

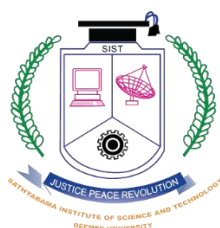
SCREENING OF MARINE ACTINOMYCETES FOR NATURAL PRODUCTS AND ITS POTENTIAL APPLICATION IN MARINE ANTI- BIOFILM ACTIVITY

Submitted in Partial Fulfillment of the requirement for the award of Bachelor of
Technology degree in Biotechnology and Engineering

By

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SATHYABAMA

**INSTITUTE OF SCIENCE AND TECHNOLOGY
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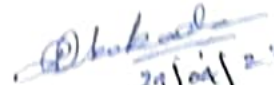
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BONAFIDE CERTIFICATE

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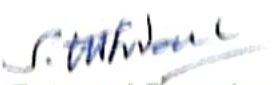

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ABSTRACT

Biofilm is the accumulation of various micro-organisms on the surfaces immersed in seawater over a period which results in various implications affecting the surfaces both physically and economically. So, various techniques have been developed to overcome the effects of marine biofilm formation. In the present study, secondary metabolites from marine actinomycetes were screened for antibiofilm activity and characterized using various methods. The research project begins with a double plating method-based preliminary screening of several marine bacteria against bacteria that create biofilms. subsequently, the effective bacterial strain is likely to be subjected to crude extraction utilizing several solvents. The crude extract extracted was tested for antibiofilm activity through the disc diffusion method, growth kinetics, and biofilm inhibition by crystal violet staining assay. The crude extract was characterized by FTIR – ATR, and the presence of various compounds in the crude extract was determined by thin-layer chromatography (TLC) and Column chromatography. The eluted fractions were tested for antibiofilm activity.

Keywords: Marine Biofilm, Actinomycetes, Secondary metabolites, TLC

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LIST OF ABBREVIATIONS

GC-MS – Gas Chromatography-Mass Spectrometry

FTIR -Fourier-Transform Infrared Spectroscopy

TLC – Thin Layer Chromatography

LB – Luria Bertani

OD – Optical Density

UV – Ultra Violet

PKS – Polyketide Synthases

CHAPTER – 1

INTRODUCTION

1.1 BIOFOULING

The term "biofilm" refers to colonies of microbial life. Biofilms might grow at liquid-air interfaces as well as solid-liquid and solid-air contacts. Most microorganisms produce biofilms, and 99 percent of all microbes on Earth are in these clumps. Because it yields a more suitable environment, surface colonization by biofilms is a crucial strategy for marine bacteria to survive in an oligotrophic environment(Jefferson, 2004). Communities of bacteria affixed to a surface are known as biofilms. It is evident that as microorganisms move from planktonic (free-swimming) creatures to cells that are a component of a complex, surface-attached community, they go through significant modifications. These modifications take place in response to various environmental cues and are represented in the new phenotypic features that biofilm bacteria have developed(O'toole et al., 2000). The attachment of barnacles, macroalgae, and microbial slimes results in marine biofouling. To control fouling, new, efficient, and ecologically friendly solutions are required. It is possible for a macrofouling population to form and outgrow the microfouling, consisting of either "soft fouling" or "hard fouling." Hard fouling is made up of invertebrates like barnacles, mussels, and tubeworms, whereas soft fouling is made up of algae and invertebrates like soft corals, sponges, anemones, tunicates, and hydroids(Maureen E Callow & James A Callow, n.d.).

1.1.1 MARINE BIOFOULING

The growth of marine organisms on the surfaces of submerged or partially submerged objects is known as marine biofouling. Any surface immersed in the sea becomes the potential home for a wide variety of marine species. Numerous creatures, ranging in size from microscopic bacteria and diatoms to shelled invertebrates like barnacles and tubeworms and kelps longer than 10 meters, can contribute to marine fouling ecosystems. Microorganisms including bacteria, fungus, and microalgae, as well as their secretions, are considered microfouling. Microfouling organisms have the ability to release persistent extracellular polymeric coatings. This is influenced by a variety of variables, including movement, pollution levels, water temperature, depth, clarity,

and salinity(Lewis, n.d.). The initial assemblage is created when naturally occurring and inorganic trash on the floor come into contact with free-living microorganisms in seawater. Second, the primary colonizing bacterial mass in biofilms is owing to their growth and reproduction, as well as the fact that they control the substratum's floor residences, which makes it the optimal environment for secondary microbial colonization. Third, bacteria from the created monolayer and other free-living bacteria interact to create a basic biofilm network. Through interactions between various bacteria and other marine micro-fouler that may be competitive or cooperative, the biofilm develops into an advanced stage(Dang et al., 2000). One of the most important unresolved issues now affecting the transportation sector and commercial equipment is marine biofouling. Biofouling is the unwanted buildup of microbes, plants, and animals on any artificial systems that may be exposed to aquatic conditions. The established order of fouling groups on a wide variety of substrata has been well explored, and the literature that has followed is extensive. Numerous ranges have been used to describe the established order of the fouling network, and many of these ranges might overlap or develop simultaneously. Any submerged surface will quickly get lined with a conditioning film made of natural and inorganic compounds that may also serve as a source of vitamins in the development of biofouling.

1.1.2 COMPOSITION

Single cells or microcolonies immersed in a matrix of microbial starter place polymers linked to a substrate make up a biofilm. A mature biofilm in wastewater treatment buildings may be extremely complex, displaying great geographical variation with respect to each organism gift and physicochemical microenvironment. These spatial gradients make it highly probable that the morphology, activities, and composition of biofilms vary with biofilm depth. Bacterial cells, extracellular polymers made by the bacteria, lysis and hydrolysis products, linked natural molecules, and a few inorganic substances are all components of biofilms. Many important properties of the biofilm, such as strength, elasticity, and adsorbent sorption capacity, are determined by the composition. The behavior of biofilms in technological buildings is determined in part by these residences. There is a decrease in pipe friction, the adsorption of particles from the majority of liquid, and biofilter clogging. It was discovered that many factors, such as shear stress, feeding pattern, substrate type and load, etc., stimulated

differences within the macroscopic biofilm shape and homes in technical structures(van Loosdrecht et al., 1995).

1.1.3 EFFECTS OF BIOFOULING

The adhesion of marine fouling organisms to artificial surfaces like ship hulls generates a slew of issues, including higher energy consumption, maintenance expenses, and corrosion. Underwater constructions such as pipelines, cables, fishing nets, and bridge pillars are particularly susceptible to biofouling. The following are some of the negative consequences of ship hull biofouling: (i) Higher fuel consumption due to increased frictional resistance caused by biofouling, which made the hull rougher and the ship heavier. Biofouling has resulted in fuel consumption increases of over 40%, and the cost of a one-way cruise from San Francisco to Yokohama has increased by about 77 percent due to ship hull biofouling. (ii) More expensive and time-consuming hull maintenance, as marine biofouling, necessitates more frequent and lengthier drydocking operations. Moreover, these cleaning techniques produce a tremendous amount of toxic waste, which is then dumped into the ocean. (iii) Increased corrosion of the ship hull when the protective covering surface deteriorates due to metabolic and other biological processes. As a result, the hull's surface is more prone to corrosion and discoloration. Biofouling is a major issue in the marine industry, and researchers are working to produce an effective antifouling remedy. However, the process of biological adherence must be elucidated before antifouling techniques may be developed.

