Investigation of Dental Biofilm-Producing Bacteria and their Disruptive Strategies

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DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation report entitled, "**Investigation of Dental Biofilm-Producing Bacteria and their Disruptive Strategies**" is based on the results of investigations carried out by me in the Biotechnology at the School of Biological Sciences and Biotechnology, Goa University under the Supervision of Ms Snigdha Sharad Mayenkar and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will not be responsible for the correctness of observations / experimental or other findings given the dissertation.

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COMPLETION CERTIFICATE

This is to certify that the dissertation report **"Investigation of Dental Biofilm-Producing Bacteria and their Disruptive Strategies"** is a bonafide work carried out by **Ms Prajyoti Pandurang Kuttikar** under my supervision in partial fulfilment of the requirements for the award of the degree of **Master of Science** in the Biotechnology discipline at the School of Biological Sciences and Biotechnology, Goa University.

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PREFACE

This dissertation examines the ongoing problem of bacteria that produce biofilms in the mouth, with a specific focus on novel methods to disrupt them. The study investigates traditional disruptors, such as Goan sea salt, and emerging alternative treatments derived from local botanical extracts, demonstrating an interdisciplinary methodology. This preface provides an overview of the reasons that motivated the research, the particular methodological approach used, and the academic setting in which the study was carried out. The objective is to actively participate in ongoing studies in dental microbiology and microbial ecology, with a primary objective of enhancing clinical outcomes and promoting improvements in public health initiatives.

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ABSTRACT

This study investigates the characteristics and disruption strategies for biofilms produced by biofilm-producing bacteria, focusing primarily on those that colonize the oral cavity and the problems caused by our dental health. Biofilms, which are complex assemblies of microorganisms encapsulated within a self-secreted polymeric matrix (EPS), present a strong resistance to standard antimicrobial therapies, posing substantial challenges in both clinical and dental settings. This research combines microbiological techniques with advanced biotechnological methods to dissect the robustness and resilience of dental biofilms. Experimental approaches are employed to assess the efficacy of emerging biofilm disruption methodologies and the utilization of botanical extracts and rock salt. The findings illuminate the mechanisms underlying biofilm persistence and suggest novel, potentially effective strategies for managing biofilm-associated infections. By advancing our understanding of biofilm dynamics and introducing innovative disruption techniques, this study contributes to the enhancement of therapeutic strategies against biofilm-related dental diseases, thereby fostering improved public health.

Chapter I: Introduction

1. INTRODUCTION

1.1 Background

Biofilm is a distinct network of microbes that grows on a surface and are known to produce extracellular polymeric substances (Flemming et al.,2016). These Biofilm-producing bacteria are known for the role they play in the environment which includes all industrial sites, medical areas, and natural settings. In terms of medical areas, Biofilms are known to cause many infections and they are resistant to antibiotics which poses a significant challenge for the treatment of these Biofilms.

Biofilm formation usually takes place by something called pellicle formation. Pellicle is a thin layer which is formed of glycoproteins which are present in the saliva of the mouth. When the oral microbes bind to these proteins, they get attached to a clean tooth surface which eventually leads to degradation of the of the teeth.

They are comprised of the microbial cells and the extracellular polymeric substances (EPS). These EPS vary distinctively like physically and chemically , but it also comprises of polysaccharides like polyanionic or neutral, as is the case of gram-negative bacterias EPS.

When Biofilm formation takes place pellicle is formed by the glycoproteins of the saliva so when the Biofilm-Producing Bacteria recognize these glycoproteins they bind to each other, and a matrix is formed after which different bacterial species will get attached to it forming a matrix called Extracellular polymeric matrix (EPS).

Oral Biofilms are microorganisms which form a network on the tooth enamel which is embedded in a polymer Matrix of bacterial origin. It is said that the mouth is a home to various species of microbes around seven hundred which certainly live inside the mouth and out of which a number of those microorganisms are said to be pathogenic.

Human teeth are the strongest structures which are made of layers of tissues namely dental enamel, dentine, and cementum. The first part is the tooth is situated above the gum line and

the root which are present below the gum line. The crown is covered by entire dental enamel which will protect the teeth because it is known as the strongest substance of a human body. Under the dental enamel is present dentine, which is kind of similar to bone, but it is harder as it surrounds the central chamber which contains blood vessels, nerves and connective tissues. In the root, dentine is further covered by cementum which is a substance which appears to be like a thin bone. .

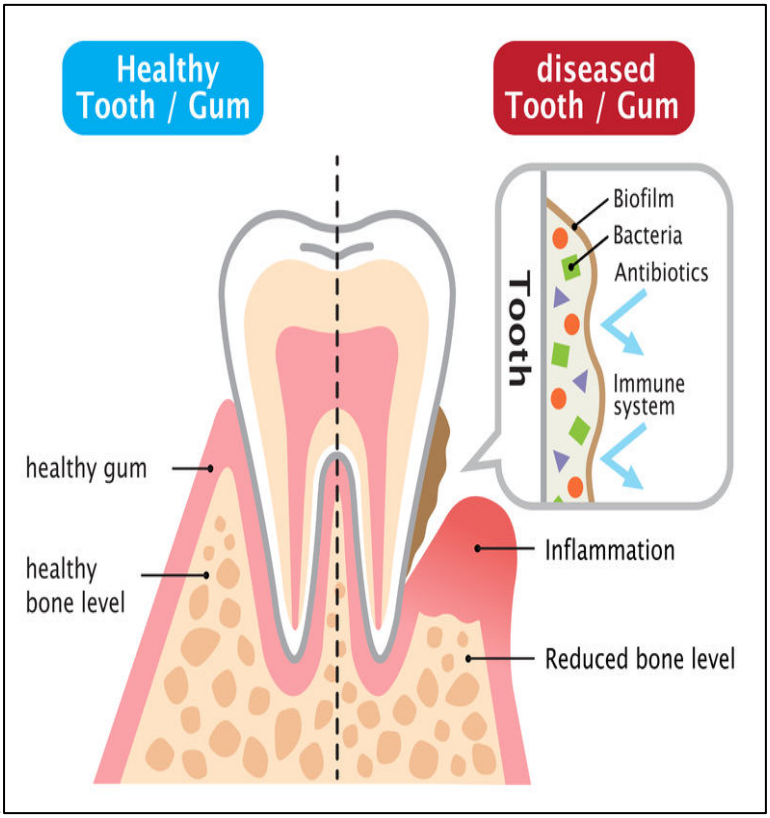


Fig 1.1: Depicts growth of biofilm on teeth

Fig 1.1 Shows the difference between a healthy tooth and a tooth affected by biofilm formation. There are lots of factors which contribute to the Growth of the biofilm-forming bacteria which can be due to the consumption of sweets and unhygienic oral health care practices. A healthy tooth has a healthy bone level where when it is compared with teeth infected by the bacteria the bone level is decreased which can also lead to falling of the teeth in some extreme cases. Microorganisms which are forming the Biofilms are not so vulnerable to antimicrobial agents and it is being said that these microbial groups can show more pathogenicity so due to the shape

of the biofilm the antimicrobial agents will not be penetrated inside even though the surface microbes are vulnerable to these antimicrobial agents.

(Kurtzman, G. M., Horowitz, R. A., Johnson, R., Prestiano, R. A., & Klein, B. I. (2022).

Teeth are surrounded by bacteria which are mostly present in saliva which itself contains extraordinary bacterial species. At an ideal environment and temperature Biofilm will grow stronger and will increase which lead to an increase in dental and periodontal diseases. Besides affecting dental health, it can also lead to financial problems as in very few countries they are known as the fourth cause due to highly-priced treatment

Oral Biofilms a house to many microbes which consist of many species of spirochetes, protozoa, fungi and viruses hence the composition of these Biofilms varies from fitness to sickness and there are also pieces of evidence stating that these biofilms can lead to many serious health-related illnesses like cardiovascular disease, rheumatoid arthritis, and lastly respiratory illnesses.

Biofilm-producing bacteria not only lead to oral diseases like dental plaque formation and dental carries but are also responsible for the decay of human dental samples followed by the alveolar bone disruption. Since Biofilms are creating many problems there is a need to disrupt them and prevent them. On a normal basis, these biofilms can be disrupted by mechanical means like brushing and by chemical means like using mouthwash or toothpaste to get rid of the bacteria where this mouthwash and toothpaste contains some content of alcohol which helps to kill microbes present in the mouth.

The development of innovative strategies to tackle mature biofilm must be formed. Maintenance of clean oral hygiene leads to no Biofilm formation and keeps the oral cavity healthy. In recent times use of developed products to prevent these Biofilms is gaining attention but there has to be a natural way out for the treatment of the same. Since the use of chemicals and mechanical methods in disruption are very common biological methods can also be used

for the same where a bacterium from the mouth which is not involved in biofilm formation can be used against the same.

Biological methods of biofilm disruption offer several advantages over physical and chemical methods. They are often more selective, targeting specific components of the biofilm or specific bacterial species, which can minimize damage to surrounding tissues or surfaces. Biological methods are also more environmentally friendly, as they typically involve biodegradable and non-toxic agents

Although individual methods may be effective, integrated approaches that integrate physical, chemical, and biological methods are often necessary due to the complexity and resilience of biofilms, resulting in more effective disruption of biofilms. Various aspects of biofilm formation and persistence can be targeted by combining different methods, such as killing the embedded bacteria, rupturing the EPS matrix, and preventing initial attachment

Ultimately, the intricate structure of biofilms and their resistance mechanisms necessitate a multimodal approach to biofilm disruption. To effectively control biofilms in medical, industrial, and environmental applications, a more comprehensive approach combining the chemical, and biological strategies is needed.

Chemical methods for biofilm disruption and inhibition, focus on the use of antimicrobial compounds, surfactants, and chemical modifications of surfaces to prevent biofilm adhesion. Surfactants and detergents disrupt biofilms by solubilizing lipids in the biofilm matrix and microbial cell membranes by targeting the hydrophobic interactions that help maintain the structure of biofilms, these agents can effectively disperse microbial communities

The use of surfactants in conjunction with antimicrobial agents can significantly improve the disruption of biofilms, highlighting their potential in cleaning and disinfection protocols.

But the use of natural substances like the use of some botanical plant extracts is also included as an innovative strategy for it to have antimicrobial and antioxidant properties. Botanical

plants have various property which makes them ideal to use as an extract to check if the bacteria can die but like wise use of minor alcohol content could be minimized to make it as natural as it can to show the ability of the plant for disruption for which use of distilled water can be done. Plants are used for many reasons especially the ones with medicinal use as this botanical extract when further implemented on oral use can be effective in killing the target microorganisms especially when a solvent like deionized water is used which will reflect all the properties of the plants.

In favorable growth conditions, Biofilm grows much stronger and more mature they develop a resistance to the antibiotics and hence attract many species which makes it difficult for the antibiotics to penetrate but this all depends up on the capsules of the biofilm-forming bacteria. Capsules are known as an important part of gram-positive and gram-negative bacteria depending on the thickness of Biofilm.

As thicker the capsule stronger the force to which they are attached as after a period of the antibiotics trapping inside the Biofilms the bacteria release some exoenzymes which will degrade the antibiotic trapped and hence it becomes ineffective.

1.2 Aims and Objectives

Aim: To conduct a study on biofilm-producing bacteria and develop various disruptive strategies for them

Objectives:

1) Isolation and screening of biofilm-producing bacteria from the oral cavity

2) To screen potential bacteriostatic biological agents isolated from the oral cavity as potential biofilm disruptive agents.

3) To investigate the potential application of three local Goan plants in disrupting dental biofilms.

4)To study the effect of traditional Goan sea salt on oral biofilm formation.

