

ISOLATION AND EXTRACTION OF MARINE NATURAL BACTERIAL COMPOUND

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

By

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DEPARTMENT OF BIOTECHNOLOGY

SCHOOL OF BIO AND CHEMICAL ENGINEERING

SATHYABAMA

INSTITUTE OF SCIENCE AND TECHNOLOGY

(DEEMED TO BE UNIVERSITY)

CATEGORY - 1 UNIVERSITY BY UGC

Accredited with Grade "A++" by NAAC | 12B Status by UGC | Approved by AICTE

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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **Keerthika.D (41770013)** who carried out the project entitled "**Isolation and extraction of marine natural bacterial compound**" under my supervision from October 2023 to April 2024.

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ABSTRACT

This study investigates the antifouling properties of deep-sea marine organisms and characterizes isolated bacterial extract for their potential antifouling nature. Bacterial isolates were cultured, and their extracts were prepared using ethyl acetate. The antibacterial activity of the extracts was tested against both Gram-positive and Gram-negative bacteria, such as *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, and *Bacillus*. Furthermore, the extracts were evaluated for their ability to inhibit biofilm formation, which was confirmed using biofilm inhibition assay (crystal violet assay) and phenol sulphuric acid assays. The results demonstrate significant inhibition of biofilm formation, highlighting promising potential for biofouling prevention.

Keywords: Biofouling, Antifouling, Deep-sea microbes, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Bacillus*, biofilm inhibition assay, phenol sulphuric acid assay.

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LIST OF ABBREVIATIONS

1. TBT - Tributyl-tin
2. DSM - Deep-sea microbes
3. NOAA - National Oceanic and Atmospheric Administration
4. ZMA – Zobell marine agar
5. ZMB – Zobell marine broth
6. CV – Crystal violet
7. BFB – Biofilm forming bacteria
8. TLC – Thin layer chromatography
9. GCMS – Gas chromatography mass spectrometry
10. μ l – Microliter
11. EPS – Extracellular polymeric substance
12. Nm – Nano meter

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CHAPTER – 1

INTRODUCTION

1.1 Biofouling

Biofouling is defined as colonization and excessive growth of microorganisms such as algae, barnacles, molluscs on submerged surfaces (*Nurul Najihah Rawi et al, 2023*). Bacterial cells are generally immersed in self- produced extracellular matrix in dense communities known as biofilms. Bacterial cells and the extracellular matrix make up the biofilms, biofilms can be harmful (*Jefferson, et al 2004*). A biofilm is a microbial community attached to a surface. It consists of microbial cells massed in the matrix of extracellular polymeric substances, which contain a large variety of biopolymers such as proteins, nucleic acids, lipids, and other substances (*Lexin Long, et al 2021*).

Biofilm formation is a natural process, it can create an ecological and economic problems. It will increase the fuel conception, slow movement of ship and corrosion of water – immersed structures (*Sheida Heidarian et al, 2019*). Biofouling is a significant problem that affects human activities, especially in the oil and gas industry. Every year, biofouling and biocorrosion contribute to enormous economic losses worldwide in industries, including heat exchange, oil and gas processing, storage and transportation, and drinking and wastewater industries.

Moreover, the persistence and transmission of harmful or pathogenic microorganisms and their genetic determinants within the marine biofilms pose a great threat to human beings. Bacteria as part of the marine microbial population, have been considered as the primary colonizers within biofilm and play a role as dominant components (*Bintang Marhaeni, et al 2011*). Among the marine biofilms, as the early colonizers, bacteria are an important factor in determining the structure and function of a mature biofilm. Therefore, bacteria from marine biofilms can be great targets for the discovery of antibiofilm compounds (*Lexin Long, et al 2021*).

1.2 Biofilm formation

Biofouling is classified into microfouling – biofilm formation and bacterial adhesion- and macrofouling – attachment of larger organisms – (*Chambers et al., 2006*). This latter can be 'soft macrofouling', comprising macroscopically visible algae and invertebrates such as soft corals, sponges, anemones, tunicates and hydroids, and 'hard macrofouling', formed by shelled invertebrates such as barnacles, mussels and tubeworms (*Braithwaite and McEvoy, 2005*). The process of biofouling formation consists of the successive colonization of a surface by a large variety of organisms (*Iratxe Menchaca et al, 2014*).

The biofilm developmental cycle is included (i) initial attachment – the biofilm formation begins with the initial attachment of planktonic bacterial cells to a surface. This attachment is facilitated by weak, reversible interactions such as van der Waals forces, electrostatic forces and hydrophobic interactions (*O'Toole & Kaplan, 2000*).

(ii) Irreversible attachment- following initial attachment, bacterial cell undergo a transition to irreversible attachment, firmly anchoring themselves to the surface. This stage involves the production of adhesion molecules like exopolysaccharides (EPS), which strengthen cell-surface interactions and stabilize attachment (*Flemming et al., 2016*). (iii) Maturation as attached bacterial cells proliferate, they begin to secrete extracellular polymeric substances (EPS), forming a matrix that encases the biofilm community. The EPS matrix provides structural integrity to the biofilm and offers protection to resident cells (*Flemming et al., 2016*). (iv) Biofilm Growth and Development: With time, the biofilm continues to grow in thickness and complexity. This growth is fueled by the recruitment of additional bacterial cells and the accumulation of EPS. The mature biofilm structure may exhibit intricate features such as channels and voids that facilitate nutrient and waste exchange within the community (*Flemming et al., 2016*). (v) Dispersal: Eventually, biofilms undergo dispersal, where cells are released into the surrounding environment. Dispersal mechanisms include active cell detachment, passive erosion of the biofilm matrix, or the production of dispersal agents such as enzymes or surfactants (*Hall-Stoodley et al., 2004*). EPS (extracellular polymeric substance), composed by polysaccharides, proteins, lipids and nucleic acids. The microbes together with the EPS increase the roughness

of the surface, facilitating the subsequent adhesion of more particles and organisms. Hence, after approximately one week, spores of macroalgae and protozoa constitute the secondary colonizers in the process of microfouling formation. Biofouling formation is a highly dynamic process, varying the degree and type of biofouling depending on the geographical location, physical and chemical factors of the substrate, species diversity and environmental factors. Among the environmental factors, temperature is undoubtedly one of the most important parameters playing a significant role in macroorganism settlement (*Iratxe Menchaca et al, 2014*).

1.3 Effects of Biofilm

Based on the size of the fouling organisms, they can be divided into two groups: (1) microfouling or biofilm, and on surfaces immersed in seawater, after its attachment, the conditioned film will develop and encourage the adhesion of larger organisms such as algae, mussels, and barnacles to the surface. Marine fouling organisms can be fixed on the surfaces of ships, buoys, offshore installations, and submarine hulls, and on the factory and power station refrigerating (2) macrofouling. Additional damage can be generated by biofouling in marine-based industries and infrastructure such as ship hulls, it will lead to deterioration of the protective coatings, increasing the frequency of dry-docking operations, increasing the fuel consumption, and reducing speed due to the roughness generated by them. The more the fuel consumption, the more the CO₂ emissions and transportation costs. Therefore, biofouling can cause both environmental and economic problems. When fixed on the surfaces of buoys, mines, offshore platforms, underwater acoustic devices, cables, and port installations or offshore platforms, their presence will accelerate corrosion processes, increase the materials repair frequency, and reduce operativity. They can also interrupt the electric current generation in power stations (*Ghazal Shineh et al., 2023*). The US Navy estimated in 2010 that the overall cost of hull fouling for the entire mid-sized naval surface ship class DDG-51 was 56 million dollars per year, and the overall cost for the entire US Navy surface fleet was 180-260 million dollars (*Schultz et al., 2011*). The anaerobic conditions found in the oil industry, combined with the abundance of microorganism substrates (e.g., hydrocarbons and organosulfur compounds), promote the formation of biofilms, resulting in microbially influenced corrosion (*Vigneron et al.,*

2016; Abu Bakar et al., 2017; Li et al., 2017). Marine Biofilms Cause Biocorrosion. The impact of corrosion was discovered to be especially significant in the oil and gas production, storage and transportation, and drinking and waste water industries.