1.2 ANTIFOULING TECHNIQUES

Coatings are applied to ship hulls as part of antifouling processes to stop marine organisms from settling. To prevent settling, toxicants like copper and tributyltin are frequently added to a paint matrix that gradually leaches the biocide from the top layer. Widespread usage of toxicants in AF paints led to high levels of environmental pollution and concerns about their impact on marine habitats. There are now non-toxic, silicone-based foul-release coatings available. These weaken the attachment strength of fouling organisms, which does not stop them from settling but makes any accumulated biofouling slough off when the vessel moves. Foul-release coatings, on the other hand, are only useful for high-speed/high-activity vessels because they can effectively self-clean. Another non-toxic tactic is to incorporate naturally occurring

antifouling chemicals from marine organisms like algae and sponges into coatings; these substances, however, are not currently commercially available. One of the most dangerous substances in the marine environment is the organotin molecule TBT (tributyltin), and there are strong scientific arguments against introducing it into marine ecosystems. One of the main sources of introduction is the TBT-containing paint that is applied to ship hulls to stop marine creatures from adhering to them, a process known as "fouling." On the other hand, the chemicals in antifouling paint slowly seep into the water and harm any organisms that are adhering to the ship's hull. These materials remain in the water, harming marine life (Bryan et al., 1988). The simplest way to handle fouling is to simply remove it mechanically, such as by using high-pressure water jets to treat the fouled surface. This technique can be used to clean sensor platforms, such as buoys, but it is not appropriate for more complex sensor components. Chlorination is a commonly used technique to prevent biofouling of sensors, water cooling systems, etc. (Whelan & Regan, 2006).

1.2.1 Natural product-based antifouling techniques

Antifouling compounds are being isolated from a variety of aquatic environment resources in various labs all over the world. Macroalgae, soft corals, bryozoans, tunicates, and sponges are frequently considered potential sources of ecologically advantageous antifouling compounds. Such invertebrates contain a variety of chemicals that have been removed and tested for their capacity to deter polluting organisms, mainly barnacles, diatoms, green algae, and bacteria (Clare, 1996). Problems with tin and copper antifouling compounds have highlighted the need for environmentally friendly antifouling coatings. In the maritime environment, bacteria isolated from living surfaces show promise as a natural source of antifouling agents. The most widely used chemical antifoulant, tri-n-butyl tin (TBT), accumulates in marine sediments and has been demonstrated in numerous studies to negatively affect a few marine organisms. Natural substances originating from marine organisms can be used in place of the chemicals often used in antifouling coatings (Yebra et al., 2004). Marine natural products have produced an antifoulant that may effectively prevent environmental damage, which is also one of the hot topics in current study. At the moment, new antifoulant research is primarily focused on the isolation and characterization of natural products. Antifouling properties may be found in a variety of natural chemicals derived from animals and plants. Antifouling action may be found

in various bioactive compounds isolated from the sea (Muralidharan & Jayachandran, n.d.).

1.3 MARINE ACTINOMYCETES

The highest genetic and metabolic diversity has been achieved by marine microbes, especially actinomycetes. So, the focus should be on investigating marine actinomycetes as a potential source for the identification of new secondary metabolites. In addition to existing in the oceans, actinomycetes are widely present in a variety of marine environments. The use of marine actinomycetes as a source for the creation of novel secondary metabolites is still relatively new. The isolation of new secondary metabolites from marine actinomycetes has enormous potential. Future success in this regard depends on our capacity to isolate novel actinomycetes from maritime habitats. Despite the dearth of isolation techniques for novel marine-derived actinomycetes, several advancements in this field have lately been made (Lam, 2006). Marine microorganisms, particularly actinomycetes, have the largest metabolic and genetic complexity. Studying actinomycetes in the marine zone, as a resource of novel bioactive molecules should hence be the emphasis of the study. Additional to being in the seas, actinomycetes are extremely prevalent in a range of aquatic settings. It is still quite new to use coastal actinomycetes as a resource for the synthesis of novel bioactive compounds. There is a lot of potential in isolating novel bioactive molecules using coastal actinomycetes. Our ability to extract new actinomycetes from marine settings will determine our future success in this area. Despite the lack of isolation methods for novel actinomycetes obtained from the sea, recent developments in this area have seen numerous improvements (Hernández et al., 2004).

CHAPTER – 2

LITERATURE SURVEY

2.1 INTRODUCTION

The best sources of medicines and therapeutic candidates remain natural substances. They are indeed fantastic small-molecule probes for studying fundamental biological processes. The natural product market has significant challenges in locating new natural products in an effective and cost-effective way (Xie et al., 2014). Chemical characteristics of substances obtained naturally and medication discovery are both seen as having a lot to gain from the study of naturally occurring compounds in the Ocean. The advancements made in the field of Chemistry of innate products derived from Oceans have also contributed to the growth of the biochemical environment, whose research has substantially improved our understanding of the ecological roles played by coastal secondary metabolites (Jensen & Fenical, n.d.). The application of innocuous or non-pollution-causing techniques to protect man-made structures from biofouling These programs supporting scientific areas will be covered, as well as properly planned research working toward the finding of novel antifouling inert ingredients. But it is necessary to first define specific rules and place these investigations in the context of an acknowledged requirement for surrogate antifoulants (Holm, 2012).

2.2 SECONDARY METABOLITES

In 1961, Bu Lock first used the word called secondary metabolite, to describe some microbiological molecules, while Bentley and Bennet questioned the phrase's usefulness in the 80's. Bioactive compounds (secondary metabolites) are substances that the microorganism can generate but which are not necessary for basic metabolic processes like growth and reproduction. However, many secondary compounds act as chemical messengers that control the producer's metabolism. Due to antibiotics' ability to prevent competitors from consuming rival ambient microbes, antibacterial drugs - producing - bacteria have an advantage in the struggle for nutrition. The number of bioactive molecules in microbes that have been recovered from the environment is often fairly low. For mass production, high-yielding strains should be picked via many genetic modifications (Bi~hal, 2000). Various species differ greatly in

their capacity to create bioactive substances. The bioactive compounds with anti-bacterial, protozoal, fungal, viral, and cancer activities are identified from microorganisms, they are referred to as antibiotics. Bioactive compound development might have happened organically like a situational response to ecological stimuli, such as the physiological and bodily surroundings. The environment has produced a wide range of strange and potent formations which have never been made in a lab, demonstrating that it was always technologically advanced, flexible, and active combinational chemist. A sizable genetic pool has grown over the span of thousands of years to maintain the metabolic synthesis of natural compounds. The bioactive compounds are unquestionably prevalent in the genomic world as multiple kinds of catalysts as a result of such a developmental system battle against severe diseases in the anticipation that this could inspire extra effort to employ these advantageous compounds against other kinds of human illness (Kim et al., 2017).

2.2.1 Peptides

Peptides are shorter versions of the amino acid's polymers. Most peptides of *Streptomyces* are cyclic in structure. In addition to other distinctive structural elements like chromophores or rare amino acids. Cyclomarins-A, a peptide with the chemical formula $C_{56}H_{80}O_{10}N_8$ and a white crystalline solid by nature, is generated by a coastal *Streptomyces* sp. Cyclomarins-B, a peptide having the molecular formula $C_{56}H_{82}O_{10}N_8$, is a naturally occurring white solid. Cyclomarins-C, a peptide having a molecular formula of $C_{56}H_{82}O_{10}N_8Na$ and a naturally acquired white solid (Renner et al., 1999).

2.2.2 Quinones

Quinones are chemical substances that have a fully connected cyclical Dione composition. These are typically present in molecules with biological significance (Dharmaraj, 2010). The solid-state fermentation of the marine *Streptomyces* sp. KS3, which was isolated from sediments from the sea, produced a new anthracycline called komodoquinone A and its aglycone, komodoquinone B (Itoh et al., 2003).

