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Faculty for biosciences, fisheries, and economy
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Selective Isolation of Actinobacteria from Marine Invertebrates: A Bio-Prospective Search for New Producer Strains

pre-treatments to selectively isolate actinobacteria from different marine invertebrate samples, in search for new producer strains.

Vincent Mary Edoziem

A master's thesis in Marine Biotechnology [Bio 3901-1] May 2024

Acknowledgment

This thesis research was done at Marbio, Norwegian College of Fisheries (NFH), UiT The Arctic University of Tromsø, from August 2023 to May 2024.
All thanks to the Almighty God for his grace and mercies in my journey thus far.

My heartfelt gratitude extends to my team of supervisors; Prof. Espen Hansen, Dr. Yannik Schneider, and Hosea Masaki (Ph.D.), for generously sharing their wealth of knowledge and practical insights in their area of expertise. Their collective guidance, attentive ear to my questions, kind corrections, and dedicated teaching has been invaluable throughout this research phase. I am deeply grateful for their unified support, and I couldn't have wished for a more exceptional team of supervisors. Thank you all immensely.

My special gratitude to Ole Christian and Robert Johnasens for your assistance in the collection of new samples. Thanks to Dr. Chun Li. and Sailesh Maharjan for your unwavering support in the lab. I want to express my appreciation to the exceptional Head of Marbio, Prof. Jeanette H. Andersen, whose guidance I deeply admire. To the research community at Marbio, Marbank, and my fellow master students, I extend my sincere thanks for making this research experience a wonderful one.
I am truly indebted to Prof. Klara Stensvåg, Head of the Department of Marine Biotechnology, for her invaluable maternal guidance and counsel. A special gratitude to Prof. Hans C. Bernstein for his unwavering belief in me and exceptional supervision. My sincere gratitude to Ander Kvanli, who was more of a big brother to me than a student adviser. To Dr. Daniela Morales and Dr. Gabriel Magno, your generous sharing of research knowledge and experience has been immensely beneficial. To the dedicated technicians and staff at the Department of Marine Biotechnology and the Norwegian College of Fisheries (NFH), I extend my sincere appreciation for your tireless efforts and support.

Finally, I want to express my sincere gratitude to my ever-supporting and loving family, "Ndi be Mr. and Mrs. G. E. EDOZIEM." Your encouragement and prayers have been my source of strength. Special thanks to my friends in Tromsø, Dr. John C. Oyem and Mr. Haraszti Rajmund, and my mentor, Mr. Prince Nnadozie, for their unwavering support.

Tromsø, May 2024
Vincent Mary Edoziem

Abstract

Actinobacteria are spore-forming Gram-positive bacteria, with high G-C content in their genome. Their ability to metabolize complex compounds helps them survive different environmental conditions and form diverse associations with different organisms. These characteristics make Actinobacteria good producers of natural products with useful bioactivity. However, Actinobacteria are slow growers and could be outcompeted by fast-dividing microbes with simple metabolic systems in a given sample. Therefore, optimizing the selective isolation of actinobacteria to increase their yield is essential in bioprospecting. This work is aimed at optimizing the selective isolation method used in Schneider *et al.*, 2022, to isolate actinobacteria from marine invertebrates, using a selective media M1 supplemented with antibiotics (1.0ml of Nalidixic acid 30mg/ml in 0.3M NaOH) and fungicide (5.0ml of cycloheximide 10mg/ml in EtOH) and 10mins heat pre-treatment at 55^0C, 65^0C, and 75^0C respectively, as well as a chemical pre-treatment with phenol alone (3%phenol) and a combination of 3% phenol and 65^0C heat treatment before culturing. At the end of this work, there was a yield of 43 bacteria isolates, 1 from the 65^0C treatment group and 42 from the control group with no treatment. Of the 43 isolates, 3 were from the class Gammaproteobacteria (7%), 5 were of the class Bacilli (12%), 7 were Alphaproteobacteria (16%), 13 were Flavobacteriia (30%), and 15 were Actinomycetes (35%).

Though there were relatively high isolates of Actinomycetes, the curiosity to investigate the high inhibition in the treatment group led to the next phase of this research. Firstly, to test the heat tolerance of the isolated Actinomycetes, 7 tentatively identified Actinomycetes genus were subjected to the heat treatment and there was a total inhibition at 75^0C except for the sample A010 (tentatively identified as *Microbacterium Sp*.) however, there was growth on the 55^0C and 65^0C in all 7 samples. This was the second phase of this work, but to further investigate the inhibition effect of the heat and phenol pre-treatment and prolonged sample storage at -80^0C in 20% glycerol, the third phase started with collecting fresh samples of breadcrumb sponge at the shorelines of Uteng, Tromsø. This is because the five animal stock samples were not enough to continue the research studies. The bread crumb samples were homogenized and separated into three conditions, unfrozen, 1 day frozen, and 15 days frozen. To aliquots from these condition groups were given the same heat treatments for 10 mins except the unfrozen sample which received three different phenol treatments (0.1%, 0.5%,

and 1%) in addition to the heat treatment received by all. There was a total inhibition on the 1% phenol and 55^0C, 65^0C, and 75^0C of all the conditions except the 65^0C of the unfrozen condition which showed growth. There was also growth in the 0.5% and 0.1% phenol, and the control groups of all conditions.

At the end of this project, we obtained a 35% yield of actinobacteria from the control group with no treatment. We observed that, though heat pre-treatment has proven to be good for the selective isolation of Actinobacteria in marine samples like soil samples and sediments as in Schneider *et al.,* 2022, it may not be suited for the selective isolation of Actinobacteria in marine animal samples, as most treatment groups showed high inhibition, but growth was observed in the control groups with no treatment. Also, Phenol treatment at 1% and 3% concentrations were observed to be bactericidal both in this research and the previous study by Scheider *et al.,* 2022, but can be used at 0.5% and 0.1% concentrations for selective isolation. Finally, we recommend the use of a sample-specific selection method like the "selective filter membrane method" described by Savitha, *et al.,* 2022 to increase the percentage yield in the isolation of Actinobacteria from marine animal samples.