1.3 Hypothesis

The study focuses on the Biofilm-Producing Bacteria in the oral cavity which is primarily a threat to the dental cavity, supportive and preventive measures are required to disrupt these biofilms to preserve enamel health. As we know biofilms from the oral cavity lead to dental plaque and carry simultaneously other health-related issues it is very important to develop a disruptive strategy which would help to stop these dental plaques from growing, various natural substances and new methods can be developed as an alternative method for disrupting these Biofilms. Explaining various natural substances for their potential Biofilm disruption abilities and leading to the development of new ways to combat growing problems of dental diseases.

1.4 Scope

The aim of this study is to study the bacteria that produce biofilms, with a particular emphasis on those often found in the oral cavity. These bacteria are significant because they are crucial in causing dental problems and are resistant to traditional antimicrobial treatments. The main goals are to isolate and identify these bacteria in order to understand the makeup of biofilms and the environmental factors that promote their growth on tooth surfaces. In addition, the study will analyze the adhesive characteristics of these bacteria and the makeup of the extracellular polymeric substances (EPS), which play a vital role in their attachment and resistance.

An essential component of this study is to search for potential bacteriostatic substances derived from the oral microbiota, which can naturally prevent the production of biofilms without causing any harm to the balance of the oral ecosystem. Furthermore, we will assess the effectiveness of botanical extracts derived from plants grown locally in Goa for their capacity

to disrupt biofilms, with a particular focus on their antibacterial properties. We will also evaluate their viability for incorporation into oral health routines. The study will examine the use of traditional Goan rock salt as a natural agent to disrupt oral biofilms, taking into account its extensive antibacterial capabilities and its possible incorporation into regular dental hygiene practices.

The study intends to develop multimodal techniques for managing biofilms, to find innovative and environmentally friendly solutions to address dental conditions related with biofilms. The objective is to gain an in-depth understanding of biofilm characteristics and investigate natural, efficient alternatives that have the potential to transform oral healthcare practices and reduce the prevalence of dental conditions associated with biofilms.

Chapter II: Literature Review

2. REVIEW OF LITERATURE

The work carried out by Marsh and Bradshaw (1995) provided a foundational understanding of the biochemical and microbial complexities of dental plaque. Their research meticulously described the plaque as a diverse microbial community embedded within a bacterial and salivary polymer Matrix. Crucially, they identified the rapid formation of things film, that acquired dental enamel pellicle, on the tooth surface post-cleaning. This pellicle facilitates the initial adhesion of microbes, thereby initiating plaque formation. Their study emphasized the dynamic interactions between early bacterial colonizers and the pellicle, detailing how these interactions leads to the stability and complexity of the developing biofilm. This research has been instrumental in framing subsequent studies on plaque management and disease prevention.

Wolff and Larson (2009) expanded upon the understanding of dental plaque by investigating its accumulation in specific oral sites. Their comprehensive analysis revealed that plaque development varies significantly across different anatomical niches, such as occlusal fissures, interdental spaces, and gingival crevices. By exploring these variations, Wolff and Larson demonstrated how localized environmental conditions influence the microbial climax communities that form. This nuanced understanding of site-specific plaque accumulation has provided a critical basis for targeted therapeutic strategies and has highlighted the need for personalized approaches in dental hygiene and care.

Further elucidating the role of biofilms in dental health, Wolff and Larson (2009) also reviewed various strategies for managing dental caries associated with biofilms. Their work critically assessed the effectiveness of traditional caries management strategies, including mechanical plaque removal, dietary modifications, and fluoride applications. Additionally, they explored the emerging role of antimicrobial therapies, which target biofilm integrity without disrupting the oral microbiota balance. This comprehensive review has underscored the importance of

integrating multiple caries prevention strategies to enhance efficacy and sustainability in oral health interventions.

introduced cutting-edge methodologies aimed at controlling oral microbial biofilms more effectively. Their research focused on the development of pH-responsive enzyme-mimic nanoparticles and antimicrobial quaternary ammonium salts. These novel agents target specific microenvironments within biofilms, particularly those prone to acid-induced demineralization. The study not only highlighted the potential of these innovative agents in disrupting biofilm formation but also suggested their utility in preventing the onset of secondary caries, marking a significant advancement in dental material sciences and therapeutic approaches.

provide a detailed analysis of how dental biofilms, when regularly disrupted, primarily consist of non-harmful oral streptococci. However, without regular disturbance, these biofilms evolve into a diverse microbial community that can lead to severe dental diseases such as caries and periodontal conditions. Their study emphasizes the necessity for consistent oral hygiene practices to maintain a balanced microbial environment that supports oral health rather than disease progression (Larsen & Fiehn, 2017).

In response to the challenges posed by dental biofilms, recent advancements in nanotechnology have been leveraged to develop innovative treatment options. review the application of nanodrugs in the dental field, highlighting how metallic nanoparticles such as silver, gold, and titanium oxide exhibit potent antibacterial and antibiofilm activities. These nanoparticles disrupt biofilm structure and function, thereby mitigating the biofilm's pathogenic potential. This study illustrates the shift towards using nanotechnology to enhance the efficacy of dental treatments, providing a promising outlook for the prevention and eradication of biofilm-associated diseases.

presented a detailed study on how biofilms affect medical devices, especially implants. Biofilms are like a thin, sticky film of bacteria that cling onto surfaces, including medical

implants. These can lead to infections that are hard to treat because biofilms protect bacteria from antibiotics and the body's immune system. The researchers looked into new ways to tackle this issue, focusing on three main strategies: discovering new antibiotics that can penetrate these stubborn biofilms, developing treatments that can be applied directly to the surfaces of implants to stop biofilms from forming in the first place, and creating coatings that make the implants themselves able to kill bacteria. This work is really important because it helps us understand better ways to stop infections before they start, which is much better than trying to treat them after they've taken hold. For people who need medical implants, these findings could be a game-changer, making their treatments safer and more effective.

Then there's the work of which dives into the world of oral biofilms—those groups of bacteria that live in our mouths and can cause tooth decay and gum disease. The team focused on finding out what makes these biofilms tick and how to control the bad bacteria that cause problems without messing up the good ones that we need for a healthy mouth. They used advanced technology that lets them look closely at biofilms and see what's happening on a really small scale. By doing this, they discovered new chemical and biological ways to stop the harmful effects of these biofilms. Their research is a big deal because it's helping us to find ways to fight common dental problems in a smart way. Instead of just trying to kill all the bacteria in our mouths, which isn't a good idea because some bacteria are actually good for us, they're working on targeted treatments. These treatments would only take out the bad bacteria and leave the good ones alone, making it possible to keep our mouths healthy without causing other problems.

In their 2020 investigation, Martínez-Hernández, Reda, and Hannig embarked on a detailed examination to understand how chlorhexidine, commonly known as CHX, can impact the formation and breakup of biofilms on teeth. They simulated an oral environment by using enamel samples from cows, which is quite similar to human enamel, to observe the effects of

CHX. Their findings were significant, revealing that rinsing with CHX had a powerful effect, not only in preventing biofilms from taking hold on the enamel but also in breaking down those that had already formed. This study is particularly relevant as it underscores the effectiveness of CHX as a preventive tool in dental care. By integrating CHX into daily oral hygiene routines, there is potential to significantly cut down the occurrence of dental diseases that are associated with biofilms, such as cavities and gum disease. The implications of these findings extend to everyday practices, suggesting that regular use of CHX could be a simple yet powerful addition to our oral health arsenal, keeping our teeth healthier and potentially saving us from more invasive dental treatments down the line.

Each referenced study contributes distinct insights into the complex interactions within biofilms, the effectiveness of various management strategies, and the implications of these strategies for dental and systemic health. The collective findings underscore the necessity of continued research and development in biofilm management to enhance both oral and systemic health outcomes.

Chapter III: Methodology

3. METHODOLOGY

3.1 Isolation and screening of Biofilm-Producing Bacteria from the oral cavity

To isolate bio-film-producing bacteria from the oral cavity consent form was given to five different individuals to fully understand the purpose of the study. The samples were then collected from these five different individuals, and they were asked to fill out the consent form which included the protocol for tooth sample collection followed by the risks and benefits.

3.1.1 Collection of Samples from the oral cavity

For the isolation of the biofilm-producing bacteria, a sterile cotton swab was carefully swabbed on the upper and lower molar and premolar teeth of five individuals in the morning hours before brushing. The swab was placed in a small tube containing sterile saline and it was closed and immediately brought to the lab for further studies.

3.1.2 Serial dilution and plating

The aim of serial dilution was to estimate the concentration by counting the number of colonies of an unknown sample. Eppendorf tubes were taken 900ul of sterile saline was added and 3 dilutions were made up to 1ml. From these prepared dilutions 100ul was spread plated on a suitable agar medium.

3.1.3 Preparation of Congo red agar medium for bacterial isolation

Congo red agar media was weighed to prepare 250ml of media. The powder was added to the flask and gradually dissolved using 250ml distilled water. A cotton plug was placed over the mouth of the conical flask and the media was autoclaved at 15psi pressure 121°C for 30 minutes. The media was cooled under the tap water and poured onto the autoclaved Petri plates. The plates were then spread-plated with the prepared different dilutions and were then labelled by mentioning the name of the media, tooth sample code and date respectively. The plates were sealed with parafilm, inverted, and kept for incubation for 3 °C and 24 hours. The petri plates

were then observed for EPS production that is the formation of black colonies on the red Congo red agar medium.

3.1.4 Purification of biofilm-producing bacteria

The plates were kept in an incubator and were checked for the number of colonies formed after the incubation period out of which one selected colony was again streaked on a fresh Congo red agar plate, labelled, inverted, and kept for incubation. The collected tooth samples were given code, and each sample was streaked separately to obtain a pure culture.

3.1.5 Preparation of biofilm-producing cultures

Purified culture plates were labelled as master plates and a total of five isolates were labelled as SM1, SM2, SM3, SM4 and SM5. Culture broth was prepared for these samples where the nutrient broth was weighed and poured into a conical flask and then the flask was autoclaved cooled and inoculated with the respective bacteria from the master plates. The conical flasks were labelled and kept on a rotatory shaker for incubation for 24 hours. The cultures were frequently sub-cultured to avoid overgrowth and contamination.

3.1.6 Characterization of bacterial isolates

The following standard bacterial characterization assay characterised purified bacterial isolates:

3.1.6.1 Colony Characteristics

The colony characteristics were examined based on the visual observations and setting the parameters as per the colony morphology protocol American Society of Microbiology. Basic characteristics like form, size, colour, elevation, opacity, and texture were recorded (Breakwell et al.,2007)

3.1.6.2 Gram staining

Gram staining is a basic technique that aims to phenotypically characterize the bacteria into Gram-positive or Gram-negative bacteria. There are two stains namely crystal violet and safranin used. Gram-positive bacteria generally have a thick peptidoglycan layer, this layer absorbs crystal violet and forms a purple complex in the presence of Grams iodine (mordant). Whereas Gram-negative bacteria have a thin peptidoglycan layer which is why they cannot form a strong complex with crystal violet and it gets destained when Grams alcohol (decolourizer) is added and gets stained by counterstain safranin, which gives Grams-negative bacteria its pinkish red colour (Gram stain protocol /ASM .org.n.d.).

The glass was taken and wiped with 70% ethanol to make it clean and grease-free. Using a sterile inoculating loop, a colony from the culture was picked and a smear was made over the slide and heat-fixed by passing the slide quickly through the flames. This slide was flooded with crystal violet for one minute and the stain was then washed with distilled water followed by the addition of a few drops of Grams iodine and was later left for 30 seconds. Decolorizer was added and the slide was again left for 1 minute. Counterstain safranin was added to the slides and kept for one minute. Using a gentle steam of distilled water, the slide was later washed and air dried. The slide was then observed under oil immersion magnification(100X).

