Tetracycline, Gentamicin, and Streptomycin. This means that these antibiotics could be effective in treating infections caused by the *Shewanella algae* RT-3 strain. However, the strain was found to be resistant to Bacitracin, Nystatin, Piperacillin, Kanamycin, Vancomycin, Cefotaxime, and Co-Trimoxazole. This indicates that these antibiotics are unlikely to be effective in treating infections caused by this strain of bacteria. It is important to note that these results are specific to the *Shewanella algae* RT-3 strain that was isolated and tested, and may not be applicable to other strains of the same species or other bacterial species. The results of the antibacterial susceptibility test can provide valuable information for guiding treatment decisions and can also be used for surveillance and monitoring of antibiotic resistance patterns in bacterial populations.

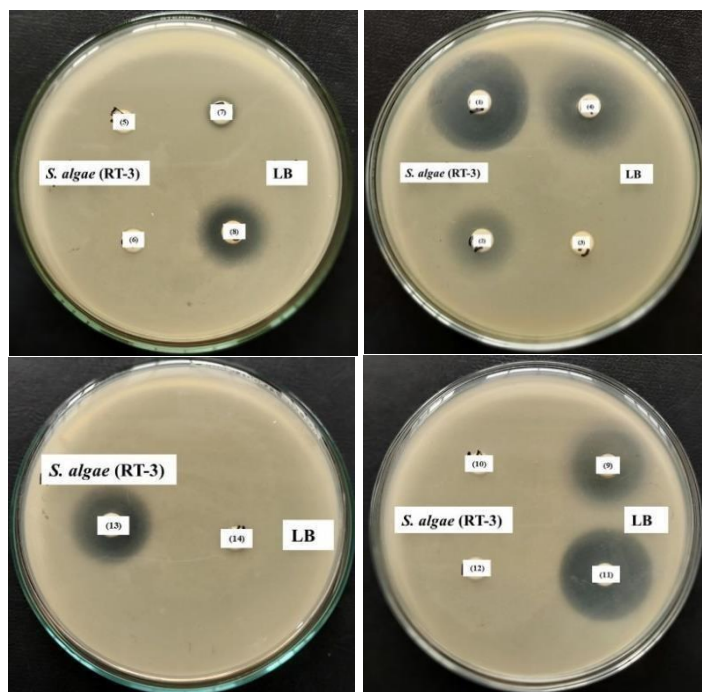


Figure 5.1: Antibiotic or Antibacterial susceptibility test

Table 5.1: Antibacterial susceptibility results of *Shewanella algae* RT-3 strain

Antibiotics	Disc Content	Unit	Sensitivity	Inhibition Zone Diameter (mm)
Amikacin	30	µg/disc	Susceptible	3cm
Ceftriaxone	30	µg/disc	Susceptible	2cm
Bacitracin	10	µg/disc	Resistant	-
Chloramphenicol	30	µg/disc	Susceptible	2.4 cm
Nystatin	100	µg/disc	Resistant	-
Piperacillin	100	µg/disc	Resistant	-
Kanamycin	30	µg/disc	Resistant	-
Doxycycline Hydrochloride	30	µg/disc	Susceptible	2.1cm
Tetracycline	30	µg/disc	Susceptible	2.4cm
Vancomycin	30	µg/disc	Resistant	-
Gentamicin	10	µg/disc	Susceptible	2.7cm
Cefotaxime	30	µg/disc	Resistant	-
Streptomycin	25	µg/disc	Susceptible	2.3cm
Co-Trimoxazole	25	µg/disc	Resistant	-

5.2 BIOFILM FORMATION

Biofilms are complex communities of microorganisms that adhere to surfaces and develop a protective matrix of extracellular polymeric substances (EPS). Studying biofilm bacterial growth at different time intervals is important for understanding the biofilm formation process. Among the methods which use non-vital dyes for biofilm detection, it is the most common for various bacteria including some of the Gram-negative bacteria like *shewanella* and *pseudomonas* (Borucki et al., 2003; Djordjevic et al., 2002; Kaplan et al., 2004; Merritt et al., 2005; Mireles et al., 2001; O'Toole G.A. et al., 2000; Pratt and Kolter, 1998; Stepanović et al., 2000; Watnick and Kolter, 1999). Although common, the main drawback of crystal violet assay is the fact that only the biofilm biomass can be measured which is less informative in comparison to viability (Pan et al., 2010; It was shown that subinhibitory concentrations of antibiotics are able to induce biofilm formation (Hoffman et al., 2005; Kaplan, 2011; Kaplan et al., 2012).