1.4 Historical antibiofilm agents

The continuous increase in economic loss and the potential threats caused by the formation of marine biofilms, effective and economic control methods are necessary. Antifouling in marine refers to the process or coating used to prevent the biofouling, on water – immersed structure. At present, antibiofilm/antifouling coatings are the widely used and are an easy control method in the marine environment (Lexin Long et al., 2021). These coatings typically contain biocides or other chemicals that deter or kill marine life, helping to maintain the vessel's performance and efficiency by reducing drag and fuel consumption (Yebra, DM& Kiil, s. et al 2019).

However, the chemical inhibitors used in traditional coatings, such as amines, amides, organic tin, and cuprous oxide, are toxic and harmful, have no favorable environmental profile, and can be bioaccumulated. Along with long-term application of tributyl-tin (TBT)- based antifouling paints, there has been an increased concerns due to the increased concentrations resulted in environmental hazards to marine ecosystems (Bintang Marhaeni et al., 2011). It was then the reason for EU countries in 1989, followed by International Maritime Organization (IMO) and the Maritime Environment Protection Committee (MPEC) to restrict the use of TBT (I. K. Konstantinou et al., 2004). Since then, the development of alternative environmentally friendly antifoulants has been carried out to find alternative replacements.

Increasing attention is focused on the use of natural products to develop effective and less hazardous coatings. Natural compounds with antibiofilm activities generated from the metabolic mechanism of microorganisms can be an ideal substitute for the traditional chemical biocides, presenting environmentally friendly properties such as low toxicity and biodegradability. However, insufficient productivity and difficulty in synthesis limit the development of naturally synthesized compounds (De Carvalho, C.C.C.R. 2018, Plaza, G. et al., 2020, Gu, and Y.; Yu, L.; Mou, J. et al., 2020). Among the marine biofilms, as the early colonizers, bacteria are an important factor in determining the structure and function of a mature biofilm. Therefore, bacteria from

marine biofilms can be great targets for the discovery of antibiofilm compounds (Dang, H.; Lovell, C.R. 2016, De Carvalho, C.C.C.R. et al., 2018, and Dang, H.; Li, T.; Chen, M. et al., 2008). The development of alternative formulations such as deep-sea microbes [DSM] using this research. The natural source of antifouling compounds is marine organisms like certain types of algae, sponges, corals and bacteria. These organisms produce chemicals to defend themselves against fouling organisms like barnacles and algae. Antifouling activity in marine environments often involves various bioactive components, including bacteria, such as *Pseudoalteromonas* species- these bacteria produce compounds such as enzymes, exopolysaccharides and small molecules with antifouling properties. *Bacillus* species - produce extracellular enzymes and metabolites. *Vibrio* species – some *Vibrio* species produce compounds that inhibit biofilm. These bacteria prevent in marine environments and contribute to natural antifouling. The production of secondary metabolites like cyclic peptides, polyketides, terpenes by bacteria, which exhibit potent antifouling properties.

1.5 Secondary Metabolites

Microbial secondary metabolites play a role in several microbial interactions and are important modulators of microbial behavior. They can mediate interference competition, virulence, and nutrient scavenging and are produced by a multitude of microorganisms. However, nature has equipped marine organisms with a fascinating arsenal of secondary metabolites that play diverse roles in combating biofouling. Secondary metabolites possess anti-bacterial, anti-algal, or anti-fungal properties. These compounds discourage the settlement and growth of unwanted organisms on the surfaces of marine organisms, thus providing a natural defense mechanism against biofouling. Many secondary metabolites exhibit potent antibacterial properties, crucial in countering the growth of bacteria that play a pivotal role in biofilm formation a precursor to biofouling. By curbing bacterial colonization, these metabolites indirectly mitigate the risks associated with biofouling, contributing to the preservation of submerged surfaces. Certain secondary metabolites directly act as antifouling agents, disrupting the settlement and attachment processes of fouling organisms. Through interference with signaling pathways or adhesive properties, these metabolites hinder the ability of organisms to firmly attach to surfaces, effectively decrease biofouling and

reducing maintenance costs for marine infrastructure. Secondary metabolites exert allelopathic effects, inhibiting the growth or settlement of neighboring organisms. This competitive advantage allows producing organisms to outcompete potential fouling organisms for space and resources, thereby enhancing their survival prospects and maintaining ecological balance in marine ecosystems (*Dobretsov, S., & Thomason, J. C. 2011 and Eguía, E., & Rivera, L. 2016*).

CHAPTER – 2

LITERATURE SURVEY

2.1 Marine microbes

Marine microbes are tiny, single-celled organisms that live in the ocean and account for more than 98 percent of ocean biomass. The term “marine microbe” covers a diversity of microorganisms, including Bacteria, Archaea, Eukaryota, and viruses. These organisms are exceedingly small—only 1/8000th the volume of a human cell and spanning about 1/100th the diameter of a human hair. Up to a million of them live in just one milliliter of seawater. We know that microbes are the Earth’s processing factories of biological, geological, and chemical (biogeochemical) interactions. Most marine microbes exist in highly organized and interactive communities that are versatile, complex, and difficult to analyze. They account for more than 98 percent of ocean biomass and possess as much variability as the environments they inhabit.

These organisms are capable of existing in practically any environment and garnering energy from a variety of sources, ranging from solar radiation to chemosynthesis. They play many different roles in the marine environment, from being the base of the food chain to controlling much of the flow of marine energy and nutrients and being essential to the ocean’s health. Marine microbe communities can evolve rapidly in response to environmental shifts and could be used as indicators of ocean change. In fact, marine microbes can be “the canary in the coal mine” for the marine environment. In addition, they are also drivers of change in the ocean. Marine microorganisms are essential players in the health of marine ecosystems. They can have a positive influence on ecosystems’ health. For example, in coral reefs, microscopic coral-specific algae live in symbiosis with the coral polyps and provide them with photosynthetic energy used by the colony for its biological functions. Over the past decades, increases in microbial abundance as well as changes in community structure and function have occurred in response to increased environmental stress mostly related to climate change and eutrophication. “Microbialization of the ocean” is the term used to describe this

phenomenon. In addition, microbialization is often associated with changes in the pathogenicity of microbes leading to increases in disease occurrence of marine organisms and changes in ecosystem function and services. Ecosystem-based management is a main focus of the National Oceanic and Atmospheric Administration (NOAA) activities. Since marine microbes are the base of the food web and the main players in ecosystem functioning and services, it is essential for NOAA to better understand their exact roles. For many decades, NOAA has been involved in marine microbial science and is presently increasing its efforts focusing on the identification of microbe diversity, number, distribution and role in the ocean. (NOAA marine development website). "Microbes comprise 98 percent of the biomass of the world's oceans, supply more than half the world's oxygen, are the major processors of the world's greenhouse gases and have the potential to mitigate the effects of climate change. They are the cause of diseases that are suspected to be spreading due to global warming, yet paradoxically compounds they produce are potential cancer cures and solutions for combating human disease. Scientists are only just beginning to understand the important environmental roles that microbes play in marine systems - from feeding ecosystems to consuming waste and sequestering carbon" (*Australian Institute of Marine Science website*).

2.2 Anti-biofilm techniques

Different antibiofilm treatments, including antifouling coatings and surface alterations, have been developed. However, they are harmful to non-target creatures and have significant negative environmental effects. The creation of natural anti-biofouling products is the main topic study. In this study first conducts a preliminary examination of a DSM strains against bacteria that produce biofilms. Next, utilizing using an Ethyl acetate solvent, a crude extraction of the deep – sea (DSM) bacterial strain will be performed. Utilizing the bacterial growth kinetics, the biofilm inhibition assay using crystal violet staining, and the phenolsulphuric acid method, the activity of the crude extract (Concentrated extract containing bioactive compounds) will be assessed. In order to obtain the crude extract using column chromatography, the crude extract will be put through the process. And then, partially purified fractions were collected and a

Thin layer chromatography was performed. The techniques stated above will also be used to analyze the bioactive compounds.

