"Microbial Marvels: Understanding Thraustochytrids Biofilm Formation"

A Dissertation for

MMI-651 Discipline Specific Dissertation

Credits: 16

Submitted in partial fulfilment of Master's Degree

M.Sc. in Marine Microbiology

by

NANDITA NAVNATH NAIK

Seat Number: 22P0390008

ABC ID: 289149232191

PRN: 201905763

Under the Supervision of

DR. VARADA SAMIR DAMARE

School of Earth, Ocean and Atmospheric Sciences

Master's in Marine Microbiology

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Seal of the School

Examined by:

DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation report entitled, **"Microbial Marvels: Understanding Thraustochytrids Biofilm Formation"** is based on the results of investigations carried out by me in the Marine Microbiology at the School of Earth, Ocean and Atmospheric Sciences, Goa University under the Supervision of Dr. Varada Samir Damare and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will be not be responsible for the correctness of observations / experimental or other findings given the dissertation.

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Naik

Nandita Navnath Naik

Seat no.: 22P0390008

Date: 02/05/2024

Place: Goa University

COMPLETION CERTIFICATE

This is to certify that the dissertation report **"Microbial Marvels: Understanding Thraustochytrids Biofilm Formation"** is a bonafide work carried out by Miss. Nandita Navnath Naik under my supervision in partial fulfilment of the requirements for the award of the degree of Masters in the Discipline Marine Microbiology at the School of Earth, Ocean and Atmospheric Sciences, Goa University.

Dr. Varada Samir Damare

Date: 02/05/2024

Sr. Prof. Sanjeev C. Ghadi

School of Earth, Ocean and Atmospheric Sciences

Date:

Place: Goa University

SEOAS, GOA UNIVERSITY TALEIGAO PLATEAU GOA

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PREFACE

The research was carried out for the dissertation topic **"Microbial Marvels: Understanding Thraustochytrids Biofilm Formation"**, to find the capability of thraustochytrid isolates for the production of biofilm.

An aggregate of surface-associated microbial cells encased in an extracellular polymeric substance (EPS) matrix is known as a biofilm. As soon as a clean surface is submerged in the sea, microorganisms that create biofilms will rapidly colonize it. The study was carried out to check the ability of thraustochytrid isolates to form the biofilm on different materials like glass, metal and plastic.

Plastics are pliable materials that are mostly made of long polymer chains that have excellent mechanical and chemical durability. Microorganisms settle on plastic's surface and create a biofilm in order to biodegrade it. This study also deals with the ability of thraustochytrids to degrade plastic in water.

The ways by which thraustochytrids create biofilms on various materials, such as glass, metal, and plastic, as well as the consequences of this biofilm formation on the biodegradation of plastics in water, is discussed in this study.

ACKNOWLEDGMENTS

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Naik

NANDITA · N · NAIK

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ABBREVIATIONS USED

Entity	Abbreviations
Atomic Force Microscopy	AFM
Acridine Orange Direct Counts	AODC
Bushnell Hass Broth	BHB
Carbon-dioxide	CO2
Crystal Violet Assay	CVS
Deoxyribonucleic Acid	DNA
Dissolved Organic Carbon	DOC
Dissolved Organic Matter	DOM
Extracellular Polysaccharides	EPS
Fourier Transform Infra-Red	FTIR
Gel Permeation Chromatography	GPC
Low Density Polyethylene	LDPE
Microplastic	MP
Modified Vishniac	MV
Polyethylene	PE
Particulate Organic Carbon	POC
Particulate Organic Matter	POM
Polystyrene	PS
Room Temperature	RT
Scanning Electron Microscopy	SEM
Transparent Exopolymeric Particles	TEP
Ultra Violet	UV
X-ray Photoelectron Spectroscopy	XPS

ABSTRACT

Thraustochytrids, a type of fungoid protist, with a diameter ranging from 3.5 to 20 µm, is bigger than that of bacterioplankton. Marine biofilms form on naturally occurring surfaces such as those of animals, plants, zooplankton, phytoplankton. Sessile bacteria, microalgae (including diatoms), tiny fungus, heterotrophic flagellates, and sessile ciliates (heterotrophic protists) are the main groups of organisms that create biofilms. There is a lack of depth in our understanding of thraustochytrids and biofilms. The purpose of this work was to comprehend the dynamics of thraustochytrid biofilm development. According to the current investigation, thraustochytrid isolates formed biofilms on plastic, metal, and glass. Various thraustochytrid isolates showed peak of biofilm formation at differing times. They create TEPs, POM during their normal metabolic processes, possess EN elements, and have a propensity to produce EPS and form a biofilm. When organic media (Modified Vishniac broth) and seawater were compared for their ability to aid thraustochytrids in biofilm formation, the former produced higher biomass as well as biofilm than the latter. Thus, the isolates OGS-2 and DB Sarg produced higher biomass and biofilm in MV broth than that in seawater. When thraustochytrids were inoculated in seawater and mineral medium containing plastic pieces, there was increase in the weight of the plastic pieces on 15 days of incubation, supporting the hypothesis of biofilm formation and decrease in weight after 60 days, indicating degradation of plastic. It is still unclear how plastic and thraustochytrids interact.

Keywords:

Biofilm; Thraustochytrids; Plastic degradation

CHAPTER 1: INTRODUCTION

1.1 Background

Thraustochytrids are unicellular eukaryotic organisms belonging to the Kingdom Stramenopila (Damare, 2019). They are obligately marine and estuarine-dwelling protists commonly found on algal surfaces (Naganuma et al., 2006). Thraustochytrids are isolated from surfaces of macroalgae and also from microalgae (Damare et al., 2021). The potential habitats of thraustochytrids in the water column are the Transparent Exopolymeric Particles (TEPs) (Damare & Raghukumar, 2008), marine snow (Lyons et al. 2005, Damare & Raghukumar 2010), salp pellets (Raghukumar et al., 1999). The fungoid protists, thraustochytrids, size range largely between 3.5 and 20 µm in diameter and are larger than bacterioplankton (<1 µm). Thraustochytrids produce polyunsaturated fatty acids such as docosapentaenoic acid and docosahexaenoic acid which may be important nutritionally for the growth of fish larvae (Kimura et al., 2001). They are likely to play a role in remineralization of particulate and dissolved organic matter, as bacteria do as they are similar to bacteria in their osmoheterotrophic mode of nutrition (Damare & Raghukumar, 2008). Thraustochytrids produce extracellular enzymes capable of breaking down several complex organic substrates (Raghukumar et al., 2001). Relation of thraustochytrids with particulate organic matter in the sea suggests that marine aggregates could be one of the potential habitats of thraustochytrids in the water column (Damare et al., 2012). In the biological role, it is reported as a decomposer of mangrove litter, crucial role in nutrient recycling of marine ecosystem, marine microbial films, and marine animals. Thraustochytrids are associated with Transparent Exopolymeric Particles (TEPs), Particulate Organic Carbon (POC) and Particulate Organic Matter (POM) (Kimura et al., 2001) which forms a Biofilm. They also produce EN elements

(Nagano et al., 2013) and they have the tendency to produce EPS (Exopolysaccharides) (Jain et al., 2005).

1.1.1 What is a Biofilm?

Surface-attached microbial agglomerations were for the first time named as a "biofilm" by William J. Costerton in 1978 (Relucenti et al., 2021). Biofilm is a sticky, firm structure formed due to communal interaction of bacteria attached to substrate surface and submerged into extracellular slimy conglomerations. The process of attachment of biofilm to surfaces, a sequential process, where bacteria firstly are transported to living or non-living surfaces and then adhere to it and formed microcolonies which then mature into aggregate in a hydrated polymeric matrix called Biofilms (Dawande et al., 2019). Marine biofilms develop on natural surfaces, including animals, plants, zooplankton, phytoplankton, micro-aggregates and macro-aggregates, and transparent exopolymer particles (Qian et al., 2022). Diverse aquatic microorganisms are capable of colonizing surfaces of various kinds, leading to the formation of biofilms and to the development of specialized processes (Dang et al., 2016). In the marine environment biofilm formation play an important role in microbial selection of the optimal habitat. Depending on the physical state in the seawater (gel, colloidal, or particulate form) POC/aggregate/marine snow can serve as a surface to which microorganisms attach (Decho et al., 2017; Damare et al., 2012). Biofilms provide protection to under-lying cells against UV radiation by restricting penetration of the radiation through the biofilm matrix, and also against other environmental stresses, such as desiccation, temperature and pH changes, competition and predation, and depleted nutrient conditions (De Carvalho, 2018).

Extracellular Polysaccharides (EPSs) are significant component of biogeochemical processes and serve a major role towards formation of microbial biofilms and aggregates

(Liu et al., 2014). Several aquatic organisms release copious amounts of dissolved organic carbon in the form of extracellular polymeric substances (Damare et al., 2012). EPS play a major role in biofilm formation and biofouling and in the localization of micro-bio-geochemical processes within aggregates and sediments (Jain et al., 2005). The EPS matrix is responsible for the integrity of the three-dimensional structure of biofilms, gluing cells together and onto surfaces. The EPS also provides protection for the microbial community from adverse environmental conditions (Liu et al.,2016). The EPS matrix surrounding the attached cells provides an effective barrier that restricts penetration of chemically reactive biocides inside the biofilm (Czaczyk et al., 2007).

Transparent Exopolymeric Particles (TEPs) are fibrillar mucopolysaccharides formed through coagulation of the increasingly refractory dissolved organic matter left behind after the action of heterotrophic bacterial processes on the biologically labile organic carbon of dissolved polysaccharide exudates released by phytoplankton and bacteria (Damare et al., 2008). TEPs are the most abundant EPS in the ocean, and are mainly formed by coagulation of dissolved polysaccharides excreted by phytoplankton and bacteria (Wurl et al., 2016). TEPs exist as discrete particles rather than as dissolved substances, capsules or surface coatings and their role in aquatic systems differs from the non-particulate forms of exopolymeric substances (EPS) because, as independent particles, they impact aggregation dynamics (Passow, 2002). They are small organic particles (less than a few hundred μm) that are visible under a light microscope only by staining with an acidic polysaccharide-specific dye, such as alcian blue, or are otherwise transparent and invisible by light microscopy. They are abundant in marine waters and formed by extracellular biopolymeric substances exuded by phytoplankton and bacteria (Dang et al., 2016).

POC (particulate organic carbon) The organic carbon in particulate form that is large enough to be retained on a filter (typically with a filter with a pore size of 0.7, 0.45, or 0.22 μm). DOC (dissolved organic carbon) the organic carbon remaining in the filtrate after the sample is filtered (typically with a filter with a pore size of 0.7, 0.45, or 0.22 μm). DOM is the dominant form of carbon in the oceans that can originate from any number of sources, much of which is produced in situ by marine microorganisms (largely eukaryotic phytoplankton and bacteria) and is derived from terrestrial inputs via transportation from river effluents and surface runoff (Decho et al., 2017). Marine snow mostly biogenic particles with a diameter of >0.5 mm. These organic particles are usually formed in the euphotic zone of the ocean and sink at high rates to serve as the principal means by which organic carbon is transported to the deep ocean and sediments (Dang et al., 2016).

1.1.2 Why is it important to study biofilm?

Biofilms are common in nature and consists of aggregates of bacteria, encased in mucoid polysaccharide structure, often growing as populations attached to surfaces and appear in our everyday life in more than one form. Bacteria have mechanisms by which they can adhere to surfaces and to each other (Choudhury et al., 2013). Biofilm formation occurs step by step, such as formation of primary bacterial adhesion, intracellular aggregation, biofilm maturation and biofilm dispersal.

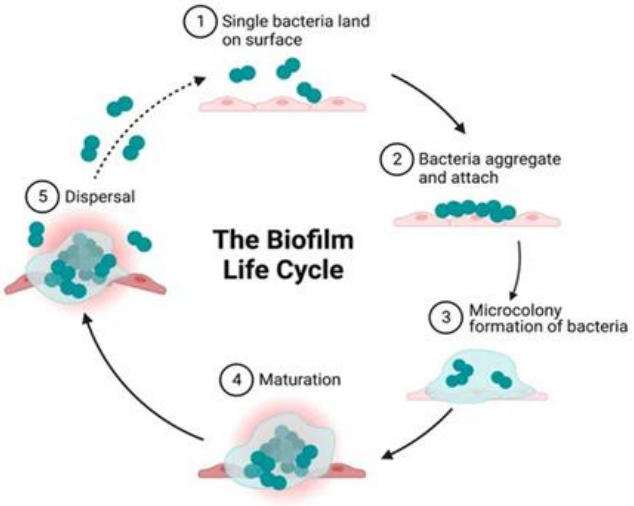


Fig 1.1 Biofilm formation (Adapted from 'Microbial biofilm: a review on formation, infection, antibiotic resistance, control measures, and innovative treatment' by S. Sharma et al., 2023, Microorganisms, 11(6): 1614, Copyright: Creative Common Licenses)

Biofilm can exist on all types of surfaces such as plastic, metal, glass, soil particles, wood, medical implant materials, tissue and food products. Biofilms have a high level of organization and they exist in single or multiple species communities and form a single layer or 3-dimensional structure (Chandki et al., 2011). Biofilm formation represents a protected mode of growth that not only allows cells to survive in hostile environments but also to colonize new niches by dispersal of microorganisms from the microbial clusters (Hall-Stoodley et al., 2004). Marine biofilms easily colonize man-made surfaces, accelerating corrosion, biofouling and may even influence the buoyance of polyethylene plastic. Together with diatoms and other microorganisms, bacteria are responsible for microfouling, allowing the adhesion of larger organisms such as algae, mussels and barnacles which cause macrofouling (De Carvalho, 2018).