2.2.3 Polyketides

PKSs (Polyketide synthases), multifunctional enzymes that can continuously join petite carboxylic acid subunits in a way that is identical to the synthesis of fatty acids, are responsible for producing the vast and chemically diverse family of organic

products referred to us as polyketides (Staunton & Weissman, 2001). The broth-made culture of the strain of *Streptomyces* sp. (CNQ-085) contained the polyketides with high cytotoxicity, Daryamide A, Daryamide B and Daryamide C (Asolkar et al., 2006).

2.2.4 Alkaloids

Alkaloids are a class of organic molecules found in nature that include nitrogen and have properties like those of an alkali. They are secondary metabolites with important biological functions, and most of them have highly complex ring structures with the greatest number of the nitrogen contained within them. Two new indolocarbazole alkaloids, 40-N-methyl-50-hydroxystaurosporine and 50-hydroxystaurosporine, were isolated from *Micromonospora* sp. L-31-CLCO-002, a marine sponge found near Fuerteventura that was connected with *Clathrina coriacea*. These substances showed cytotoxicity against different tumour cell lines (Abdelmohsen et al., 2014).

2.2.5 Fatty acids

Fatty acids' antifouling ability was first discovered around 20 years ago, but up until recently, its antifouling potential was ignored. With an EC50 value of 0.6 mg/mL and a very low LD50 of 80 mg/mL, 12-methylmyristic acid from the actinomycete *Streptomyces* sp. isolated from deep-sea sediments (5774 m) demonstrated potent antilarval activity against the polychaete *Hydroides elegans*, indicating that its therapeutic ratio is >133.54. 12-methylmyristic is a promising nontoxic antifoulant (Fusetani, 2011).

2.3 NATURAL ANTIFOULING COMPOUNDS

In laboratory tests, the bulk of the chemicals either are added to something like a plate of bacterial isolates or adhered to plastics. The efficacy of chemical compounds, when coupled with paint to produce a possible anti-fouling layer has not been thoroughly studied. The only compound-infused paint test that has been reported so far used rudimentary extracts of sponges which had some effectiveness in battling barnacles (Willemssen, 1994). The findings emphasize the importance of microbes as producers of advantageous bioactive molecules and consequently of possible anti-fouling compounds. Estimates place the number of microbial sources from the aquatic environments from 1 to 5 percent. If that is the case, there are still numerous bacterial species in the sea whose ability to produce helpful compounds hasn't been investigated. There is still much research that has to be undertaken on collecting and

growing marine microorganisms in order to fully utilize their capabilities (Armstrong et al., 2000). Various species differ greatly in their capacity to create bioactive substances. The bioactive compounds with anti-bacterial, protozoal, fungal, viral, and cancer activities are identified from microorganisms, they are referred to as antibiotics. Bioactive compound development might have happened organically like a situational response to ecological stimuli, such as the physiological and bodily surroundings. The environment has produced a wide range of strange and potent formations which have never been made in a lab, demonstrating that it was always technologically advanced, flexible, and active combinational chemist. A sizable genetic pool has grown over the span of thousands of years to maintain the metabolic synthesis of natural compounds. The bioactive compounds are unquestionably prevalent in the genomic world as multiple kinds of catalysts as a result of such a developmental system battle against severe diseases in the anticipation that this could inspire extra effort to employ these advantageous compounds against other kinds of human illness (Kim et al., 2017).

Table 2.1: Natural products derived from Marine Actinomycete *Nocardiosis* Sp.

S. No	Compound	Characterization	Molecular formula	Reference
1	Nocardiopsin C	Colourless oil	$C_{20}H_{20}N_2O_3$	(Raju et al., 2013)
2	Nocardiopsin D	Colourless oil	$C_{28}H_{43}NO_7$	(Raju et al., 2013)
3	Nocardiopyrone A	White powder	$C_{32}H_{49}NO_7$	(Raju et al., 2013)
4	Nocarimidazole A	amorphous, white	$C_{13}H_{23}N_3O$	(Leutou et al., 2015)
5	Nocarimidazole B	amorphous, white solid	$C_3H_3N_3O_2$	(Leutou et al., 2015)
8	Nocapyrone E	colourless oil	$C_{12}H_{16}O_2$	(Fu et al., 2011)
9	Nocapyrone G	colourless oil	$C_{12}H_{16}O_3$	(Fu et al., 2011)

10	Nocazine A	colourless needles	$C_{22}H_{22}N_2O_4$	(Fu et al., 2011)
11	Nocazine B	yellow, amorphous powder	$C_{21}H_{20}N_2O_3$	(Fu et al., 2011)
12	Nocazine C	yellow, amorphous powder	$C_{20}H_{20}N_2O_3$	(Fu et al., 2011)
13	Nocazoline A	yellow oil	$C_{10}H_{11}NO_3$	(Fu et al., 2011)

CHAPTER – 3

AIM AND OBJECTIVES

3.1 AIM

- To extract a Natural product from a marine actinomycete and check its application in anti-biofilm activity.

3.2 OBJECTIVES

- To isolate and culture the marine actinomycetes
- To Prepare Crude extract from actinomycete
- To check the bioactivity of the crude extracts against biofilm formation
- To Characterize the crude extract
- To partially Purify the crude extract for antibiofilm activity

CHAPTER-4

MATERIALS AND METHODS

4.1 Bacteria culture maintenance

4.1.1 Actinomycetes culture

Marine actinomycetes culture were used for the extraction of secondary metabolites. All actinomycetes culture were streaked on to actinomycetes isolation agar added with glycerol. Later, they were transferred on to Luria Bertani agar and broth culture for further processing of secondary metabolites.

4.1.2 Biofilm bacterial culture

Biofilm forming bacterial culture were isolated from the biofouling samples in the different marine environment. All samples were spread on to the Zobell marine agar plates. Once, visible colonies were developed they were streaked into new Zobell marine agar plates to obtain pure cultures. To ensure the biofilm forming ability, biofilm static assay was performed using crystal violet stain. All biofilm samples were characterized using various biochemical test like gram staining, catalase, lipase and motility test.

4.2 Preliminary screening for anti-biofilm activity

Actinomycetes culture were initially screened for its anti-biofilm activity using double plating method. Biofilm forming bacteria were spread on the LB plates using soft agar. Once the agar gets solidified completely, supernatant from the actinomycetes culture were spotted on the plate surface. Plates were incubated under room temperature for 24 H. Zone of clearance/inhibition were observed on the consecutive day and activity was noted.

4.3 Crude extraction

Potent actinomycetes cultures were selected and subjected for crude extraction. Actinomycetes culture were inoculated on Luria Bertani broth and incubated under room temperature for 5-6 days. Once maximum growth has been obtained, cells were centrifuged to separate pellets and supernatant. Then, supernatant was collected and transferred into fresh flask. Equal volume of solvent was added to the supernatant and

kept in shaker for 24 H. Next day, supernatant and solvent were separated using separating funnel. The solvent was dried and crude was extracted using the rotary evaporator.

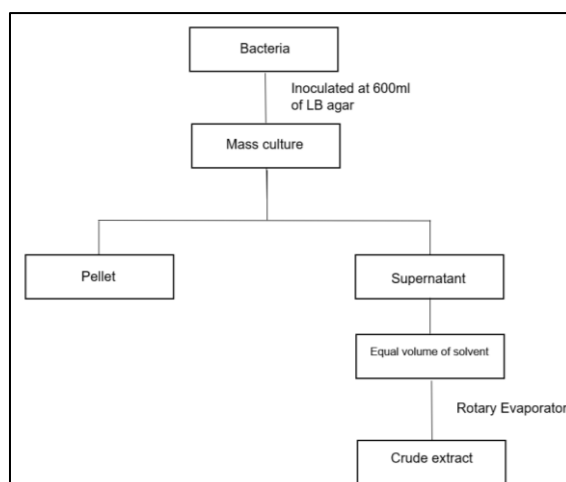


Fig 4.1 Crude extraction of marine actinomycetes

4.4 Characterization of crude extract

4.4.1 UV-Visible spectrophotometry

UV-vis spectrophotometry was used to analyze the maximum absorbance present in the crude extract extracted from the marine actinomycetes strain. Various peaks will be determined using ethyl acetate as the blank and readings will be plotted in form of graph.

