

**STUDIES ON EXTRACTION OF SECONDARY METABOLITES FROM
MARINE BACTERIAL STRAINS FOR ANTIBIOFILM APPLICATION.**

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

By

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DEPARTMENT OF BIOTECHNOLOGY

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INSTITUTE OF SCIENCE AND TECHNOLOGY

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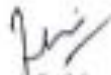
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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of KAVINMALAR.M
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Internal Guide

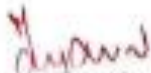
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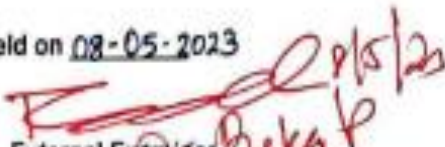

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ABSTRACT

Biofilm is the accumulation of various microorganisms on different surface immersed in sea water for a long period of time. Biofilm formation seems to be the major problem in marine environment with adverse effects like structural damage to pillars and oil rings, economic losses to shipping industry due to increased fuel consumption and so on. Various antifouling techniques have been developed which includes different anti-biofouling paints and surface modification. But they have a serious disadvantage on the environment and toxic to nontarget organisms, And hence this study focuses on development of natural product based antifouling coatings to minimize the damage caused by biofouling. The study starts with the preliminary screening of various marine bacteria against biofilm forming bacteria using double plating method. Then the potent bacterial strain will be subjected for crude extraction using various solvents. The activity of crude extract will be evaluated using well diffusion method, bacterial growth kinetics and biofilm inhibition assay using crystal violet staining method. The crude extract will then be subjected for characterization to determine the presence of functional groups by using FTIR – ATR for analyzing the various compounds using GC-MS. The crude extract will then be processed to obtain partial purified fractions by using column chromatography technique. The activity of the partial purified fractions will also be analysed using the same above-mentioned methods.

Keywords: Biofilm, Marine Bacteria, Crude Extract, HPLC, Pure Compound.

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LIST OF ABBREVIATIONS

- LB-Luria Bertani
- % -percentage
- Cm - centimeter
- OD-optical density
- UV-Ultra Violet
- CV-crystal violet
- PBS-phosphate Buffer Saline
- BFB - Biofilm Forming Bacteria
- TLC - Thin layer chromatography
- μ L - Microliter

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CHAPTER - 1

INTRODUCTION

Bacterial cells are generally immersed in self-produced extracellular matrix in dense communities known as biofilms. Due to the potential applications of these interactions in biological, medicinal, and industrial systems, competitive interactions between strains within the biofilm setting are being researched. Biofilms attach to the surfaces, for instance by bacterial adhesion. Cells and the extracellular matrix make up the biofilms. In some locations, such as on medical devices or inside pipelines, biofilms can be harmful. Surface colonization by biofilms is an important means of marine bacteria, that survives in an oligotrophic environment because it provides favorable conditions for its growth (Jefferson, 2004).

Biofilms are additional polymeric matrices that self-assemble from a sessile microbial community and are distinguished by the cells' adhesion to either biotic or abiotic surfaces. The extra cellular, slime-like layer completely encloses the microbial cells and shields them from numerous outside influences. The biofilm's constituent parts play a crucial role in both its structural and functional characteristics. Major groups of macromolecules are found in microbial biofilms include nucleic acids, polysaccharides, proteins, enzymes, lipids, humic compounds, and ions.

Most microbes are not free floating in reality as they are found in biofilms. A community comprising colonies are from the species or either from other genera and they adhere to surfaces. Microorganisms move from free floating to anchored state such as planktonic microorganisms to a sessile surface-attached one. Contact with a surface causes changes in gene expression as well as robust micro colony adhesion. This happens after a few hours. Matrix formation is related to biofilm maturation. The matrix is significant because it ensures stability and protects against environmental damages.

Polysaccharides, water, lipids, proteins, and extracellular DNA make up its composition.

Biofilms can be found in the environment, water systems, and other places. The existence of these components makes them tough and allows them to survive in harsh

environments. Different types of forces of attraction are responsible for binding the microbial cells together in a biofilm, and the interstitial spaces and water channels play an important role in the circulation of nutrients to every cell in the biofilm. This study adds a remark on bacterial biofilms and seeks to provide information on elements ranging from their detrimental effects on the human community.