2.3 Antibacterial activity

2.3.1 Crude extraction

The crude extract is the freshly obtained product acquired from the extraction process of the natural substance. According to the crude extract definition, the obtained extract is formed in liquids, semi-solids, and powder form. Crude extraction in the context of bacteria typically refers to a method used to isolate and extract various cellular components or biomolecules from bacterial cells in a relatively unrefined form. This process is often the initial step in more detailed biochemical or biotechnological studies aimed at characterizing the properties or functions of specific molecules within the cell. One common application of crude extraction in bacteria is for the isolation of proteins and compounds.

This process involves disrupting the bacterial cells to release their contents, followed by the separation of proteins from other cellular components such as nucleic acids, lipids, and carbohydrates. Crude extracts can then be further purified using techniques such as chromatography or electrophoresis for more detailed analysis. Common techniques for crude extraction of bacterial proteins include sonication, homogenization, or mechanical disruption to break open the cells, followed by centrifugation to separate the cellular debris from the soluble protein fraction (*Sambrook, J., & Russell, D. W. 2001*).

In order to achieve the necessary concentration, crude extracts, fractions, or pure chemicals are dissolved in a suitable solvent and serially diluted. The initial stage in separating the desired natural products from the base materials is extraction. According to the extraction principle, there are several extraction procedures, including solvent extraction, distillation, pressing, and sublimation. The technique with the highest usage is solvent extraction.

The process of extracting natural products involves the following steps: (1) allowing the solvent to permeate the solid matrix; (2) allowing the solute to dissolve in the solvents; (3) allowing the solute to diffuse out of the solid matrix; and (4) collecting the extracted solutes. The extraction will be made easier by any element that increases the above steps' diffusivity and solubility. The qualities of the extraction solvent, the particle size of the raw materials, the solvent-to-solid ration, the extraction temperature and the extraction period will affect the extraction efficiency.

For solvent extraction, the choice of the solvent is essential. The selection of solvents should take into account selectivity, solubility, cost, and safety. According to the principle of similarity and intervisibility (like dissolves like), solvents whose polarity values are close to those of the solute's are likely to perform better, and vice versa. In solvent extraction, alcohols (EtOH and MeOH) are the standard solvents (*CH. Ramesh et al.,2016*).

2.3.2 GROWTH KINETICS

Growth kinetics in bacteria refers to the study of the rate and pattern of bacterial growth over time under specific environmental conditions. This field of study examines various parameters such as population size, growth rate, generation time, and growth phases to understand how bacterial populations proliferate and interact with their environment. Growth kinetics typically involves measuring changes in bacterial population density over time, often represented by a growth curve, which typically includes lag phase, exponential (log) phase, stationary phase, and death phase. Understanding growth kinetics is crucial for various applications, including microbiology, biotechnology, food science, and medicine, as it provides insights into bacterial physiology, metabolism, and responses to external stimuli.

2.3.3 GROWTH CYCLE OF BACTERIA

Binary fission, which divides bacteria into two daughter cells, is the most popular mode of bacterial division. The length of time it takes a bacterium to complete one division is known as the generation time, and it varies depending on the type of bacteria and the characteristics of the media.

2.3.3.1 Lag Phase:

The single cell inoculation into the liquid media does not initiate cell division at the rate expected for the generation time. The bacteria expand in size rather than dividing into daughter cells during this phase as they get used to the new media. In this stage, bacteria produce the most important co-enzymes or enzymes, which are traces of substances but are essential for healthy growth and multiplication. A significant amount of protoplasm is being synthesized by the cell, which is also metabolically active. Every bacterial cell divides at the end of this phase and begins the subsequent phase of active multiplication.

2.3.3.2 Log Phase

After the substrate reaches its limit, the growth's logarithmic phase starts to progressively slow down while maintaining a constant cell count, resulting in a straight line. Since there are exactly as many divisions as there are deaths, the population does not change. Since there are only so many cells in the body, when an old cell dies, enough new cells can develop and multiply to keep the number constant.

2.3.3.3 Stationary Phase

After the substrate reaches its limit, the growth's logarithmic phase starts to progressively slow down while maintaining a constant cell count, resulting in a straight line. Since there are exactly as many divisions as there are deaths, the population does not change. Since there are only so many cells in the body, when an old cell dies, enough new cells can develop and multiply to keep the number constant.

2.3.3.4 Death Phase

When the substrate provided by dying cells is insufficient, the rate of bacterial death outpaces that of bacterial development, which causes a significant fall in the number of bacteria.

2.4 Biofilm inhibition assay

The microtiter plate biofilm assay is a useful method for assessing bacterial attachment by measuring the staining of the adherent biomass. Because it utilizes a 96-well plate format, it is suitable as a tool for screening large numbers of bacterial strains or species. The principle of a biofilm inhibition assay involves evaluating the ability of various compounds or treatments to prevent or reduce biofilm formation by microorganisms, a biofilm inhibition assay involves exposing biofilm-forming microorganisms to test compounds or treatments and assessing their ability to inhibit biofilm formation (*Karla Gutierrez-Almada et al.,2020*). This assay serves as a valuable tool for screening potential antimicrobial agents, elucidating mechanisms of biofilm inhibition, and developing strategies to combat biofilm-related problems. There are several methods for conducting biofilm inhibition assays. (1) Preparation of Biofilm: Microorganisms are cultured under conditions that promote biofilm formation. This usually involves inoculating the microorganisms into a suitable growth medium and allowing them to adhere to a surface or form aggregates, leading to biofilm development. (2) Exposure to Test Compounds: Once the biofilm has formed or is in the process of forming, the microorganisms are exposed to the test compounds or treatments being evaluated for their ability to inhibit biofilm formation. These compounds can include antibiotics, antimicrobial agents, natural products, chemical compounds, or physical treatments. (3) Incubation Period: After exposure to the test compounds, the microorganisms are typically incubated under controlled conditions for a specified period. This allows time for the compounds to exert their effects on the biofilm-forming microorganisms. (4) Assessment of Biofilm Formation: Following the incubation period, the extent of biofilm formation is assessed. This can be done using the dye crystal violet (CV) in this experiment to evaluate the degree of biofilm development. However, a number of other colorimetric and metabolic stains have been described for the microtiter plate assay for quantifying biofilm formation. The microtiter plate test is an essential tool for the research of biofilms because of its simplicity, low cost, and adaptability. (5) Comparison and Analysis: The degree of biofilm inhibition caused by the test compounds is compared to control samples, which may include untreated biofilms or biofilms treated with known inhibitors or antimicrobial agents. Statistical analysis may be performed to

determine the significance of any observed differences between experimental groups.

(6) Interpretation of Results: The results of the biofilm inhibition assay provide information about the effectiveness of the test compounds or treatments in preventing or reducing biofilm formation. Compounds that demonstrate significant inhibition of biofilm formation may be further evaluated for their potential as antimicrobial agents or for use in preventing biofilm-related issues in various applications (*Lexin Long et al., 2020*).

2.5 Phenol-sulphuric acid method

The phenol-sulfuric acid assay is a common method used to quantify the total concentration of carbohydrates in a sample. Its purpose is to determine the amount of reducing sugars (mono- and disaccharides) or polysaccharides present in a solution. This assay is sensitive to mannose from 1 – 100 μg but is sensitive towards other carbohydrates. This assay was used for the quantification of complex carbohydrates that constitutes bacterial cell wall. Whole Gram-positive bacterial lysates can be detected using this assay providing a fast, colorimetric alternative to bacterial quantification (*Jason Fan., 2015*).