From previous studies it was known that bacteria are able to produce biofilm on different materials and this biofilm are sometimes beneficial and sometimes harmful (Choudhury et al., 2013). Biofilms are of industrial and ecological significance and can be used for water treatment, in that they can break down undesirable compounds, thereby purifying the water. Many sewage treatment plants include a treatment stage, in which waste water

passes over biofilm grown on filters, which extract and digest harmful organic compounds (Choudhury et al., 2013). Ability of bacterial biofilms in degrading the industrial contaminants of chemical origin which are considered to be recalcitrant by using them as carbon source. Microbial biofilms play a vital role in breaking down the unwanted debris formed from the dead fish and aquatic plants and absorb the heavy ions from the water without depleting the oxygen content and in this manner, it contributed positively towards the ecological balance (Vasudevan et al., 2014). Biofilms provide protection against UV radiation by restricting penetration of the radiation through the biofilm matrix. This protection also provided safety from the extreme and fluctuating temperature, pH and UV radiation of the primitive Earth (De Carvalho, 2018).

The physical presence of biofilm either damages surface or causes obstruction so that the efficiency of the surface is reduced. This kind of surface damage is collectively termed as “biofouling”. It causes corrosion or deterioration of the interior of metal pipelines, storage tanks or vessels, pharmaceutical and medical products, equipment failure, energy loss through inefficient energy transfer and decreased productivity. The biofilms on floors and counters can make sanitation difficult in food preparation areas. Microorganisms like bacteria or algae can form a microfilm on the hull of a ship. This biofilm can then serve as an attractive substrate for the attachment of macro-organisms like seaweed or barnacles. This macro-coating fouls the hull, and can retard the efficiency of the vessel (Choudhury et al., 2013). Biofilm formation poses a significant problem to the drinking water industry as a potential source of bacterial contamination, including pathogens, and, in many cases, also affecting the taste and odour of drinking water and promoting the corrosion of pipes (Liu et al., 2016).

There is no depth of knowledge with respect to biofilms and bacteria. However, there remains a big void in research on biofilms by thraustochytrids in the marine environment

though thraustochytrids are an important part of marine food-web and ecosystems. This study was therefore done to understand the dynamics of biofilm formation by thraustochytrids. As thraustochytrids are found to be associated with TEPs, POC and POM (Kimura et al., 2001), do they survive in nature as free-living form or always as associated form? Production of EN elements for attachment to substrates, and exopolysaccharides (EPS) may bestow them with the advantage of forming a biofilm on any surface (Nagano et al., 2013; Jain et al., 2005). If these organisms can successfully form biofilm on any surface including plastic, then these may prove biotechnologically important. A biofilm on plastic may help in the degradation of plastic as biofilm formation is a prerequisite for plastic degradation. It is not known if thraustochytrids can degrade plastics. Due to all these constraints the present study was carried out with the following aim and objectives.

1.2 Aim and Objectives

Aim:

Characterize the ability of thraustochytrids to form biofilms on different materials including natural substrates (e.g., marine debris, sediments) and synthetic surfaces (e.g., plastics, glass) and assess the effectiveness of these biofilms in degrading plastic materials through morphological analyses. With this aim in mind the following objectives were carried out:

Objectives:

1) To check the potential of Thraustochytrids isolated from different marine habitats for producing biofilm.
2) To see if biofilm production can occur on different materials such as plastic, metal.
3) To assess the dynamics of biofilm formation by Thraustochytrids along with bacteria.

4) To evaluate if thraustochytrids can degrade plastic.

1.3 Hypotheses

1) Irrespective of the source of isolation (free-living/associated) all thraustochytrid isolates should produce biofilm.
2) Thraustochytrids should produce biofilm on different types of materials.
3) Thraustochytrid isolates should have the potential to degrade plastic material present in water.

1.4 Scope

- Understanding the influence of environmental factors such as temperature, pH, salinity and nutrient availability on thraustochytrid biofilm formation in aquatic ecosystems.
- Investigating the interactions between thraustochytrids and other microbial species within aquatic biofilms, including symbiotic relationships, competition for resources and biofilm mediated transformations.
- Evaluating the potential applications of thraustochytrid biofilms in bioremediation, biotechnology, aquaculture and environmental monitoring and developing innovative strategies for their utilization in water management and conservation efforts.
- Understanding the process of biofilm formation by thraustochytrids on plastic surfaces, including the initial attachment, colonization and maturation stages and how these processes are influenced by environmental factors such as temperature, nutrient availability and water chemistry.
- Elucidating the mechanism by which thraustochytrid biofilms degrade plastic materials, including enzymatic degradation, physical fragmentation and chemical modification and identifying the key enzymes and metabolic pathways involved in plastic biodegradation.

CHAPTER 2: LITERATURE REVIEW

2. Literature review:

2.1 Introduction:

A biofilm is an agglomeration of surface-associated microbial cells that is enclosed in an extracellular polymeric substance (EPS) matrix (Donlan, 2002). The term "biofilm" comprises of both, organisms growing attached to a surface and organisms which grow as aggregates, where the cells are sticked together by EPS (Moreno et al., 2021). Biofilms are primarily composed of microbial cells and EPS. EPS may account for 50% to 90% of the total organic carbon of biofilms and can be considered as the primary matrix material of the biofilm (Donlan, 2002). Biofilm formation is a natural process in which no chemicals are required (Wang et al., 2022). The moment a clean surface is submerged in the sea, biofilm forming micro-organisms will quickly colonize it and eventually form highly complex, dynamic three-dimensional (3D) surface structures (Salta et al., 2013). Biofilms have been referred to by several terms, including periphyton and mycrophytobenthos. In 1924, Behnin proposed the term periphyton and was used to describe organisms growing attached to artificial surfaces in water. Periphyton is defined as a complex community mainly composed of heterotrophic bacteria, photoautotrophic algae, fungi, metazoans, protozoans, and viruses, and inorganic and organic detritus, which is attached to inorganic or organic benthic substrates (De Carvalho et al., 2018). Biofilms usually start with the adhesion of bacterial cells which modify the surface physicochemical properties, thus influencing the adhesion of successive colonizers such as algae, cyanobacteria, and protists (De Carvalho et al., 2018). Major advantage is the protection that biofilm provides to the colonizing species from competing micro-organisms, environmental factors such as host defence mechanisms and potentially toxic

substances like lethal chemicals or antibiotics (Chandki et al., 2011). Surfaces of microplastics (MPs) act as substrata, as well as a carbon source including the easily bioavailable dissolved organic carbon (DOC), which promote the formation of microbial biofilms (Sooriyakumar et al., 2022). Man-made structures as well as natural surfaces (both inanimate and living) are affected by biofilm attachment and growth (Salta et al., 2013).

2.2 History of Biofilms:

Biofilms are nothing new. "The term 'Biofilm' was coined by Bill Costerton in 1978" (Chandki et al., 2011). In 2002, Donlan and Costerton stated that biofilm is "a microbially derived sessile community identified by cells that are irreversibly attached to a substratum or combine or to each other, embedded in a matrix of EPS that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription (Socransky et al., 2002)." A biofilm is a layer of biological material that consist of a mixture of bacteria, fungi and protozoa which are naturally present in the environment (Farkas et al., 2012). Biofilms comprise of a single microbial species as well as multiple microbial species and can form on a range of biotic and abiotic surfaces (O'Toole et al., 2000). In the biofilm, microbes are grouped together in a polysaccharide matrix with other organic and inorganic materials (Chandki et al., 2011).

2.3 Biofilm formation by different microorganisms:

Diverse aquatic microorganisms are capable of colonizing surfaces of various kinds, leading to the production of biofilms and the development of specialized processes within these structures (Dang et al., 2016). Biofilm forming organisms are mainly represented by sessile bacteria, microalgae including diatoms, microscopic fungi, heterotrophic flagellates and sessile ciliates (heterotrophic protists) (Salta et al., 2013).

2.3.1 Biofilm formation by bacteria:

Bacteria play a pivotal role in marine environments, including driving biogeochemical cycles and supplying materials and energy to higher trophic levels. Bacterial cells produce an extracellular polymeric substance (EPS) matrix, establishing the formation of a biofilm (De Carvalho et al., 2018). Bacteria have been studied in great detail with relation to the process of microbial film formation and are known to play an important role in this process (Raghukumar et al., 2000). The biofilm matrix and the development of specific microenvironments promote the maintenance of extracellular enzyme structural integrity and activities as well as improved opportunities for physiological homeostasis of the bacteria (Dang et al., 2016).

2.3.2 Biofilm formation by microalgae:

Microalgae are a vast group of oxygen producing photosynthetic organisms that live using heterotrophic, mixotrophic or autotrophic metabolic strategies. Algal biofilms can be established on any surface that receives sufficient light and moisture. Microalgal biofilms, composed of cyanobacteria and/or green microalgae, are ubiquitously distributed in almost all the photic aquatic environments (Moreno et al., 2021). EPS is made from various chemical substances, which help microalgal cells bind to surfaces, thereby forming a diffusion barrier against the environment (Wang et al., 2022). Microalgae biofilms have promising applications on toxicity measurements (biosensors), CO2 capture, as well as polycyclic aromatic hydrocarbons accumulation and degradation (Moreno et al., 2021). Microalgal-substrata biofilms and microalgal-bacterial biofilms can be successfully applied to treat nutrient-rich wastewaters (Wang et al., 2022).

2.3.3 Biofilm formation by Thraustochytrids:

The presence, and often dense populations, of thraustochytrids single-celled microorganisms has been reported from numerous habitats, including living algae, marine detritus, phytoplankton aggregates, water column, invertebrates and numerous other habitats. The mode of reproduction in these protists is by means of motile zoospores (Raghukumar et al., 2000). The thraustochytrids zoospores would settle rapidly on freshly submerged substrata in the sea. Such materials have received a great deal of attention focused on the primary film that forms on them (De Carvalho et al., 2018). In the laboratory, thraustochytrids grew to varying population densities on surfaces of glass, aluminium and fibre glass (Raghukumar et al., 2000).

2.4 Biofilm formation on different materials:

Biofilms are ubiquitous, they form on virtually all surfaces immersed in natural aqueous environments (Chandki et al., 2011). Stainless steel, glass, rubber, and polypropylene (plastic) surfaces can be contaminated either by pathogenic microorganisms or spoilage that, under certain conditions, are deposited, adhered to, and interact with the surface, initiating cellular growth, and therefore leading to biofilm formation (Marques et al., 2007).

2.4.1 Biofilm formation on metal:

Biofilm formation on metals in marine environments is considered distinct from other materials and conditions. Metal substrates in marine environments assess unique challenges that ultimately shape the community and physical structure of biofilms. Biofilm formation on metals is influenced by the structure of metallic materials. It is proposed that unique conditions offered by metals in seawater, such as surface structure

and microstructure, electrochemical properties and chemical composition provide a niche for biofilm development (Tuck et al., 2022).

2.4.2 Biofilm formation on glass:

Raghukumar et al. (1995) isolated thraustochytrids from mangrove leaf detritus that had been in water for 4 d. Raghukumar et al. (2000) study, presented for the first-time information on the rapid settlement of thraustochytrids on marine surfaces and their presence in a few substrata immersed in the sea. The study indicates that thraustochytrid protists can settle on inorganic particulate material in the sea within 24 h. In Kwon et al. (2002) study bacterial cultures were inoculated with glass slides and the number of attached bacteria on a glass slide was enumerated under a light microscope after staining with 0.3% methylene blue.

2.4.3 Biofilm formation on plastic:

Plastic can be used as a surface to determine the biofilm-forming microbial communities by growing microbial films on the surface, a community known as 'plastisphere'. Based on the previous studies, the most common marine plastic associated bacterial communities belonged to Gammaproteobacteria, Alphaproteobacteria and Bacteroidota classes whereas the archaeal community belonged to Euryarchaeota and Crenarchaeota phyla (Kumar et al., 2022). Sooriyakumar et al. (2022) research shows that the preliminary microbial attachment on plastic surfaces and formation of biofilms might occur within a few days. Bacteria's ability to attach to and form a biofilm on plastic could increase the local concentration of the enzyme around the target substrate and maintain the enzymes in this location for longer by trapping them in the biofilm matrix, therefore increasing the overall rate of plastic degradation (Howard et al., 2023). The physicochemical weathering (UV-induced, thermal, etc.), and microbial biofilm

formation are considered to be the two basic processes that can affect the behaviour and circumstance of plastics in the environment (Bhagwat et al., 2021).

2.5 Biodegradation of plastic:

Plastics are flexible materials mainly composed of long polymer chains with superior chemical stability and mechanical properties, which are broadly used in the automotive industry, agriculture, packaging, textiles and construction. Plastics can be divided into degradable and non-degradable ones based on their degradability in natural environments (Yang et al., 2023). Plastic pollution in the ocean has major impacts on both marine and human ecosystems. The accumulation of these plastics can persist for decades in the ocean (Banihashemi et al., 2022). The long-lasting nature of plastics makes them complicated to biodegrade. Several species of fungi and bacteria have been identified that are responsible for degrading plastics in aquatic environments (Sooriyakumar et al., 2022). In order to biodegrade plastic, microorganisms colonize its surface and form a biofilm. The colonizing microorganisms secrete extracellular enzymes that depolymerize the polymer, yielding shorter chains as well as oligomer, dimer and monomers, which are assimilated by microorganisms (Rüthi et al., 2023). Plastic biodegradation is a stepwise process, where the physical breakdown of plastic fragments is followed by the formation of microbial biofilms on the surface of plastic and the involvement of bacterial enzymes in the degradation process. The time-consuming nature of the biodegradation of plastics is the major challenge of investigating plastic biodegradation using traditional microbiological methods, such as substrate depletion or microbial growth (Sooriyakumar et al., 2022). The collection of plastic waste is a major global issue threatening the environment, animals, and human health. Plastic often breaks down into persistent microplastics in the environment, entering into the food chain and posing unknown long term affects to ecosystems and health. The plastic-associated microbiota can undertake a

variety of functions such as plastic degradation, xenobiotic degradation, horizontal gene transfer including transfer of antibiotic resistance or metal resistance, nitrogen fixation, sulfur reduction, quorum sensing, etc. Therefore, biofilms associated with plastic are the key to unlock our understanding of the behaviour and fate of plastics in the environment (Bhagwat et al., 2021). There are various reports available on different types of plastic degradation by microbes are listed in Table 1 (Tania et al., 2023).