1.1 Biofilm formation

Biofilm formation is a process with distinct stages. The biofilm developmental cycle is thought to include (i) initial attachment of microbes to a surface or each other, (ii) formation of microcolonies, (iii) biofilm maturation, and (iv) biofilm dispersal. Bacterial physiology and phenotypic responses suggestive of the presence of a distinct biofilm are among the various biofilm stages. Such structures can form on a variety of abiotic and biotic surfaces (Lasa et al., 2005) and serve as the primary bacterial reservoir in the majority of ecosystems (Costerton et al., 1999). A biofilm is made up of single cells or microcolonies embedded. Bacterial cells are included in biofilms. Extracellular polymeric substances (EPS) produced by microorganisms serve similar functions in both marine snow and biofilms: in addition to adsorbing and transporting solutes and microorganisms, they also serve as a protective "roof" for cells, particularly in harsh environments. The organization provided by the EPS aids in (i) nutrient and toxic compound entrapment, (ii) extracellular enzyme activity and stabilization by buffering pH and salinity fluctuations, (iii) quorum sensing, (iv) genetic information exchange, (v) physical anchorage, and (vi) predation protection (de Carvalho and Fernandes, 2010; Decho and Gutierrez, 2017). (Karpov et al., 2012) proposed the following types of biofilms observed during the first stage of fouling: Type I is a surface film containing only living and dead bacterial cells; type II is a diatom film deposited on top of the bacterial layer; and type III is formed when related species settle on top of the diatom layer (Karpov et al., 2012)

1.2 Secondary Metabolites

Marine habitats are a rich source of molecules of biological interest. Marine bacteria, in particular, have drawn attention due to their ability to synthesize structurally diverse classes of bioactive secondary metabolites with high biotechnological potential. The last few decades have seen numerous discoveries of biomolecules from bacterial symbionts, which were previously thought to be metabolites of marine animals. Many compounds isolated from marine bacteria are unique in their structure and biological activity.

On the contrary, metabolites from microorganisms is a rapidly growing field, owing, at least in part, to the suspicion that a number of metabolites obtained from algae and invertebrates may be produced by associated microorganisms. Bacteria and fungi isolated from seawater, sediments, algae, fish, and, most notably, marine invertebrates such as sponges, mollusks, tunicates, coelenterates, and crustaceans are being studied. Although it is still too early to define trends, it can be stated that metabolites produced by microorganisms are in most cases quite different from those produced by invertebrate hosts.

1.3 Effects of Biofilm

Bacteria form multispecies biofilms with other microorganisms. Bacteria and microalgae cause microfouling and allow larger organisms such as algae, mussels, and barnacles to adhere, resulting in macrofouling. They adhere to almost all seawater-immersed surfaces, such as pipes and ship hulls. The costs associated with biofilms attached to ship hulls are primarily due to increased fuel consumption caused by higher frictional drag, though hull cleaning, coating, and painting may also be significant. 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).

Although organisms such as mussels and barnacles are responsible for macrofouling and thus for a ship's reduced hydrodynamic performance, microorganisms contribute to

microfouling and play an important role in surface colonization. Bacteria can also easily colonize pipes and heat exchangers, where they stimulate the settlement of invertebrate larvae (Raikina, 2003). According to a review, biofilms in general induce larval and spore settlement of macrofoulers, though negative and neutral effects have also been reported (Salta et al., 2013). Microbes play an important role in the bioremediation of oil compounds; however, microbial contamination of oil and natural gas facilities is undesirable because it can metabolize hydrocarbons, change sulphur content, and influence oil density and viscosity. 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.

CHAPTER – 2

LITERATURE SURVEY

2.1 MARINE MICROBES

In the ocean, there is a teeming world of bacteria with complexity and diversity that exceeds all other life on Earth. Bacteria, viruses, archaea, protists, and fungi are examples of microorganisms. If you weighed all the living organisms in the water, microorganisms would make up 90% of the weight. The fact that these bacteria cannot be seen does not imply that they are inconsequential. Microbes are frequently the motors of ecosystems that would otherwise lack access to food and resources. Many are also stewards of healthy ecosystems, cleaning up trash in the ocean and often protecting against disease rather than spreading it. Microbial communities that are complex and diverse mediate the ocean. Over the last decade, marine ecosystem and biogeochemistry models have begun to address some of this diversity by resolving numerous categories of (mainly autotrophic) plankton distinguished by biogeochemical function. Microbes are frequently the motors of ecosystems that would not otherwise have access to the food and resources they require. Many are also custodian of healthy ecosystems, cleaning up pollution in the ocean and often guarding against disease rather than spreading it. Microbes can be found in some of the most hostile habitats, including boiling hydrothermal vents and deep glacial lakes in Antarctica. They were even the earliest life on Earth, existing in a primordial sea without oxygen. They have the ability to shine, manipulate clouds, and produce one of the world's most lethal toxins.

Microbes are necessary for a healthy ocean ecology. The world as we know it would not exist without them. An interconnected metropolis of microbes, such as bacteria, algae, protists, and viruses, known as a biofilm develops on a hard surface, such as a rock or seafloor. Microbes, which are unicellular organisms, frequently float alone or in small

colonies in open water. However, if they drift near a surface like a rock or seaweed, they may attach and stick to the surface once the initial microbe is trapped, a domino effect occurs in which subsequent microorganisms continue to cling to one another nearly like they are caught in adhesive. As the microorganisms accumulate, they may form layers of distinct microbe communities that may eventually develop into microbial mats that are several millimeters thick. Once a microbe attaches to a biofilm, it will go through a sequence of alterations that will make it best adapted for an immobile existence surrounded by a network of other organisms. The creation of gluing cement secretions, which bind the bacteria together, is the primary modification.