Among many colorimetric methods for carbohydrate analysis, the phenol–sulfuric acid method is the easiest and most reliable method. It has been used for measuring neutral sugars in oligosaccharides, proteoglycans, glycoproteins, and glycolipids. This method is used widely because of its sensitivity and simplicity. Moreover, our method is more convenient, requiring neither shaking nor covering, and takes less than 15 min to complete. The speed and simplicity of this method would make it most suitable for analyses of large numbers of samples such as chromatographic fractions (*Masuko, T et al., 2005*).

2.6 Column chromatography

Column chromatography is simple and the most popular separation and purification technique. Both solid and liquid samples can be separated and purified by column chromatography. Column chromatography consists of a stationary solid phase that adsorbs and separates the compounds passing through it with the help of a liquid

mobile phase. On the basis of their chemical nature, compounds get adsorbed and elution is based on differential adsorption of a substance by the adsorbent. Silica gel and alumina are thought of as the two most typical examples of mobile phases for column chromatography, and different solvent compositions based on the nature of compounds to be separated and isolated, are used in column chromatography. In column chromatography, a cylindrical glass tube, which is plugged at the bottom by a piece of glass wool or porous disc, is filled with slurry (adsorbent) and a suitable solvent. Samples to be separated are mixed with silica and introduced at the top of the column and allowed to move with the solvent. With polarity differences, compounds are adsorbed at different regions and desorbed with suitable solvent polarity. The compound of higher adsorption ability will be adsorbed at the top and that with the lower one will be at the bottom. By adding the solvent at the top, compounds get desorbed and pass through the column and this process is called elution. Compounds are separated based on the polarity of the molecules and differential adsorption to the adsorbent. Depending on their polarity, molecules flow through the column at different rates, effectively separating them. Under pressure or gravity, the organic solvent (mobile phase) passes through a narrow tube enclosing the stationary phase. A mixture is purified by collecting the eluted mobile phase in test tubes as it elutes from the column. Column chromatography is a versatile and easy-to-use method for purifying compounds (*Nishi Srivastava, ... Ravindra N. Kharwar.,2021*).

2.7 Thin Layer Chromatography

Thin Layer Chromatography (TLC) is a type of chromatography which is based upon the distribution of biomolecules between two immiscible phases. TLC was originally developed to separate lipid molecules and can be used to identify components in a sample, and for preparative purposes. In TLC the stationary phase is a polar absorbent, like finely ground alumina (Al_2O_3) or silica (SiO_2) particles which are coated on a glass slide or plastic sheet to create a thin layer of the particular stationary phase. Silica contains some free – OH groups which form hydrogen bonds or other Van-der-Waals interactions with the analyte components and as a result adsorption takes place. Sometimes a small amount of a binder such as plaster of Paris is mixed with the

absorbent to facilitate the coating. The mixture to be separated is dissolved in a solvent and the solution is spotted at one end of the coated TLC plate. The plate is placed with spotted end down in a covered jar containing a shallow layer of suitable solvent. The solvent (mobile phase) is allowed to move up the plate by capillary action through the adsorbent at its own rate and as a result differential partitioning occurs between the components of the mixture dissolved in the solvent and the stationary adsorbent phase. The more strongly a given component of a mixture is adsorbed onto the stationary phase, the less time it will spend in the mobile phase and the more slowly it will migrate up the plate. When the solvent front has moved to within about 1 cm of the top end of the adsorbent, the plate should be removed from the developing chamber. The R_f values strongly depend upon the nature of the adsorbent and solvent.

2.8 Gas Chromatography-Mass Spectrometry (GC-MS)

Gas chromatography (GC) is the separation technique of choice for smaller volatile and semi-volatile organic molecules such as hydrocarbons, alcohols and aromatics, as well as pesticides, steroids, fatty acids and hormones, making this analytical technique common in many application areas and industry segments, particularly for food safety and environmental testing. When combined with the detection power of mass spectrometry (MS), GC-MS can be used to separate complex mixtures, quantify analytes, identify unknown peaks and determine trace levels of contamination.

CHAPTER – 3

3.1 Aim

To isolate & extract the natural bacterial compound from marine source

3.2 Objective

- 1) To isolate marine bacteria from different marine source.
- 2) To extract compound from marine bacteria.
- 3) To perform characterization studies to confirm compound.

Techniques:

1. Column Chromatography
 2. Thin layer Chromatography
 3. GCMS Chromatography
- 4) To check the inhibition efficiency of isolated compound against biofilm bacteria.

CHAPTER – 4

MATERIALS AND METHODS

4.1 Isolation of biofilm bacteria

The bacterial samples were collected from boats and ships. Then serial dilution was performed to ($10^{-1} - 10^{-5}$) it will decrease the concentration of the sample. Followed by spread plate method was performed using Zobell marine agar (ZMA) agar, after 24 hrs incubation the individual colonies were picked and streak into another ZMA plate. The DSM strain isolates was inoculated into ZMA to activate the bacteria from glycerol stock and incubated overnight at room temperature in the incubator. The pure bacteria colony was isolated via repeated streaking.

4.2 Extraction of Bioactive Compounds

The antibacterial potential of DMS strain was extracted with active solvent of ethyl acetate by liquid- liquid extraction method. The DMS strain was inoculated in ZMB broth and the broth was incubated at room temperature for 3 to 5 days.



Fig 4.2.1 Bacterial inoculation

4.2.1Centrifugation:

After fermentation the broth was centrifuged at 7500rpm for 20minutes and the supernatant was filtered by Whattman No.1 filter paper. Equal volume of ethyl acetate was added with filtrate of supernatant in the ratio of 1:1(w/v) and shaken vigorously 1hr for complete extraction. The organic phase was separated from aqueous phase using separating funnel and transferred into a clean flask. Following this, the extract was concentrated by using rotary evaporator at 40°C under vacuum pressure. And kept in freezer until antifouling activity was performed (*Nurul Najihah Rawi et al., 2023*).



Fig 4.2.1.1 Bcterial culture after centrifugation

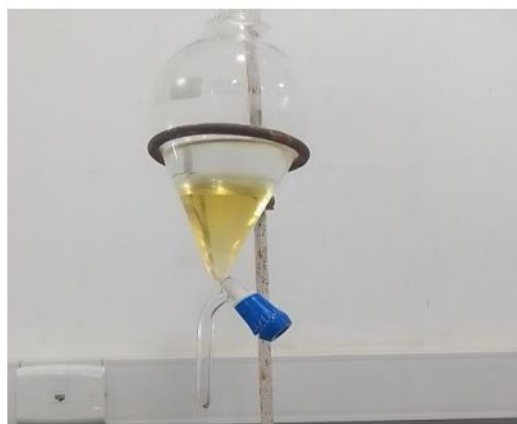


Fig 4.2.1.2 Liquid liquid extraction

4.3 Biochemical identification test

4.3.1 Gram staining

The gram's staining was performed to determine the isolated bacteria was gram positive or negative. A small amount of the bacterial colony was transferred to a glass slide using a sterile loop. The slide can be either air dried or dried with help of heat over a gentle flame. Crystal violet stain is added over the fixed culture. After 1 minute the stain is poured off, and the excess stain is rinsed with water. Iodine solution is used to cover the seamer for 30 to 60 seconds. Rinse off the iodine solution with water. Add the decolorizer for 5 to 10 seconds. Rinse off decolorizer with water. Flood the slide with safranin. Allow it for 30 seconds. Rinse with distilled water. Dry the slide on absorbent paper and place the slide under the microscope and note the observation.

Gram positive bacteria:

It has a thick peptidoglycan cell wall. It retains the dye in gram testing, causing them to have a blue color, which can be seen under the microscope.

Gram negative bacteria:

It has an outer membrane they have a thinner peptidoglycan cell wall. They do not hold the blue dye used in cell gram testing and do not appear blue. Instead, they appear red or pink in color.