Table 2.1: Plastic biodegradation by bacteria (Tania et al., 2023).

Microorganism	Type of plastic tested	Evaluated parameters	References
Pseudomonas citronellolis; *Bacillus flexus*	Polyvinyl chloride	FT-IR spectra, GPC permeation chromatography analysis, weight loss	Giacomucci et al., 2019
Paenibacillus sp.	Polyethylene, LDPE	FT-IR, SEM, weight loss	Bardají et al., 2019
Pseudomonas sp.	Polyphenylene sulfide	FT-IR, XPS, weight loss	Li et al., 2020
Pseudomonas aeruginosa RD1-3; *Pseudomonas knackmussii* N1-2	Polyethylene	AFM, SEM, viability test, whole genome characterisation, weight loss	Hou et al., 2022
Clostridium thermocellum	Polyethylene terephthalate	SEM, UV, weight loss	Yan et al., 2021

In, Howard et al. (2023) study it was found that when biofilm was increased, polyester degradation was also increased for a well characterised polyester-degrading enzyme. By using biofilm as a modification, Howard et al. (2023) hypothesised that increased biofilm formation in the culture could enhance the rate of plastic degradation. Recently, different bacteria, algae, actinomycetes, and fungi with the potential to biodegrade various plastic polymers have been investigated. To date, more than 56 species of bacteria and fungi

belonging to 25 genera have been reported for polyethylene biodegradation (Yang et al., 2023).

2.6 Detection of biofilm formation:

Biofilm formation can be detected using SEM analysis, biofilm development can be measured using viable counts, acridine orange direct counts (AODC), and a colorimetric method (Marshall et al., 1971; Raghukumar et al., 2000 & Tang et al., 1998).

2.6.1 Detection of biofilm formation on glass:

As early as 1971, Marshall et al. provided evidence based on scanning electron microscopy (SEM) that attached bacteria were associated with the surface by a fine extracellular polymeric fibril. Among the techniques used to determine the biofilm formation, SEM is better indicated to evaluate the microbial interaction in the biofilm matrix. SEM of the growth of *Ulkenia profunda*, isolated from the primary film, on surfaces of glass and aluminium proclaimed that the thraustochytrid cells directly attaches to the surfaces. Microscopic examinations confirmed the presence of thraustochytrids on glass surfaces (Raghukumar et al., 2000). This method (SEM) preserves the associated structures maintained under hydrated and viable conditions. Samples are fixed with the help of a chemical agent, such as paraformaldehyde, glutaraldehyde and osmium, or cryo-fixed through quick freezing, to prevent cell damage by ice crystals (Marques et al., 2007). Tang et al. (1998) reported that the development of biofilms of *Pseudomonas aeruginosa* PAO-1 was studied using modified Robbins devices. Biofilm development was measured using viable counts, a colorimetric method for exopolysaccharide (EPS) and acridine orange direct counts (AODC).

2.6.2 Detection of biofilm formation on plastic:

Banihashemi et al. (2022) investigation of bacterial degradation employed multiple approaches to provide quantitative and qualitative evidence that plastic degradation was mediated by microorganisms. Biofilm biomass was quantified with crystal violet staining, plating, and DNA extraction. Crystal violet is a positively charged, basic dye which binds to the negatively charged peptidoglycan and proteins in the bacterial cell wall. Crystal violet staining has been used to provide a quantitative measure of bacterial growth over time in a way that is relatively quick, with little preparation and materials required (Banihashemi et al., 2022).

2.7 Conclusion:

The earlier studies suggested that bacteria are able to form biofilm on different materials like glass and plastic (Marques et al., 2007; Sooriyakumar et al., 2022). The bacteria are able to degrade the different forms of plastic present in the water (Yang et al., 2023).

The existence and importance of thraustochytrid protists in primary film formation of freshly immersed surfaces in the sea have not been investigated very well. There is very limited published information about marine biofilm formation by thraustochytrids in different habitats of water. This present study describes the processes of thraustochytrids biofilm formation on different materials like glass, metal and plastic and the implications of biofilm formation biodegradation of plastics in water.

CHAPTER 3: METHODOLOGY

3. Methodology:

3.1 Culture and Growth Conditions

Eighteen isolates of thraustochytrids were provided for this study. The axenic cultures of thraustochytrid were maintained on Modified Vishniac (MV) agar plates. For the biofilm studies, thraustochytrid isolates were grown in MV medium at room temperature (RT) on a shaker at 105 rpm. Three-day-old cultures were used as inoculum. A volume of 10 ml of MV medium was inoculated with 100 µL inoculum and incubated for 3 days.

3.2 Biofilm Formation

The potential of thraustochytrids to produce biofilms on different surfaces such as glass, metal and plastic, was examined. This was carried out by crystal violet staining (CVS) as mentioned below. All thraustochytrid isolates i.e. 5 Long Padina, ZB-6, MC-1, MC-4, DB Sarg, OMD-1, OMD-2, OMD-3, OMD-4, OMS-2, OMS-4, RD-1, OGS-2, AKN3 sed, 8B red, A3 brown, A8 Ulva, and 9B were tested for biofilm formation on glass slides. Biofilm formation on nail was examined using four thraustochytrid isolates OMS-4, OMS-2, RD-1 and MC-4. Similarly, biofilm formation on plastic was examined using two thraustochytrid isolates, DB Sarg and 5 Long Padina, as those two isolates were obtained from macroalgae *Sargassum* and *Padina*, respectively. Biofilm formation on plastic was done in two ways, one using multi-well plates and the other using autoclavable plastic pieces, both described in separate sections.

3.2.1 Biofilm Assay i.e. Crystal Violet Staining (CVS)

The specimens were stained with an aqueous solution containing 0.1% crystal violet (CV). The glass (slides) and metal (nail) were then stained with CV for 10 min and

washed with distilled water. The amount of biofilm present from stained specimens were then immersed in 95% ethanol for 10 min. Absorbance was measured at a wavelength of 540nm that indicate biofilm formation.

3.3 Effect of bacterial biofilm on Thraustochytrids abundance

In 24 well plate the 5 µL of bacterial culture (associated with *Sargassum* species and obtained from Ms. Gayatri Kerkar) was inoculated into plastic wells and incubated at RT for 24 h. Next day the bacterial cultures were removed from the wells and the wells were washed with distilled water. Then, 30 µL of thraustochytrid isolates were immersed into wells for 24 h, 48 h, 72 h and 96 h at RT. The thraustochytrids abundance was determined using haemocytometer counting method at 24 h, 48 h, 72 h and 96 h. With the help of micro-pipette 15 µL of a sample were filled into the haemocytometer chamber. To minimize sample errors the cell suspensions were mixed before filling into the counting chamber. The cell suspension was loaded into the chamber without overflowing it and ensuring there are no air bubbles. The cells were allowed to settle a few minutes before counting. Using the 20X objective of compound microscope the cells in 1-mm squares were counted (fill both sides of chamber, and count the four corner and the middle squares of each side).

3.3.1 Biofilm Assay by Crystal Violet Staining

Along with the abundance of thraustochytrids in the above experiment, the amount of bacterial biofilm produced and/or used up by thraustochytrids were also measured.

3.4 Effect of biofilm produced by one thraustochytrid on the abundance of another thraustochytrid i.e. Biofilm formation on plastic (I)

Two thraustochytrid isolates 5 long Padina and DB Sarg were used to produce biofilm in multi-well plate. In 24 well plate the 5 µL of thraustochytrid culture was inoculated into

plastic wells and incubated at RT for 24 h. Next day the cultures suspensions were removed from the wells with the help of micropipette and the wells were washed with sterile seawater. Then, 30 µL of Thraustochytrid isolates were immersed into wells for 24 h, 48 h, 72 h and 96 h at RT. Thraustochytrids abundance was determined using haemocytometer counting method as mentioned above at 24 h, 48 h, 72 h and 96 h.

3.4.1 Biofilm Assay by Crystal Violet Staining

Along with the abundance of thraustochytrids in the above experiment, the amount of bacterial biofilm produced and/or used up by thraustochytrids was also measured by CVS mentioned earlier. An aqueous solution containing 0.1% crystal violet (CV) was prepared to stain specimens. The glass (slides) and metal (nail) were stained with CV for 10 min, then washed with distilled water that resulted in stained specimens that correspond to the amounts of biofilm present and immersed in 95% ethanol for 10 min. Absorbance indicating biofilm formation was measured at a wavelength of 540 nm. The same method was used for 24 well plate.

3.5 Biofilm formation on plastic (II)

All Thraustochytrid isolates used for this experiment were maintained and grown in MV broth. Pre-weighed (1cm*1cm) plastic pieces (HiMedia bag) were added in flask containing MV broth, Bushnell Haas Broth (BHB) and Seawater were autoclaved at 1210C for 20 min. The thraustochytrid isolates were inoculated to each flask and kept for incubation at RT. The incubation period of isolate OMD-2, RD-1 and MC-1 in MV broth with plastic pieces was 15 days and OGS-2 was for 8 days at RT. In BHB and seawater the plastic pieces were incubated with isolate OGS-2 and DB Sarg for 2 months each. After incubation the plastic pieces were weighed again and dipped in 1%

paraformaldehyde or 1% glutaraldehyde and stored in refrigerator and proceeded for Scanning Electron Microscope (SEM) analysis.

3.5.1 Sample processing for SEM analysis

The plastic pieces primarily fixed in 1% paraformaldehyde or 1% glutaraldehyde were taken and immersed in increasing concentrations of ethanol (70%, 85%, 95% and 100%) for 10 mins each. After which they were air dried and kept in a desiccator till further processing. Next the samples were sputter coated and analysed using the Scanning Electron Microscope (Carl Zeiss EVO18).

3.5.2 Biomass determination

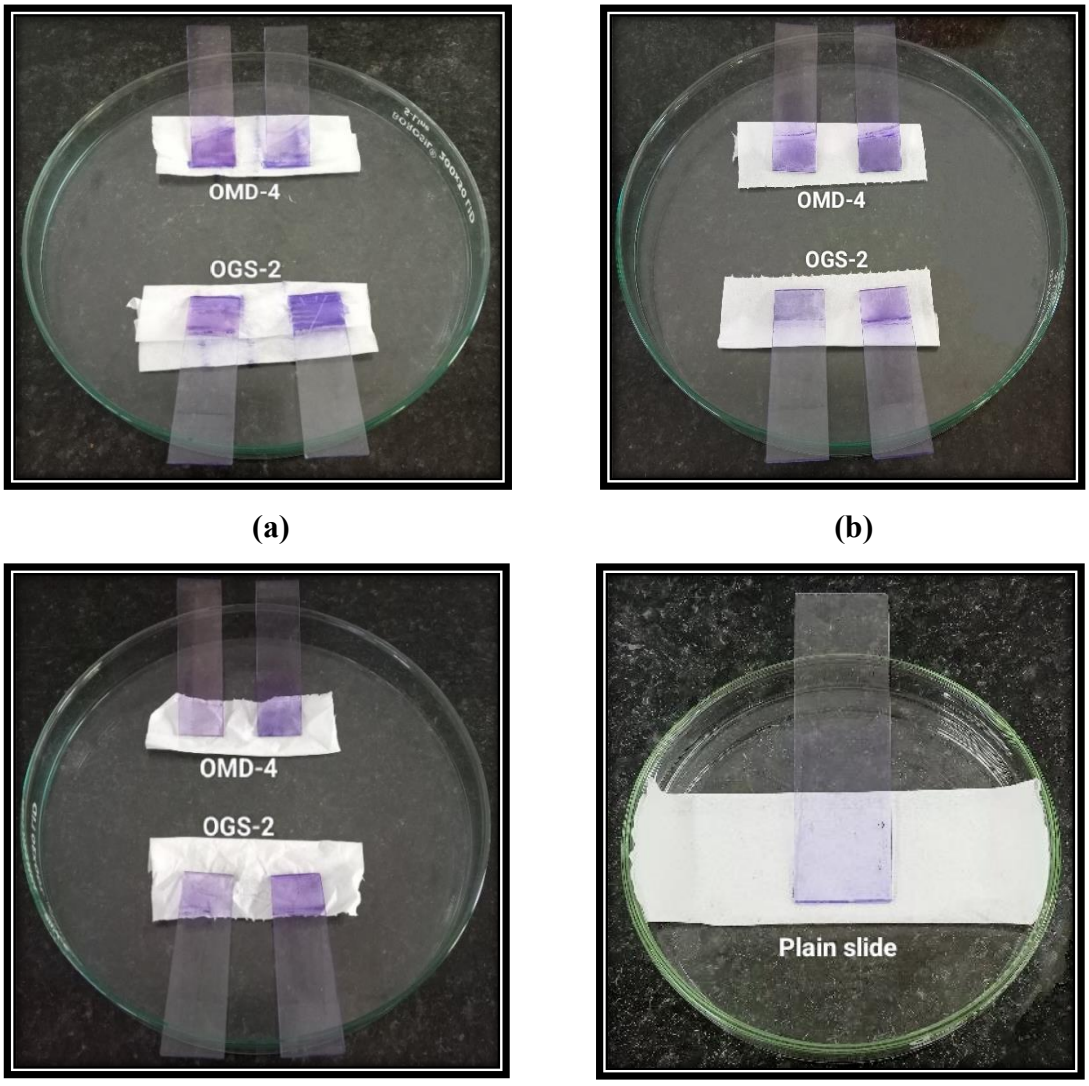
Dry weight biomass determination was carried out by transferring the entire contents of the flasks containing plastic in MV, BHB and seawater along with thraustochytrid isolates into pre-weighed 50 ml centrifuge tubes and centrifuged at 4500 rpm for 20 min. The supernatant was discarded and the wet thraustochytrid cells (pellet) were kept for drying for 24 h. The pellets were weighed and expressed as g per mL.