2.2 ANTIBIOFILM

However, bacterial microcolonies that have connected to a surface and hidden themselves in an extracellular matrix of polysaccharide, protein, and nucleic acids are the primary source of biofilms, which can be produced by a wide variety of microorganism species.

Many researchers have started to extract and analyze natural compounds from a wide range of plants and marine species in an effort to uncover reliable sources of antibiofilm agents (Armstrong, E; Boyd, KG; Burgess, JG). Numerous of these chemicals are secondary metabolites produced by the host organism in response to external stresses like competition for resources and potential predators. In a marine environment, antibacterial and antifouling metabolites are essential for many sessile species to ensure that they do not harbor harmful biofilms on their exposed surfaces, especially considering that the vast majority of the world's microbial biomass likes to be in a biofilm state.

2.3 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. Using the twofold plating method, this study first conducts a preliminary examination of a variety of marine bacteria against bacteria that produce biofilms. Next, utilizing a variety of solvents, a crude extraction of the powerful bacterial

strain will be performed. Utilizing the well diffusion method, bacterial growth kinetics, the biofilm inhibition assay using crystal violet staining, and the phenolsulphuric acid method, the activity of the crude extract will be assessed. In order to obtain partially purified fractions using column chromatography, the crude extract will be put through the process. The same techniques stated above will also be used to analyze the activity of the partially purified fractions. The chemical will be used for antibiofilm applications after being refined.

2.4 ANTIBACTERIAL ACTIVITY

2.4.1 Crude extraction

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.

2.4.2 Agar well diffusion method

Well diffusion method is frequently used to assess the antibiotic activity of microbial samples using the agar well diffusion method (Magaldi S., Mata-Essayag S., Hartung de Capriles C.). A volume of the microbial inoculum is dispersed throughout the entire agar surface to inoculate the agar plate surface. Next, a volume (20-100 L) of the antimicrobial agent or extract solution is put into the well by aseptically drilling a hole with a diameter of 6 to 8 mm utilizing a sterile cork borer or tip. The test microorganism is then placed on an appropriate agar plate, and the incubation process is continued. The antibiotic components spread across the agar media and inhibits the tested microbial strain from growing. In comparison to loading a disc, the well diffusion assay has the benefit that you can typically load more sample at once.

2.4.3 GROWTH KINETICS

One of the fundamental tools in microbiology is growth kinetics, which studies the relationship between a given growth rate and the concentration of a substrate. The validity and application of growth kinetics as seen in the lab to environmental growth circumstances, however, remain the subject of numerous fundamental questions that have not been fully resolved despite more than 50 years of research. The growth kinetic data presented for pure cultures growing on a single substrate exhibits significant discrepancies. The kinetic data that have been collected so far for the growth of pure cultures in continuous cultures under carbon control with defined mixtures of two or more carbon sources (including pollutants) show unmistakably that simultaneous substrate utilization lowers the residual steady-state concentrations of all substrates. This should give a cell that can grow on both single and mixed substrates a competitive edge because it can grow considerably more quickly at low substrate concentrations than would be predicted by single-substrate kinetics. In addition, the discussion covers the applicability of the kinetic principles discovered in defined culture systems with single, mixed, or multicomponent substrates to the kinetics of pollutant degradation in complex environmental systems when it occurs in the presence of alternative carbon sources. (J. Monod, 1949).

2.5 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.

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. Log Phase:

During this phase, the bacterial cell population is actively dividing and is largely homogeneous in terms of its chemical make-up, physiology, and metabolic activity. The number of cells (in log scale) plotted versus time yields a straight line. Up till the substrate concentration is not limiting, the growth of the bacterial cell population increases steadily.

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.

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.

2.6 Biofilm inhibition assay

The microtiter dish assay, which has been used primarily to research bacterial biofilms, is a crucial tool for the investigation of the early phases of biofilm formation. This assay does not support the development of mature biofilms, which are often related to flow cell systems, because it employs static, batch-growth conditions. However, the experiment has been successful in locating numerous components necessary for the start of biofilm formation, including genes that produce extracellular polysaccharide and flagella, pili, adhesins, and enzymes that bind and metabolize cyclic-di-GMP. A biofilm can grow on the wall or bottom of a microtiter dish using this straightforward microtiter dish assay. Due to the assay's high throughput, it can be used to examine the development of biofilms by diverse strains under varied growing conditions as well as for genetic screening. A variety of microorganisms have been evaluated using this assay's variants to determine early biofilm formation. The dye crystal violet (CV) is used 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.