4.4 Characterization studies to confirm compound

4.4.1 Column chromatography

The compound mixture that needs to be separated is added from the top of the column in the first step without disturbing the top level. The process of adsorption on the surface of the silica starts as soon as the tap is opened. Touching the glass column's sides carefully adds the stationary phase solvent mixture without causing any disturbance. Over the course of the experiment, the solvent is added as needed. To begin moving the compounds in the mixture, the tap is turned on. Molecular polarity of the sample is what causes the movement. Comparing the polar and non-polar components, the non-polar component moves faster.

4.4.2 Purification of bioactive compound by thin layer chromatography

A pre-made TLC plate was trimmed to the proper size and the samples were found at the TLC plate's one end. The plates were held in the mobile phase-containing beaker so that the end closest to the sample application should touch the mobile phase. Set the TLC plate to the 3/4 level and let the chromatogram run for roughly 1-2 hours. For band visualization, the plates were dried and kept in crystal iodine strains. A formula can be used to calculate the Rf value of the samples.

$$R_f = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent}}$$

4.4.3 Gas Chromatography-Mass Spectrometry (GC-MS)

Gas chromatography (GC) is the separation technique of choice for smaller volatile and semi-volatile organic molecules such as hydrocarbons, alcohols and aromatics, as well as pesticides, steroids, fatty acids and hormones, making this analytical technique common in many application areas and industry segments, particularly for food safety and environmental testing. When combined with the detection power of mass spectrometry (MS), GC-MS can be used to separate complex mixtures, quantify analytes, identify unknown peaks and determine trace levels of contamination.



Fig 4.4.3.1 GCMS QP 2010

4.5 To check the inhibition efficiency of isolated compound against biofilm bacteria

4.5.1 Growth kinetics

A 96- well plate was used to hold several biofilm bacterium samples and ZMB broth was prepared as the growth medium. Different bacterial strains that can form biofilms were collected. Add biofilm bacteria to each well at the rate of 100 μ l. For the wells, add crude extract at a rate of 100 μ l and the first columns were used as a control. And the growth kinetics of the bacteria were monitored for 24 hours using a microplate reader (D. Inbakandan et al., 2013).

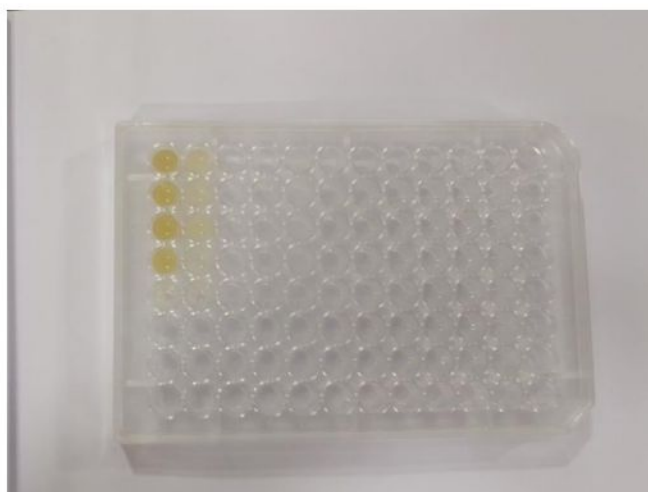


Fig 4.5.1 Growth kinetics of crude extraction

4.5.2 Biofilm inhibition assay (CV assay)

Cell suspension was inoculated in 5ml of ZMB medium. After 2 days incubation 150 μ l of cell suspension and 150 μ l of DSM strains were inoculated in sterile 96 well plate. After 48 hours incubation the plate was washed to remove planktonic cells, with 99% methanol. Plates are washed twice times and air dried. Then 200 μ l of Crystal violet solution was added to all well, after 5 minutes the excessive crystal violet was dissolved in 33% acetic acid. Biofilm growth was monitored in terms of OD 520 nm using micro plate reader.

4.5.3 Phenol sulfuric acid assay

Create a 5% w/v phenol in water solution. Add 50 μ l of sample into 96 well plate. Eject 150 μ l concentrated H_2SO_4 into each well. Sugar present in the sample will react with the acid to quickly change into a yellow- brown colour. Add 30 μ l of 5% phenol. Let stand for 10 minutes at room temperature. Read absorbance at a wave length of 490 nm

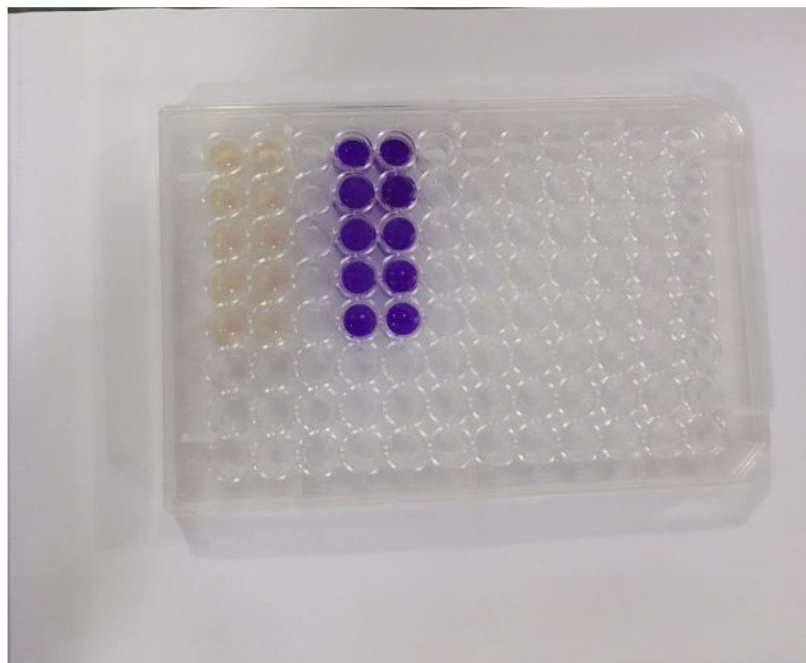


Fig 4.5.3.1 Phenol sulphuric acid assay and CV assay

CHAPTER – 5

RESULT AND DISCUSSIONS

5.1 Isolation of biofilm bacteria:

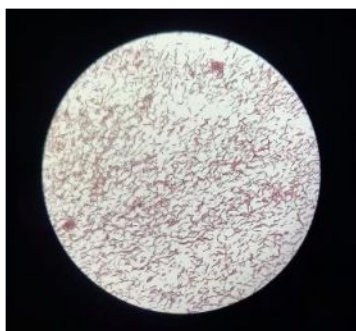
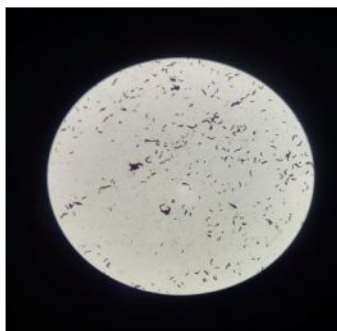
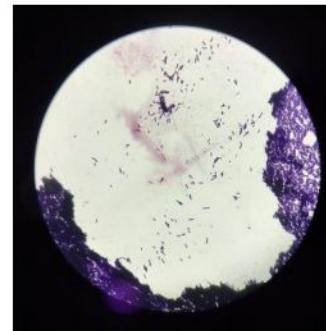


Fig 5.1(a) Gram positive



5.1(b) Gram negative



5.1(c) Gram negative

The morphology of bacteria colonies and biochemical characterization can help in the partial identification of marine isolates. The bacterial colonies were cultured on marine agar and the further characterization were observed under a light microscope.

Gram staining: The biochemical analysis was performed to identify the biofilm forming bacteria using gram staining test. **Gram positive bacteria:** It have a thick peptidoglycan cell wall. It retains the dye in gram testing, causing them to have a blue color, views them under a microscope. **Gram negative bacteria:** It have an outer membrane they have a thinner peptidoglycan cell wall. They do not hold the blue dye used in cell gram testing and do not appear blue. Instead, they appear red or pink in color.