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1 Introduction

Actinobacteria are a diverse group of Gram-positive bacteria with high C-G base pair content. They are known for their prolific production of bioactive compounds with significant pharmaceutical potential. Actinobacteria, specifically the Streptomyces genus, have contributed significantly to the pool of natural products developed into pharmaceutical drugs (János, 2005; Jose and Jha, 2016). Numerous antibiotics and drugs in use today have their origins in Streptomyces. For instance, actinomycin for cancer treatment (Sakula and Sakula, 1988), the immunosuppressant, rapamycin (Vezina *et al*., 1975; Arriola Apelo and Lamming, 2016), and the pancreatic lipase inhibitor, lipstatin (Hochuli *et al*., 1987) are all from streptomyces. Apart from Streptomyces, natural products have also been isolated from other phyla of actinobacteria like thiocoraline, from *micromonospora marina,* demonstrated anticancer activity on cell lines LoVo and SW620 (human colon cancer cell lines) (Erba, Bergamaschi *et al*., 1999). Indolocarbazoles and staurosporines with antibiotic activity were isolated from *salinispora sp.* and *verrucosispora sp.* (Steinert *et al*.,2015).

Gram-positive bacteria hold a prominent position in producing bioactive natural substances, particularly antibiotics. According to Gupta (2011), the evolution of the cell wall in Gram-negative bacteria may be attributed to the pressure exerted by antibiotics, which are primarily synthesized by Gram-positive bacteria that have an earlier evolutionary history. This notion could provide an evolutionary rationale for the role of Gram-positive bacteria as the primary producers of antibiotics (Schinke *et al*., 2017).

Beyond the evolutionary rationale, environmental factors to a high extent, influence the adaptation and survival mechanism of actinobacteria within their respective environment. Some actinobacteria are conditioned to form various associations with several animals, plants, and other organisms to compensate for what they need but can't produce naturally to survive certain difficult environmental conditions. In most symbiotic associations of actinobacteria, they synthesize natural products that protect the host from predators while they get nutrients from the host. Piel (2009) reported that studies on secondary metabolites previously attributed to the host organisms have been shown to be produced by organisms associated with them. Besides the interaction with other organisms, the conditions of their physical environment can influence the production of secondary metabolites by bacteria for better adaptation and survival.

So, extreme environmental conditions such as high altitudes, volcanic areas, mangroves, and marine environments are attracting the attention of researchers because it is expected that adapting to physiological or biochemical pressures in such harsh environmental conditions can lead to the production of diversified natural compounds among microbes. Hence, environments with extreme conditions like the marine environment, draw more attention to bioprospecting for discovering novel natural products with biological potency (Dhaka *et al.*, 2017). The marine environment is the largest ecosystem on earth with a high level of biodiversity, yet little is known of this versed ecology (Kurtböke *et al.,* 2015). Due to the highly varied biodiversity in different sections of marine ecology and the little that is known of this diverse ecosystem, the marine environment is seen as an untapped potential and viable hotspot for the discovery of new bioactive compounds (Luna, 2015). The marine environment harbors a vast and untapped reservoir of Actinobacteria associated in a symbiotic relationship with marine invertebrates. Marine invertebrates are known to host complex microbial communities (Kurtböke *et al.,* 2015). They have been studied to engage in symbiotic relationships with these microbes to digest complex polysaccharides in food for easy absorption/digestion while the host animal provides shelter for the microbes and in some cases produces compounds that would keep harmful pathogens at bay (Luna, 2015). This could possibly contribute to their ability to produce bioactive compounds.

Bacteria interact within themselves (within their colony), other colonies, and their environment using chemical compounds or signals. A process known as quorum sensing (Aminov 2009). These chemical compounds could be released internally or externally. Bacteria within certain environmental conditions produce compounds to help them communicate with others, adapt, and survive varying conditions (Aminov 2009). Some of these compounds are produced as a preventive measure to repel predators, some as a response to stressor factors like osmotic stress due to changes in solute concentration like salinity (osmotic stress), dehydration, high acidity, change in pH, *etc*., some as a chemical signal to the colony (externally) for biofilm formation or within the cell (internally) for hibernation/spore formation in unfavorable conditions (like food scarcity or drought) and to reproduce (cell division) in time of abundance and sometimes, as secondary metabolites from by-products of everyday cell metabolism. These compounds could be beneficial or toxic to humans, bacteriostatic or bactericidal (antibiotics). These compounds are said to be bioactive if we detect their usefulness/functionality. However, an axenic culture of the bacteria of interest is needed to investigate it for potential bioactive secondary metabolites. This is

normally achieved by making a culture of various dilutions (eg 10 times, 100 times, and 1000 times dilution) of a sample with a high microbial load and a mixture of different species. The next step will be to subculture observed distinct colonies based on their unique morphological features, identify each isolated colony using molecular makers, and finally select the colonies of interest and discard others as biowaste. The critical question becomes; what if a treatment that could be selectively bactericidal to the unwanted bacteria species is applied to optimize the process and to achieve less colony with a high yield of the desired bacteria? Which is the main aim of this study.

In this work, we try to improve the existing protocol at Marbio in order to isolate actinobacteria more specifically. This work aims to selectively isolate and identify Actinobacteria from marine invertebrates. Isolated marine actinobacteria are the starting material for the discovery of new bioactive metabolites. For the specific cultivation, different pre-treatment conditions and a selective media are used, in order to isolate actinobacteria from samples of marine invertebrates. For the identification of the bacterial isolates, colony PCR of the 16S rRNA subunit in combination with Sanger sequencing and bioinformatics analysis were used. The main focus of the study is to evaluate the selectivity of different sample pre-treatment methods to optimize selective isolation of actinobacteria. In previous studies at Marbio, heat shock (at 55^0C) has been employed as a pre-treatment for Actinobacteria isolation, within this work, different temperature conditions (with range 55^0C, 65^0C, and 75^0C) and chemical concentration (phenol) were investigated in order to optimize the actinobacteria isolation.