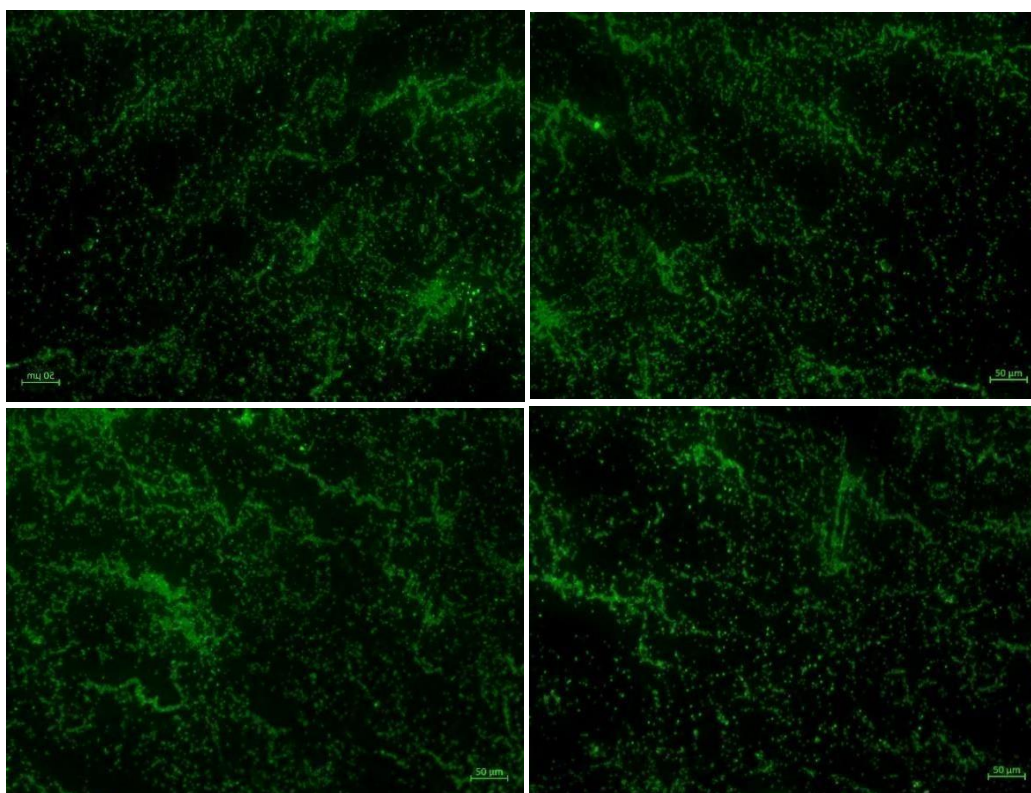


Figure 5.2: Biofilm formation of *Shewanella* at different time intervals

5.3 FTIR ANALYSIS OF THE EPS EXTRACT

In this study, Fourier Transform Infrared (FTIR) spectroscopy was used to analyze the EPS extract from *Shewanella algae*. The FTIR spectrum showed eight prominent peaks, which were assigned to specific functional groups. The first peak at 847.96936 cm^{-1} corresponded to an alkene, the second peak at 989.60820 cm^{-1} corresponded to an alkane, and the third peak at 1079.06430 cm^{-1} corresponded to an alkyne. The fourth peak at 1233.74882 cm^{-1} corresponded to an amine, the fifth peak at 1543.11786 cm^{-1} corresponded to an alcohol, and the sixth peak at 1656.80167 cm^{-1} corresponded to a carboxylic acid.

The seventh peak at 2983.73394 cm^{-1} corresponded to a ketone, and the eighth peak at 3216.69255 cm^{-1} corresponded to nitro compounds. These functional groups are important in biogeochemical processes, and their identification provides insight into the biochemical composition of the EPS extract from *Shewanella algae*. Overall, the FTIR analysis of the EPS extract from *Shewanella algae* revealed the presence of several important functional groups that may play a role in the microbial ecology and biogeochemistry of aquatic ecosystems.