CHAPTER 4: ANALYSIS AND CONCLUSIONS

4.1 Biofilm formation using Crystal Violet Stain (CVS) assay

4.1.1 Biofilm formation on glass

All thraustochytrid isolates (OGS-2, OMD-1, OMD-3, OMD-4, OMS-4, A8 Ulva, AKN3 sed and A3 brown) produced biofilm on glass at 48 h, 72 h and 96 h (Fig.4.1, 4.2, 4.3).



(a) **(b)**

(c) **(d)**

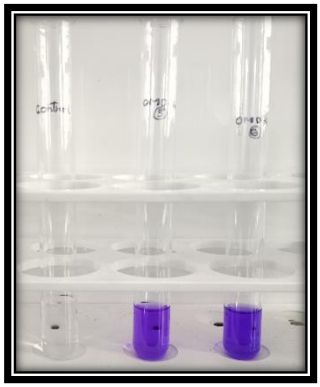
Fig 4.1: Biofilm formation on glass slide by OMD-4 and OGS-2 (a) 48 h (b) 72 h (c) 96 h (d) Blank

Crystal Violet Assay at 540nm

OMD-4

(a)

(b)



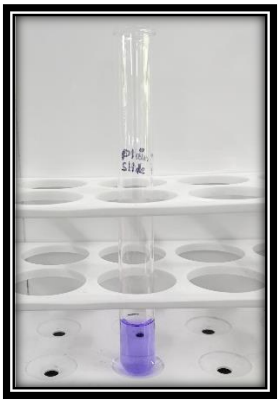
(c)

OGS-2

(d)

(e)

(f)



(g)

Fig 4.2: Crystal Violet Assay of OMD-4 (a-c) and OGS-2 (d-f) at 540nm (a, d) 48 h (b, e) 72 h (c, f) 96 h (g) Blank

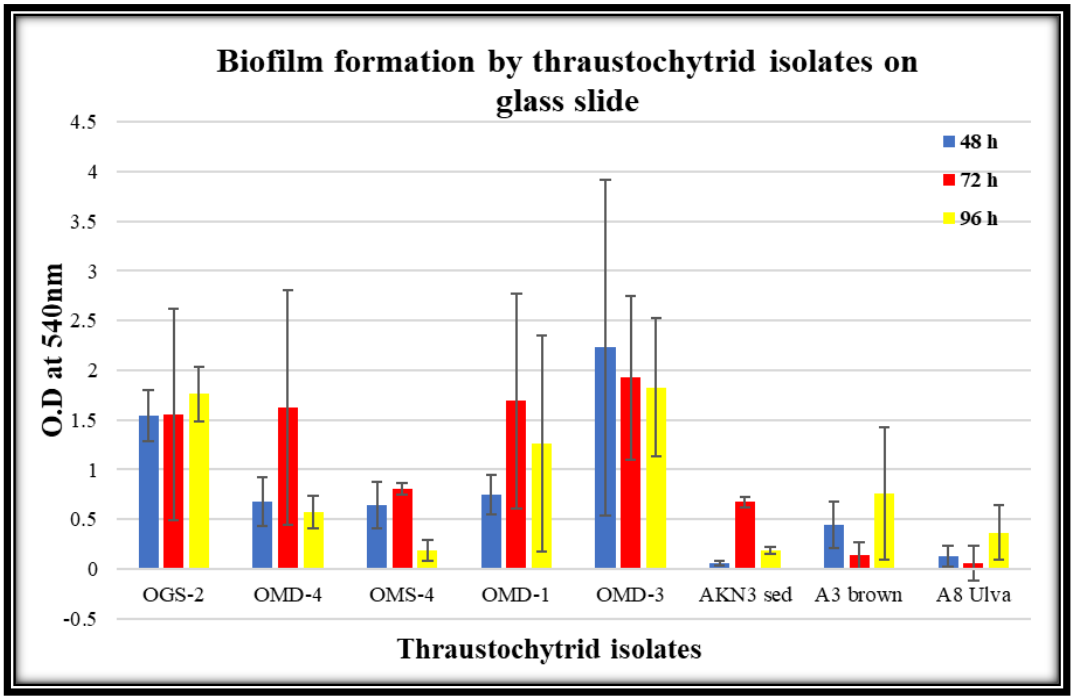


Fig 4.3: Biofilm formation on glass slide using Crystal Violet Assay at 540nm

Thraustochytrid isolates (OGS-2, OMD-4, OMS-4, OMD-1, OMD-3, AKN3 sed, A3 brown and A8 Ulva) gave positive spectrophotometric readings, thus indicating production of biofilm. OMD-3 showed highest biofilm forming ability on glass at 48 h followed by 72 h and 96 h. OGS-2 showed biofilm forming ability on glass at 96 h followed by 72 h and 48 h whereas AKN3 sed, A3 brown and A8 Ulva showed least biofilm forming ability as seen by very low readings (Fig 4.3). Biofilms produced on glass slides by a few representative isolates was assessed by SEM. Most of them showed production of extracellular polymers causing the adherence of cells (Fig 4.4, 4.5). SEM of OGS-2 isolate showed increase in the size of clump or cluster of cells with increasing incubation time (Fig 4.6 b-d). The cluster of cells seen at 24 h on SEM represented the cells from the inoculum adhering to glass (Fig 4.6 a).

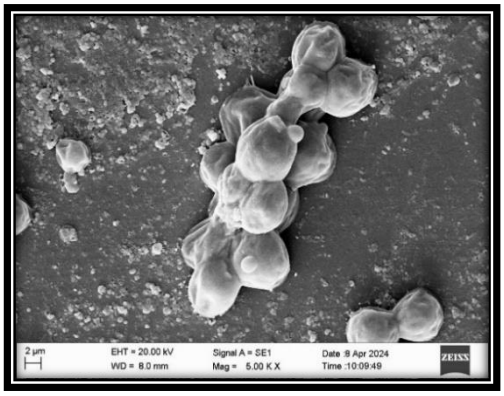


Fig 4.4: SEM of biofilm of OMD-2 on glass at 48 h incubation

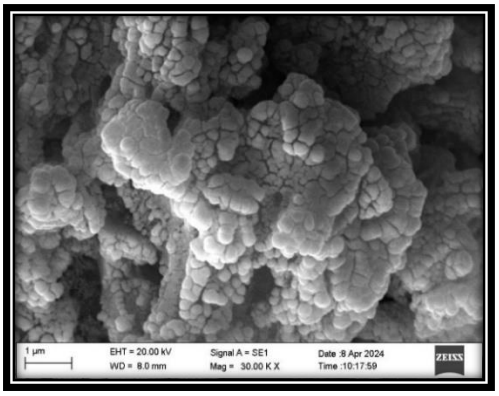
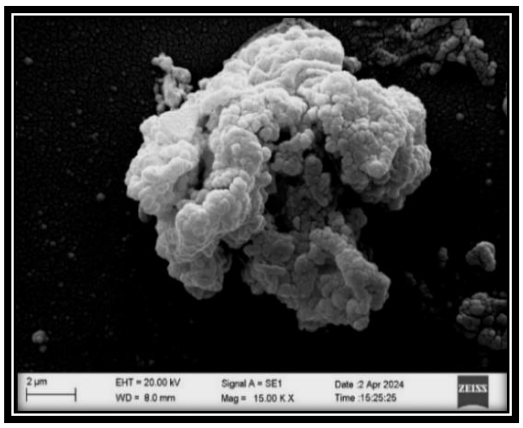
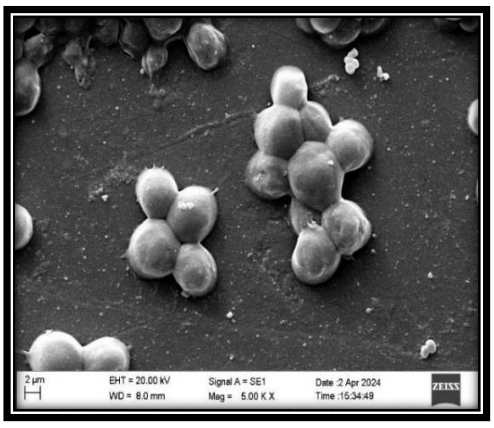


Fig 4.5: SEM of biofilm of ZB-6 on glass at 96 h incubation

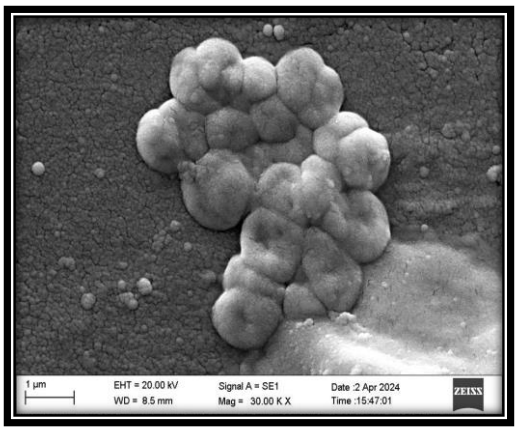
OGS-2



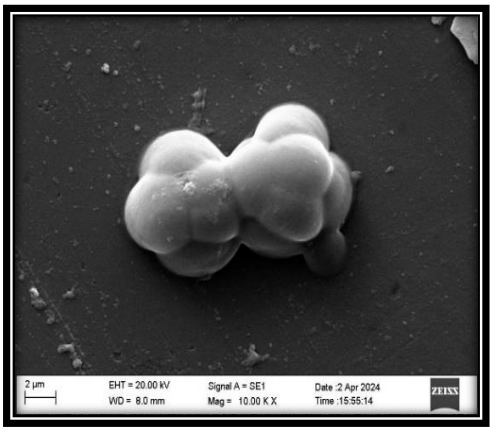
(a)



(b)



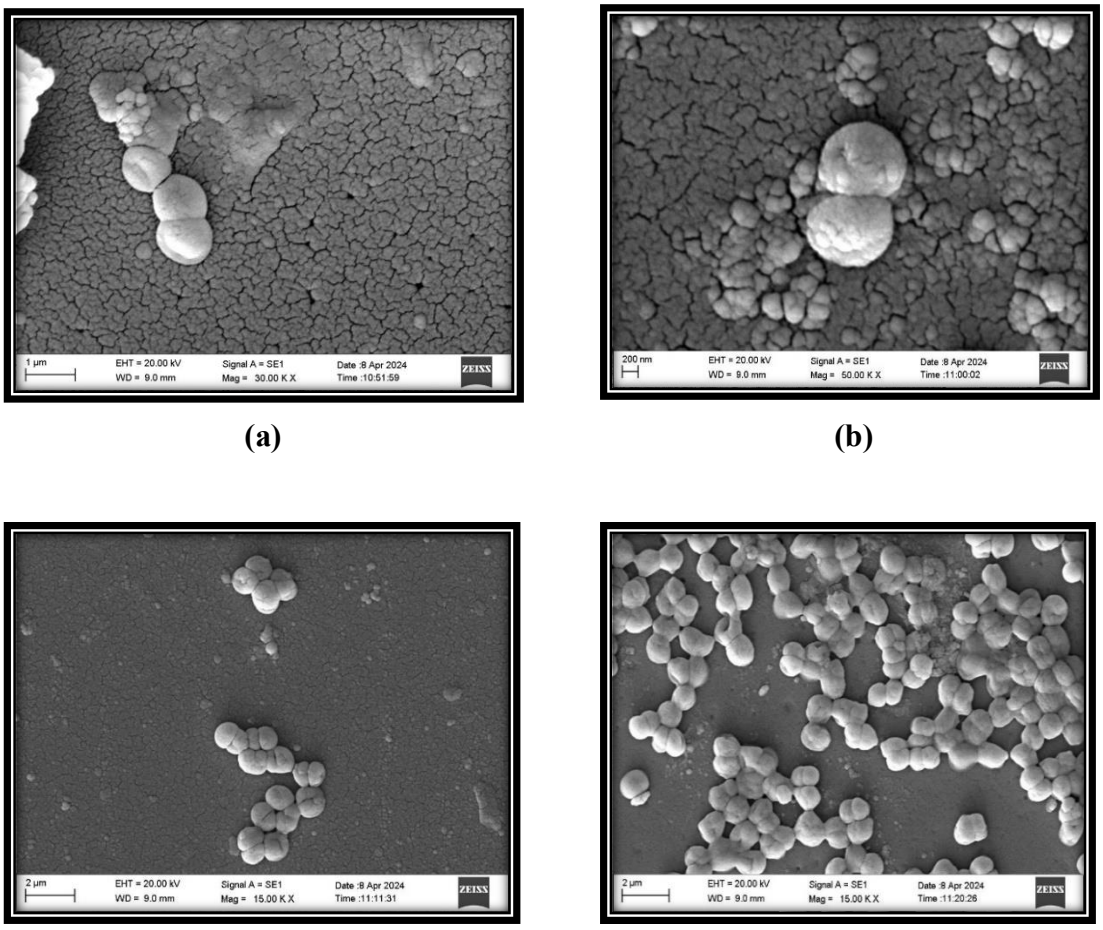
(c)



(d)

Fig 4.6: SEM of biofilm of OGS-2 on glass at (a) 24 h (b) 48 h (c) 72 h (d) 96 h of incubation

DB Sarg



(a) **(b)**

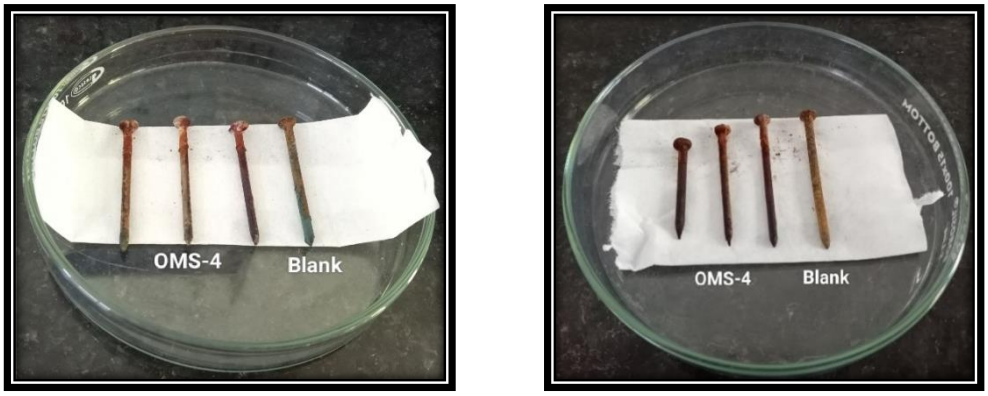
(c) **(d)**

Fig 4.7: SEM of biofilm of DB Sarg on glass at (a) 24 h (b) 48 h (c) 72 h (d) 96 h of incubation

SEM of DB Sarg isolate also clearly showed increase in the size of clump or cluster of cells with increasing incubation time (Fig 4.7 a-d).