1.1 Taxonomy and Classification of Actinobacteria

Initially, the classification and taxonomy of Actinobacteria relied heavily on phenotypic criteria, which involved the examination of limited traits such as morphology, specific proteins/enzyme activity, chemistry, and physiology (Mohammadipanah and Dehhaghi, 2017). Although aspects like colony morphology, spore chain nature, substrate color, aerial mycelium, and diffusible pigments remain significant for differentiating genera, they often lacked the depth required for the accurate and precise classification of this phylum because of their diversity and complexity (Mohammadipanah and Dehhaghi, 2017). However, the current classification of actinobacteria is based on the 16S rRNA gene because it has been considered a significant molecular marker in the taxonomy of prokaryotes due to it being universal, relatively stable, and highly conserved. From the 16S rRNA sequencing, there are

six classes in the phyla Actinobacteria including Rubrobacteria, Thermoleophilia, Coriobacteriia, Acidimicrobiia, Nitriliruptoria, and Actinobacteria, 5 subclasses, 6 orders, and 14 suborders (Ludwig *et al*., 2012). The class Actinobacteria has 43 families while the other 5 classes constitute only 10 families (Ludwig *et al*., 2012). The Actinobacteria class consists of orders that were previously considered as suborders within the order *Actinomycetales,* and the orders within the class Actinobacteria are grouped into two clades, the first clade includes orders, *Actinopolysporales, Micromonosporales, Glycomycetales, Jiangellales*, *Corynebacteriales*, *Propionibacteriales*, and *Pseudonocardiales*, while the second clade consists of the orders *Bifidobacteriales*, *Micrococcales*, *Actinomycetales*, and *Kineosporiales*.

However, some of the limitations of the 16S rRNA gene as a molecular marker apart from highly conserved include nucleotide variations among multiple rRNA operons, and the possibility of horizontal gene transfer (HGT) of these genes between taxa (Ramasamy *et al*., 2014) but with the application of the new generation sequencing of the whole gene sequencing, an even better and more precise phylogenetic taxonomic classification of actinobacteria that will assist researchers to distinctively differentiate each bacteria strain.

1.2 General Overview

In every ecological system, living organisms have to adapt to survive over time by forming feeding associations with other organisms to make up for what they can't produce naturally and offering what they have little or no use for, or what they have in excess. In the marine environment, microbes have adapted in various associations (both symbiotic, parasitic, and commensal) with themselves and other animals both vertebrates and invertebrates. According to Wilkins *et al.*, the microbial community could be specific to a given animal and could also change during the developmental stages of the animal. This microbial community could also vary from one animal to another (Wilkins *et al.*, 2019). In a study of microbiota in the life circle of a sea cucumber by Yu *et al.*, (Yu, Sakai *et al*., 2022) the result showed a significant change in the microbial community from the early life stages of *Apostichopus japonicus* to the late *auricularia* stage. Also, Li *et al*., (2022) proposed a correlation between the symbiotic microbial community and their host genetic background. Also, these microbial communities may be transmitted both horizontally and vertically. the vertical and horizontal transmission of the microbiome was explained by Wale *et al.*, (2021) In a study of crab's larval stages. He speculated that symbiotic microbiomes facilitate the transition of crabs from living in water to land (Wale, Daffonchio *et al*., 2021). this could be because of the crucial role they play in the

daily functionality of the host. From the production of protective compounds (antibiotics) to the breakdown of complex food sources *etc.* Jiang *et al*., (2022) explored the evolution of the physiological and genomic properties of microbes associated with marine invertebrates. Jiang *et al*. compared the genomes of type strains of *Halioticoli clade* species in Vibrionaceae with different lifestyles and found more carbohydrate metabolism-related genes in the genome of free-living Vibrio (Jiang, *et al.,* 2022). Furthermore, in the interaction between microbial communities and their invertebrate host, Actinobacteria form diverse associations with several host organisms and play several roles owing to their complex metabolic system, production of bioactive compounds, and adaptation to various environmental conditions. In their associations with various higher organisms, they play important roles as symbionts (*e.g*., *Frankia* spp.), gastrointestinal commensals (*e.g.*, Bifidobacterium spp.), and pathogens (notably, species of *Corynebacterium, Mycobacterium, Nocardia, Propionibacterium, and Tropheryma*) to sustain the ecological balance in nature. They form these relations with different plants and animals including humans, but notably with invertebrates.

The various feeding associations and the adaptation to diverse environmental conditions that actinobacteria are capable of, play a significant role in shaping the evolution of secondary metabolic repertoires, driven by both vertical inheritance and horizontal gene transfer (Ziemert, *et al*., 2014). A study of variations in biosynthetic gene clusters (which have been correlated to natural product production) with respect to demographic expansion, using *Salinispora* spp., demonstrates strong conservation within clades in a given environment owing to vertical inheritance but a significant difference was observed in BGCs with respect to the demographic expansion of the same species owing to horizontal gene transfer (Adamek, *et al*., 2018). Similarly, with Amycolatopsis, BGC occurs within clades but not between them. Suggesting that these clusters may be crucial for adaptation rather than mere survival (Adamek, *et al.*, 2018).

As bacteria associate and adapt to diverse environmental conditions, they produce compounds or secondary metabolites that are not essentially needed for growth but are necessary for adaptation and survival (Seipke *et al.*, 2012; Van der Meij *et al.*, 2017). When discovered, these compounds could have some activity like bactericidal effect, anti-inflammatory, immune-suppressant, anticancer, cytotoxicity, *etc.* which could be of economic value to humans when tested. Such a compound is called a bioactive compound. Actinobacteria especially the Streptomyces produce 2/3rd of the known natural bioactive compounds from

bacteria. Of this, Streptomyces produce 80%. ranging from antibiotics, immuno-suppressants, antifungals, insecticides, *etc.*