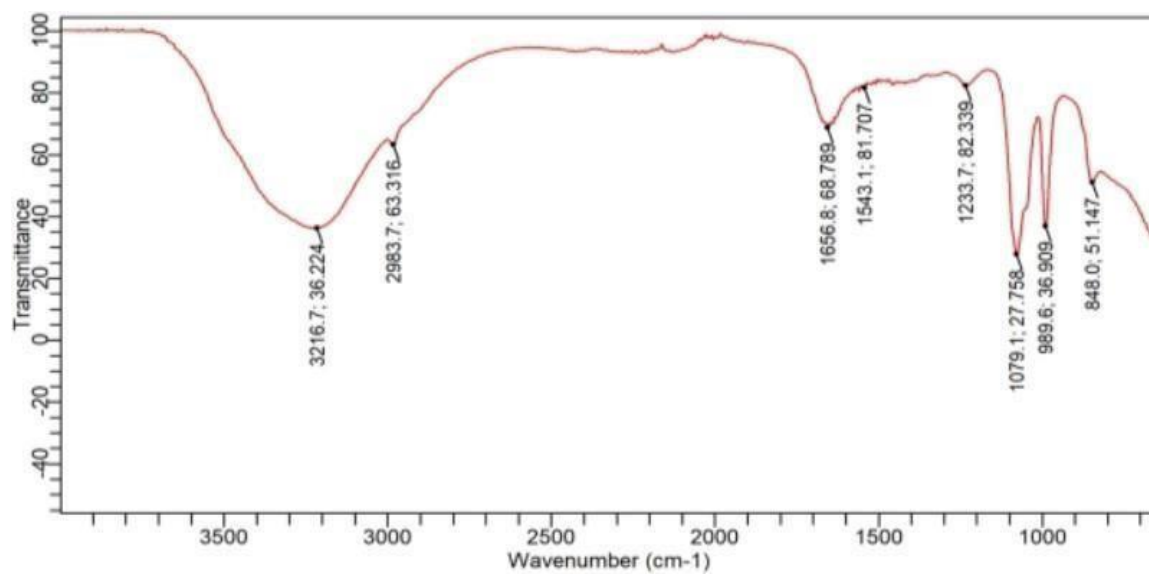


Figure 5.3: FTIR spectrum of *Shewanella* algae RT-3 strain

Table 5.2: FTIR results of *Shewanella algae* RT-3 strain

Peak number	Wavenumber (cm ⁻¹)	Intensity	Functional groups
1	847.96936	51.14684	Alkene
2	989.60820	36.90935	Alkane
3	1079.06430	27.75777	Alkyne
4	1233.74882	82.33890	Amine
5	1543.11786	81.70718	Alcohol
6	1656.80167	68.78919	Carboxylic acid
7	2983.73394	63.31638	Ketone
8	3216.69255	36.22385	Nitro compounds

5.4 FAME ANALYSIS

The GC-MS analysis of *Shewanella algae* indicated the presence of 21 compounds respectively. The name of the compound with their retention time, and abundance (peak area in %) are presented in Table 5.3 respectively. The major compounds in *Shewanella algae* were 3-benzylhexahydropyrrolo[1,2-A]pyrazine-1,4-dione-(17.99%), formic acid, (2-fluoro-5-nitrophenyl)methyl-(14.61%), bis(2-ethylhexyl)phthalate-(10.06%), cyclo(L-prolyl-L-valine)-(8.44%), pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-(6.72%), cyclo-(l-leucyl-l-tyrosyl)-(5.85%), perhydro-hlx-8-one,2-depenty-(3.87%), 4-methoxy PV8-(3.48%), 1-heneicosanol-(3.45%), 2,5-piperazinedione,3-benzyl-6-isopropyl-(2.27%).