4.1.2 Biofilm formation on nail

All thraustochytrid isolates (OMS-4, OMS-2, RD-1 & MC-4) produced biofilm on nail at 24 h and 48 h (Fig 4.8-4.10). MC-4 showed highest biofilm forming ability on nail at 48 h but at 24 h it produced least biofilm. Among all the isolates RD-1 showed least biofilm formation on nail.



(a) (b)

Fig 4.8: Biofilm formation on nail by OMS-4 isolate (a) 24 h (b) 48 h

(a) (b) (c)

Fig 4.9: Crystal Violet Assay of OMS-4 at 540nm (a) 24 h (b) 48 h (c) Blank

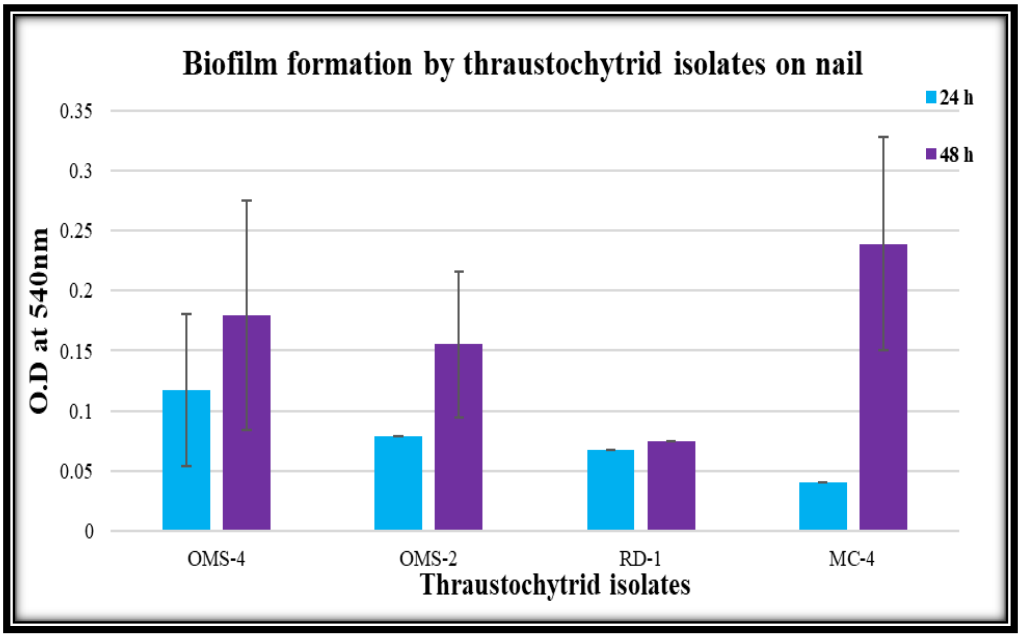


Fig 4.10 Biofilm formation on nail using Crystal Violet Assay at 540nm

4.2 Effect of bacterial biofilm on thraustochytrids abundance

In the presence of bacterial biofilm ZB-6 abundance increased from 24 h to 96 h. However, the cell numbers were less in seawater as compared to MV (Fig 4.11). CVS showed that bacterial biofilm was used up for thraustochytrid growth, except at 72 h in MV where biofilm formation was maximum (Fig 4.12).

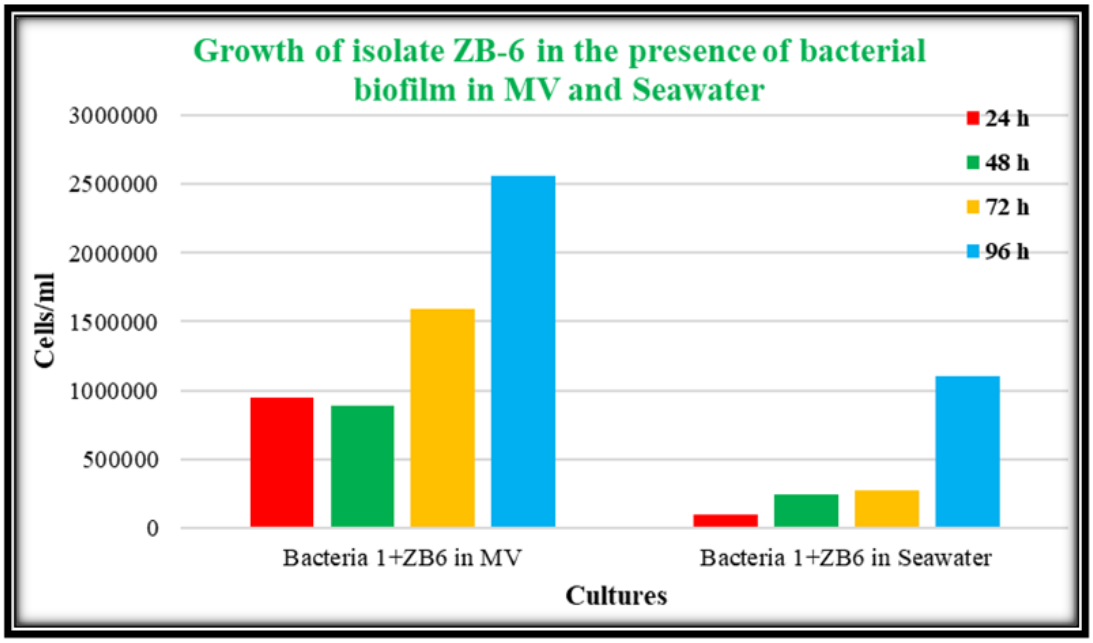


Fig 4.11: Abundance of ZB-6 in the presence of bacterial biofilm

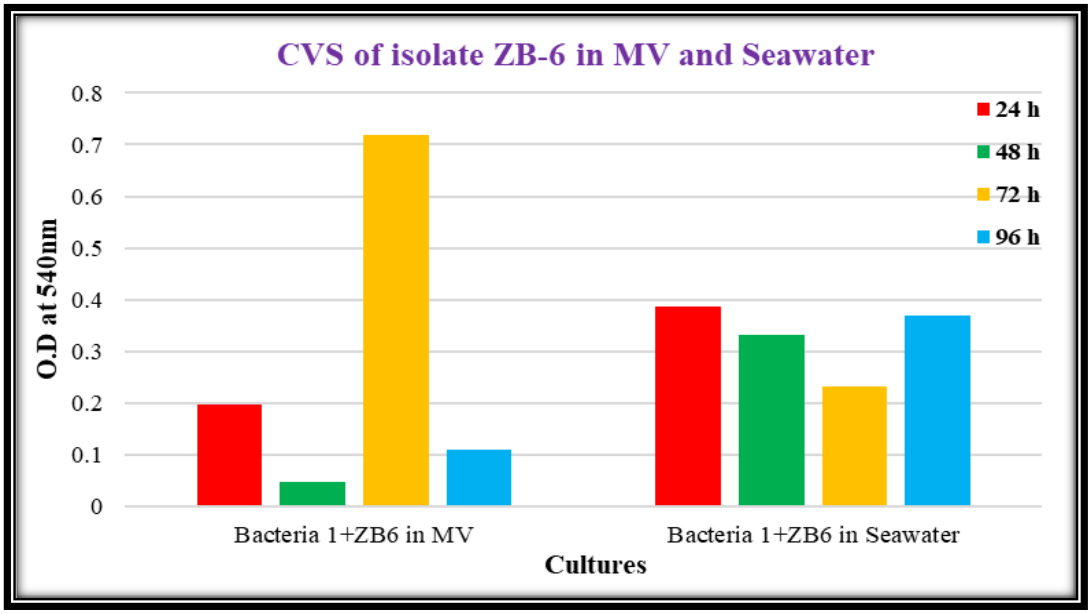


Fig 4.12: Biofilm formation by isolate ZB-6

Similar to ZB-6, the abundance of 5 Long Padina also increased with incubation time in the presence of bacterial biofilm and it was much higher in MV than in seawater (Fig 4.13). CVS showed highest biofilm formation at 72 h than the other times (Fig 4.14).

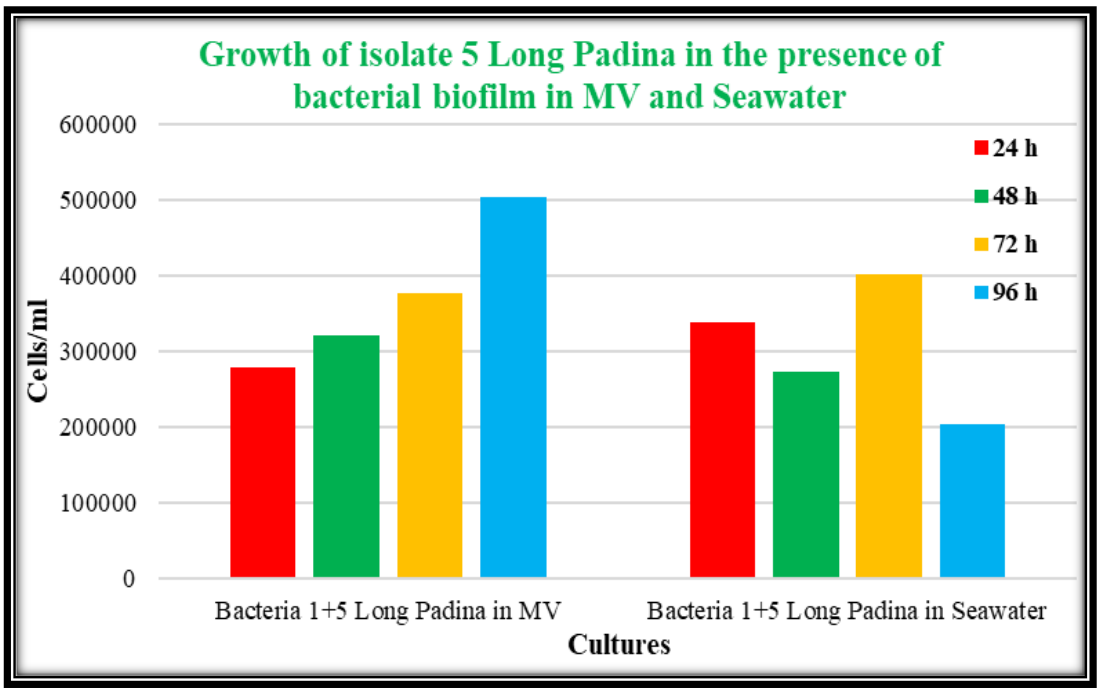


Fig 4.13: Abundance 5 Long Padina in the presence of bacterial biofilm

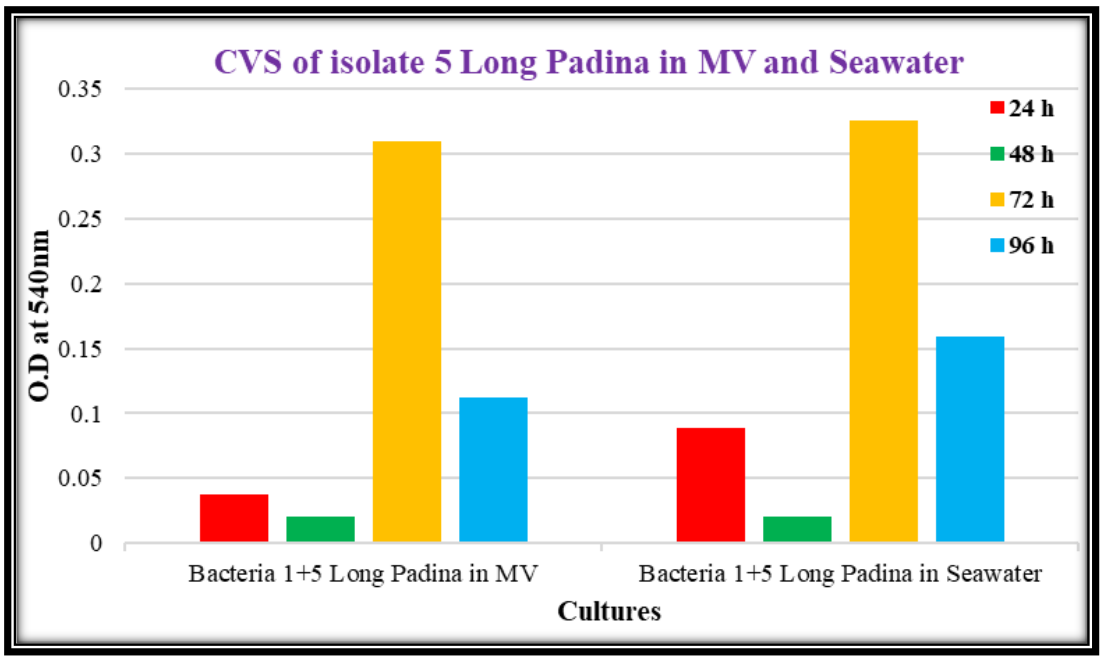


Fig 4.14: Biofilm formation by isolate 5 Long Padina

4.2.1 Effect of bacterial biofilm on the growth of thraustochytrid isolate OMD-4

In the presence of bacterial biofilm, the abundance of OMD-4 decreased from 24 h to 96 h in MV as well as seawater. In the absence of bacterial biofilm OMD-4 abundance increased with time in MV broth and remained more or less constant in seawater (Fig 4.15). CVS showed the highest biofilm at 96 h in all conditions except in the absence of bacterial biofilm in seawater (Fig 4.16).