4.4.2 ATR-FTIR

FTIR is abbreviated as Fourier Transform infrared Spectroscopy. Maleki H et al., were able to confirm the ability of *Streptomyces* species to aid in the production of antibiotic compounds by performing FTIR, coupled with UV-visible spectroscopy, on 35 samples of soil brought all the way from Iran (Maleki & Mashinchian, 2011).

4.4.3 GC-MS

A study conducted using GC-MS on *Nocardiopsis dassonvillei* (MAD08), showed the presence of 26 peaks of which 11 were of substances, that had promising antibiotic performance (Selvin et al., 2009). Bioactive compounds like trimethyl cyclohexane with an area of 13 percent and time of retention of 20.54 and dichloromethane with an area of 76% and time of retention of 5.04 and 2.79 were reported in the GC-MS analysis of

VITBKA3 strain of actinomycetes (Bhakyashree & Kannabiran, 2020). The ethyl acetate extract of the isolate RAM25C4 which revealed the qualitative presence of macrolides, terpenoids, and quinolones in HPTLC was analyzed by GC–MS using the FISIONS Instruments model GC 8000 series and Mass Spectra is MD 800, with fused silica capillary column; GC column dimension: 30 m × 0.25 mm × 0.5 mm AB-35MS. With the following GC conditions: Injector temperature 250 °C column temperature isothermal at 100 °C then programmed to rise up to 250 °C at 6 °C/min and be held at this temperature for 10 min. The ion source temperature is 200 °C and the interface temperature is 250 °C. Helium gas is engaged as a carrier gas at the rate of 1 ml/min. The spectra were obtained in the EI mode with 70 eV ionization energy. The compounds were identified by comparison with the inbuilt Wiley and NIST libraries (Wahaab & Subramaniam, 2018).

4.5 Partial purification of crude extract

4.5.1 Thin Layer Chromatography (TLC)

In-situ identification of active metabolites can be carried out using thin-layer chromatography (Bavya et al., 2011). The strain of actinomycetes crude extract which is dried and mixed with DMSO, was partially purified with the help of thin-layer chromatography using silica gel on the plates (Kumar et al., 2018). Among the 5 solvent systems selected for TLC (Thin layer chromatography), chloroform-Methanol, at a ratio of 24:1 in (v/v) solution was shown to be the most suitable system of solvent for separating the metabolites of antibiotic agents (Atta, 2015).

4.5.2 Column Chromatography

Sunil et al., 2012., have suggested using dichloromethane as the mobile phase that carries the molecules and ethyl acetate as the stationary phase to separate the molecules to perform Column chromatography and they were also able to confirm the presence of active compounds in marine actinomycetes (Sunil et al., 2012b). The extract obtained from liquid-liquid fermentation was subjected to centrifugation and the supernatant was allowed to be purified with the mixture of chloroform and methanol using column chromatography. 30 fractions, each containing 20 ml of actinomycete (A11) strain tested for antimicrobial activity and it was reported that it showed a positive response (SUNARYANTO & MARWOTO, 2010).

4.6 Activity of eluted fractions

4.6.1 Growth inhibition property of eluted fractions

Growth kinetics, which examines the relationship between a growth rate and substrate concentration, is one of the core tools in microbiology. Significant differences can be seen in the growth kinetic data for pure cultures grown on a single substrate. The kinetic data for the growth of pure cultures in continuous cultures under carbon control with defined mixtures of two or more carbon sources exhibit completely that simultaneous substrate utilization decreases the residual steady-state concentrations of all substrates. Given that it can expand significantly more quickly at low substrate concentrations than would be anticipated from single-substrate kinetics, a cell that can grow on both single and mixed substrates should have an advantage over others in its field (Monod, n.d.). Different biofilm bacteria samples were transferred to 96-well plate and Crude extract of different concentrations were added and growth kinetics were observed for 24h using a microplate reader.

4.6.2 Biofilm Inhibition assay

Biofilm inhibition assay was performed to determine how extent different concentrations of crude extract were inhibiting the biofilm formation. Different biofilm-forming bacteria samples were cultured into a 96-well plate and crude extracts of different concentrations (10 mg/L, 20 mg/L, 30 mg/L, 40 mg/L, 50 mg/L) were added and incubated. Four different plates were used to assess the biofilm inhibition ability on different days. On specific days, cells were removed and crystal violet was added and incubated for 30 minutes. Then, the stain was removed and washed with distilled water to remove the excess stain. Plates were left to dry to remove off excess water content then 80% acetic acid was added to dissolve the dye-stained cell. O.D values were taken at 600nm.

4.6.3 Phenol-sulphuric acid assay

Phenol-sulphuric acid assay was used to determine the carbohydrate content in the cell. Biofilm forming cells were capable of producing extracellular polysaccharides at different conditions. To asses the carbohydrate content of the biofilm forming bacterial cells treated with the crude extract, cells were grown and inoculated with the crude extract with different concentrations. Later, cells were discarded and phenol was

added and conc. HCL was added to visualize the formation of brown colour and readings was taken at 580 nm.

CHAPTER-5

RESULTS

5.1 ACTINOMYCETE CULTURE

Actinomycetes culture were grown on actinomycetes isolation agar with glycerol as supplement. All cultures were grown properly under optimum lab conditions. Growth was monitored properly and stored in the refrigerator for future use.



Fig 5.1 Actinomycete strains streaked in Luria Bertani Agar plates

5.2 BIOFILM BACTERIAL CULTURE

Samples isolated from various fouling sites have been purified to obtain pure culture of biofilm forming bacteria. Eight different biofilm forming bacterial strains have been isolated and purified. Out of eight, three were gram positive and rest five were gram negative and are rod shaped. Biochemical characterization and biofilm forming ability were listed in the below mentioned table (Table 5.1).

5.3 Preliminary screening for anti-biofilm activity

Different marine actinomycetes strain were subjected to preliminary screening for anti-biofilm activity using double plating method. In this 4 actinomycetes strain has more activity compared to the rest. So, these four marine actinomycetes were further scrutinized and one actinomycetes strain was selected for further studies based on their activity against various biofilm forming bacterial strains. The results of preliminary screening of actinomycetes strain for anti-biofilm activity was listed in the table (Table 5.1)