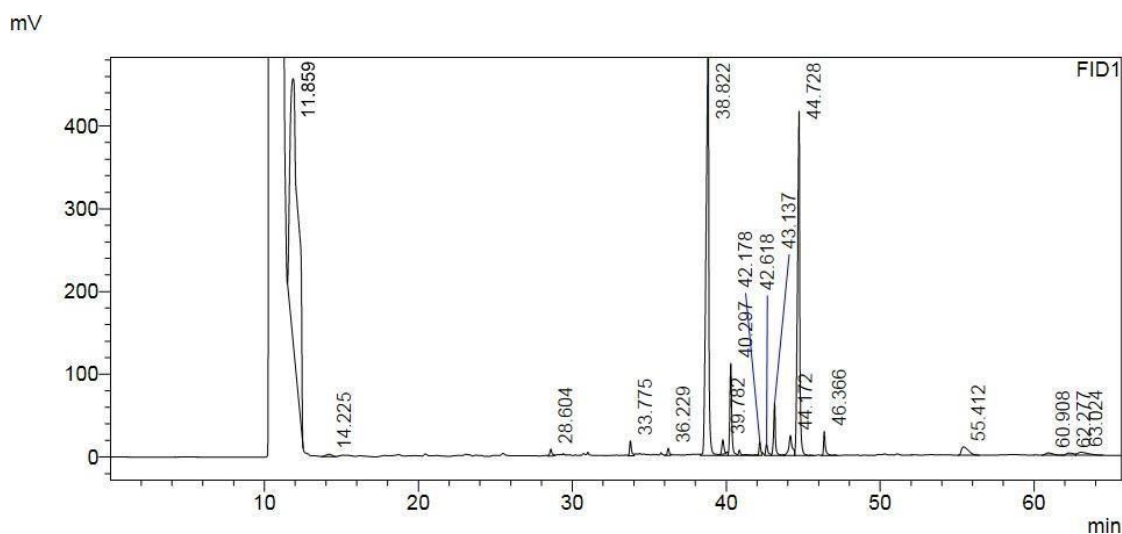


Figure 5.4: GC-MS chromatogram of *Shewanella algae* RT-3

Table 5.3: FAME results of *Shewanella algae* RT-3 strain

Peak	Ret. Time	Area	Height	Concentration unit	Mark	Name
1.	11.859	12399404	318132	0.000%		Butyric Acid Methyl Ester (C4:0)
2.	14.225	73913	2750	0.000%		Caproic Acid Methyl Ester (C6:0)
3.	28.604	82258	8217	0.000%	V	Lauric Acid Methyl Ester (C12:0)
4.	33.775	147704	17880	0.000%	V	Myristic Acid Methyl Ester (C14:0)
5.	36.229	77744	8811	0.000%	V	Pentadecanoic Acid Methyl Ester (C15:0)
6.	38.822	6348301	510847	0.000%	SV	Palmitic Acid Methyl Ester (C16:0)
7.	39.782	163144	17479	0.000%	T	Palmitoleic Acid Methyl Ester (C16:1)
8.	40.297	1019971	111574	0.000%	SV	Palmitoleic Acid Methyl Ester (C16:1)
9.	42.178	118574	16785	0.000%	V	cis-10-Heptadecenoic Acid Methyl Ester (C17:1)
10.	42.618	136870	13949	0.000%	V	cis-10-Heptadecenoic Acid Methyl Ester (C17:1)

11.	43.137	517524	63813	0.000%	SV	Stearic Acid Methyl Ester (C18:0)
12.	44.172	369026	24249	0.000%	V	Elaidic Acid Methyl Ester (C18:1n9t)
13.	44.728	4275423	416122	0.000%	SV	Oleic Acid Methyl Ester (C18:1n9c)
14.	46.366	240121	29447	0.000%	SV	Linoleic Acid Methyl Ester (C18:2n6c)
15.	55.412	311143	9916	0.000%		cis-5,8,11,14,17-Eicosapentaenoic Acid Met
16.	60.908	86908	2539	0.000%		cis-4,7,10,13,16,19-Docosahexaenoic Acid M
17.	62.277	86555	2670	0.000%		cis-4,7,10,13,16,19-Docosahexaenoic Acid
18.	63.024	175216	3818	0.000%	SV	cis-4,7,10,13,16,19-Docosahexaenoic Acid
Total		26629796	1578998			

CHAPTER- 6

SUMMARY&CONCLUSION

This study focused on the molecular characteristics and the fatty acid profiling of *Shewanella algae* RT-3. Molecular characterization and fatty acid profiling are important methods for understanding the metabolic and functional capabilities of microorganisms such as the marine bacteria *Shewanella algae*. Through molecular analysis, the researchers identified the species as *S. algae*. The study also revealed the presence of Antibiotic resistance and observed the Biofilm formation.

Isolation of EPS (Ex polymeric substances) and In addition, Fourier transform infrared (FTIR) spectroscopy was used to examine the functional composition of the representative bacterial strain and the intensities of various peaks from FTIR give us additional information related to the bacteria concentration. A novel and quick DNA extraction method from the *Shewanella algae* was reported. In addition, fatty acid profiling revealed that the bacterial strain was rich in unsaturated fatty acids, particularly monounsaturated fatty acids, which are important for maintaining membrane fluidity and functionality. Overall, the study highlights the potential of *S. algae* as a valuable resource for biotechnological applications and underscores the importance of molecular and biochemical characterization for understanding the metabolic capabilities of microorganisms.

CHAPTER- 7

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