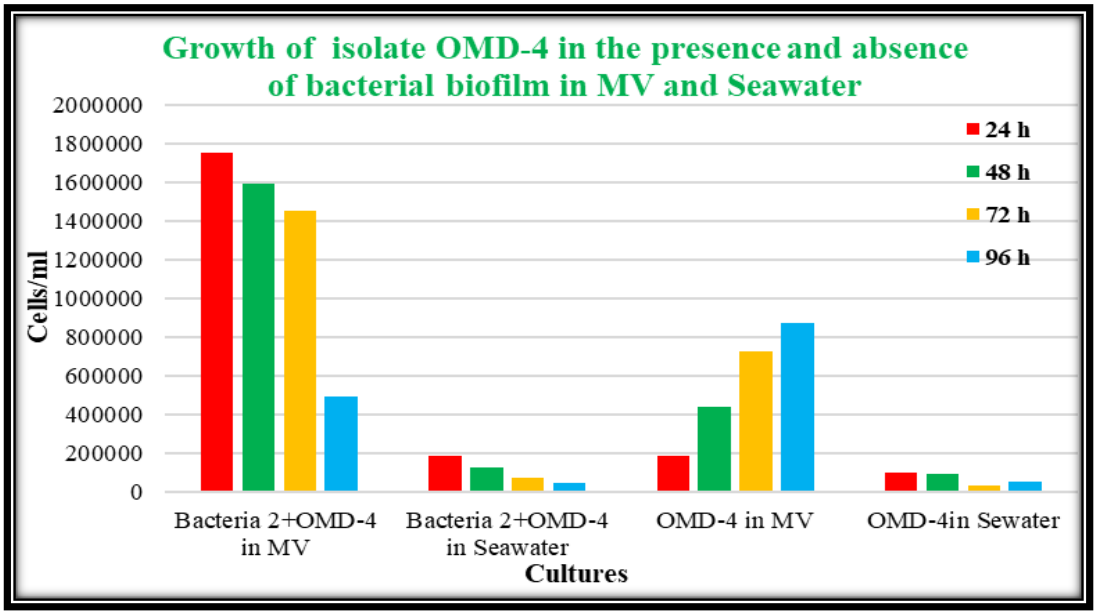


Fig 4.15: Abundance of OMD-4 in the presence and absence of bacterial biofilm

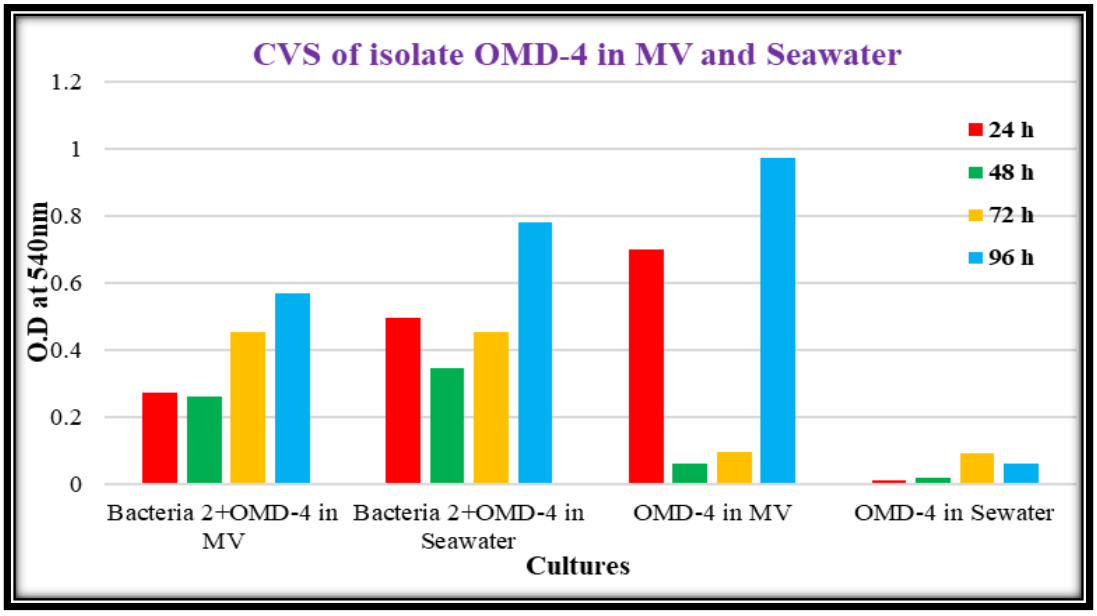


Fig 4.16: Biofilm formation by isolate OMD-4

4.2.2 Effect of bacterial biofilm on the growth of thraustochytrid isolate DB Sarg

In the presence of bacterial biofilm, the abundance of DB Sarg decreased from 24 h to 96 h in MV as well as seawater. In the absence of bacterial biofilm DB Sarg abundance increased with time in MV broth and remained more or less constant in seawater (Fig 4.17). CVS showed increasing biofilm in the presence of bacteria in seawater whereas almost constant biofilm in other conditions (Fig 4.18).

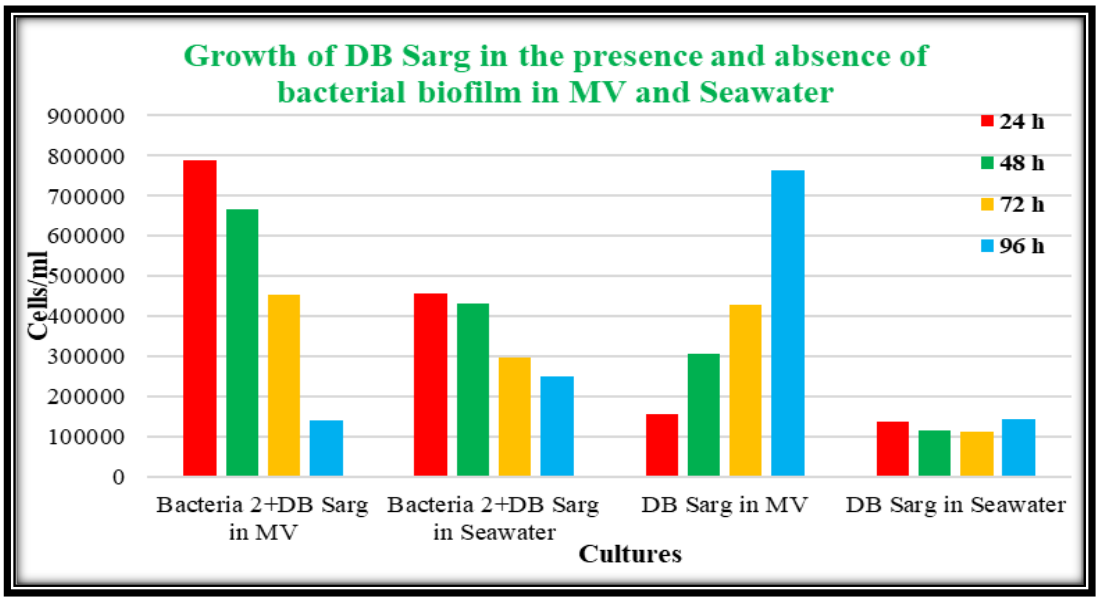


Fig 4.17: Abundance of DB Sarg in the presence and absence of bacterial biofilm

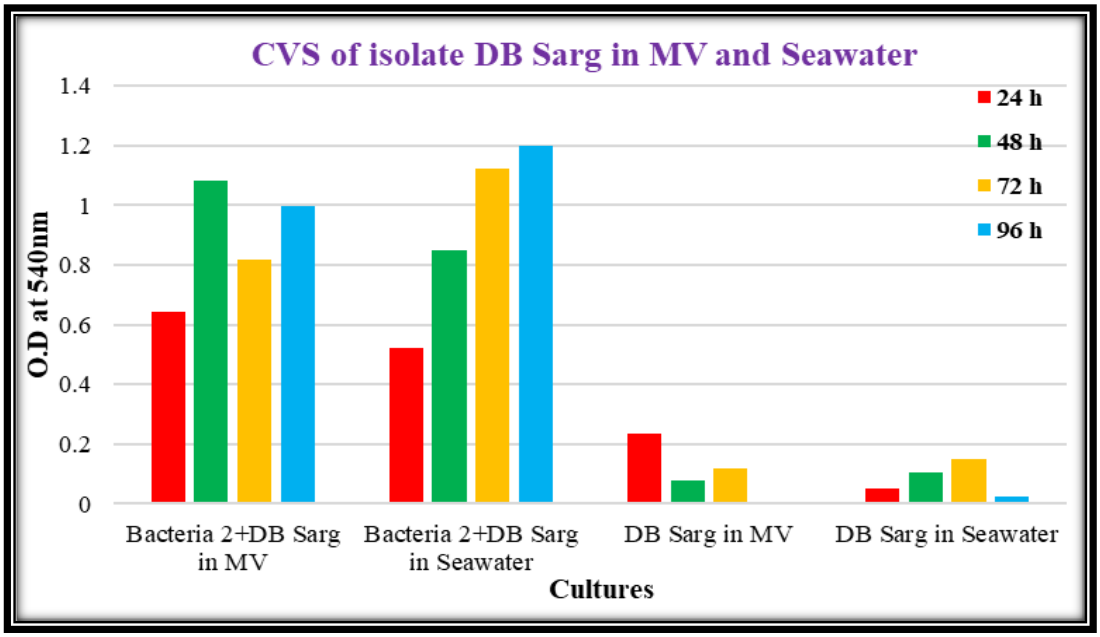


Fig 4.18: Biofilm formation by isolate DB Sarg

4.3 Effect of thraustochytrid biofilm on the growth of thraustochytrid isolate i.e. Biofilm formation on plastic (I)

5 Long Padina biofilm on the growth of OGS-2: The graph (Fig 4.19) shows decrease in the abundance of OGS-2 in the presence of 5 Long Padina biofilm from 24 h to 96 h in MV and Seawater. In the absence of 5 Long Padina biofilm the OGS-2 abundance increases in MV and decreases in Seawater. Lesser biofilm was produced in MV and seawater in the absence of bacteria than in their presence.

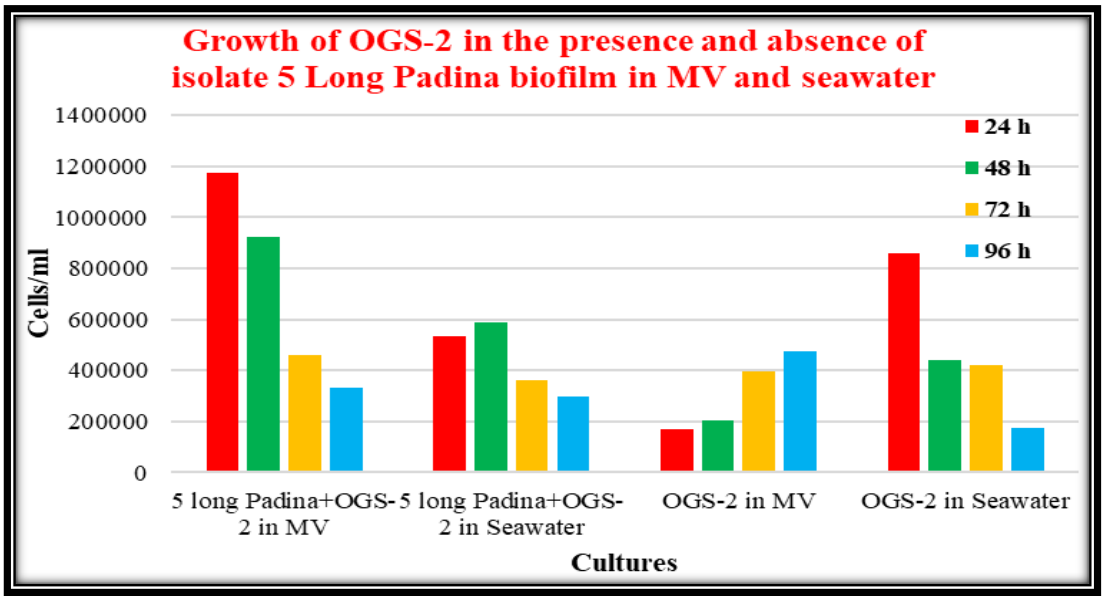


Fig 4.19: Abundance of 5Long Padina in the presence and absence of OGS-2 biofilm

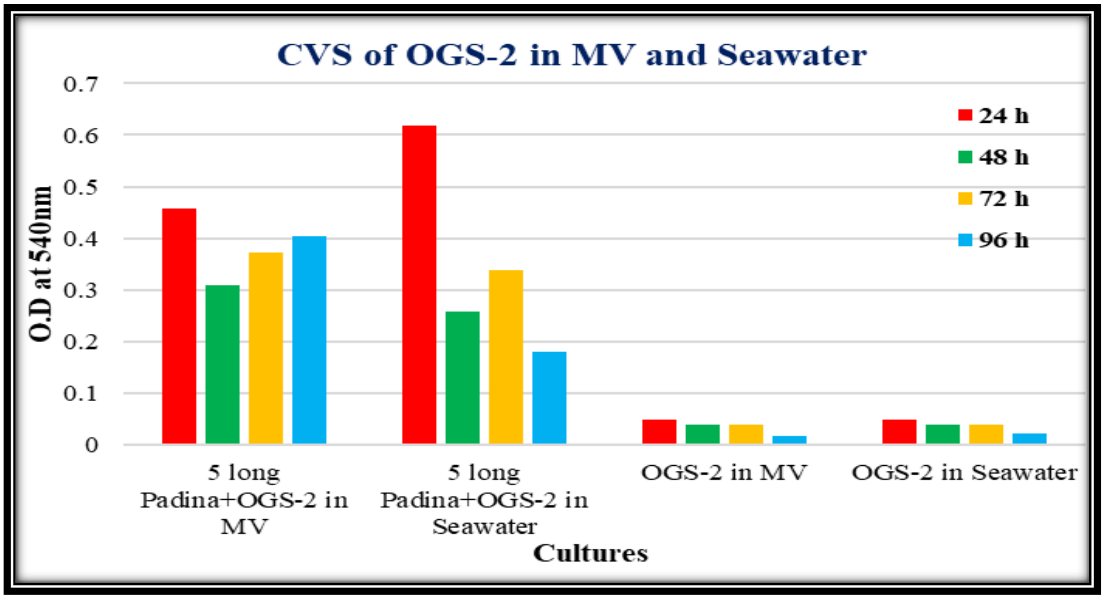


Fig 4.20: Biofilm formation by isolate OGS-2 with 5 Long Padina biofilm

DB Sarg biofilm on the growth of OMD-4: The graph (Fig 4.21) shows the decrease in OMD-4 abundance in MV and Seawater in the presence of DB Sarg biofilm from 24 h to 96 h. Whereas in the absence of DB Sarg biofilm the abundance of OMD-4 increases in MV and remains constant in Seawater. The graph (Fig 4.22) shows highest biofilm formation by OMD-4 in the presence of DB Sarg biofilm in MV and Seawater. Whereas biofilm formation by OMD-4 in the absence of DB Sarg biofilm is highest at 24 h in MV and 72 h in Seawater.