5.2 Characterization studies to confirm compound

5.2.1 Column chromatography

The compound mixture to be separated by using column chromatography. Molecular polarity of the sample is what causes the movement. Comparing the polar and non-polar components, the non-polar component moves faster. Successfully the fractions collected from the column chromatography.



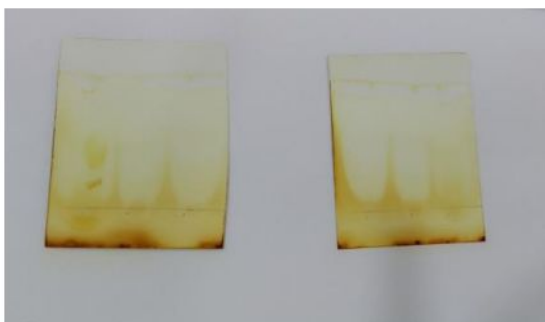
5.2.1.1(a) Column



5.2.1.1(b) Fractions collected from column

Using column chromatography 10 fractions of bioactive compounds were collected, for the antibiofouling activity. And the fractions collected were subjected to other characterization tests such as TLC and GCMS.

5.2.2 Purification of bioactive compounds by thin layer chromatography



5.1.3 TLC

The crude bioactive compound extracted using ethyl acetate displayed maximum activity and was selected for further purification. In the present study, methanol: ethyl acetate (30:70) was chosen as suitable solvent system based on the fractionation of spots on the TLC plate. The visible fractions separated were visualized by placing the silica gel plate in iodine vapor in a glass chamber. The fractions with similar R_f values on TLC plate were calculated.

$$R_f = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent}}$$

Upon fraction with TLC, the crude extract of DSM revealed the presence of bioactive compounds with the R_f value of 0.76.

5.2.3 Gas Chromatography-Mass Spectrometry (GC-MS)

Table 5.2.3.1 Compounds identified by GCMS

PEAK	RETENTION TIME	AREA	AREA%	NAME
1	3.621	17743379	13.86	9-Octadecenoic Acid (Z)-
2	32.833	1289086	1.01	Hexadecanoic Acid, 2-Hydroxy-1,3-Propanediyl Ester
3	37.059	23384086	1.83	15Hydroxypentadecanoic acid
4	38.010	6690556	5.22	9,12-Octadecadienoic acid (Z, Z)-
5	38.127	20154189	15.74	cis-9-Hexadecenal
6	40.091	1961911	1.53	cis-9-Hexadecenal
7	40.775	7664641	5.99	Glycidol stearate
8	42.849	903027	0.71	9,12-Octadecadienoic Acid (Z, Z)-, 2,3-Dihydroxypropyl Ester
9	43.642	25929463	20.25	(R)-(-)-14-Methyl-8-hexadecyn-1-ol
10	43.734	23191206	18.11	9-Octadecenoic acid, 1,2,3-propanetriyl ester, (E,E,E)-
11	43.849	2813329	2.20	14-Methyl-8-Hexadecyn-1-Ol #
12	44.148	3441261	2.69	Glycidol stearate
13	46.000	5279873	4.12	2-(Dodecanoyloxy)-1-(Hydroxymethyl)Ethyl Laurate #
14	46.189	6038051	4.72	Dodecanoic Acid, 1,2,3-Propanetriyl Ester
15	46.325	2616949	2.04	Dodecanoic Acid, 1,2,3-Propanetriyl Ester

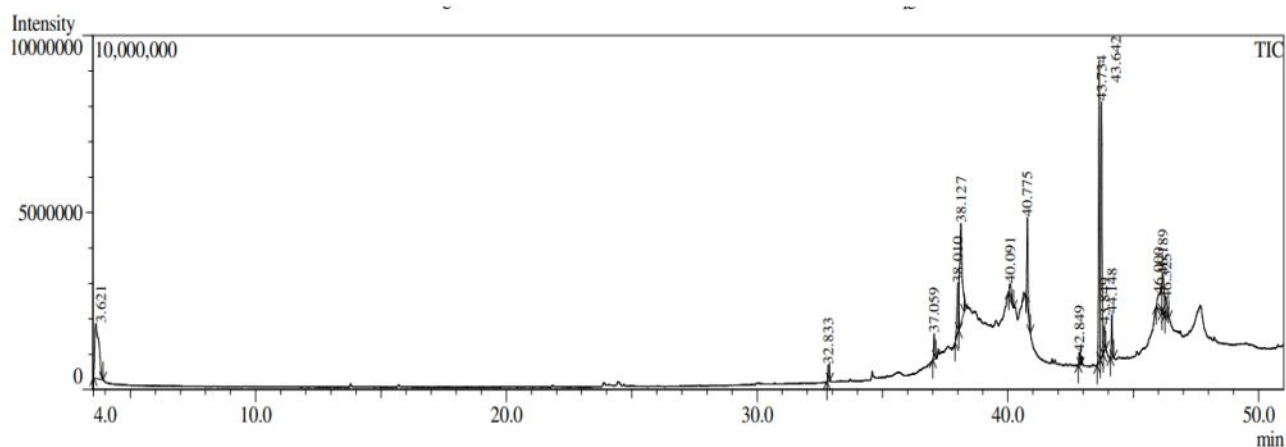


Fig 5.2.3.1 Peaks of compounds analyzed under GCMS

In the present study, the investigation of crude extract from deep sea microbes revealed the presence of various bioactive compounds such as 9-Octadecenoic acid, Hexadecanoic Acid, 2-Hydroxy-1,3-Propanediyl Ester, 15-Hydroxypentadecanoic acid, 9,12-Octadecadienoic acid (Z,Z), cis-9-Hexadecenal, Glycidol stearate, 2,3-Dihydroxypropyl Ester, (R)-(-)-14-Methyl-8-hexadecyn-1-ol, 9-Octadecenoic acid, 1,2,3-propanetriyl ester, (E,E,E)-, 2-(Dodecanoyloxy)-1-(Hydroxymethyl)Ethyl Laurate, Dodecanoic Acid, 1,2,3-Propanetriyl Ester. The 9-Octadecenoic acid bioactive compound shows the antibiofilm activity against the bacterial symbionts of gastropods (Viju, N., Punitha et al., 2020). Hexadecanoic Acid shows the maximum level of inhibition against the seaweeds such as *Padina tetrastromatica*, *Caulerpa taxifolia*, and *Amphiroa fragilissima* (Suresh, M., Iyapparaj, P et al., 2016). 2-Hydroxy-1,3-Propanediyl Ester and 9,12-Octadecadienoic acid (Z, Z) compounds show the antibiofouling activities against marine sponges (Lee, O.O et al., 2007). cis-9-Hexadecenal bioactive compound carried out to evaluate the antagonistic efficacy of *Aspergillus versicolor* against the soil and seed inhibiting destructive plant pathogen *Macrophomina phaseolina*. The tested antagonist was confirmed by rDNA sequencing (Khan, I. H., & Javaid, A. 2022). Cis-9-Hexadecenal has been previously reported by Mensah-Agyei, G. O et al., 2020, used for the antibacterial activity. 15-hydroxypentadecanoic acid, along with other related compounds, could serve as potential markers for nonalcoholic steatohepatitis (Harris, et al., 2014). Chen, J., Li, X., 2017 according to them Glycidol stearate compound used for synthesis of soyabean

oil-based vinyl chloride. 2,3-Dihydroxypropyl Ester compound used for Nematicidal activity has been reported to the Sirisha Tadigiri et al., 2020. According to the Jamal, M. T., & Satheesh, S. (2022) Dodecanoic Acid used for the antifouling activity. Followed by the Propanetriyl Ester used for the antimicrobial activity.