3.1.7 Crystal violet assay

The Crystal violet assay is an important tool for the study of the early stages of biofilm formation and has been applied primarily for the study of bacterial biofilms because this assay uses static, batch growth conditions and doesn’t allow for the formation of the mature biofilms. A microtiter plate is used for this assay as this microtiter plate allows for the formation of a biofilm on the wall and or bottom of a microtiter plate and the extent of biofilm formation is measured using the crystal violet dye as it stains the adhered biofilm biomass. (O’Toole, G.A.(2011).

3.1.7.1 Preparation of the microtiter plate

In a microtiter plate containing 2ml of nutrient broth, 20ul of 24-hour-old culture was inoculated and a well with uninoculated culture was kept as control. This plate was then incubated on a rotatory shaker at 37 °C for 24 hours at the speed of 90 rpm. After the incubation period was over the microtiter plate was inverted and the content was discarded, and the plate was blotted on a tissue paper for a time period of 15 minutes. The wells were later washed in saline and the plate was again kept in an inverted position on a tissue paper for blotting for 15 minutes. 100 ul of 0.1% crystal violet dye was added into all these wells and the microtiter plate was kept incubated at 37 °C for 15 minutes on a rotatory shaker at 90 rpm speed.

The dye was then discarded, and the wells were washed with saline to remove excess stain followed by the addition of 30% acetic acid which was added in all the wells up to the brim. The microtiter plate was then kept for incubation at 37 °C at the speed of 90rpm on a rotatory shaker for 15 minutes and later the acetic acid was discarded as this was done to elute out the stain taken by the biofilm-forming cells. The final step was to read the plate under the Elisa plate reader at the wavelength of 595nm and acetic acid was used in the control wells kept as blank. The graph was plotted according to the absorbance reading obtained.

3.1.8 Optimization assay for growth media

The aim of carrying out an optimization assay was to check for the suitable growth medium the biofilm-producing bacteria needs as a supporting growth medium. In this assay, a minimal agar medium was supplemented with growth factors like sucrose as sugar and peptone as a nitrogen source making it completely specific for the biofilm-producing bacteria to grow. The objective was to check if the biofilm-producing bacteria uses the particular sugar and nitrogen source for their growth. Sucrose was supplemented because it contains glucose and fructose and all these sugars occur in the certain food we consume. Peptone is considered to provide nutrients to the bacteria and helps in rapid cell growth.

3.1.8.1 Preparation of minimal agar medium with the addition of sugars and carbon source

Four flasks were made and labelled; accordingly, In flask 1 minimal agar medium was weighed for preparing 250ml of total medium and only minimal agar was added to the flask. Flask 2 was supplemented with sucrose which already contained minimal agar followed by flask 3 containing minimal agar and peptone and flask 4 containing minimal agar with sucrose and peptone. All the contents were weighed accordingly and gradually added to the respective flasks and then the conical flask was autoclaved at 121psi pressure for one hour. After the autoclave was done the media was cooled and poured in petri plates and kept.

After the plates were prepared the plates were streaked with respective cultures and they were wrapped and incubated at 37°C for 24 hours. The bacterial growth was observed after the period was complete.

3.1.9 IMViC Test

The biochemical test was done in a kit which was a combination of 12 tests for the differentiation of Enterobacteriaceae species. The kit used contained sterile media for Indole, Methyl red, Voges Proskauer's, and Citrate utilization tests, as well as eight different carbohydrates: Glucose, Adonitol, Arabinose, Lactose, Sorbitol, Mannitol, Rhamnose, and Sucrose.

The tests were based on the principle of pH change and substrate utilization. Upon incubation, the organisms underwent metabolic changes, which were indicated by a colour change in the media. The colour changes could be interpreted visually or after the addition of a reagent.

The kit was opened aseptically followed by peeling off the sealing tape. Each well was inoculated with 50 µl of the above inoculum by surface inoculation method.

3.1.9.1 Carbohydrate utilization test

The carbohydrate test was done using a kit which involved a combination of 35 tests. The used kit contained 3 Parts A, B, and C, each kit had different carbohydrate utilization tests.

The kit with Part A consisted of 12 carbohydrate utilization tests namely Lactose, Xylose, Maltose, Fructose, Dextrose, Galactose, Raffinose, Trehalose, Melibiose, Sucrose, L-Arabinose, and Mannose.

The kit with Part B contained 12 carbohydrate utilization tests: Inulin, Sodium gluconate, Glycerol, Salicin, Dulcitol, Inositol, Sorbitol, Mannitol, Adonitol, Arabitol, Erythritol, and alpha-Methyl-D-glucoside.

The kit with Part C included 11 sugar utilization tests and 1 control: Rhamnose, Cellobiose, Melezitose, alpha-Methyl-D-Mannoside, Xylitol, ONPG, Esculin, D-Arabinose, Citrate, Malonate, Sorbose, and 1 control.

The kit was opened aseptically followed by peeling off the sealing tape. Each well was inoculated with 50 µl of the above inoculum by surface inoculation method.

3.2 To screen potential bacteriostatic biological agents forming bacteria isolated from the oral cavity as potential biofilm disruptive agents.

The biological strategy involves the use of other bacteria to kill pathogenic bacteria. Potential bacteriostatic biological agents are bacteria which does not play a role in biofilm formation but also, do not allow the biofilm-producing bacteria to grow. These bacteria which belong to the

same environment will prevent the growth of the biofilm if they have antimicrobial properties against them. Furthermore, no harm will be caused if they are tested in the same environment as the use of other biological agents against this biofilm-producing bacteria can be dangerous to health as it can't be used practically on oral surfaces.

3.2.1 Sample collection of potential bacteriostatic biological agent.

For the isolation of potential bacteriostatic biological agents like point 3.1.1, a sterile cotton swab was carefully swabbed on the upper and lower molar and premolar teeth of five individuals in the morning hours before brushing. The swab was placed in a small tube containing sterile saline and it was closed and immediately brought to the lab for further studies. Then after preparing serial dilution and plating them on Congo red agar medium, they were checked for no EPS production on the plate and then were streaked on nutrient agar for purification and for further studies.

3.2.3 Giant colony technique

The giant colony technique is a type of technique which is used for determining the antibiotics which can be diffused in the solid medium. In this type of technique, MHA plates were used where the plate was first streaked in a vertical line using the potential bacteriostatic biological agent and was incubated at 37°C for 24 hours. The biofilm cultures were streaked on the next day in a horizontal pattern close but not touching to the vertically streaked bacteria and kept for incubation at 37°C for 24 hours. The aim here was to check if there was inhibition of growth of the Biofilm-Producing Bacteria.

3.2.4 Spot inoculation

Spot inoculation is a technique mainly used to see the zone of inhibition formed by the spotted bacteria. For this technique, MHA plates were spread-plated with respective biofilm-producing cultures and the potential bacteriostatic biological agents were spot-inoculated at the centre of the plate and kept for incubation for 24 hours at 37 °C.

3.2.5 To study the antimicrobial activity of potential bacteriostatic biological agent by agar well diffusion assay

To disrupt the biofilm-producing bacteria the potential bacteriostatic biological agents were isolated from the same culture plate and the bacterial colony showing no EPS production was streaked on a nutrient agar plate and then purified on a Congo red agar plate. To check for the antimicrobial properties MHA(muller Hilton agar) plates were made as this medium allows for better diffusion of the bacteriostatic agent than most other media.

An agar well diffusion assay was also carried out to check for the zone of inhibition where the MHA plates were spread-plated with respective cultures and then with the help of cork borer wells were made. In these wells 100ul of the potential bacteriostatic biological agent was added and the plates were later stored at 37 °C for incubation. Results were checked on the next day after the incubation period was over.

3.3 To investigate the potential application of three local Goan plants in disrupting dental biofilms.

Plant extracts are mainly used as they are known to have antimicrobial and antioxidant properties in them. Goan plants are very rich in antimicrobial properties and the use of these extracts is quite a large scale for curing many diseases which also include dental diseases out of which three plants were used namely Plectranthus amboinicus (Votellav), Andrographis paniculate (karate) and Azadirachta indica (neem). The aerial parts, roots and shoots have been used over centuries for making plant extracts and mainly solvents like water, chloroform, ether alcohol, etc are used. The extract made for this study was purely of autoclaved distilled water and the reason behind using this autoclaved distilled water was to fully focus on the plant properties to disrupt the biofilm as many extracts made consist of some amount of alcohol content and the plant extract made of alcohol solvent cannot be directly used on the oral surface as it may have certain disadvantages.

3.3.1 Sample collection of botanical plant extracts

Three local Goan plants were chosen namely Coleus aromaticus (Wattelaw), Androgeaphis paniculata and Occimum sanctum (Tulsi). These plants were collected from the place marked according to the map. The plant was first identified and then the marked part was used for making the extracts, so the marked part was separated with the help of scissors and then placed inside a clean container and taken to the lab immediately for further studies.

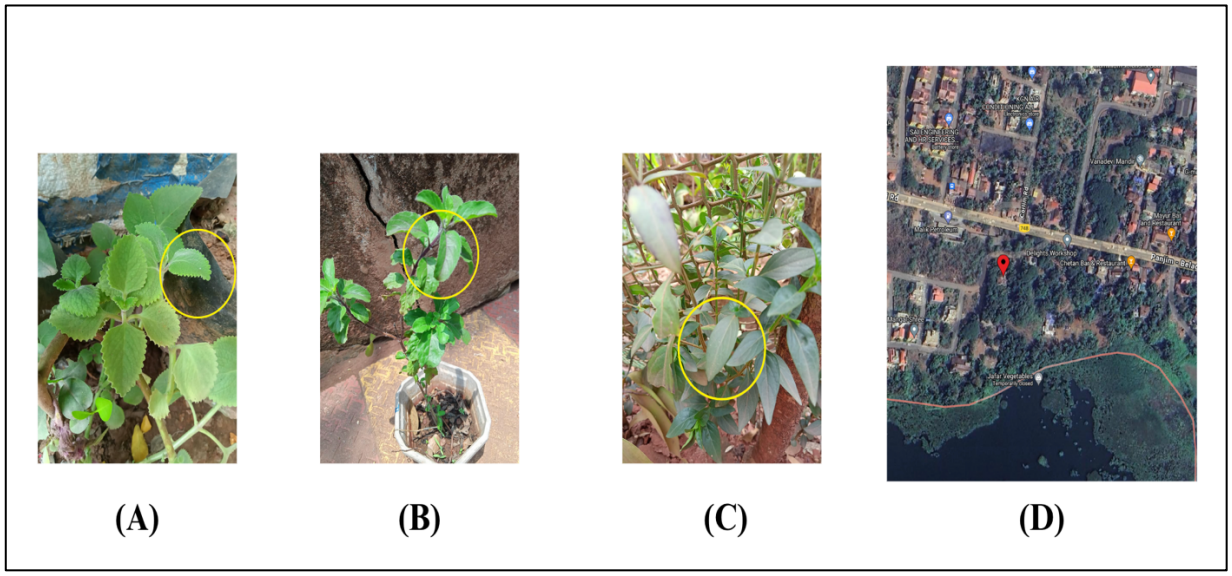


Figure 3.31: (A) Plant of *Coleus aromaticus* (Wattelaw) (B)Plant of *Occimum sanctum* (Tulsi) (C)Plant of *Andrographis paniculate* (Kirayate) (D) Sampling site, Corlim, Goa, India

3.3.2 Preparation of Goan botanical plant extracts

The Plant leaves were washed using distilled water cleaned to remove excess solids and dried for ten minutes. The mortar and pestle were washed and wiped with alcohol followed by autoclaving distilled water for about 30 minutes and after autoclaving it was cooled and kept. The freshly dried leaves were mixed with this autoclaved water and a fine paste was made with mortar and pestle. The Whatman filter paper 41 was cut in a cone shape and placed inside a funnel under which a clean beaker was placed. The freshly ground paste was through passed the filter paper and the filtered solution was stored in an ambered coloured bottle.