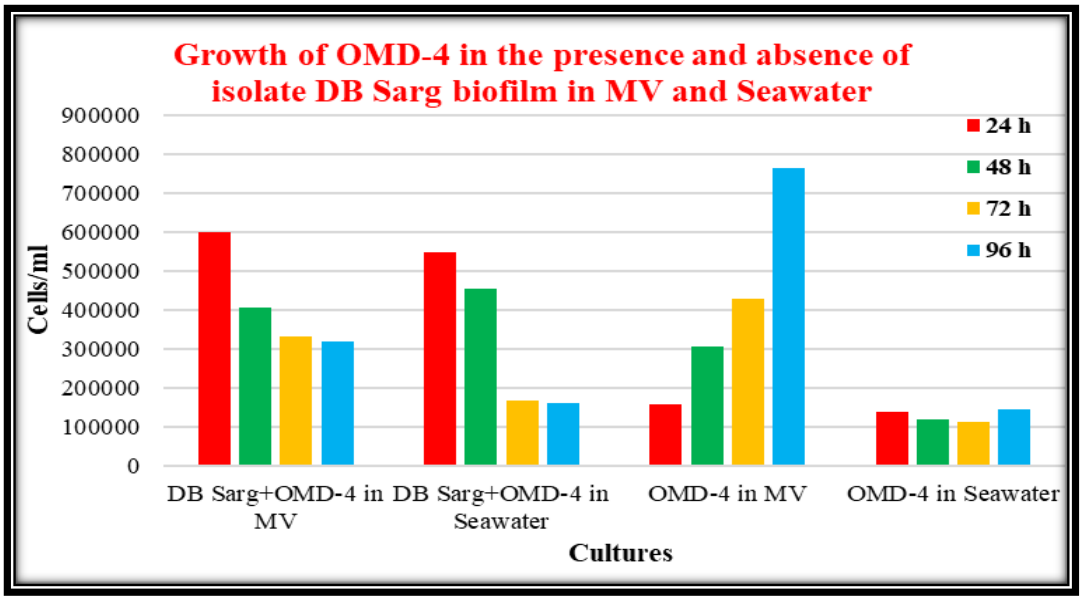


Fig 4.21: Graph of isolate DB Sarg abundance in the presence and absence isolate OMD-4 biofilm

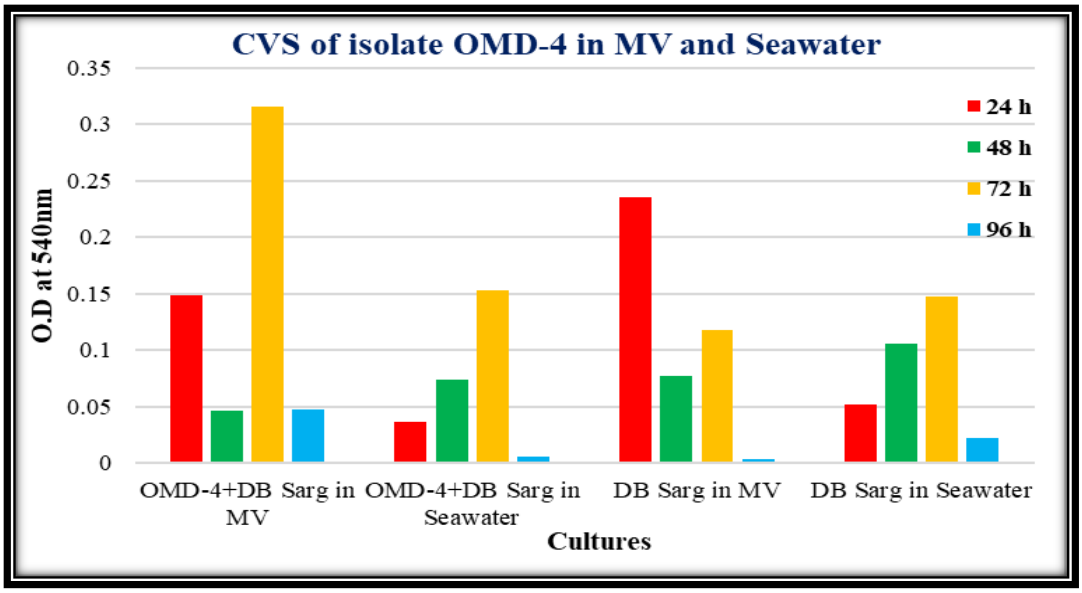


Fig 4.22: Biofilm formation by OMD-4 with DB Sarg biofilm

4.4 Biofilm formation on plastic (II)

The weight of all plastic pieces increased within 15 days of incubation indicative of biofilm formation on them (Fig 4.23). This was clearly visible in SEM images (Fig 4.24).

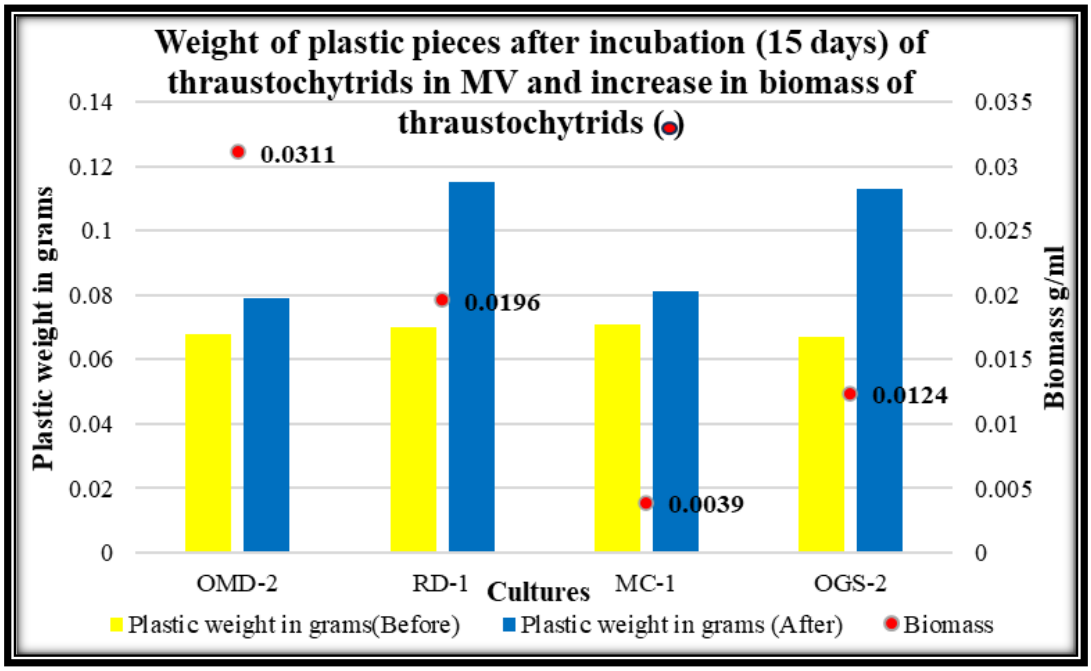


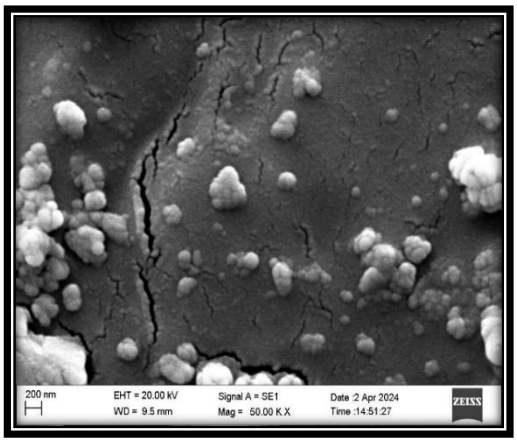
Fig 4.23: Weight of plastic pieces after incubation (15 days) with thraustochytrids in MV and biomass of the isolates

4.4.1 SEM of Plastic in MV broth

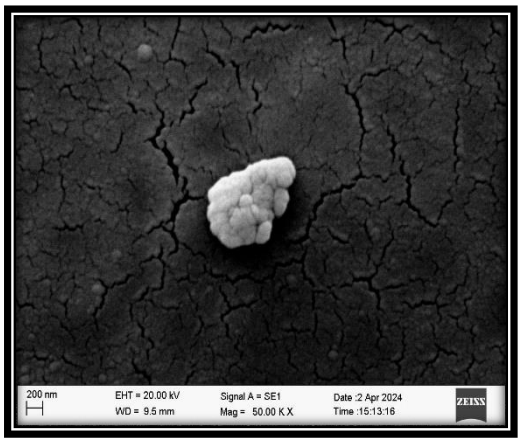
On incubation for 60 days, the weight of plastic pieces in seawater decreased by 65 % in the presence of OGS-2 and 5 % in the presence of DB Sarg (Fig 4.25). The increase in biomass was minimal, OGS-2 increased up to 0.04 g/ml while DB Sarg increased up to 0.01 g/ml. The biomass in BHB was greater than that in seawater. In BHB, biomass increased up to 0.03 g/ml for OGS-2 and 0.01 g/ml for DB Sarg (Fig 4.26). The decrease in the weight of plastic pieces in BHB was comparable to that in seawater. A thick mass of cells of OGS-2 was observed after 60 days in seawater in SEM image. In case of DB Sarg the mass or film of cells was thinner than OGS-2. However, in the presence of BHB,

OGS-2 cells formed a thin layer of cells with a peculiar pattern, while DB Sarg showed a thick mass of cells (Fig 4.27).

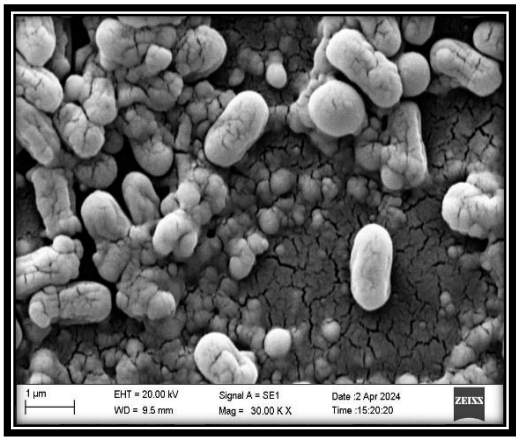
OMD-2



RD-1



MC-1



OGS-2

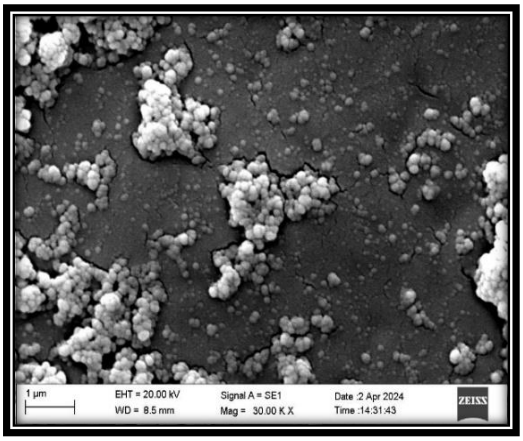


Fig 4.24: SEM of plastic pieces in MV broth (a) OMD-2 (b) RD-1 (c) MC-1 (d) OGS-2 for 15 days of incubation