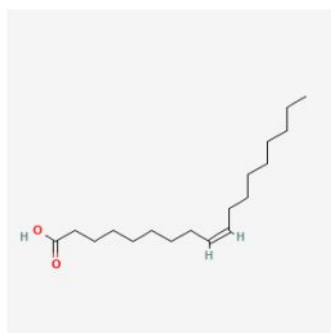


Fig: 5.2.3.2(a) 9-Octadecenoic acid
C₁₈H₃₄O₂

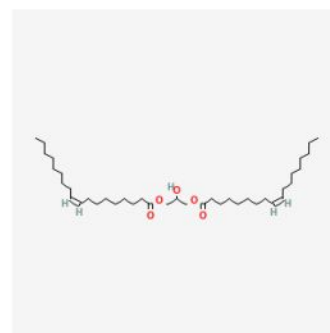


Fig 5.2.3.2(b) 2-Hydroxy-1,3-Propanediyl Ester
C₃₇H₇₂O₅

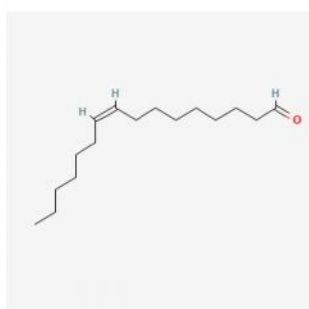


Fig 5.2.3.2(c) Cis9Hexadecenal C₁₆H₃₀O

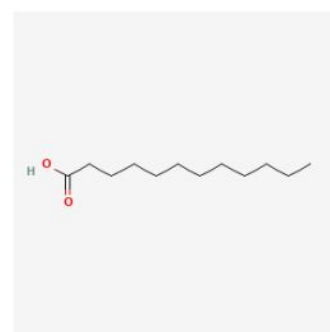


Fig 5.2.3.3 (d) Dodecanoic Acid C₁₂H₂₄O₂

5.3 Growth kinetics

Table 5.3.1 Optical density(520nm) value for *Bacillus* species

Time (Hrs)	A1-Control OD Value	A2-Sample OD Value
0	1.707	1.049
2	1.719	1.175
4	1.676	1.286
6	1.647	1.337
8	1.631	1.404
10	1.677	1.452
12	1.699	1.483
14	1.713	1.562
16	1.737	1.585
18	1.792	1.572

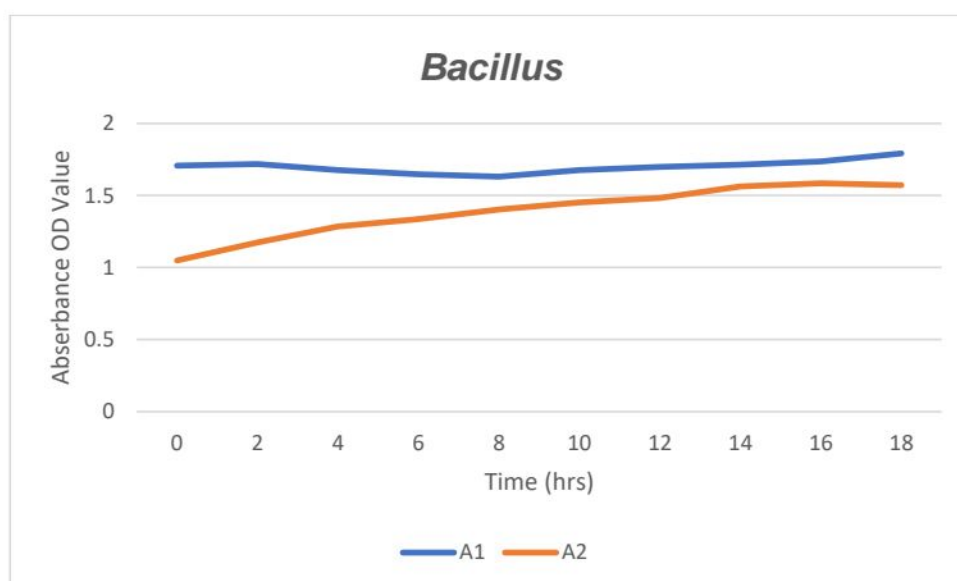


Fig: 5.3.1 Percentage of growth inhibition

Table 5.3.2 Optical density (520nm) value for *Pseudomonas fluorescens*

Time (Hrs)	Control-B1 OD Value	Sample-B2 OD Value
0	1.63	0.841
2	1.713	0.486
4	1.65	0.932
6	1.648	0.822
8	1.672	0.929
10	1.697	0.943
12	1.703	1.077
14	1.727	1.14
16	1.746	1.17
18	1.833	1.192

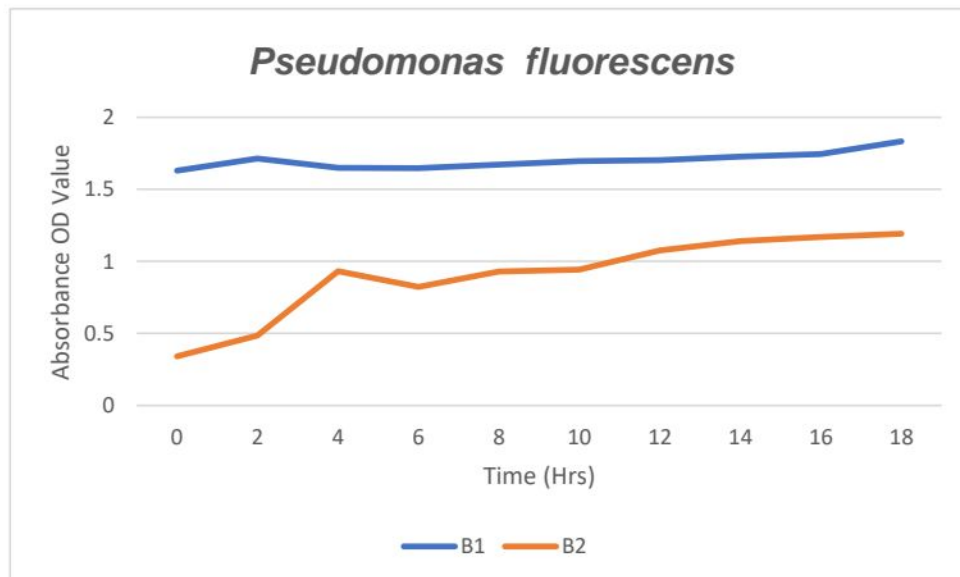


Fig: 5.3.2 Percentage of growth inhibition

Table 5.3.3 Optical density (520nm) value for *Pseudomonas aeruginosa*

Time (Hrs)	Control-C1 OD Value	Sample-C2 OD Value
0	1.649	0.759
2	1.71	0.723
4	1.731	0.926
6	1.809	1.058
8	1.863	1.357
10	1.902	1.421
12	1.925	1.477
14	1.972	1.515
16	2.031	1.519
18	2.048	1.5