3.3.3 Preliminary test for antimicrobial activity against Biofilm forming bacteria.

Muller Hilton agar medium was prepared, and plates were made. The plates were spread-plated with biofilm-producing culture and kept for 5 minutes. After the time period was over, autoclaved cork borer wells were punched on the plate and the wells were filled with 100ul of the plant extracts. The plates were then stored under 37 °C for 24 hours for incubation and after 24 hours they were checked for zone of inhibition.

3.4 To study the effect of traditional Goan sea salt on oral Biofilm formation.

Salts are known for many of their benefits but the major benefit which they possess in terms of dental health care is the disruption of the oral microbiota because of which salts are used in toothpastes. Goan salts are very useful in terms of many things but the kind of property it has makes it more important. Rock salts are not refined salts as they are directly collected after the drying process from the salt pans and sold to people which to use them for scientific purposes it has to pass under various stages to get properly filtered and autoclaved for final use.

3.4.1 Sample collection of traditional Goan rock salt

Goan rock salt was collected from a salt pan located in Curca Goa according to the location marked on the map. The salt sample was collected and placed inside a clean container and the container was immediately taken to the lab for further studies for the test to carry out.

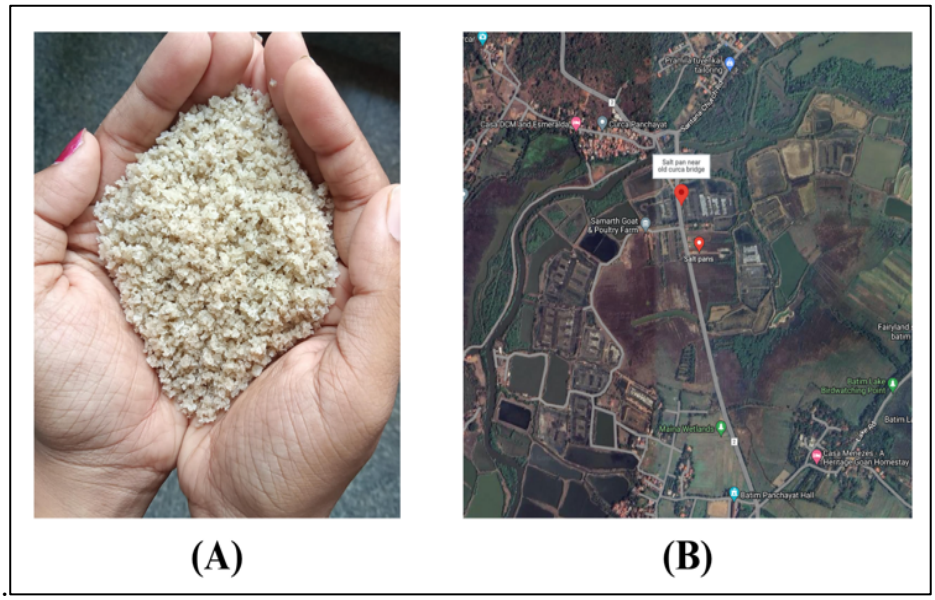


Fig 3.4.1: (A)Traditional rock salt sample from Curca, Goa, India (B) Sampling site, Curca, Goa, India

3.4.2 Preparation of rock salt solution of different concentrations

Rock salt was first cleaned to remove unwanted debris and different concentration of the rock salt was prepared, for which four different concentrations were made ranging from 10% to 40%. Four beakers were taken and labelled and in each respective concentrations were added by weighing, 10g was weighed and added in 100 distilled water same way 20g in 100ml distilled water, 30g in 100ml distilled water and 40g in 100l distilled water accordingly. The weighed salt was mixed with the help of a glass rod until the salt completely dissolved. After the salt was mixed four funnels were taken and Whatman filter paper was placed inside it the salt solution was poured and it was done until the solution was filtered. After the solution was filtered the process was repeated twice to make sure that no debris remained, and the final content was stored and stored.

3.4.3 Pellicle assay

A thin layer of bacteria is called the pellicle and pellicle assay is a type of assay used to check pellicle formation by certain species of bacteria. In this type of assay 24 well microtiter plate

whose maximum volume was 2ml. The wells were labelled on top of the cover plate where two wells were kept as control, control 1 with the culture and control 2 without the culture. The remaining 4 wells were filled with 10% to 40% salt solution and minimal agar medium supplemented with sucrose and peptone. The wells were then inoculated with 20 ul of a culture where 4 cultures that are SM1, SM2, SM3, and SM4 were used for the same. The protocol was performed under sterile conditions and after inoculation.

3.4.4 Textural colony assay

The assay includes the formation of textural colonies which are formed when they are treated with specific salt solutions. In this assay supplemented minimal agar medium was prepared which consisted of salt solutions so four conical flasks were prepared with 10% to 40% concentrations the media was autoclaved and plates were prepared. The plates were spotted with the biofilm-producing bacteria and plates were stored in an incubator for 24 hours at 37 °C for growth. After the time period was over the plate was observed under a microscope at 4X magnification to observe for the wrinkles formed. The plate was then again kept in an incubator to check the plate after 48 hours of growth. The plate was observed under a microscope and sterile conditions a sterile toothpick was through the colony the reason behind doing so was to check if the biofilm-producing bacterial colony was strong or weak enough and if it was weak the toothpick easily passed it meant that the salt solution has affected the growth.

3.4.5 Growth curve to study the effect of rock salt solution on biofilm-producing bacteria.

To check if the salt solution prepared had any effect on the biofilm-producing bacteria growth curve was studied for a total period of 8 hours. In this 96-well microtiter plate where four cultures were used the plate was divided into four halves and one well was kept has control which consisted of 300ul of minimal agar supplemented medium with inoculated 20ul culture

and 4 wells were marked as 10% to 40% which consisted of the salt solutions and supplemented minimal agar medium and 20ul of culture. The plate was kept under an ELISA plate reader and readings were taken at zero time in the same way after every hour reading was taken to observe the effect of salt solution on the biofilm bacteria. The reading obtained was tabulated and the growth curve of absorbance versus the time was plotted.

Chapter IV: Results and Discussion

4. RESULTS AND DISCUSSION

4.1 Isolation and screening of biofilm-producing bacteria from the oral cavity

The microbial cultures obtained from swabbing the oral cavity were allowed to grow on a Congo red agar medium to understand the bacterial diversity. They were purified on a nutrient agar medium. These plates were incubated for 24 hours at 37°C and then master plates were prepared on Congo red agar plates for the biofilm-producing bacteria.

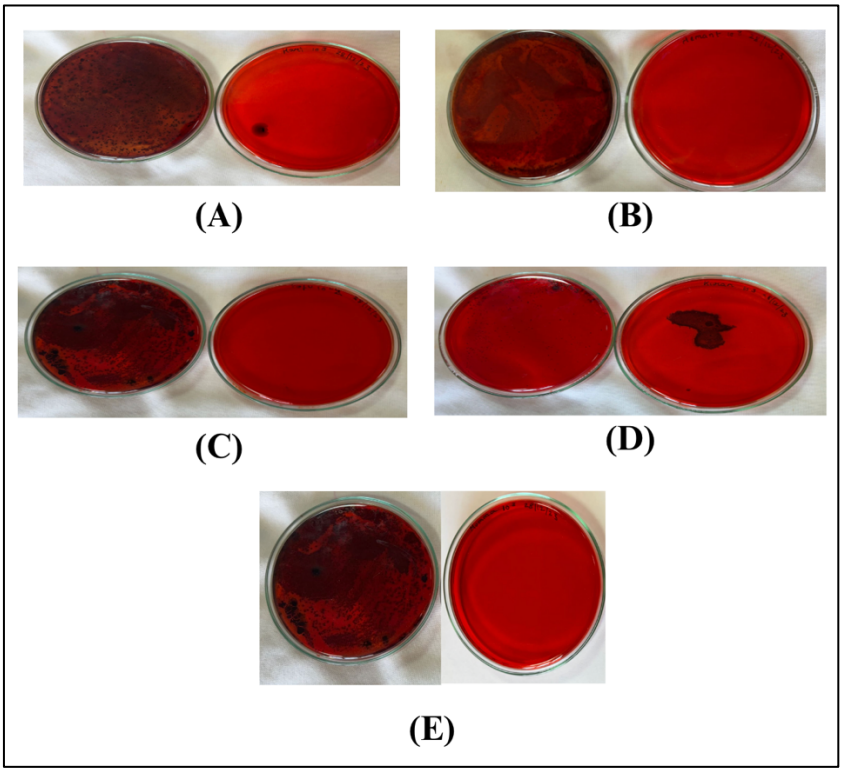


Figure 4.1: Congo red agar medium spread plated by the samples isolated from five individuals and were labelled.

(A) SM1 from sample 1

(B) SM2 from sample 2

(C) SM3 from sample 3

(D) SM4 from sample 4

(E) SM5 from sample 5

4.1.1 Purification of the biofilm-Producing Colonies

The biofilm-producing bacteria from the master plate were streaked on nutrient agar medium to observe for the colony characteristics and the plates were then incubated.

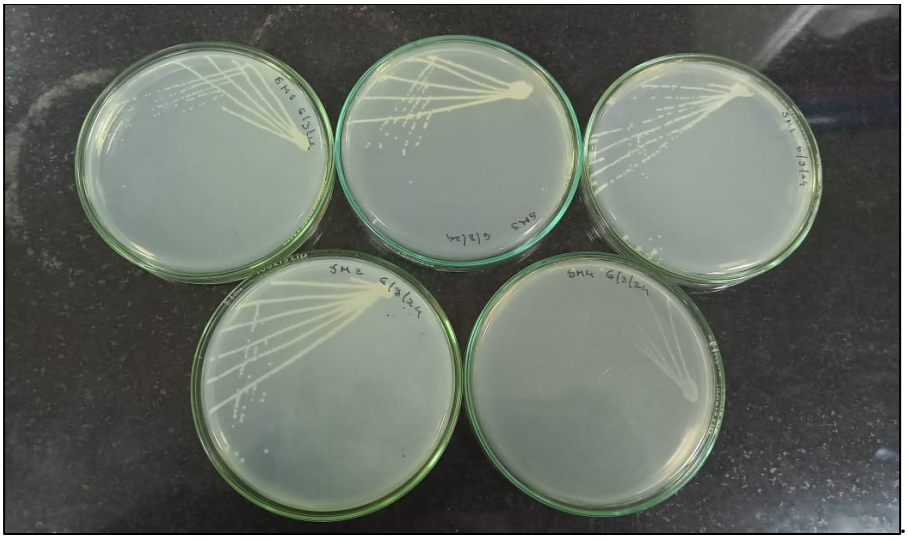


Fig 4.1.1: Biofilm-producing bacteria streaked on nutrient agar (NA) plates.