2.7 Phenol-sulphuric acid method

The phenol-sulfuric acid method is a quick and easy colorimetric technique for figuring out how many total carbohydrates are present in a sample. The technique can identify almost all types of carbohydrates, such as mono-, di-, oligo-, and polysaccharides. Although practically all carbohydrates are detected by the approach, the absorptivity of the various carbohydrates varies. The results must therefore be represented arbitrarily in terms of one carbohydrate unless a sample is known to contain only one carbohydrate.

All polysaccharides, disaccharides, and oligosaccharides in this procedure are able to be converted into monosaccharides by the concentrated sulfuric acid. These pentoses, which have five carbons, are later dehydrated to produce furfural, and the hexoses, which have six carbons, produce hydroxymethyl furfural. An orange-gold colour results from the compounds' reaction with phenol. It is advised to utilize xylose to produce the standard curve used in the test and then determine the absorption at 480 nanometers if the product is exceptionally high in xylose (a pentose), such as bran from wheat or maize. Glucose is frequently utilized to construct the standard curve when the product contains a lot of hexose carbohydrates. 490 nm is the wavelength at which it absorbs. In the right circumstances, the reaction's precision is within $\pm 2\%$, and the colour it produces lasts for a very long period. This study indicates Strong acid, heat, and the reaction of carbohydrates (simple sugars, oligosaccharides, polysaccharides, and their derivatives) result in furan derivatives that condense with phenol to create persistent yellow-gold compounds that may be detected spectrophotometrically at 490 nm.

2.8 Column chromatography

Column chromatography is defined as a useful method in which the substances to be isolated are presented onto the highest point of a column loaded with an adsorbent (stationary phase), move through the column at different rates depending on the affinities of each substance for the adsorbent and the solvent or mixture, and are typically gathered in solution as they move through the column at different times. Silica gel and alumina are thought of as the two most typical examples of mobile phases for column chromatography, whereas organic solvents are thought of as the most typical stationary phases. The primary idea behind column chromatography is the adsorption of the solution's solutes with the aid of a stationary phase, which then separates the mixture into individual components. The movement of the mixture's distinct components occurs at different speeds as the mobile phase and the mixture that has to be isolated are lowered from the top of the column. Comparatively speaking, the components with lower adsorption and affinity to the stationary phase leave the mixture more quickly than those

with higher adsorption and affinity. The elements that move quickly are removed first, while the elements that move slowly are removed last. Molecules of solute adsorb to the column in a reversible process.

CHAPTER - 3

3.1 AIM

To study the extraction of secondary metabolites from marine bacterial strains against anti-biofilm application.

3.2 OBJECTIVE

- To examine the anti-biofilm potential of various marine bacterial strains.
- Potent marine bacterial strains to do crude extraction.
- To utilize column chromatography to obtain partially pure fractions.
- To evaluate the crude extract's antibacterial activity.

4.3 Crude extraction

Prepare 600 ml of luria bertani broth and then pour it into 4 (1000 ml) conical flasks. Then take **BS3-la-2**, a potent bacterial culture. Then scrape the bacterial culture with sterilized pipette tips or a toothpick. Add the bacterial culture in the conical flask and rest it for it to grow for a weeks' time. Well-developed cultures are transferred to falcon tubes for centrifugation process.



Fig: 4.3.1 Bacterial inoculation

Centrifugation:

The polar and non-polar solvents should be added with four different types of solvents. One bacterial strain *Staphylococcus saprophyticus subsp. Boydi* (**BS3-la-2**) has been chosen for further processing out of 15 bacterial strains since it has been demonstrated superior anti-biofilm activity against a range of biofilm producing bacteria. The chosen bacteria *Staphylococcus saprophyticus subsp. boydi* (**BS3-la-2**) were exposed to crude extract using four different solvents: n-Hexane, dichloromethane, methanol, and chloroform and was spun at 8000 rpm with a 4°C temperature and followed by 24 hrs incubation. After 24hrs the bacterial culture were subjected to rotary evaporator for removal of solvents from the samples by evaporation.



Figure: 4.3.2 Solvent separation

4.4 Characterisation of crude extraction

4.4.1 ATR - FTIR

In combination with conventional infrared spectroscopy, the sampling method known as attenuated total reflection (ATR) enables samples to be viewed directly in either a solid or liquid condition, without the need for further preparation. Fourier-transform infrared spectroscopy, or FTIR, is a technique that can be used to obtain an infrared spectrum of either the emission or absorption of a liquid, solid, or gas sample. The sample is put in a tiny droplet form onto the ATR crystal for liquids and pastes. The crystal can then be cleaned using a gentle solvent if necessary once the measurement has been taken. In order to provide the best possible contact between the sample and the ATR crystal, a swivel press is used to push down on solid samples such as powders, thin films, or other solids. It is best for low-volume or expensive samples to recollect the sample after the measurement.