1.2.1 Biosynthetic Potentials of Actinobacteria

From the discovery of streptomycin from actinobacteria in 1939 by the WAKSMAN group until this current day, Actinobacteria have kept delivering bioactive compounds with pharmaceutical use and other applications (Schneider, 2020). However, one would think it is time to search elsewhere but a current genomic study has revealed the numerous presences of biosynthetic gene clusters (BGCs) in actinobacteria genomes which are not yet traced to any known compound produced. BGCs are tightly linked sets of mostly non-homologous genes participating in a common, discrete metabolic pathway and their expression is often co-regulated (Schläpfer *et al.*, 2017). These clusters of genes are believed to code for catalytic enzymes or proteins/peptides. Four drugs have been isolated from the model organism *S. coelicolor* namely the plasmid-encoded methylenomycin (Mmy), actinorhodin (Act), undecylprodigiosin (Red), and calcium-dependent antibiotic (Cda) (Bentley, *et al.,* 2002; Ikeda, *et al.,* 2003; Ohnishi, *et al*., 2008). but the exploration of its BGCs led to the discovery of a new antibiotic; coelimycin P1(Gomez-Escribano, *et al.*, 2012). This means, there are more untapped potentials locked up within actinobacteria and in the quest for new antibiotics for resistant pathogens, lots of work is ongoing on how these gene clusters can be activated or expressed for potential bioactive sources (Lilya and Andriy, 2017). One of the currently tried techniques is cryptic biosynthesis which refers to the activation of previously silent or "orphan" biosynthetic pathways in microorganisms (Challis & Hopwood, 2003). These pathways remain dormant under standard laboratory conditions but can be triggered to yield the respective products through various strategies. To trigger these pathways, bacteria are co-cultured with other microbes, and animal species, and sterilized body parts of the animal (Wu *et al.,* 2015). This gave a positive result when the study model *S. coelicolor* was co-culture with the fungus *Aspergillus niger*. this gave rise to a novel branch of the polyketide actinorhodin biosynthetic pathway (Wu *et al.,* 2015) and substantial changes in the secreted metabolome when the study model *S. coelicolor* was co-cultured with five other Actinobacteria (Traxler, *et al.,* 2013). This indicates that natural products are also produced during the association of Actinobacteria with other animals but are not captured in the laboratory because only the pure strain is cultured.

1.2.2 Strategies for New Compounds from Actinobacteria

Understanding the mechanism behind the production of natural products by these organisms will help to exploit other ways of activating these gene clusters. Another method is to mimic the environmental conditions on which antibiotics are produced by these bacteria. These compounds are produced at their vulnerable state to protect them from motile saprophytic bacteria (van Bergeijk, Terlouw *et al*., 2020). In stress conditions like nutrient starvation or drought, programmed cell death is activated for an autolytic degradation of old mycelia to liberate needed nutrients to fuel the synthesis of an arial hyphae which is their mode of propagation (van Bergeijk, Terlouw *et al*., 2020). This is practically like extracting the nutrients from the old cell materials they can't afford to keep due to a shortage in nutrient supply. Furthermore, the development of new aerial hyphae correlates temporally to the production of antibiotics. Rationally, this could be to protect the extracted nutrients they can't afford to lose since the cell-wall peptidoglycan is recycled to release the amino sugars N-acetylmuramic acid (MurNAc) and N-acetylglucosamine (GlcNAc) (van Bergeijk, Terlouw *et al*., 2020). GlcNAc is converted to GlcN-6P, which functions as a ligand for the regulator DasR (which is known to repress the activation of BGCs). The Binding of GlcN-6P to the repressor DasR makes it inactive, resulting in the activation of pathway-specific activators of antibiotic biosynthetic gene clusters (BGCs) (Gomez-Escribano, *et al.*, 2012). This results in the production of antibiotics which serves as the first line of defense to protect the nutrient from programmed cell death against motile saprophytic bacteria, as the propagation hyphae are developed simultaneously (van Bergeijk, Terlouw *et al*., 2020). This mechanism can be mimicked in the laboratory by cultivating actinobacteria in stress conditions that would lead to the production of GlcN-6P since the presence of a sugar source would not lead to the conversion of GlcNAc to GlcN-6P.

The demand for new antibiotics continues to grow due to the rapid emergence of multiple antibiotic-resistant pathogens causing life-threatening infections. Although considerable progress is being made within the fields of chemical synthesis and engineered biosynthesis of antibacterial compounds, nature remains the richest and the most versatile source for new antibiotics (Baltz, 2006; Pelaez, 2006). Of which, Actinobacteria have gained significant attention due to their diverse biological activities and potential for producing bioactive compounds of pharmaceutical interest. In marine ecosystems, Actinobacteria are known to play crucial roles in the breakdown of complex organic matter and the production of secondary metabolites. Therefore, the selective isolation and identification of marine

Actinobacteria holds promise for exploring their biotechnological applications. This project aims to test a pretreatment that could be selective in isolating actinobacteria from marine invertebrate samples. owning to the facts that actinobacteria are Gram-positive bacteria and most are spore-forming. For this study, we propose to try treatments that would inhibit non-spore-forming and Gram-negative bacteria. A pre-treatment with phenol at different concentrations, a pre-heating treatment of 55^0C, 65^0C, and 75^0C, and a selective media (M1-media) will be used which will contain an anti-fungi; cycloheximide 10mg/ml (because fungi are also spore-forming and can withstand high-temperature treatment) and a narrow spectrum antibiotics; Nalidixic acid 30mg/ml (inhibits Gram-negative bacteria) as the selective adjunct to increase the high chance of a selective isolation of actinobacteria. some molecular and biotechnological techniques were combined to successfully identify the isolated bacteria colonies, the principles and process behind these techniques are discussed below:

1.3 Techniques for Bacteria Identification.

Actinobacteria have been identified using various techniques. Initially, this was based on morphological features, by observing the differences in their phenotypical characteristics like nature of spore, substrate color, aerial mycelium, and diffusible pigments. Another method was chemically, by testing bacteria colonies for different enzyme activities to identify the bacteria present. Most bacteria groups produce specific enzymes and the catalytic action of those enzymes indicates the presence of a member of the group. However, these two methods can indicate the bacteria group, but most times it is difficult to get a clear distinct grouping as members of different groups produce similar enzymes and may have similar morphological appearance. These methods are not precise and accurate, but with the advancement in biotechnology, bacteria can be identified using their genetic material to distinguish different species with a very high accuracy. There are many molecular techniques used for identifying bacteria colonies, some used for this work are: Selective isolation, Polymerase chain Reaction (PCR), Gel electrophoresis, 16S rRNA sequencing, Sanger Sequencing, search on genetic database banks like the National Center for Biotechnology Information (NCBI) and analysis with gene editing software like BioEdit and MEGA 11.