4.1.2 Colony Characteristics

The following table depicts the colony characteristics of bacterial isolates:

Characters of bacteria	Bacteria 1	Bacteria 2	Bacteria 3	Bacteria 4	Bacteria 5
	SM1	SM2	SM3	SM4	SM5
Form: Punctiform/Circular/Filamentous/Irregular/ Rhizoid/Spindle	Round	Irregular	Round	Irregular	Round
Colour	White	White	White	White	White
Elevation: Flat/Raised/Convex/Pulvinate/Umbonate	Raised	Umbonate	Raised	Umbonate	Raised
Margin: Entire/Undulate/Lobate/Erose/Filamentous	Entire	Undulate	Entire	Undulate	Entire
Opacity: Opaque/Translucent/Transparent	Opaque	Opaque	Opaque	Opaque	Opaque

Table 4.1.2: Colony characteristics of bacterial isolates

4.1.3 Gram staining

Gram staining was carried out of isolates SM1, SM2, SM3, SM4 and SM5 and viewed under a microscope. It was found that bacterial isolates SM1, SM4 and SM5 were gram-positive, and SM2 and SM3 were gram-negative.

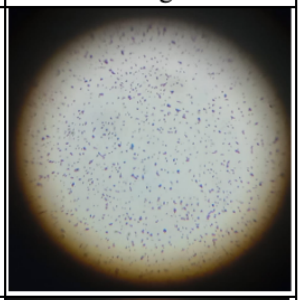
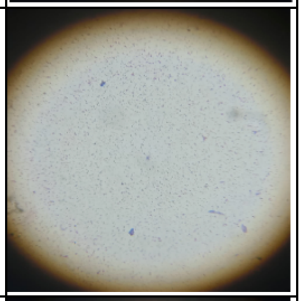
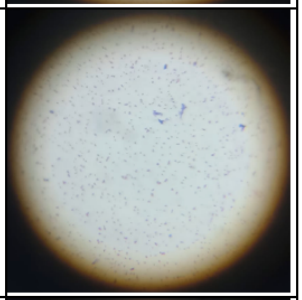
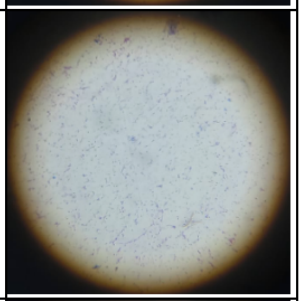
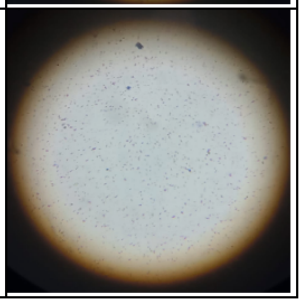
Bacterial Isolates	Gram Characteristics	Image
SM1	Gram positive	
SM2	Gram negative	
SM3	Gram negative	
SM4	Gram positive	
SM5	Gram positive	

Table 4.1.3: Gram characteristics of bacterial isolates SM1, SM2, SM3, SM4, & SM5

4.1.4 Crystal Violet Assay

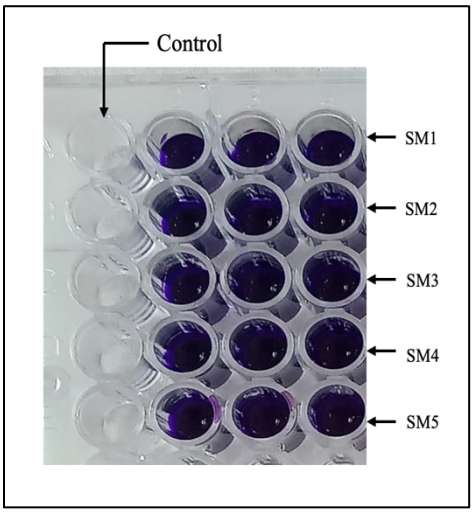


Fig 4.1.4: Crystal violet assay being carried out in 96 wells microtiter plate.

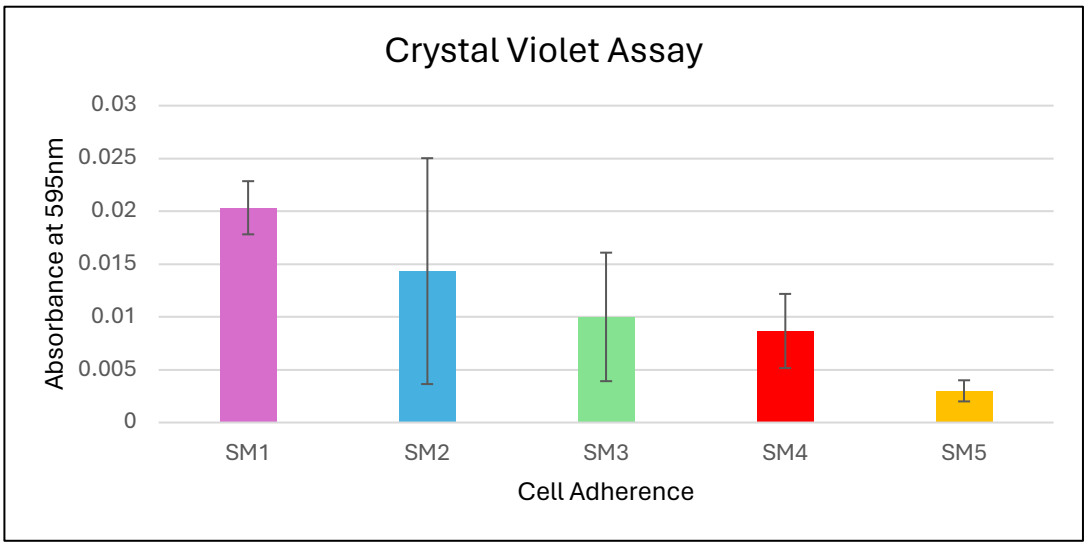


Fig 4.1.4.1: Graphical representation of Crystal violet assay, in terms of absorbance Vs cell adherence.

4.1.5 Optimization Assay for growth media.

Optimization assay was carried out on minimal agar to understand the nutrient dependency of peptone and sucrose of biofilm-producing bacteria.

All the bacterial isolates except SM5 showed no growth in Minimal agar which was not supplemented with peptone and sucrose.

All the bacterial isolates except SM4 showed growth in Minimal agar which was supplemented with peptone only.

All the bacterial isolates except SM1 showed growth in Minimal agar which was supplemented with sucrose only.

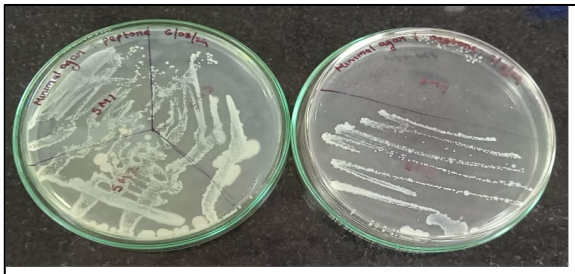


Fig: Minimal agar with peptone as nitrogen source

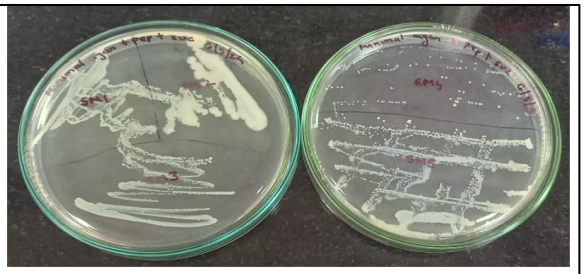


Fig: Minimal agar with peptone as nitrogen source and sucrose as sugar

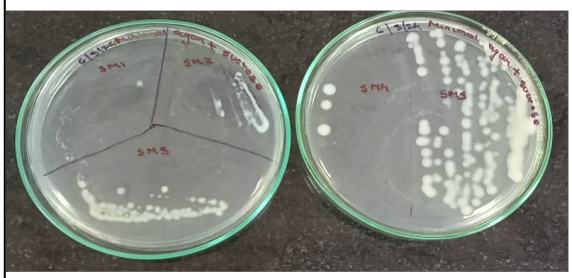


Fig: Minimal agar with sucrose as sugar source

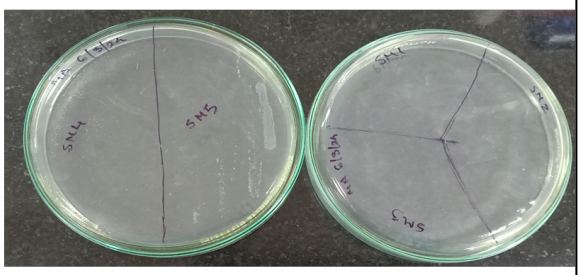


Fig: Minimal Agar plate with no peptone and sucrose

4.1.6 Biochemical test – IMViC test

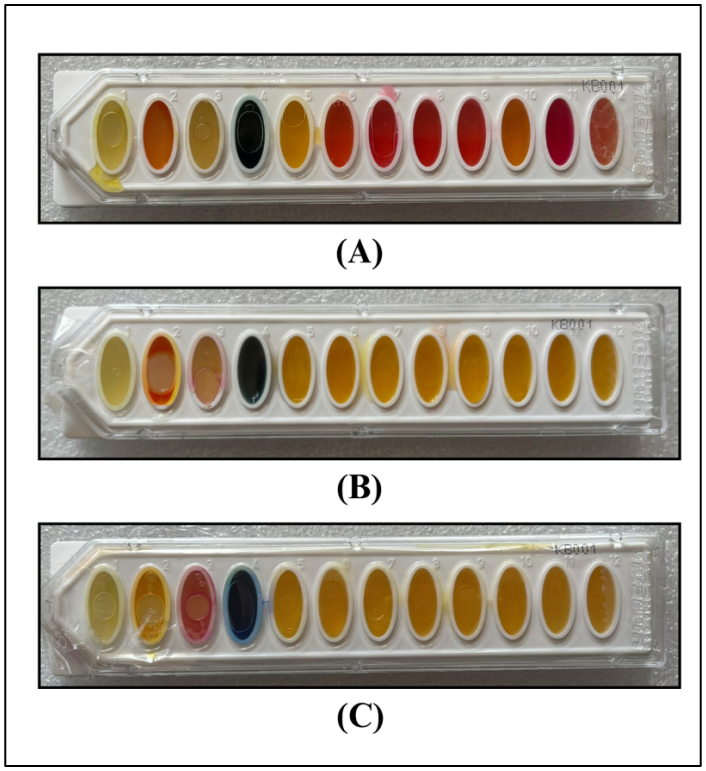


Fig 4.1.6: (A) Tabular representation of the results of SM1, SM2 & SM3 observed in IMViC test kit

SM1			SM2			SM3		
Sr. No	Component	Results	Sr. No	Component	Results	Sr. No	Component	Results
1	Indole	-	1	Indole	-	1	Indole	-
2	Methyl Red	-	2	Methyl Red	-	2	Methyl Red	+
3	Voges proskauer	+	3	Voges proskauer	+	3	Voges proskauer	+

4	Citrate Utilization	-	4	Citrate Utilization	-	4	Citrate Utilization	-
5	Glucose	+	5	Glucose	+	5	Glucose	+
6	Adonitol	-	6	Adonitol	+	6	Adonitol	+
7	Arabinose	-	7	Arabinose	+	7	Arabinose	+
8	Lactose	-	8	Lactose	+	8	Lactose	+
9	Sorbitol	-	9	Sorbitol	+	9	Sorbitol	+
10	Mannitol	+	10	Mannitol	+	10	Mannitol	+
11	Rhamnose	-	11	Rhamnose	+	11	Rhamnose	+
12	Sucrose	-	12	Sucrose	+	12	Sucrose	+

Table 4.1.6: Table representing the results of SM1, SM2 & SM3 found in IMViC test kit