S.No	Bacteria Strain	1S	2S	3S	Black 1	Black 2	VP 1	V.S	KP
1	At - 2 - 1	No	Yes	No	Yes	No	No	No	Yes
2	At - 2 - 2	Yes	No	No	No	No	No	No	Yes
3	At - 2 - 3	Yes	Yes	No	No	No	No	No	No
4	At - 2 - 4	No	Yes	No	No	No	Yes	No	No
5	At - 2 - 5	No	No	No	Yes	Yes	Yes	No	No
6	At - 2 - 6	No	No	No	Yes	No	Yes	Yes	No
7	At - 2 - 7	No	Yes	No	No	No	No	No	No
8	At - 2 - 8	Yes	No	Yes	No	No	No	No	No
9	At - 2 - 9	No	Yes	No	Yes	No	Yes	No	No
10	At - 2 - 10	No	Yes	Yes	No	No	No	No	No
11	At - 2 - 11	Yes	Yes	No	No	No	No	No	No
12	At - 2 - 12	Yes	No	No	No	No	No	No	No
13	At - 2 - 13	Yes	Yes	No	Yes	No	Yes	Yes	Yes
14	At - 2 - 14	Yes	No	No	No	No	No	No	Yes
15	At - 2 - 15	Yes	Yes	No	No	No	No	No	No
16	At - 2 - 16	No	Yes	No	No	No	Yes	No	No
17	At - 2 - 17	No	No	No	Yes	Yes	Yes	No	No
18	At - 2 - 18	No	No	No	Yes	No	Yes	Yes	No
19	At - 2 - 19	No	Yes	No	No	No	No	No	No
20	At - 2 - 20	Yes	No	Yes	No	No	No	No	No
21	At - 2 - 21	No	Yes	No	Yes	No	Yes	No	No
22	At - 2 - 22	No	Yes	Yes	No	No	No	No	No
23	At - 2 - 23	Yes	Yes	No	No	No	No	No	No
24	At - 2 - 24	Yes	No	No	No	No	No	No	No
25	At - 2 - 25	No	Yes	Yes	Yes	No	Yes	Yes	Yes
26	At - 2 - 26	No	No	No	Yes	No	Yes	Yes	No
27	At - 2 - 27	No	Yes	No	No	No	No	No	No
28	At - 2 - 28	Yes	No	Yes	No	No	No	No	No
29	At - 2 - 29	Yes	Yes	No	No	No	No	No	No
30	At - 2 - 30	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes
31	At - 2 - 31	Yes	No	No	No	No	No	No	Yes
32	At - 2 - 32	Yes	Yes	No	No	No	No	No	No
33	At - 2 - 33	No	Yes	No	No	No	Yes	No	No

TABLE 5.1 Results of preliminary screening of actinomycetes strain for anti-biofilm activity

5.4 Crude extraction

One potent actinomycetes strain was subjected to crude extract and the compound was obtained using the rotary evaporator. The weight of the extracted compound was determined to be 76 μg . Crude extraction was performed again and again to extract more active compounds. The compound was dissolved again in ethyl acetate to the final concentration of 100 $\mu\text{g}/\text{ml}$.

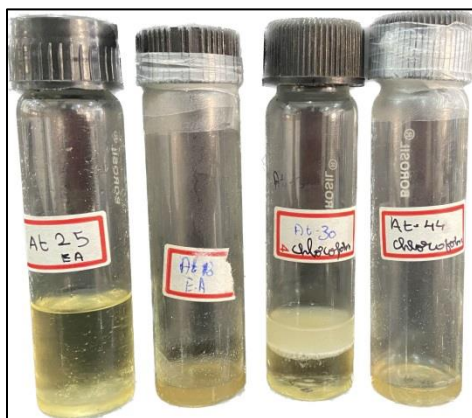


Fig 5.2 Crude extract of actinomycetes

5.5 Characterization of crude extract

5.5.1 UV-Vis spectrophotometer

The crude extract was subjected to UV-vis spectrophotometry to determine the maximum absorbance of the particular compound present in the crude extract. UV characterization was done under the range of 200 – 700 nm. The peaks were obtained at 256 nm and 342 nm.

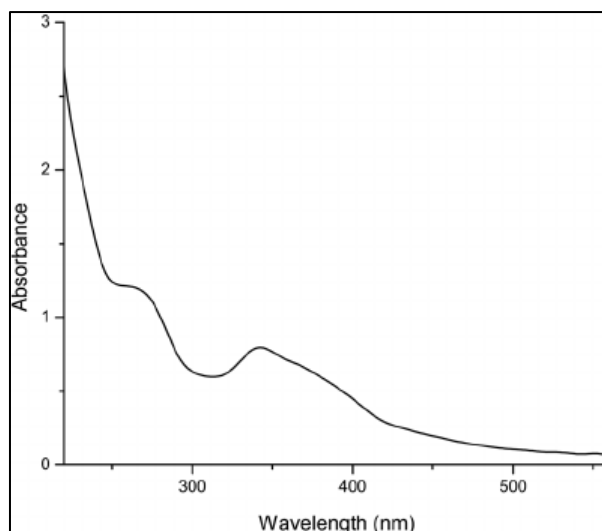


Fig 5.3 UV characterization of crude extract

5.4.2 ATR-FTIR

The crude analyzed with the ATR-FTIR showed peaks in the range of 2983, 2628, 1736, 1373, 1006 indicated the presence of carboxylic acid, aldehydes, ether, ester, hydroxyl and amine groups.

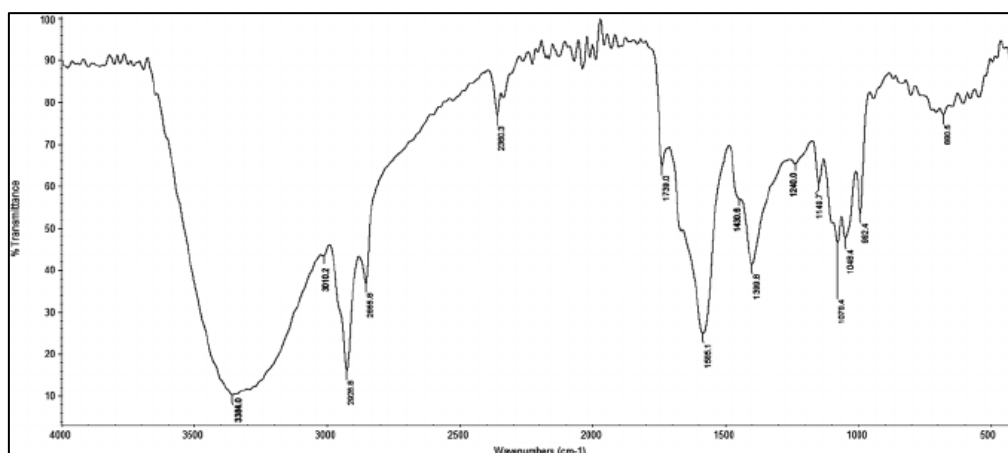


Fig 5.4 ATR-FTIR analysis of the crude extract

5.4.2 GC-MS

GC-MS result for the crude extract indicated the presence of various compounds. The major peak compound was found to be pyrrolo and tetracosanol. Other compounds are also present in varying composition.

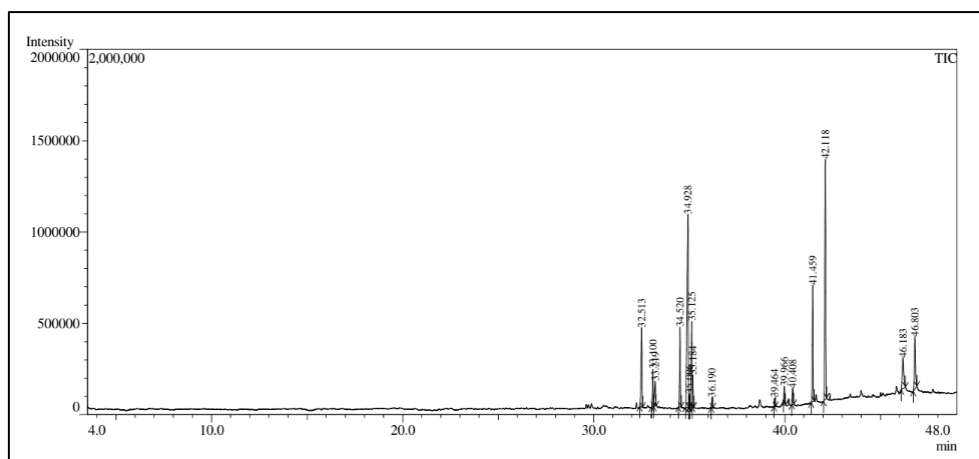


Fig 5.5 GC-MS analysis of the crude extract

5.5. Partial elution of crude extract

5.5.1 Thin Layer Chromatography

Crude extract was subjected to partial separation using thin layer chromatography. The compounds were separated based on their polarity of the solvent. Two bands were visualized when hexane and ethyl acetate were used as solvent and methanol, sulphuric acid were used as a staining agent. Rf value were determined accordingly.