Workflow of Techniques

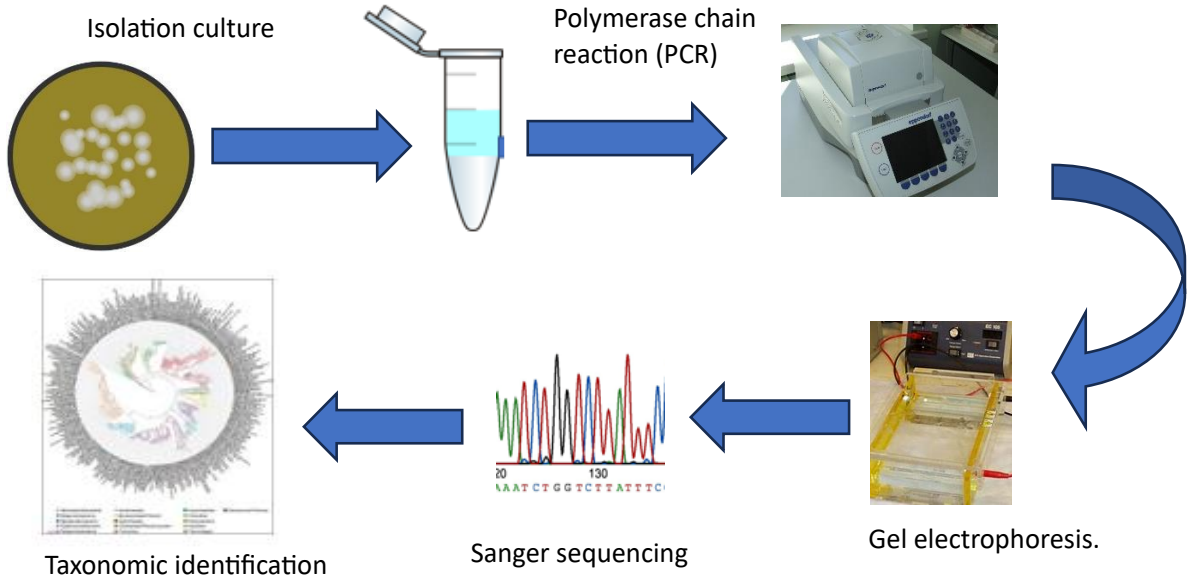


Figure 1: The workflow of techniques used in the selective identification of actinobacteria. Cultures where isolated using isolation M1 agar and sub-cultured on FMAP media, colony PCR was used to amplify the 16S rRNA gene. The PCR results were analyzed with gel electrophoresis before sequencing the 16S rRNA gene using Sanger sequencing and subsequently taxonomic identification of the sequenced result.

1.3.1 Specific/Selective Isolation

This includes any measure taken to inhibit the growth of unwanted organisms based on their known growth conditions. This can be done using a selective media, or a pre-treatment or a combination of both. The selective media could contain complex polymers (that only the organism of interest can degrade like chitin, cellulose, *etc.*) or inhibition agents like narrow-range antibiotics, antifungal agents, *etc.* that are not (or less) harmful to the organism of interest but inhibit other competitors *e.g.* Mac Conkey agar (selective for Gram-negative bacteria), mannitol salt agar (selective for *S. aureus* and some halophilic bacteria), *etc.*

The pre-treatment could be physical or chemical. Physical treatment may include heat shock, hot air drying/drying on laminar flow for organisms that can withstand a high desiccation. The chemical treatment may include; high salt concentration for halophilic organisms, the use of harsh chemicals like phenol, in a concentration that the organism of interest can survive but which inhibits unwanted organisms.

1.3.2 16s rRNAs Colony PCR (Polymerase Chain Reaction)

Polymerase Chain Reaction (PCR) is a basic molecular biology technique. It is used to amplify a specific DNA/gene region prior to sequencing or other molecular analysis that requires more gene copies. Specific regions of the 16S rRNA gene are amplified using PCR primers that target conserved regions flanking variable regions of the gene. This is done to increase the chances of a good sequencing result. When a bacteria culture plate contains mixed colonies with different morphology, it is recommendable to subculture these colonies based on their unique morphological appearance, before amplifying their genes to end up with a sequence for an axenic colony. This amplification is done in a thermocycler machine (Figure 2) which runs in 40 cycles of regulated temperatures necessary for DNA amplification. In each cycle, three basic reaction stages occur, namely: Denaturation, annealing, and elongation of the DNA.