4.1.7 Carbohydrate Utilization Test

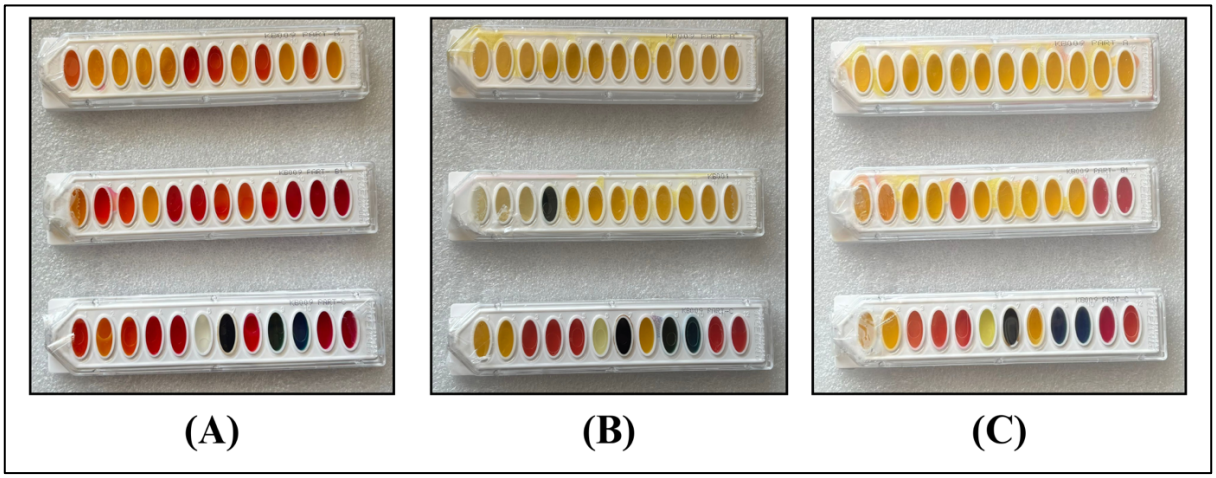


Fig 4.1.7: (A) Results of SM1 culture for carbohydrate utilization A/B/C test kit (B) Results of SM2 culture for carbohydrate utilization A/B/C test kit (C) Results of SM3 culture for carbohydrate utilization A/B/C test kit

SM1 Culture								
Part A			Part B			Part C		
Sr. No	Component	Results	Sr. No	Component	Results	Sr. No	Component	Results
1	Lactose	-	1	Inulin	+	1	Rhamnose	+
2	Xylose	+	2	Sodium gluconate	-	2	Cellobiose	+
3	Maltose	+	3	Glycerol	-	3	Melezitose	-
4	Fructose	+	4	Salicin	+	4	alpha-Methyl-D-Mannoside	-
5	Dextrose	+	5	Dulcitol	-	5	Xylitol	-
6	Galactose	-	6	Inositol	-	6	ONPG	-
7	Raffinose	-	7	Sorbitol	-	7	Esculin	+
8	Trehalose	+	8	Mannitol	-	8	D-Arabinose	+
9	Melibiose	-	9	Adonitol	-	9	Citrate	-
10	Sucrose	+	10	Arabitol	-	10	Malonate	-
11	L-Arabinose	-	11	Erythritol	-	11	Sorbose	-
12	Mannose	+	12	alpha-Methyl-D-glucoside	-		Control	-

SM2 Culture								
Part A			Part B			Part C		
Sr. No	Component	Results	Sr. No	Component	Results	Sr. No	Component	Results
1	Lactose	+	1	Inulin	+	1	Rhamnose	+
2	Xylose	+	2	Sodium gluconate	+	2	Cellobiose	+
3	Maltose	+	3	Glycerol	+	3	Melezitose	-
4	Fructose	+	4	Salicin	+	4	alpha-Methyl-D-Mannoside	-
5	Dextrose	+	5	Dulcitol	-	5	Xylitol	-
6	Galactose	+	6	Inositol	+	6	ONPG	-
7	Raffinose	+	7	Sorbitol	+	7	Esculin	+
8	Trehalose	+	8	Mannitol	+	8	D-Arabinose	+
9	Melibiose	+	9	Adonitol	+	9	Citrate	-
10	Sucrose	+	10	Arabitol	+	10	Malonate	-
11	L-Arabinose	+	11	Erythritol	-	11	Sorbose	-
12	Mannose	+	12	alpha-Methyl-D-glucoside	-		Control	-

SM3 Culture								
Part A			Part B			Part C		
Sr. No	Component	Results	Sr. No	Component	Results	Sr. No	Component	Results
1	Lactose	+	1	Inulin	+	1	Rhamnose	+
2	Xylose	+	2	Sodium gluconate	+	2	Cellobiose	+
3	Maltose	+	3	Glycerol	+	3	Melezitose	-
4	Fructose	+	4	Salicin	+	4	alpha-Methyl-D-Mannoside	-
5	Dextrose	+	5	Dulcitol	+	5	Xylitol	-
6	Galactose	+	6	Inositol	+	6	ONPG	-
7	Raffinose	+	7	Sorbitol	+	7	Esculin	+
8	Trehalose	+	8	Mannitol	+	8	D-Arabinose	+
9	Melibiose	+	9	Adonitol	+	9	Citrate	-
10	Sucrose	+	10	Arabitol	+	10	Malonate	-
11	L-Arabinose	+	11	Erythritol	-	11	Sorbose	-
12	Mannose	+	12	alpha-Methyl-D-glucoside	-		Control	-

Table 4.1.7: Table representing the results of SM1, SM2 & SM3 found in carbohydrate utilization A/B/C test kit.

4.2 To screen potential bacteriostatic biological agents isolated from the oral cavity as potential biofilm disruptive agents.

4.2.1 Giant Colony Technique

A giant colony technique was conducted to test if the biofilm-producing bacteria produced any antibiotic substance against the test non-biofilm-producing bacteria.

No antibiotic substance was produced against the non-biofilm-producing bacteria.

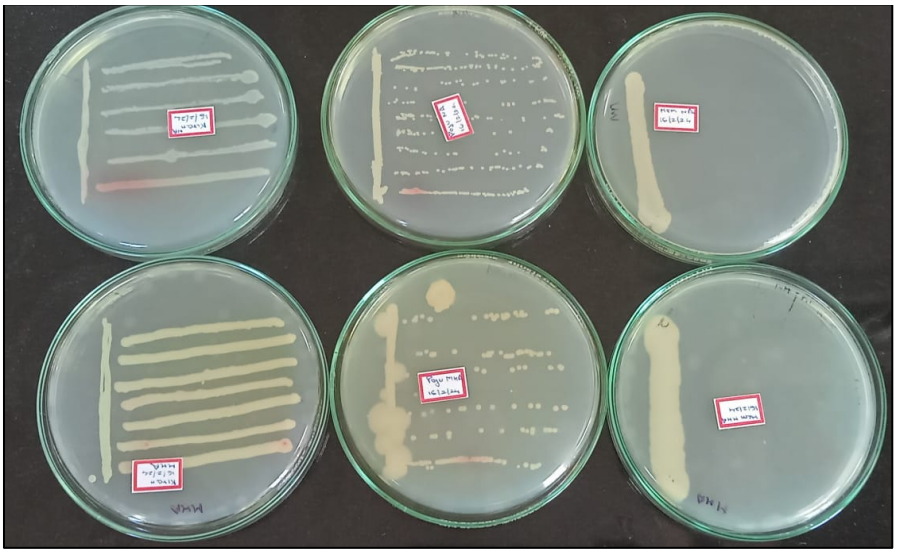


Figure 4.2.1: Depicts giant colony technique performed on biofilm-producing bacterial isolates.

4.2.2 Spot Inoculation

Spot inoculation technique was conducted to observe if a zone of inhibition would be formed by spotted biofilm-producing bacteria against the test organism non-biofilm-producing bacteria, It was observed that no zone of inhibition was observed in any of the plates.

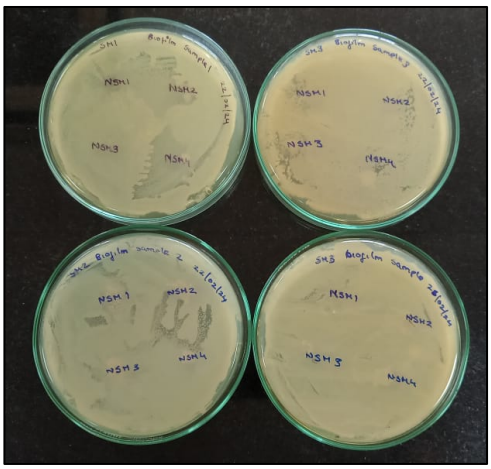


Fig 4.2.2: Spot inoculation was carried out to check for any bacteriostatic activity by non-biofilm-producing bacteria against biofilm-producing bacteria.

4.2.3 Agar well diffusion assay to check the antimicrobial activity.

Agar well diffusion assay was conducted to test the antimicrobial activity of plant extracts obtained from local plant species *Andrographis paniculate* (Kirayate), *Coleus aromaticus* (Wattelaw) and *Occimum sanctum* (Tulsi)) and of rock salt from Curca, Goa, India.

It was observed that it showed a negligible zone of clearance in plant extracts and rock salt concentrations in the following assay:

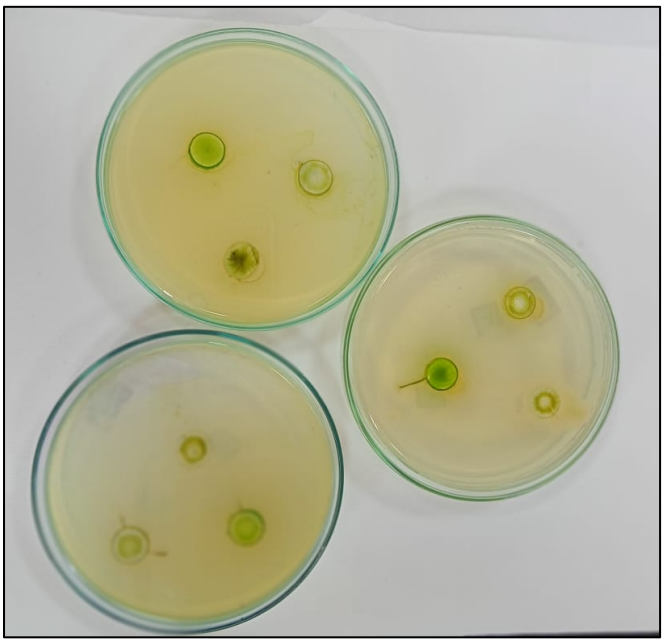


Fig 4.2.3: Agar well diffusion assay using plant extracts of *Andrographis paniculate* (Kirayate), *Coleus aromaticus* (Wattelaw) and *Occimum sanctum* (Tulsi) on biofilm-producing bacteria.

4.3 To study the effect of traditional Goan sea salt on oral biofilm formation.

4.3.1 Pellicle Assay

A pellicle assay was conducted to observe pellicle formation in the biofilm production isolates SM1, SM2, SM3, and SM4 and it was observed that there was no pellicle formation in 24 of all the isolates.

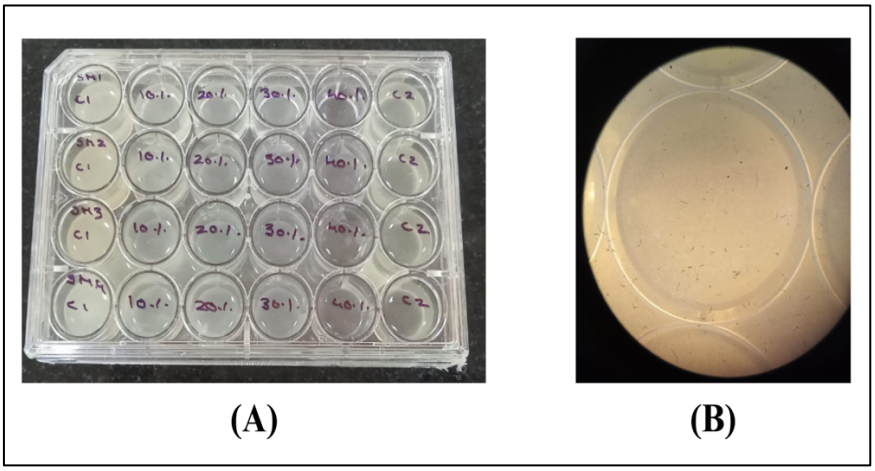


Fig 4.3.1: (A) Pellicle assay carried out in 24 wells plate.