Fig 5.6 Crude extract spotted TLC plate with separated bands

5.5.2 Column chromatography

Column chromatography was used to elute fractions from the crude extract. Chloroform and methanol were used in varying composition and different fractions

were eluted at different time interval. Around 11 fractions have been eluted and activity of all 11 fractions have been determined.

Fraction	BFB 1	BFB 2	BFB 3	BFB 4	BFB 5	BFB 6	BFB 7	BFB 8
E-0	Yes	No	No	Yes	Yes	Yes	Yes	Yes
E-1	Yes	Yes	No	No	Yes	No	Yes	No
E-2	No	Yes	Yes	No	No	No	No	No
E-3	No	Yes	Yes	No	Yes	Yes	Yes	No
E-4	No	Yes	No	Yes	No	No	No	Yes
E-5	Yes	No	Yes	No	Yes	No	Yes	No
E-6	No	Yes	No	Yes	No	Yes	No	Yes
E-7	Yes	No	Yes	No	Yes	No	Yes	No
E-8	No	Yes	Yes	Yes	Yes	Yes	No	No
E-9	Yes	No	Yes	Yes	No	No	Yes	No
E-10	No	Yes	No	Yes	Yes	Yes	No	Yes

Table 5.2 Activity of eluted fractions from crude extract by column chromatography

5.6 Activity of eluted fractions

5.6.1 Growth inhibition property of eluted fractions

Eluted fraction inoculated with the biofilm bacteria has inhibited the bacterial growth to different extent depending upon the concentration of eluted fraction. Three different conc. have been used against eight different biofilm forming bacteria. Higher conc. has shown more activity in inhibiting the bacterial growth. And in control, where no crude extract has been added cells undergo normal cell growth without any disruption in their growth mechanism.