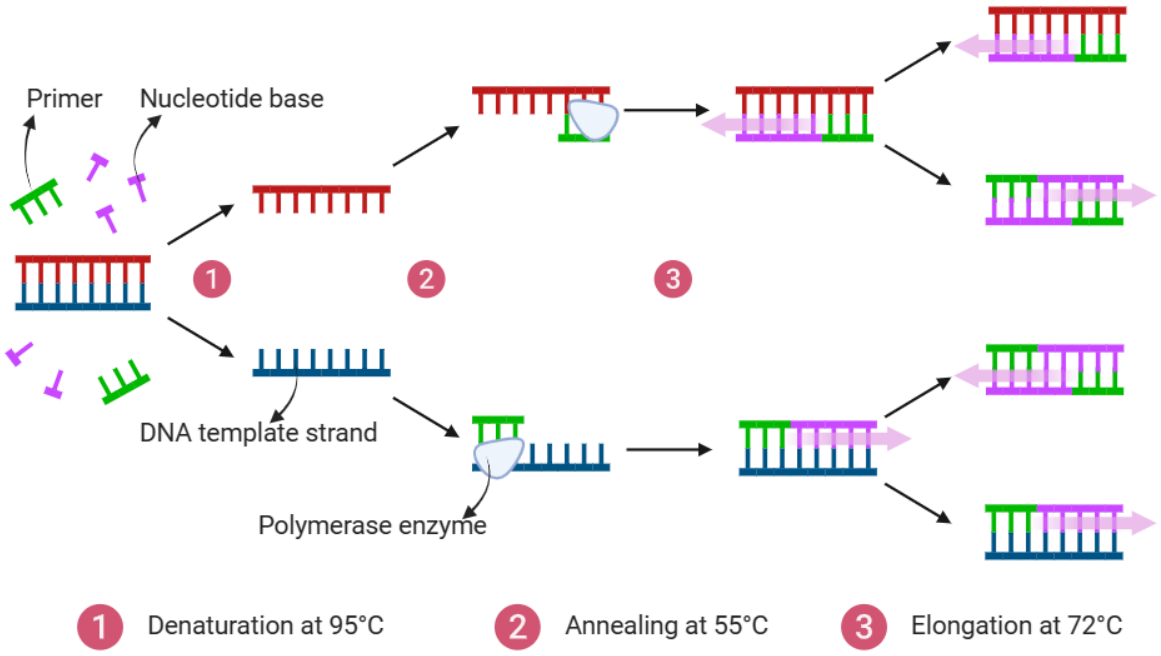


Figure 2: A flow diagram of the Polymerase chain reaction process (PCR). Each PCR cycle consists of three main stages; the denaturation at stage1 separates the double-stranded DNA at 95°C and stage 2 starts with the annealing of the forward and reverse primers to the corresponding sides of each strand at 55°C in a 5' to 3' direction. The polymerase enzyme binds to elongate the primer with nucleotide bases that are complementary to the template strand. This continues until it is terminated by the denaturation stage of the next cycle. A total of 40 cycles is possible in a PCR reaction. This flow chart was edited with biorender.com.

In the denaturation stage at 95°C, the hydrogen bonds that hold together the two strands of the double-stranded nucleic acids are broken and the double-stranded DNA unwinds into single strands. on cooling to 55°C, the primers bind to the complimentary segment of each DNA template there are high chance of the primers binding each strand of the DNA at either end

because of the high ratio of primer to DNA. This is known as Annealing. It is worthy of note that this stage is highly affected if there are significantly more DNA strands than primer present in the respective reaction. The binding of the primer initiates the binding of the DNA polymerase enzyme, which elongates the primer by adding nucleotide bases that are complementary to the base sequence of the template strand. This step is known as elongation, and it is done in the 5' to 3' direction at 72°C this step will continue until it is interrupted/by the denaturation stage of the next cycle. Each cycle gives twice the previous starting number of gene copies, and it runs for 40 cycles. For each cycle, the number of DNA copies is $C_{DNA} = 2^n$ where n is a number of copies in the previous cycle. The number of DNA copies after a certain number of PCR cycles can be calculated as $C_{DNA} = I * 2^n$ where I stands for the initial number of DNA copies and n is the number of PCR cycles.

Figure 3: PCR-Thermocycler (picture: Edoziem V.M.)

1.3.3 Gel Electrophoresis

After the completion of the PCR amplification, the reaction products are analyzed using gel electrophoresis to determine the presence or absence of the target DNA amplicon. Gel electrophoresis is commonly used to separate and visualize DNA or DNA fragments, such as PCR products. The agarose gel stained with DNA-specific dyes is loaded with PCR samples and subjected to an electric field in a buffer solution. the DNA fragments migrate through the gel based on size towards the anode because they are negatively charged (Deoxyribose-phosphate-backbone of the DNA). A Positive PCR will yield DNA bands of the expected size corresponding to the target sequence with respect to the bands of the DNA leather (a buffer

that contains DNA fragments of known size and is used to read the size of the DNA fragments in the sample), while negative reactions will show no bands. Positive PCR products are further sequenced to analyze the identity of the amplified DNA sequence. Sanger sequencing, next-generation sequencing (NGS), and single molecule sequencing techniques can be employed to determine the nucleotide sequence of the PCR product. This is used to confirm if the genes were amplified or not *i.e.* if the PCR was positive or negative. Positive PCR reactions will yield DNA bands, while negative reactions will show no bands.

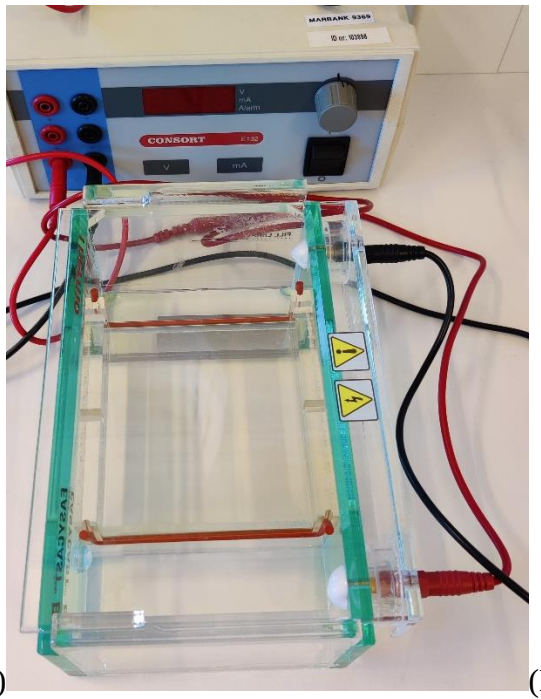
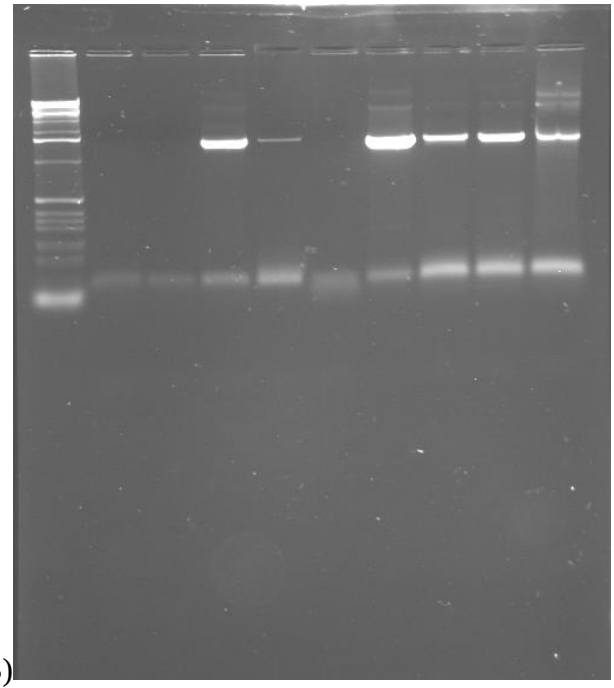
(A)  (B) 

Figure 4: Gel Electrophoresis setup: from left to right is the (A) electrophoresis glass case which contains the buffer, agarose gel, and the conducting terminals (red +ve terminal and black -ve terminal), and the voltage regulator. On the left (B) is the agarose gel with wells for loading the marker buffer and the PCR samples. On exposure to an electric field, the DNA fragments, migrate to the +ve terminal according to their weight. the buffer on the first well contains DNA fragments with known weights which helps to know the weight of the DNA in the sample (picture: Edoziem V.M.)