4.4.2 UV – Visualization spectroscopy

The technique of ultraviolet-visible (UV-Vis) spectroscopy is widely utilized in a variety of scientific fields, including chemical research, bacterial culture, drug identification, nucleic acid purity testing, and beverage industry quality control. In contrast to a reference or blank sample, the number of distinct wavelengths of UV or visible light that are absorbed by or transmitted through a sample can be determined using the analytical technique known as UV-Vis spectroscopy.

Sample analysis

The light then travels through a sample using whichever wavelength selection is employed in the spectrophotometer. It is essential for all analyses to measure a reference sample, often known as the "blank sample," like a cuvette full of the same solvent used to create the sample. The aqueous buffered solution devoid of the target substance is utilized as the reference if an aqueous buffered solution containing the sample is used for measurements. The sterile culture media would be used as a guide when analyzing bacterial cultures. The device will afterwards employ the reference sample signal automatically to assist in obtaining the accurate absorbance values of the analytes.

4.5 Partial Purification

4.5.1 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.

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

4.6.2 Growth kinetics

A 96-well plate was used to hold several biofilm bacterium samples. Crude extracts of varying concentrations were added, and the growth kinetics of the bacteria were monitored for 24 hours using a microplate reader.

A 96-well plate was employed for the growth kinetics approach, and Lb broth was prepared as the growth medium. Hexane, chloroform, methanol, and dichloromethane were taken as four distinct crude extracts. Different bacterial strains that can form biofilms were collected. For the wells, add crude extract at a rate of 25 or 50 μ L and the first columns were used as a control. The solvents should be dried by keeping it in the UV for 1 hour. After that, add biofilm bacteria and broth to each well.

$\ln N_t - \ln N_0 = \mu(t - t_0)$ the natural log of the number of cells at time t minus the natural log of the number of cells at time zero (t_0) equals the growth rate constant multiplied by the time interval



Fig: 4.6.2 crude extract of different concentration

4.6.3 Biofilm inhibition assay

A biofilm inhibition assay was carried out to ascertain the degree to which various concentrations of crude extract inhibited the production of biofilm. Different biofilm forming bacteria samples were cultivated in a 96-well plate with crude extracts at various concentrations (25 mg/L, 50 mg/L) were then added, and the mixture was then incubated.

Cells were removed at the appropriate times, followed by a 30-minute incubation with crystal violet. After that, the extra stain was cleaned off and the wells rinsed with distilled water or pbs. Then O.D values were taken at 600nm.

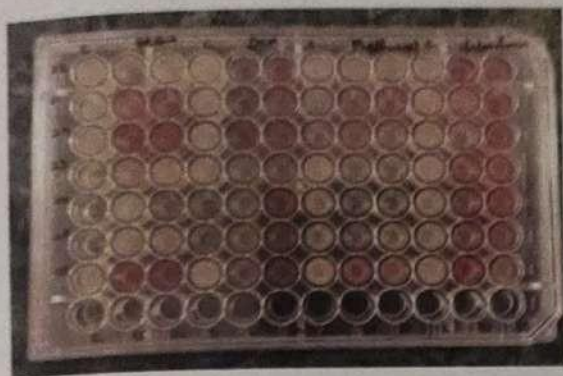


Fig 4.6.3.1 Growth of bacteria

4.6.4 Crystal violet staining

To determine how much different concentrations of crude extract inhibited biofilm development, a cv staining was done. In a 96-well plate, various biofilm-forming bacteria species were grown. The combination was then added, and crude extracts of varying quantities (25 mg/L, 50 mg/L) were added. At the appropriate periods, cells were extracted, then crystal violet was incubated for 30 minutes. The wells were then rinsed with distilled water or pbs once the additional discoloration had been removed. O.D. measurements were then made at 600 nm.

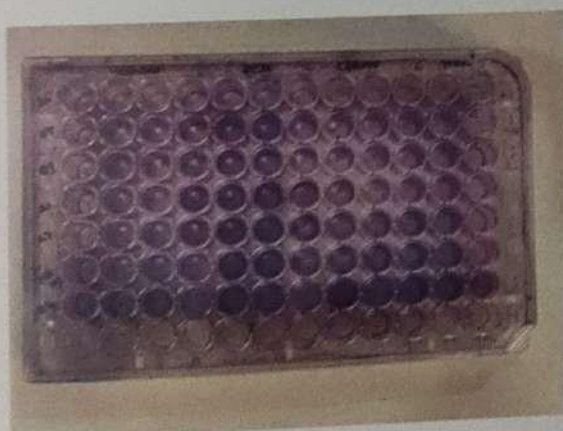


Fig 4.6.3.1: Crystal violet staining

4.6.5 Phenol sulphuric acid assay

In a well with a range of crude extract and LB broth concentrations, the bacteria were cultured and inoculated. The control group didn't receive any care. When the incubation period was through, the microtiter plate was incubated for 24 hours. A sterile phosphate buffer was used to clean the wells. 5% of phenol and strong sulfuric acid are then added to each well. The coloration changes to brown after 30 minutes of incubation.