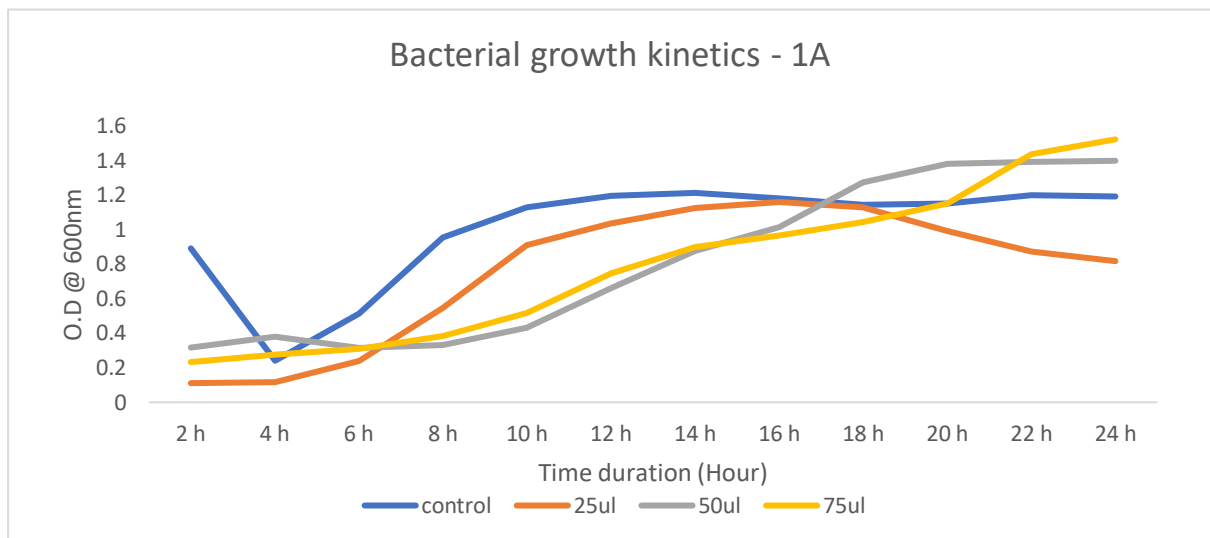


Fig 5.7 Growth kinetics of bacteria – 1A

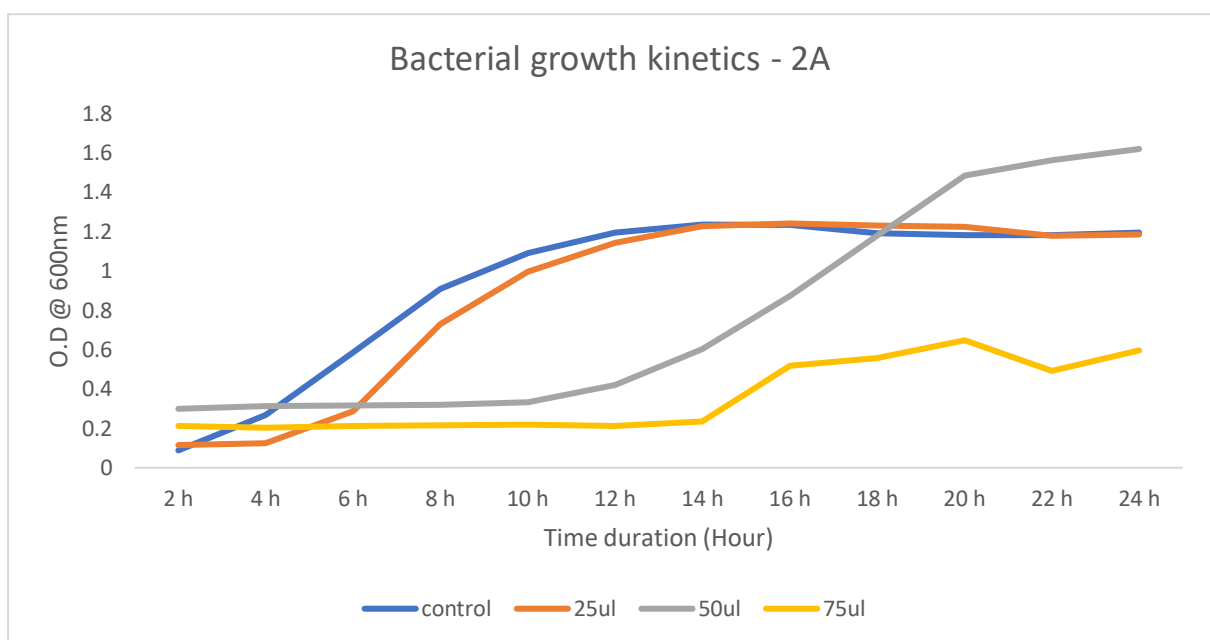


Fig 5.8 Growth kinetics of bacteria –2A

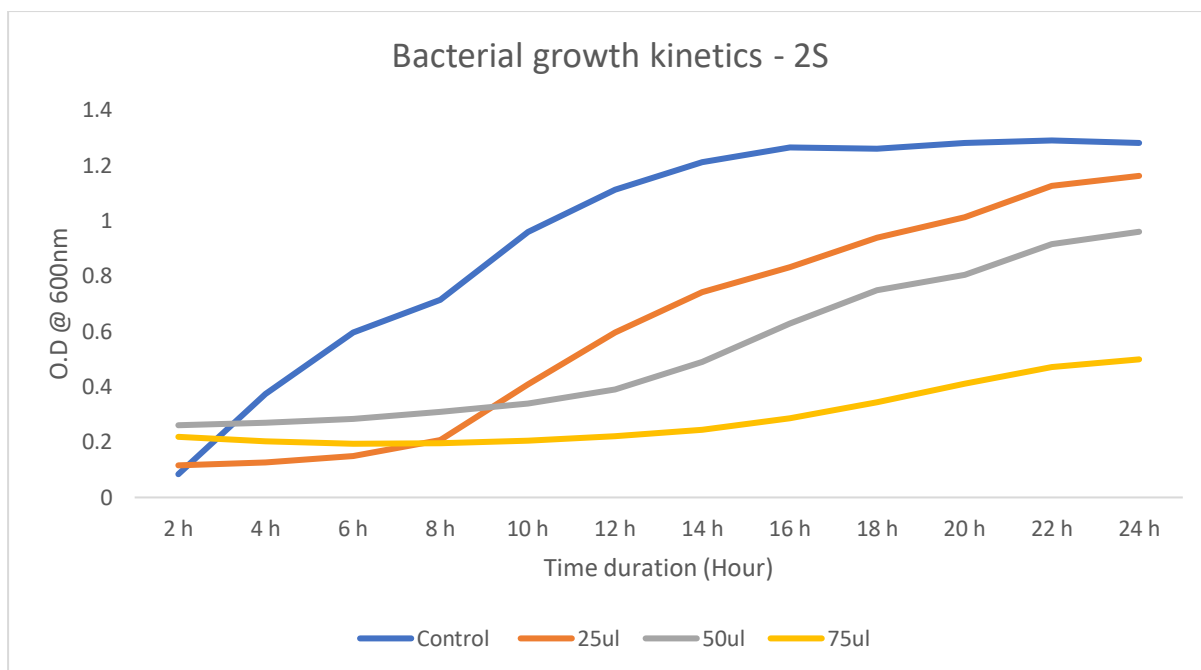


Fig 5.9 Growth kinetics of bacteria –2S

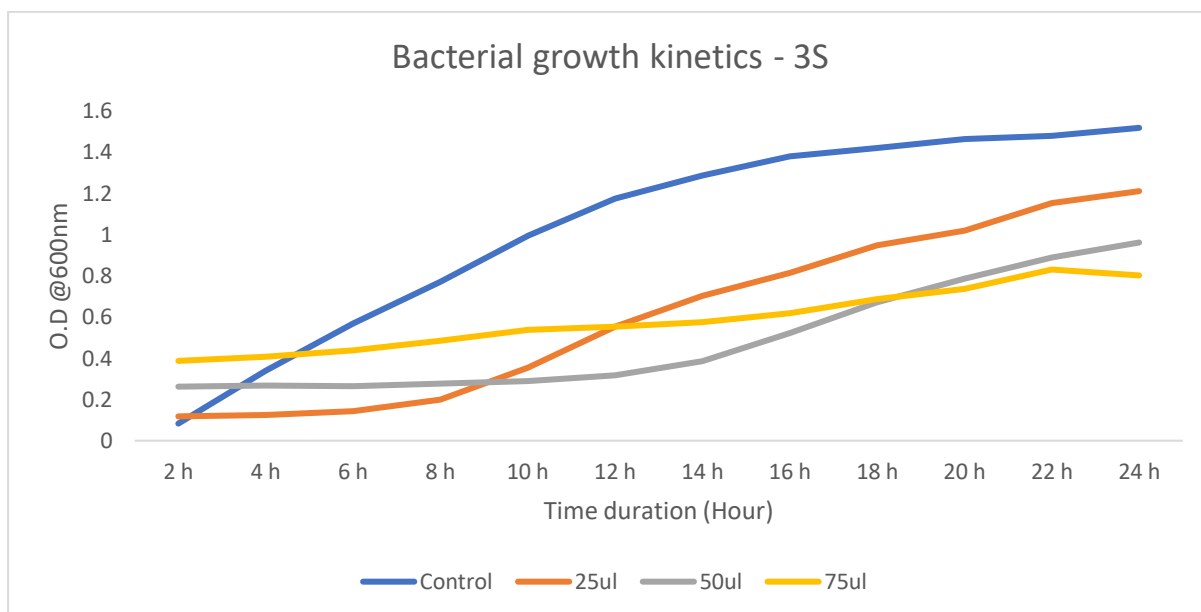


Fig 5.10 Growth kinetics of bacteria –3S

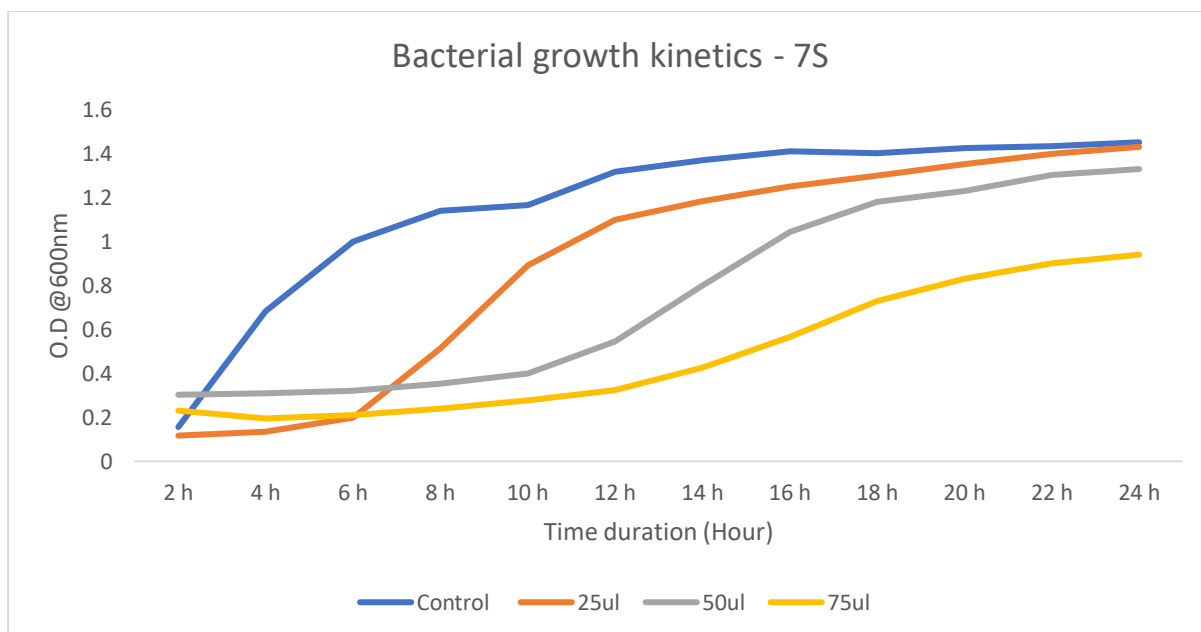


Fig 5.11 Growth kinetics of bacteria –7S

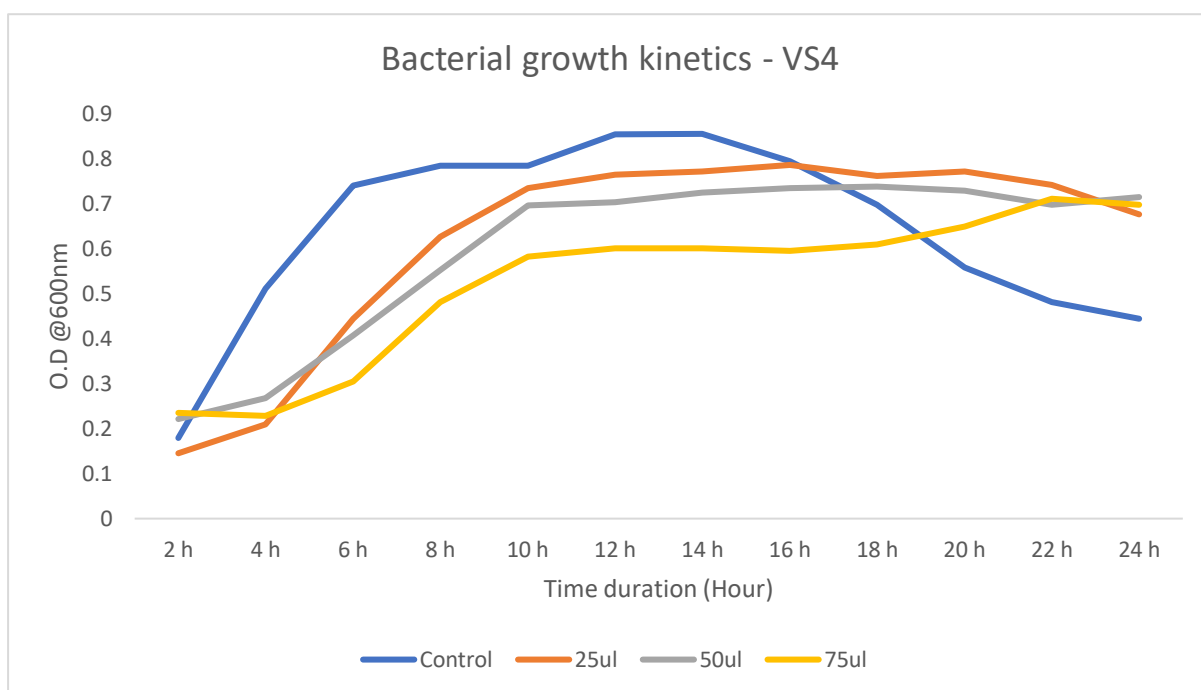


Fig 5.12 Growth kinetics of bacteria –VS4

5.6.2 Biofilm inhibition assay

Biofilm inhibition assay was performed using crystal violet staining to quantify the biofilm formation of bacteria. Cells inoculated with the crude extract has shown diminishing formation in the biofilm whereas the cells with no crude extract continuous to form biofilm at the normal rate. Dose dependent inhibition of biofilm formation has been observed in the assay.

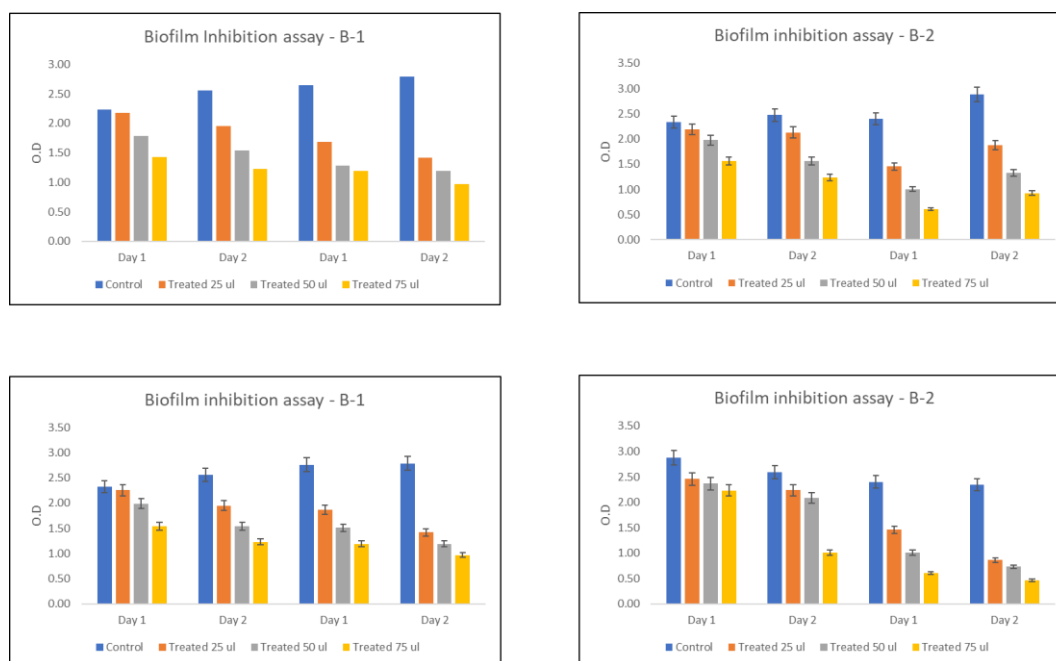


Fig 5.13. Biofilm inhibition assay – B-1, B-2

5.6.3. Phenol sulphuric acid assay