1.3.4 16S rRNA Gene as a Molecular Marker

16S sequencing is a molecular biological technique used to analyze the diversity and composition of bacterial communities based on the sequencing of the 16S ribosomal RNA (rRNA) gene. The ribosome is an organelle present in all living organisms with the specific function of protein synthesis. The bacterial ribosome is primarily composed of rRNA (65%) and ribosomal protein (35%). The bacterial rRNA consists of two subunits: the large 50S

subunit and the small 30S subunit. The main function of the 30S subunit is to decode the genetic information on the mRNA and it consists of approximately 1,500 nucleotides with a sedimentation rate of 16S (Svedberg, 10^{-13}sec.). The 50S subunit consists of two RNA molecules of different sizes; the larger RNA molecule has a nucleotide sequence of 2900bp, with a sedimentation rate of 23S, and the major function of adding new amino acids from the respective tRNA to the newly formed peptide chain (elongation of peptide chain). It does this by forming an amino linkage bond between the last amino acid in the peptide chain and the new amino acid from tRNA. Furthermore, the second RNA molecule has a nucleotide sequence of 120bp and a sedimentation rate of 5S, with the main function of stabilizing the ribosomal structure.

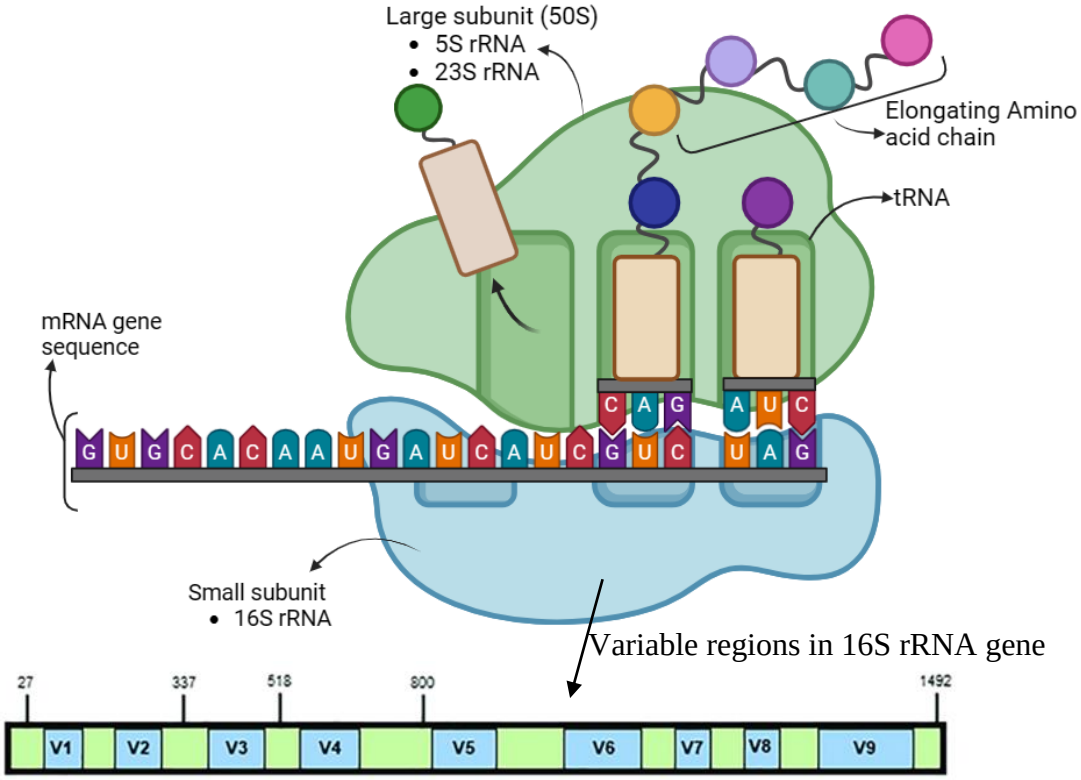


Figure 5: The structure of the prokaryotic ribosomal rRNA; depicting the two subunits (large and small) and their genetic makers (5S rRNA, 23S rRNA, and 16S rRNA) and the variable regions on the 16S rRNA gene. The mRNA is read in the small subunit and the amino acid chain is synthesized in the large subunit. The diagram was edited with biorender.com

The 50S subunit contains two rRNA molecules which were either too big to sequence (the 23S with 2900bp) or too small for molecular identification (the 5S with 120pb). This makes the 16S rRNA with a moderate nucleotide sequence of 1500bp the best-suited molecular maker and easy target to sequence. Beyond its moderate base pair sequence, it has highly conserved regions interspersed with hypervariable regions. The 16S rRNA has 9 variable

regions, which contain genetic information unique to specific bacterial groups. The unique configuration of 16S rRNA, allows for the design of primers (see Table 1) that target conserved regions for PCR amplification, followed by sequencing of the variable regions. By sequencing these regions, researchers can precisely detect the taxonomic and phylogenetic classification of bacteria even at the species or strain level.

Table 1: The variable regions of the 16S rRNA gene and their primer sequence for both forward and reverse primers (Baker et al., 2003; Klindworth, et al., 2013)