CHAPTER-5

RESULTS AND DISCUSSIONS

5.1 Bacteria culture maintenance

The culture of marine bacteria is periodically maintained and sub cultured under the following temperature 24- 27°C, these parameters were made sure in order to achieve the best possible development of the cultures after 24-27 hours of incubation.



Fig: 5.1 streaking

5.2 Double plating method

In double plating method the zone of inhibition is measured. Based on its zone of inhibition the bacterial strain has been selected for further process as it showed better anti-biofilm activity. This selected bacterium has been subjected to crude extract.



Fig: 5.2 Double plating

5.3 Crude extraction

Four different solvents (chloroform, dichloromethane, methanol, and n-hexane) were used to perform crude extract on the chosen bacterium. By using the double plating method and the growth kinetics method, the isolated crude extract was examined for its antibacterial activity.

S.NO	BACTERIAL STRAIN	SOLVENT USED	Conc. Of crude extract.
01	BS3-LA-2	Chloroform	72 ug/ml
		Dichloromethane	63 ug/ml
		n-Hexane	81 ug/ml
		Methanol	43 ug/ml



Fig: 5.3 Crude Extract using solvents

5.4 Partial Purification

5.4.1 Thin - Layer chromatography



Fig: 5.4: TLC

In TLC plates the solvents used are considered to be as mobile phase. For this experiment Ethyl acetate and hexane solvents were used as mobile phase. 5.5 Activity of eluted fraction.

5.5 Disc diffusion assay

In order to check the antibacterial activity of the selected bacterial sample, The discs were placed on the agar of the previously inoculated test bacterium. The crude extract was poured the on discs, followed by 24 hr incubation for measurement zone of inhibition.



Fig 5.5 Antibacterial activity

5.5.2 Growth kinetics

In growth kinetics method, the percentage of bacterial growth inhibition was studied against various biofilm bacteria. It has been observed that 75ul of chloroform had high percentage of bacterial growth inhibition.

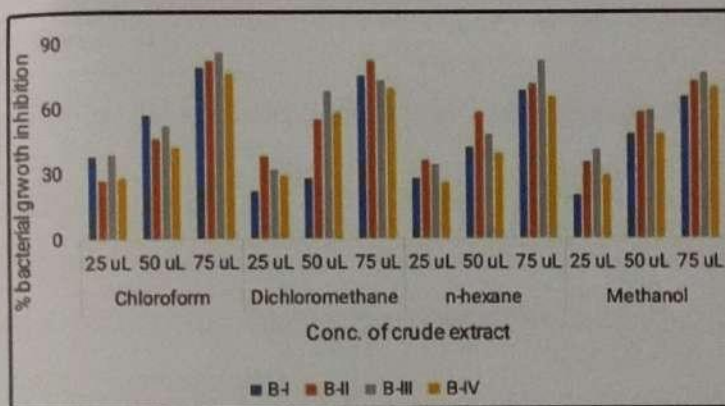


Fig:5.6: Percentage of growth inhibition after 24hr

5.5.3 Biofilm inhibition assay

A biofilm inhibition test was conducted to evaluate the effectiveness of different concentrations of crude extract in inhibiting various biofilm bacteria. Study samples that had been treated and those that hadn't were compared. Treatment-embedded cells were subjected to varying concentrations of crude extract (25µl, 50 µl, and 75 µl). Quantifying the biofilm is done using crystal violet staining. Crystal violet binds to the EPS that wells'

surface secretes, which is subsequently broken down by ethanol and measured at 600 nm with a multimode reader. The comparison between treated and untreated samples was shown using values presented as a bar graph. As time goes on, practically all biofilm-forming bacteria see a significant drop in biofilm amount due to greater concentrations of crude extract. After some time, biofilm in control wells typically stays the same. In all treated samples of all concentrations, a decrease in the amount of biofilm can be noted after day 2. This might be as a result of bacteria being inhibited over time when exposed to larger concentrations of crude extract.