(B) 24 Wells plate under stereo microscope, showing no pellicle formation at 10% concentration

4.3.2 Textural Assay of Bacterial Colony

A colony assay was conducted to observe textural changes in the colony:

Minimal agar with a 10% salt concentration showed wrinkled colony formation.

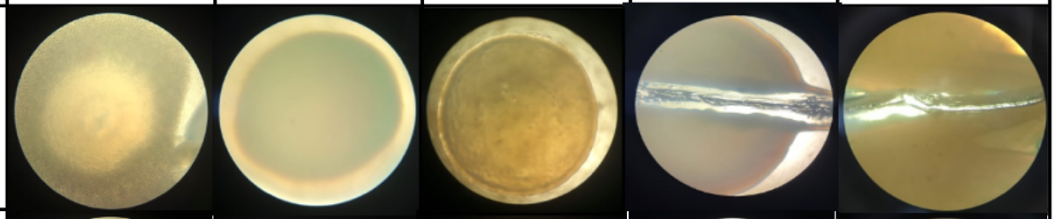
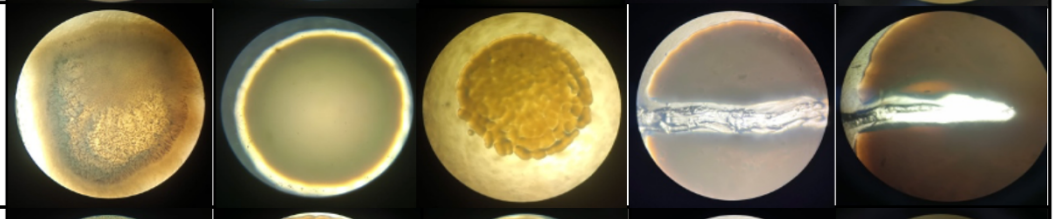
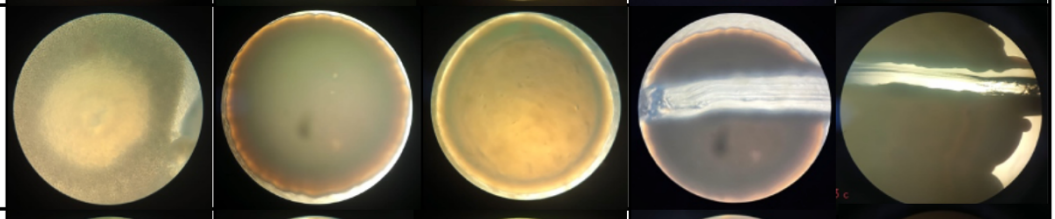
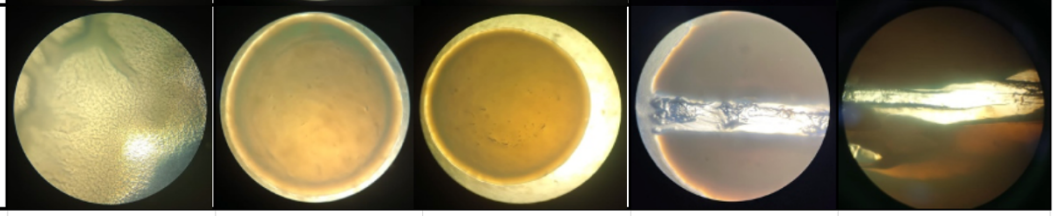
Bacterial Isolate	CONTROL	Colony grown with 10% salt concentration after 24 hours	Colony grown with 10% salt concentration after 48 hours	Toothpick passed through colony grown with 10% salt after 48 hours	Toothpick passed through colony grown without salt (control) after 48 hours
SM1					
SM2					
SM3					
SM4					

Table 4.4.2: Textural colony assay data representation.

4.3.3 Growth curve to study the effect of rock salt solution on biofilm-producing bacteria

The growth curves indicate the effect of varying salt concentrations on a culture's growth over time. The control group displays robust growth, suggesting optimal conditions without added salt. At lower salt concentrations (10%), growth is sustained and comparable to the control, indicating tolerance to a degree of

salinity. As salt concentration increases to 20% and 30%, growth diminishes, showing that higher salt levels start to inhibit growth. At 40% concentration, growth is severely hindered or completely stalled, suggesting a high level of salt toxicity to the culture. This pattern is consistent across the datasets, with some variations

in the degree of inhibition, which could be due to experimental or biological variability.

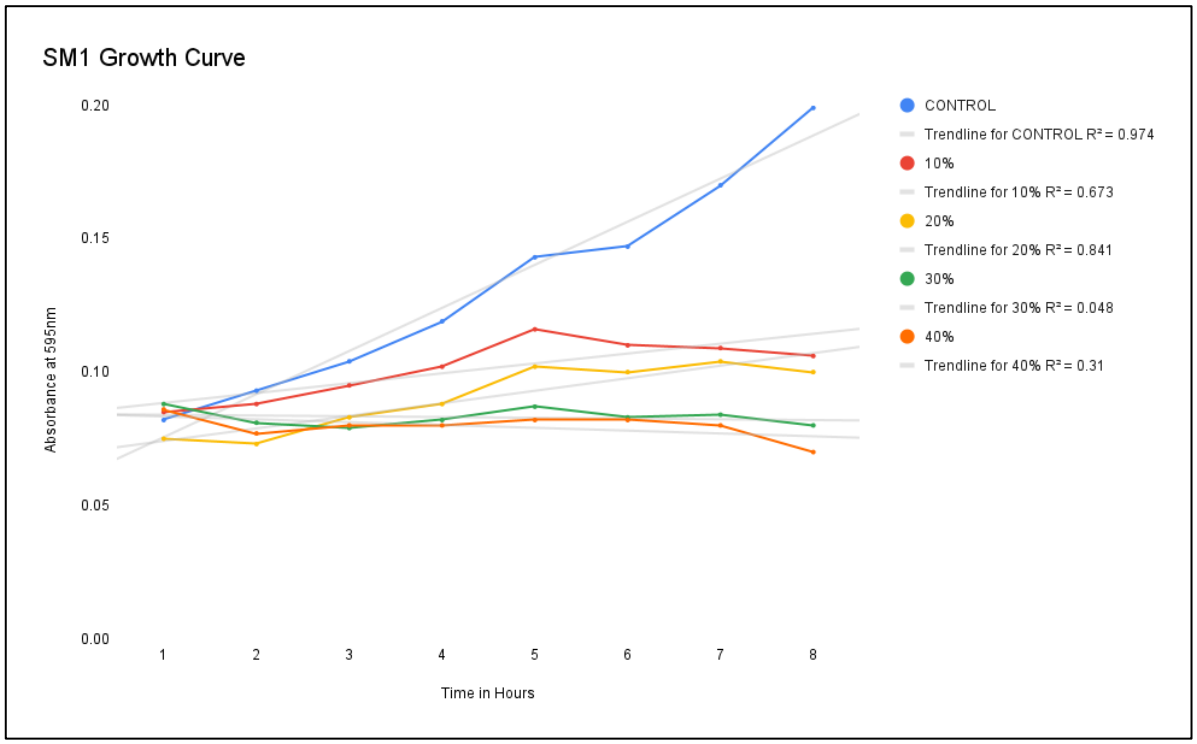


Fig 4.3.3.1:Graphical representation of SM1 Growth curve.

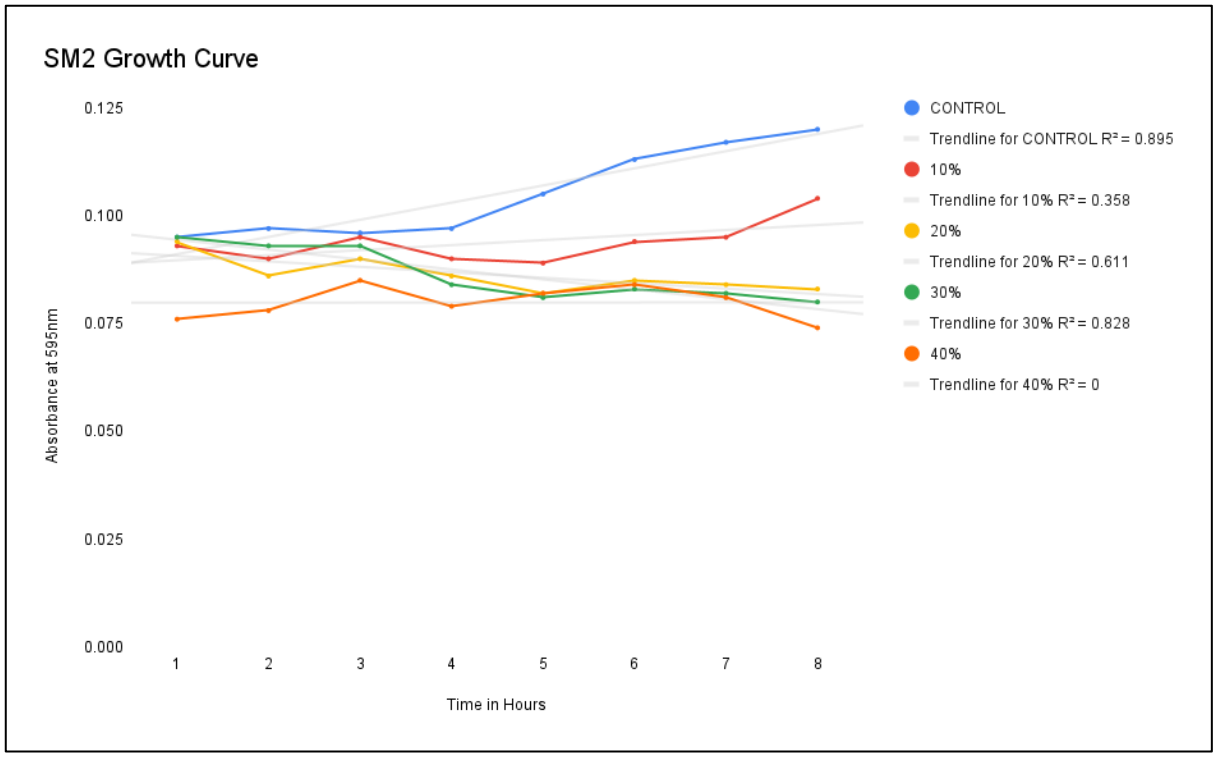


Fig 4.3.3.2:Graphical representation of SM2 Growth curve.