Biofilm forming bacteria inoculated with different concentration of eluted fraction has been determined for its carbohydrate content using phenol-sulphuric acid assay. Eluted fractions with higher concentration have limited the carbohydrate content to the higher extent which directly emphasizes the inhibition of biofilm forming ability of the different biofilm bacteria.

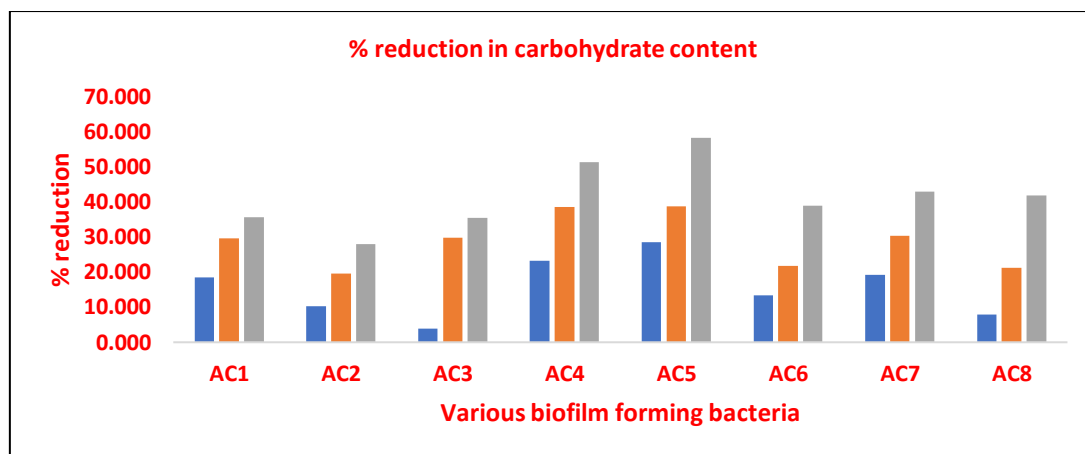


Fig 5.14 Phenol sulphuric acid assay of various biofilm forming bacteria

CHAPTER – 6

CONCLUSIONS

Though bio-fouling cannot be completely inhibited from the marine scenario, it can be controlled to some extent by trying out novel ways. The results obtained from the various screening procedures confirm the hypothesis that marine-based actinomycetes can be used for inhibiting biofouling in the ocean and hence have been suggested to be tested for the said application based on the evidence provided. Actinomycetes have the function of preventing the formation of marine micro fouler while also being careful not to harm undesired organisms. Applying the crude extract to various surfaces will be used in future research to better understand the biofouling and to track the long-term release of actinomycete into the marine environment.

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