5.5.3.1 Chloroform

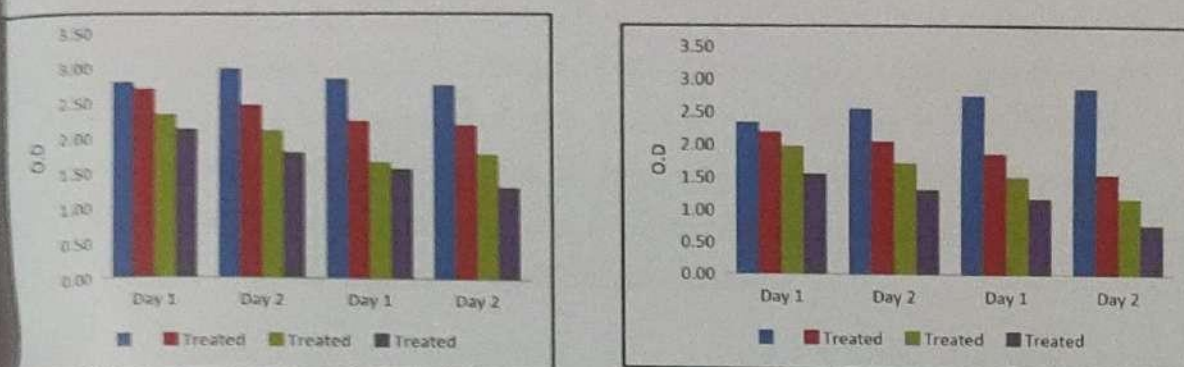


Fig: 5.7 a) Biofilm Inhibition assay B-1&B-2

The bar chart illustrates the comparison of treated and untreated samples. The first column was treated as control. It has been depicted that 70mg/l concentration of crude extract had been decreasing in the consecutive days which shows that there is a decrease in the amount of biofilm forming rate.

5.5.3.2 Dichloromethane

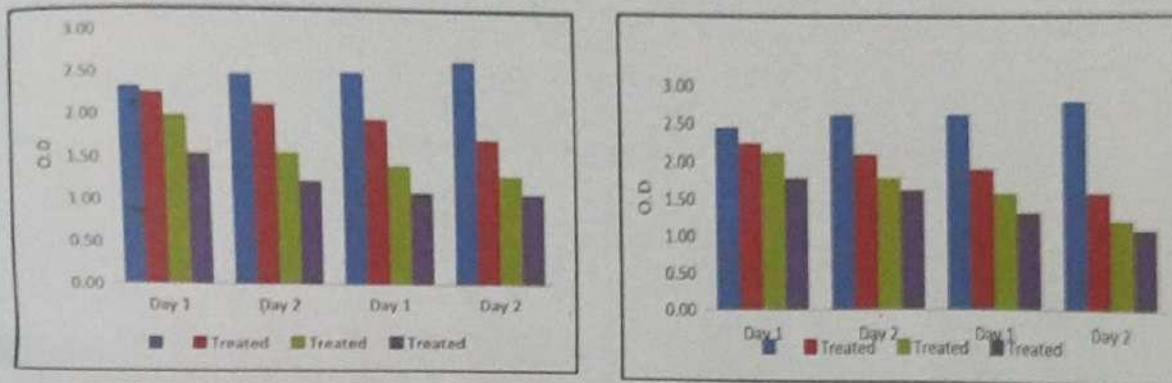


Fig 5.8 Biofilm inhibition assay B-1 & B-2

The bar graph (B1) illustrates the reduction of biofilm forming bacteria, as there is an increase in control and its inhibition rate of the bacterial sample at (50mg/l, 75mg/l) concentrations remains the same. The bar graph (B2) illustrates the inhibition of biofilm bacteria in which 75mg/l concentration of crude extract shows lower bacterial density than day 1, due to its higher concentration of bacterial compound.

5.5.3.3 n-Hexane

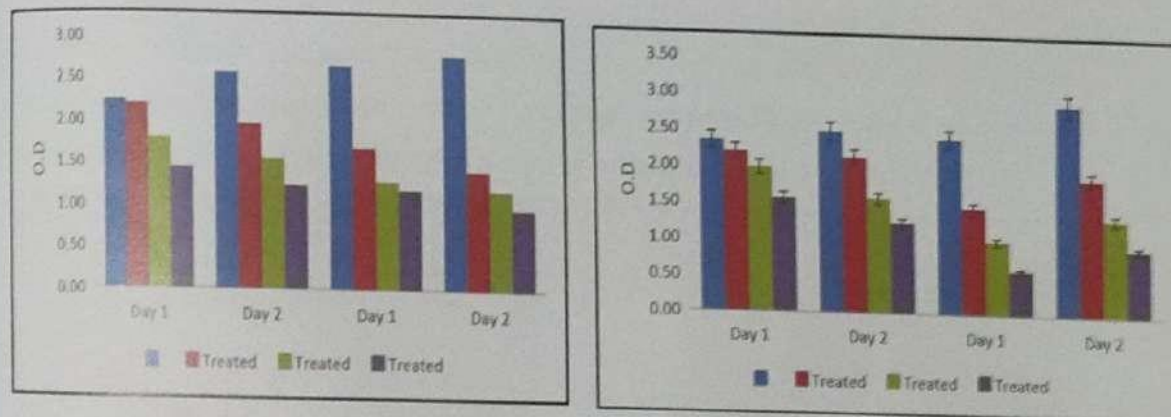


Fig: 5.9 a) Biofilm inhibition assay B- 1 & B-2

The chart illustrates to assess the inhibiting efficiency of different concentrations of crude extract against the biofilm forming bacteria. After day-1 there is a reduction in biofilm quantity in all the treated sample. This diagram evaluates the effectiveness of various crude extract concentrations for inhibiting the growth of bacteria found in biofilms. A

subsequent reduction has been observed in the biofilm concentration of 75mg/l in the following days.