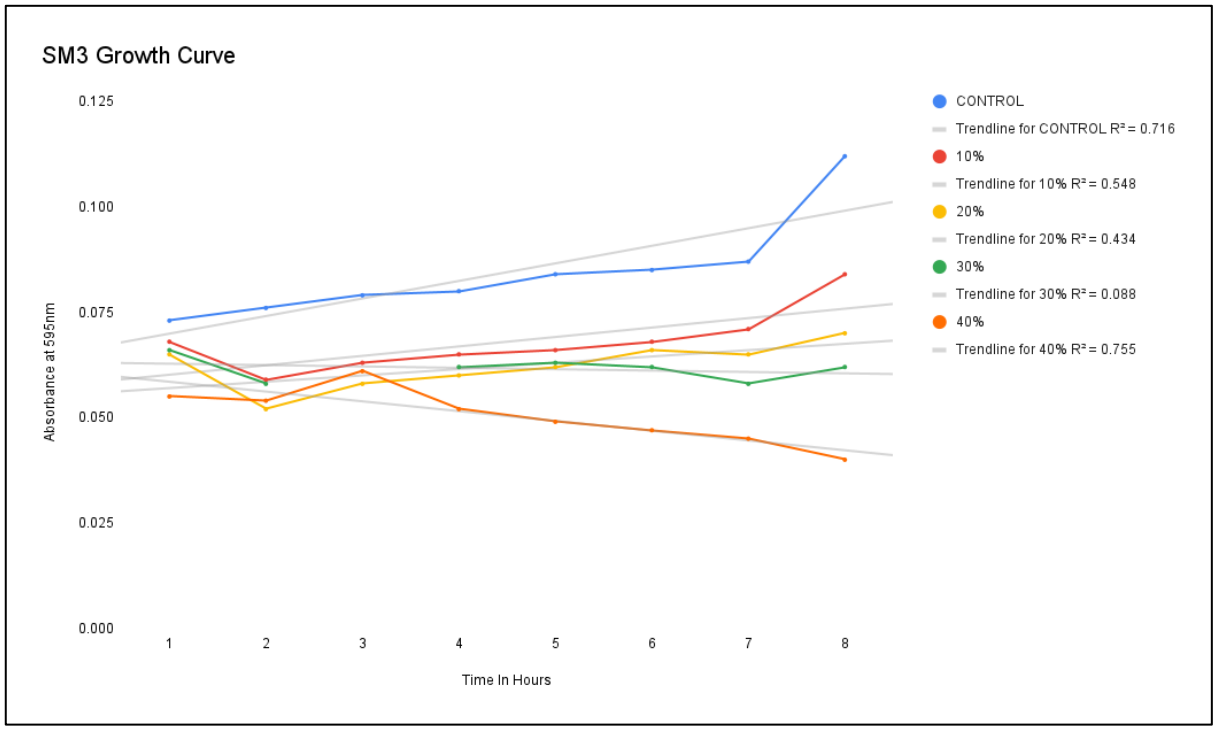


Fig 4.3.3.3:Graphical representation of SM3 Growth curve.

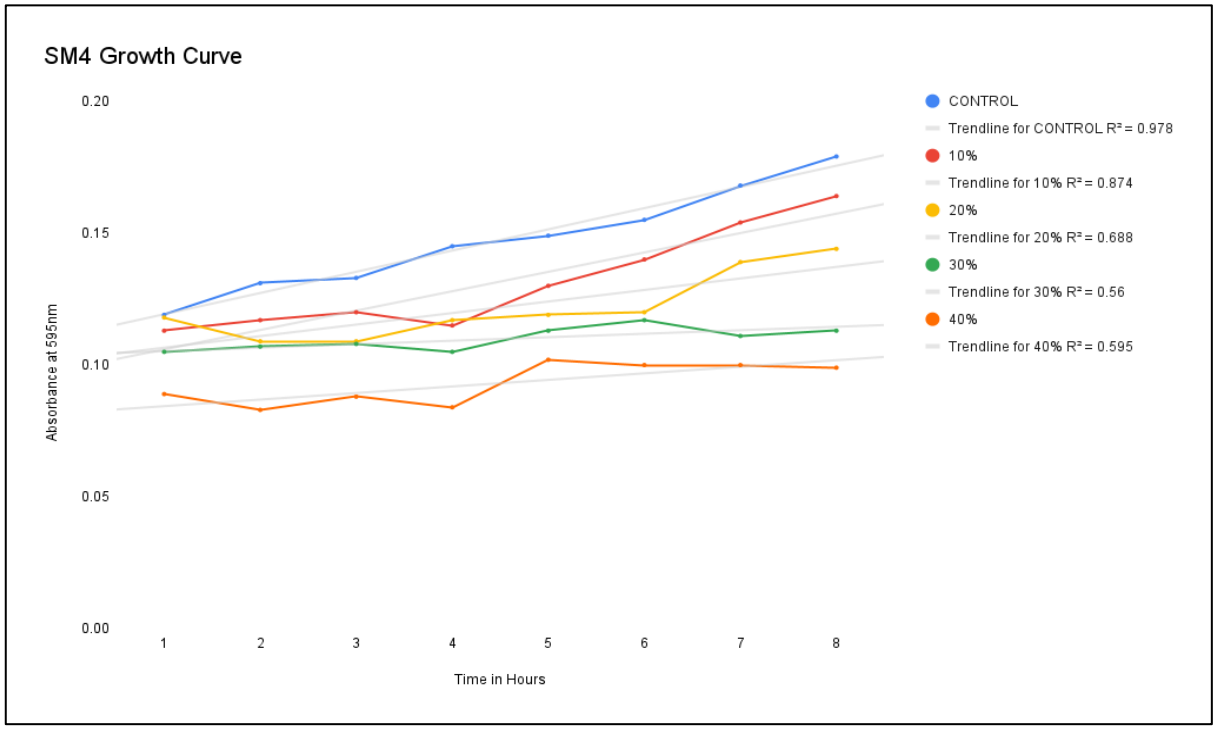


Fig 4.3.3.4:Graphical representation of SM4 Growth curve.

CONCLUSION

The study on bacteria that produce biofilms and the strategies to disrupt them has made a significant contribution to our understanding of how biofilms behave in the mouth and the potential ways to control them. The results of our study highlight the wide range and strong resistance of bacteria that form biofilms, emphasizing the difficulty of effectively addressing their long-lasting presence in dental settings.

The results obtained demonstrated the diverse responses of various bacterial isolates to growth media optimization, emphasizing the significance of customized strategies and the effectiveness of disruptive techniques. The utilization of traditional Goan sea salt in disrupting biofilms demonstrated encouraging yet insufficient effectiveness, suggesting that although these natural substances hold promise, their implementation may require additional optimization and specific improvements to ensure consistent outcomes.

In addition, the growth curve analyses confirmed the ability of biofilm-producing bacteria to withstand various levels of salt. The results of the pellicle assays and wrinkled colony assays yielded additional information regarding the physical characteristics and response of the biofilms when subjected to stress. This information can be utilized to enhance the development of more efficient strategies for managing biofilms in clinical settings.

REFERENCES

- Amankwah, S., Abdusemed, K., & Kassa, T. (2021). Bacterial Biofilm Destruction: A Focused Review On The Recent Use of Phage-Based Strategies With Other Antibiofilm Agents. *Nanotechnology, Science and Applications*, *Volume 14*, 161–177. https://doi.org/10.2147/NSA.S325594
- Berger, D., Rakamimova, A., Pollack, A., & Loewy, Z. (2018). *Oral Biofilms: Development, Control and Analysis*. https://doi.org/10.20944/preprints201808.0174.v1
- Donlan, R. M. (2002). Biofilms: Microbial Life on Surfaces. *Emerging Infectious Diseases*, *8*(9), 881–890. https://doi.org/10.3201/eid0809.020063
- Flemming, H.-C., & Wingender, J. (2010). The biofilm matrix. *Nature Reviews Microbiology*, *8*(9), 623–633. https://doi.org/10.1038/nrmicro2415
- Larsen, T., & Fiehn, N. (2017). Dental biofilm infections – an update. *APMIS*, *125*(4), 376–384. https://doi.org/10.1111/apm.12688
- Li, J., Helmerhorst, E. J., Leone, C. W., Troxler, R. F., Yaskell, T., Haffajee, A. D., Socransky, S. S., & Oppenheim, F. G. (2004). Identification of early microbial colonizers in human dental biofilm. *Journal of Applied Microbiology*, *97*(6), 1311–1318. https://doi.org/10.1111/j.1365-2672.2004.02420.x
- Liu, D., Huang, Q., Gu, W., & Zeng, X.-A. (2022). A review of bacterial biofilm control by physical strategies. *Critical Reviews in Food Science and Nutrition*, *62*(13), 3453–3470. https://doi.org/10.1080/10408398.2020.1865872
- Marsh, P. D. (2006). Dental plaque as a biofilm and a microbial community – implications for health and disease. *BMC Oral Health*, *6*(S1), S14. https://doi.org/10.1186/1472-6831-6-S1-S14
- Marsh, P. D., & Bradshaw, D. J. (1995). Dental plaque as a biofilm. *Journal of Industrial Microbiology*, *15*(3), 169–175. https://doi.org/10.1007/BF01569822
- Martínez-Hernández, M., Reda, B., & Hannig, M. (2020). Chlorhexidine rinsing inhibits biofilm formation and causes biofilm disruption on dental enamel in situ. *Clinical Oral Investigations*, *24*(11), 3843–3853. https://doi.org/10.1007/s00784-020-03250-3

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- O'Toole, G. A. (2011). Microtiter Dish Biofilm Formation Assay. *Journal of Visualized Experiments*, *47*, 2437. https://doi.org/10.3791/2437
- Sonkusale, K. D., & Tale, V. S. (2015). *Isolation and Characterization of Biofilm Forming Bacteria from Oral Microflora*. *2*.
- Sujana, K., Abraham, K. P., Mikkili, I., & Prabhakar, K. V. (2013). *BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF BIOFILM-PRODUCING BACTERIA*.
- Welch, K., Cai, Y., & Strømme, M. (2012). A Method for Quantitative Determination of Biofilm Viability. *Journal of Functional Biomaterials*, *3*(2), 418–431. https://doi.org/10.3390/jfb3020418
- Wolff, M. S., & Larson, C. (2009). The cariogenic dental biofilm: Good, bad or just something to control? *Brazilian Oral Research*, *23*(suppl 1), 31–38. https://doi.org/10.1590/S1806-83242009000500006

APPENDIX

1. CHEMICALS AND REAGENTS USED

Reagents	Source
Gram's crystal violet	HI media laboratories Pvt
Gram's iodine	HI media laboratories Pvt
Gram's decolorizer	HI media laboratories Pvt
Safranine	HI media laboratories Pvt
Congo red dye	HI media laboratories Pvt
Peptone	HI media laboratories Pvt
Ethanol	HI media laboratories Pvt
Sucrose	HI media laboratories Pvt
Sodium hydroxide	HI media laboratories Pvt
Hydrochloric acid	HI media laboratories Pvt

2.. MEDIA USED FOR ISOLATION

1) Nutrient agar (Hi Media Laboratories Pvt ltd)

Ingredients	Gms/ litres
Petone	5.00
HM PEPTONE B#	1.50
Yeast extract	1.50
Sodium chloride	5.00
Agar	15.00

2) Brain heart infusion agar (Hi Media Laboratories Pvt)

Ingredients	Gms/ liters
HM infusion powder	12.50

BHI powder	5.00
Protease peptone	10.00
Dextrose (glucose)	2.00
Sodium chloride	5.00
Disodium hydrogen phosphate	2.50
Agar	15.00
Sucrose	5.00

Note: After mixing all the contents in distilled water in the order given make sure the pH has been adjusted to 7.4

3) Muller Hinton agar (Hi Media Laboratories Pvt)

Ingredients	**Gms/ litres**
HM infusion B from	300.00
Acicase	17.50
Starch	1.50
Agar	17.00

4) Minimal broth

Ingredients	**Gms/liters**
Dipotassium hydrogen phosphate	7.00
Potassium dihydrogen phosphate	2.00
Sodium citrate	0.50
Magnesium sulphate	0.10
Ammonium sulphate	1.00
Petone type 1	5.00
Sucrose	5.00