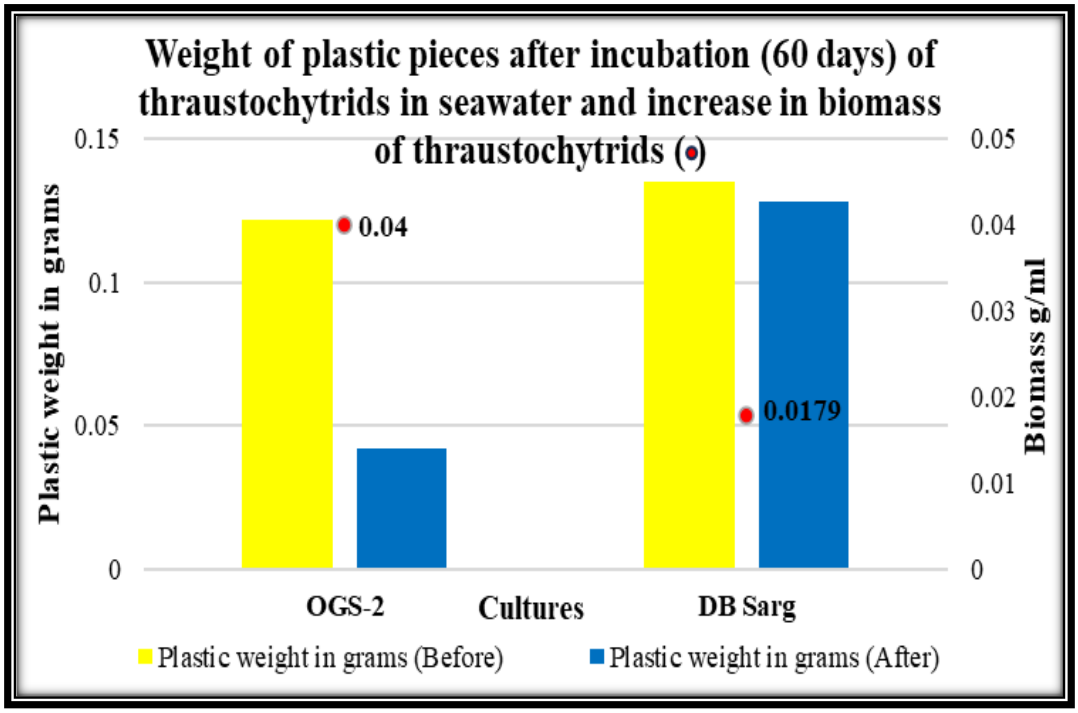


Fig 4.25: Weight of plastic pieces after incubation (60 days) with thraustochytrids in seawater and biomass of the isolates

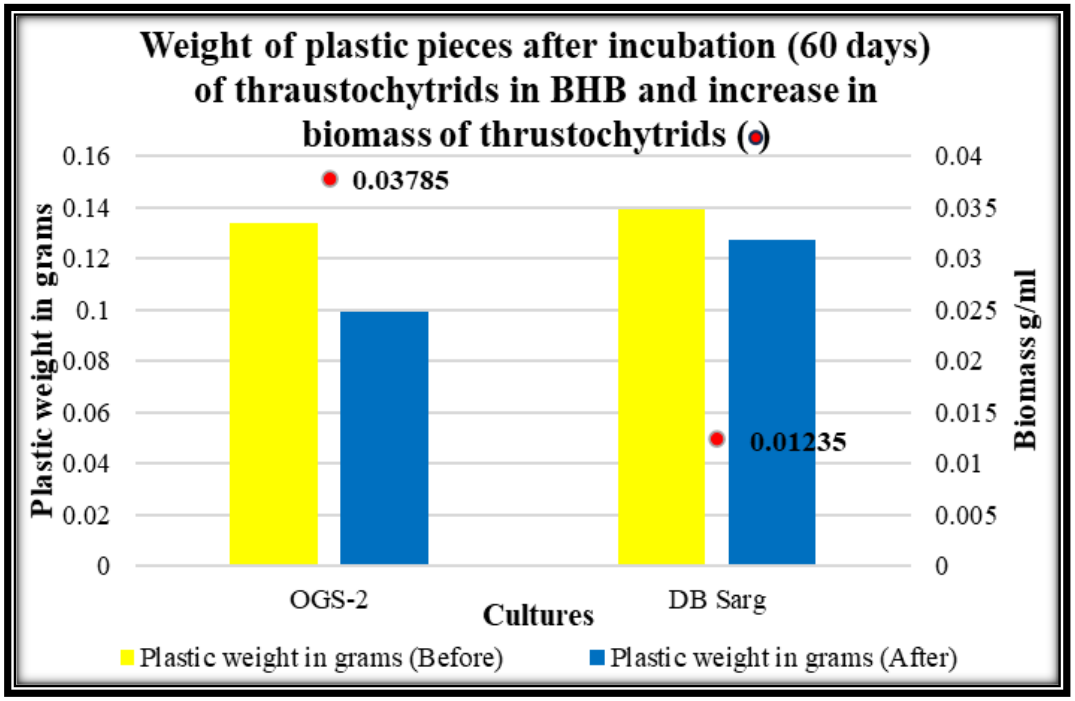
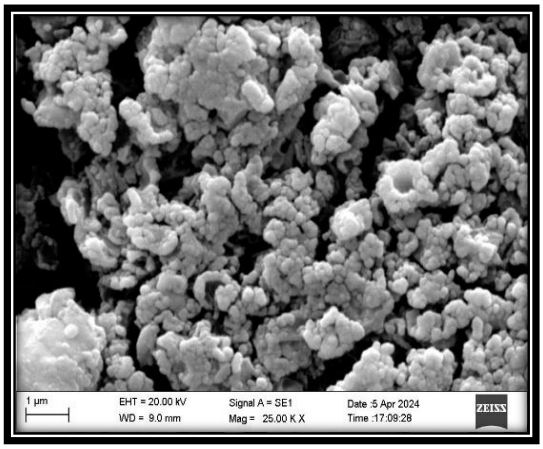


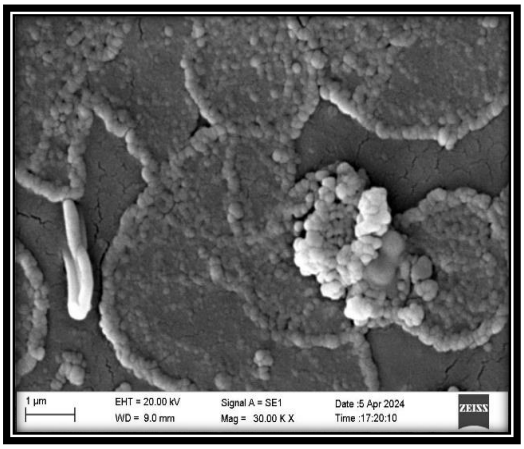
Fig 4.26: Weight of plastic pieces after incubation (60 days) with thraustochytrids in BHB and biomass of the isolates

4.4.2 SEM of plastic in Seawater and BHB

OGS-2

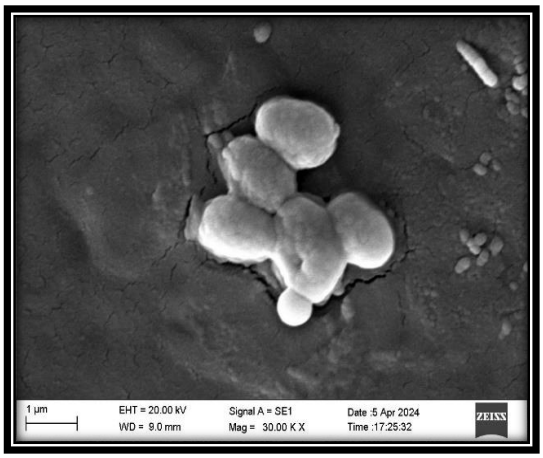


(a)

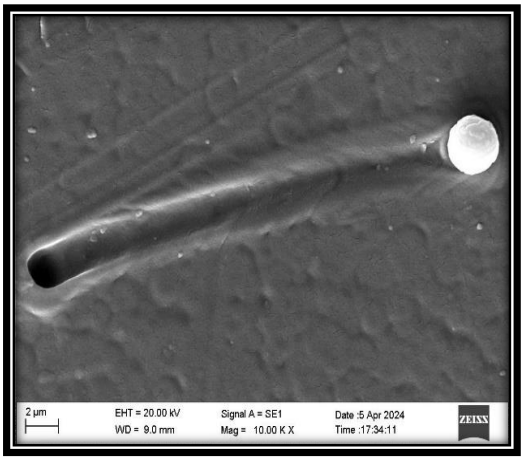


(b)

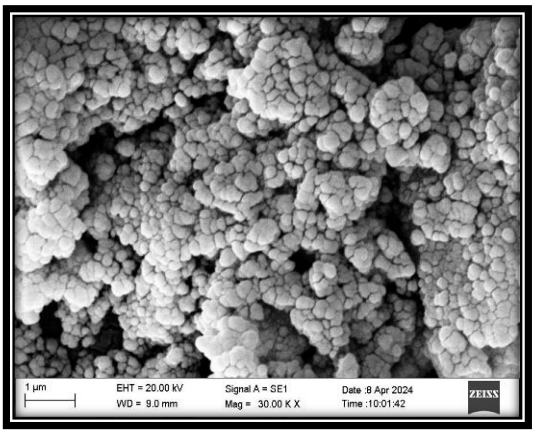
DB Sarg



(c)



(d)



(e)

Fig 4.27: SEM of plastic pieces (a)OGS-2 in seawater (b)OGS-2 in BHB (c, d) DB Sarg in seawater (e) DB Sarg in BHB for 60 days of incubation

4.5 Discussion:

The present study proclaimed that thraustochytrid isolates showed biofilm formation on glass, metal and plastic. The peak in biofilm production was seen at different hours by different thraustochytrid isolates. Thraustochytrids form a biofilm as they produce TEPs, POC, POM (Kimura et al., 2001), EN elements (Nagano et al., 2013) and also, they have the tendency to produce EPS (Jain et al., 2005). TEPs might serve as an important determinant as a substrate for thraustochytrids for settlement and growth and it plays a major role in the formation of aggregates (Damare et al., 2012). Earlier study by Raghukumar et al. (2000) demonstrated that these protists can settle on inorganic particulate material in the sea within 24 h. The experiment was carried out on fibre glass, aluminium, mild steel, copper and cupro-nickel and glass, and showed that under laboratory conditions, glass, aluminium and fibre glass panels supported settlement and growth of the thraustochytrid isolates (Raghukumar et al., 2000).

Thraustochytrids are generally found to be substrate specific in occurrence rather than free-living in the water column (Damare 2015). This substrate-specific nature helps these protists to grow on bacteria associated with *Sargassum* species as epibionts (Susilowati et al., 2015). In the present study thraustochytrids multiplied in numbers and formed biofilm, despite of dense bacterial populations. The thraustochytrid isolates were able to grow in the presence of bacterial biofilm in a multi well plate. Crystal violet assay showed that thraustochytrids utilized bacterial biofilm for their growth leading to greater abundance of thraustochytrids which in turn contribute to biofilm formation of thraustochytrids.

The ultrastructural characterization of a biofilm can be carried out by different microscopy methods however, SEM method provide the most detailed images at the

highest magnifications (Relucenti et al., 2021). Microscopic examinations have confirmed the presence of thraustochytrids on glass surfaces (Raghukumar et al., 2000). In the present study SEM images showed some extracellular polymer causing the adhesion of thraustochytrid cells to each other to form clumps. It also showed increase in the size of clump with increasing incubation time on glass as well as on plastic. Cell walls of these protists are known to be composed of sulphated polysaccharides, predominantly made of galactans and proteins (Raghukumar et al., 2000).

Raghukumar et al. (2001) showed that thraustochytrids were often related to chlorophyll 'a' and POC, suggesting that they might be important in the degradation of autochthonous oceanic material. The present study revealed that OGS-2 and DB Sarg showed more biomass and biofilm production in MV broth than that in seawater. In the experiment with plastic pieces, the weight of plastic after incubation with thraustochytrids were more than before within 15 days but lesser than before within 60 days. The initial increase in weight within 15 days of incubation could be because of the formation of biofilm on the plastic pieces. The latter decrease in weight after 60 days of incubation could be indicative of the degradation of plastic with prolonged exposure to thraustochytrids. This could be seen in SEM images (Fig 4.27 a-e)

In a recent study, biofilm formation on Polyethylene (PS) and Polystyrene (PE) was investigated and it was found that after 2 weeks of incubation microplastics were covered by assemblages, and bacteria from the genus *Erythrobacter* were found on the microplastics. It was suggested that members of *Erythrobacter* were able to degrade plastic (Urbanek et al., 2018). Hence, from the earlier study it was known that bacteria have the capability of degrading plastic (Li et al., 2020) but no studies have dealt with the capability of thraustochytrids to degrade plastic. The interactions between plastic and thraustochytrids are still poorly known.

4.6 Conclusion

The study demonstrates the robust biofilm-forming capabilities of thraustochytrid isolates across different surfaces such as glass, metal, plastic and the biofilm production peak observed at different times. SEM imaging provided visual confirmation of biofilm formation and revealed the presence of extracellular polymers contributing to cell adhesion and cluster formation. Moreover, the study highlights the resilience of thraustochytrids in the presence of dense bacterial populations, as evidenced by their ability to utilize bacterial biofilms for growth. It also explores the impact of thraustochytrid biofilms on plastic degradation. Thraustochytrids demonstrated the ability to form biofilms on plastic surfaces, with SEM imaging revealing changes in biofilm morphology over time. This study marks a pioneering exploration into thraustochytrids' involvement in plastic degradation, suggesting a novel avenue for further research.

In conclusion, the study underscores the multifaceted capabilities of thraustochytrids, from biofilm formation on diverse surfaces to their potential role in plastic degradation. Further research in this area is needed to elucidate the mechanisms underlying thraustochytrid biofilm formation and plastic degradation, as well as to explore their practical implications in environmental management.

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Appendix I: Media

MV

Ingredients	Gms/100ml
Liver infusion powder	0.001g
Yeast extract	0.01g
Peptone	0.15g
Dextrose	0.4g
Agar	0.9g
Seawater	100ml

BHB

Ingredients	Gms/1000ml
Magnesium sulphate	0.200
Calcium chloride	0.020
Monopotassium phosphate	1.000
Dipotassium phosphate	1.000
Ammonium nitrate	1.000
Ferric chloride	0.050
Final pH (at 25°C)	7.0+/-0.2

Appendix II: Stains

Crystal Violet

Crystal violet is the monochloride salt of crystal violet cation. In cell culture, crystal violet finds its application in staining the nuclei of adherent cells. It is an intercalating dye, thus, can be used for determining cell number colorimetrically.

Ingredients	Gms/100ml
Crystal Violet	0.1g
Deionized water	100ml