5.5.3.4 METHANOL

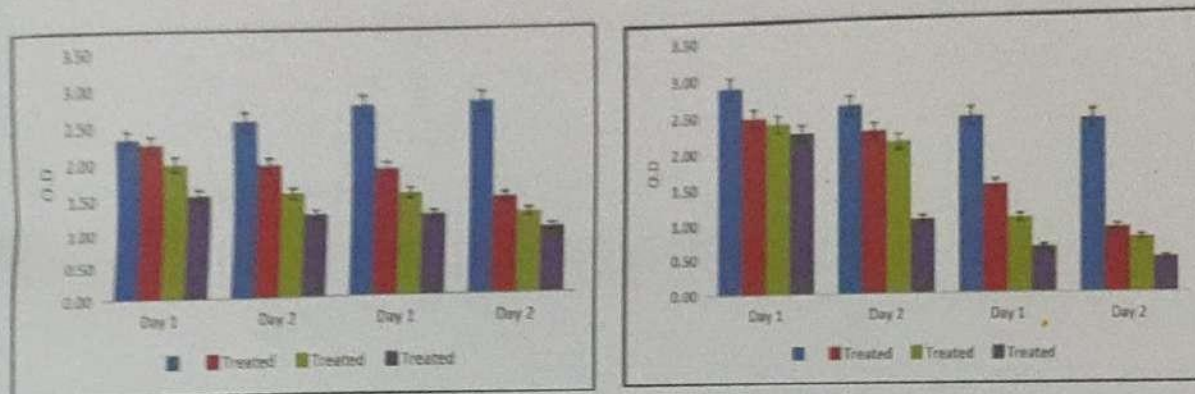


Fig 5.10 a) Biofilm Inhibition Assay B-1 & B-2

The efficiency of different crude extract concentrations was performed to prevent the development of bacteria that are present in biofilms and also shows the effectiveness of different quantities of crude extract which whereby prevents the formation of biofilm.

6.1 Crystal violet staining

The above diagram represents the bacterial attachment or inhibition of bacteria on different concentration of crude extract. It has been observed that if there is an increase in the concentration of crude extract it shows an increase in rate of its inhibition

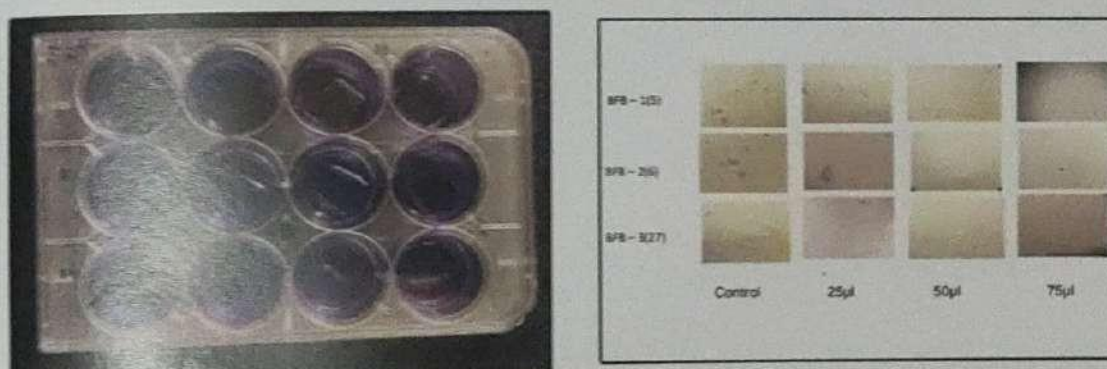


Fig 5.11: Crystal violet staining using different concentrations of crude extract

6.2 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 that a sample is known to contain only one carbohydrate, and its results are expressed arbitrarily in terms of one carbohydrate. The colour for this reaction was stable for several hours, and its accuracy of the method was estimated under proper conditions.



Fig: 5.12 Carbohydrate estimation using Phenol -sulphuric acid method

CHAPTER 6

CONCLUSION

The effectiveness of natural bacterial compounds extraction against algae, bacteria that form biofilms, and other surface organisms like barnacles has been examined. In ships and undersea pipes, among other places, biofilm growth results in a variety of issues. Antifouling coatings, which kill non-target species, are employed to stop these harms. Hence the usage of this extraction method in anti-Biofilm treatments were carried out, as it will be employed in a maritime setting. Cell viability was inhibited as a result of the interaction between the bacteria that form biofilms and the crude extraction. The purpose of natural substances is to slow the growth of marine microfoulers while also ensuring that they do not harm the unintended organisms.

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