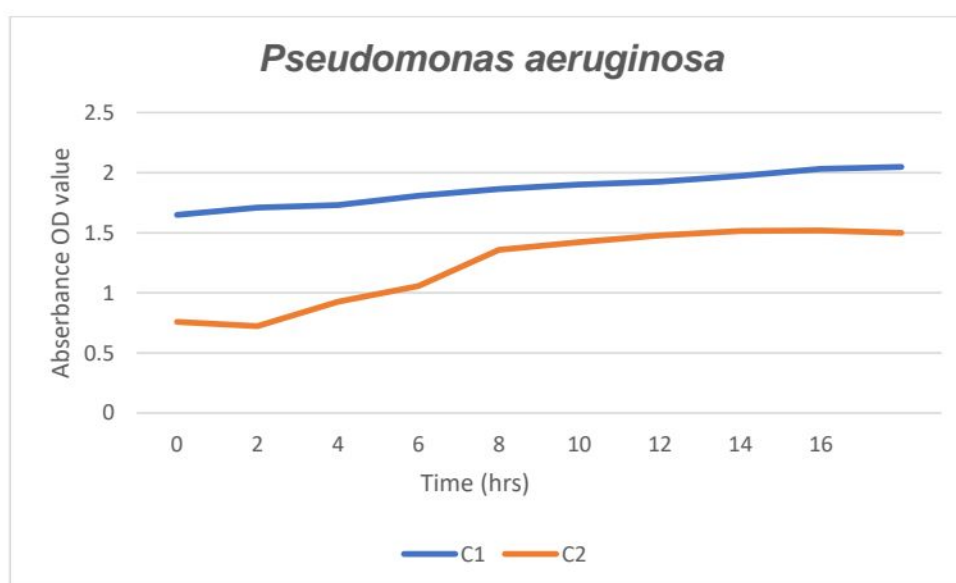


Fig: 5.3.3 Percentage of growth inhibition

$$\text{Percentage} = \left(\frac{\text{Final value} - \text{Initial value}}{\text{initial value}} \right) \times 100$$

Among the tested microorganisms, *Pseudomonas aeruginosa* exhibited the maximum growth inhibition of about 97.63%. *Bacillus* and *Pseudomonas fluorescens* showed

49.90% and 41.73% inhibition respectively, as indicated in Tables 5.3.1, 5.3.2, and 5.3.3, respectively.

5.4 Biofilm inhibition assay (CV assay)

A biofilm inhibition test was conducted to evaluate the effectiveness of concentrations of crude extract in inhibiting various biofilm bacteria. Quantifying the biofilm is done using crystal violet staining. Crystal violet binds to the EPS that wells', measured at 600 nm with a multimode reader.

Table 5.4 Optical density values for CV assay

Assay	OD Value		
	M1	M2	S3
Control (Only BS)	3.555	3.33	3.451
Sample (BS treated with DSM 13)	3.458	3.11	2.931

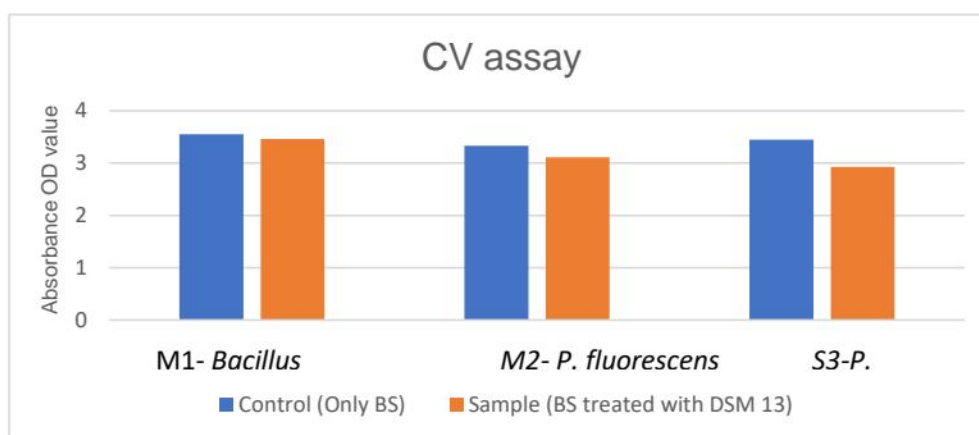


Fig 5.4.1 Biofilm inhibition assay (CV assay)

To calculate the OD value percentage of the CV assay, typically use the formula,

$$\text{Percentage \%} = 100 - \left(\frac{\text{OD Value of Sample}}{\text{OD Value of Control}} \right)$$

The bar graph comparing treated (with DSM strains) and untreated (without DSM) samples illustrates a consistent reduction in biofilm levels across all treated

concentrations. This decline suggests that the concentrated crude extract potentially inhibited bacterial growth. Specifically, *Bacillus* exhibited a 99.03% inhibition, *Pseudomonas fluorescens* showed 99.07% inhibition, and *Pseudomonas aeruginosa* demonstrated 99.15% inhibition.

5.5 Phenol sulphuric acid assay

In this method the concentrated sulfuric acid breaks down the polysaccharides, oligosaccharides and disaccharides to monosaccharides. The absorptivity of the different carbohydrates was detected and it has been studied.

Table 5.5 Optical density values for phenol sulphuric acid assay

Assay	OD Value		
	M1	M2	S3
Control (Only BS)	0.251	0.123	0.173
Sample (BS treated with DSM 13)	0.106	0.111	0.117

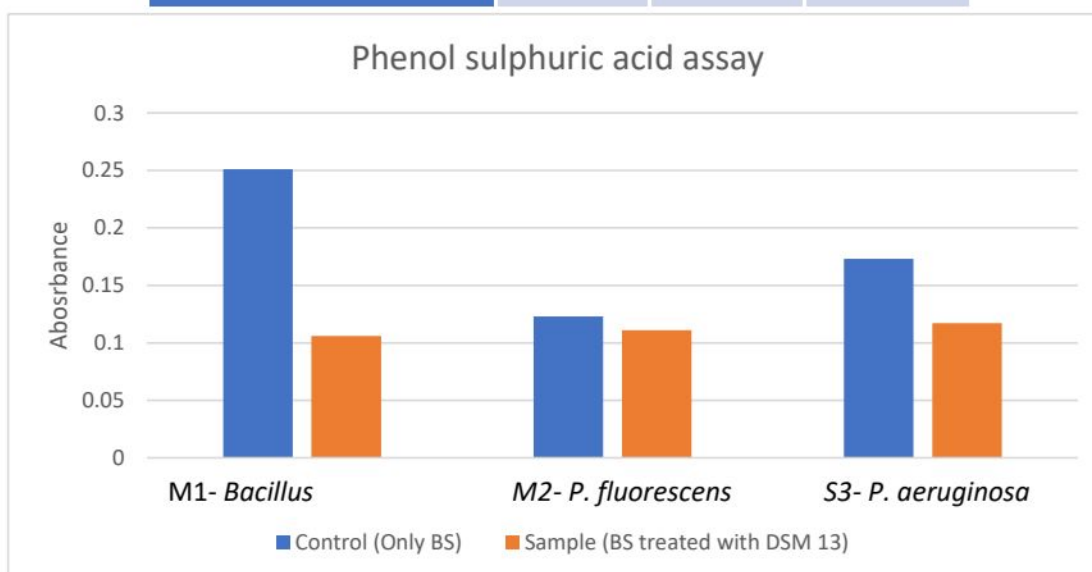


Fig:5.5.1 Phenol sulphuric acid assay

The comparison between treated (with DSM strain) and untreated (without) samples was shown using values presented as a bar graph. In all treated samples of all

concentrations, a decrease in the amount of biofilm can be noted this might be as a result of bacteria being inhibited by the concentrated crude extract. For *Pseudomonas aeruginosa* shows the 99.57% of inhibition level, for *Pseudomonas fluorescens* shows 99.09% inhibition and *Bacillus* shows 99.32% of inhibition.

CHAPTER – 6

CONCLUSION

The crude extract from the marine deep-sea bacteria showed good inhibition against the biofilm forming bacterias such as *Pseudomonas aeruginosa*, *Pseudomonas fluorescens* and *Bacillus*. This is confirmed using the assay like Biofilm inhibition assay (CV assay) and Phenol sulfuric acid assay. Over the above-mentioned strains *Pseudomonas aeruginosa* showed highest biofilm inhibition percentage of about 97.63% to 99.57% when compared to other two strains. Through GCMS analyses we found that antibiofilm efficient compounds like 9-Octadecenoic acid, 2-Hydroxy-1,3-Propanediyl Ester, Cis9Hexadecenal, Dodecanoic Acid can be produced from deep sea microbes. Hence for future studies these 4 compounds can be produced in pure form with confirmation techniques like HPLC and NMR, followed by bulk production of compounds for large scale antifouling applications.

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