SN	Variable Region	Primer	Sequence (5'--3')
01	V1	27F (Forward)	5'-AGAGTTTGATCMTGGCTCAG-3'
		1492R (Reverse)	5'-GGTTACCTTGTTACGACTT-3'
02	V2	338F (Forward)	5'-ACTCCTACGGGAGGCAGCAG-3'
		518R (Reverse)	5'-ATTACCGCGGCTGCTGG-3'
03	V3	338F (Forward)	5'-ACTCCTACGGGAGGCAGCAG-3' (same as V2)
		518R (Reverse)	5'-ATTACCGCGGCTGCTGG-3' (same as V2)
04	V4	515F (Forward)	5'-GTGCCAGCMGCCGCGGTAA-3'
		806R (Reverse)	5'-GGACTACHVGGGTWTCTAAT-3'
05	V5	799F (Forward)	5'-AACMGGATTAGATACCCKG-3'
		1392R (Reverse)	5'-ACGGGCGGTGTGTRC-3'
06	V6	926F (Forward)	5'-AAACTYAAAKGAATTGRCGG-3'
		1392R (Reverse)	5'-ACGGGCGGTGTGTRC-3' (same as V5)
07	V7	1100F (Forward)	5'-AGYTGCCAGCMGCCGCGG-3'
		1392R (Reverse)	5'-ACGGGCGGTGTGTRC-3' (same as V5)
08	V8	1391F (Forward)	5'-GACGGGCGGTGWGTRCA-3'
		1492R (Reverse)	5'-GGTTACCTTGTTACGACTT-3' (same as V1)
09	V9	1391F (Forward)	5'-GACGGGCGGTGWGTRCA-3' (same as V8)
		1492R (Reverse)	5'-GGTTACCTTGTTACGACTT-3' (same as V1)

1.3.5 Sanger Sequencing (16S rRNA Sequencing)

Sanger Sequencing was named after the English biochemist, Frederick Sanger who developed this method with his colleagues in 1977. This sequencing is done in two sections: the cycle sequencing and the capillary electrophoresis. The cycle sequencing occurs in the same stages as explained in the PCR reaction (section 1.3.2 above) but differs in the termination stage as it is terminated by the addition of a fluorescently dyed dideoxynucleoside (ddNTPs). This method is also called the chain termination method or the dideoxynucleoside triphosphate (ddNTPs) sequencing because the products are DNA fragments terminated by ddNTPs. During DNA replication, the polymerase enzyme synthesizes new strains from the template, by the formation of a phosphodiester linkage between the hydroxyl group (-OH) at carbon

three (3) of the last added nucleotide and the phosphate group at carbon (5) of the next nucleotide to be attached. But unlike deoxynucleotides (dNTPs) which are used to elongate the DNA chain, di-deoxynucleotides (ddNTPs) have the phosphate group at carbon five of the ribose sugar (5) which allows it to be attached to the previous base but lacks the hydroxyl group (-OH) at carbon (3) which stops further addition of a nucleotides. So, the addition of ddNTPs terminates the new chain and the process continues again. Resulting in DNA fragments of different sizes with ddNTPs at their terminal. These ddNTPs contain fluorescent dyes (the big dye terminators) which are necessary for their detection during capillary electrophoresis.

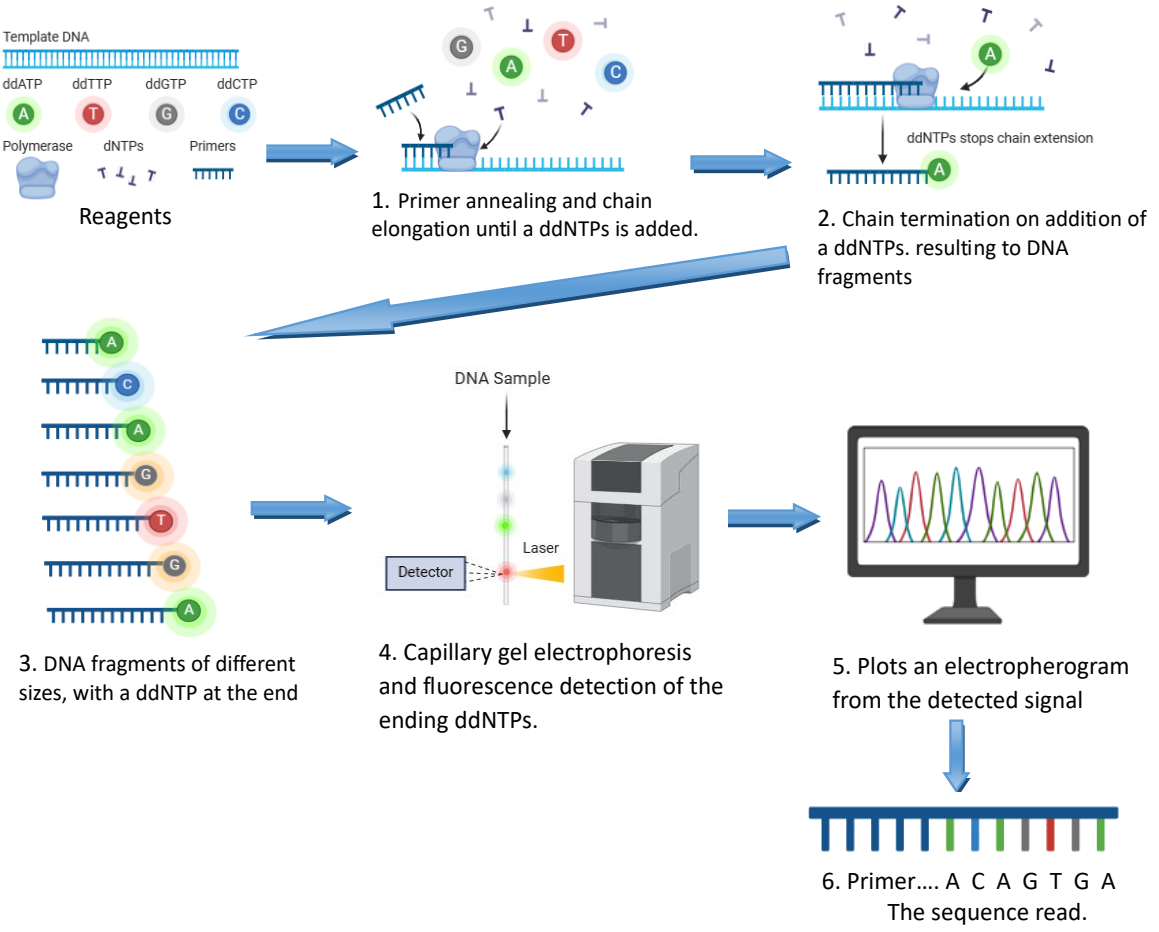


Figure 6: A flow chart of the Sanger sequencing with the same stages as the PCR as explained in Figure 2, except for the termination. In Sanger sequencing, the chain elongation is terminated by the addition of a ddNTPs, and the fluorescence of each ddNTPs when hit by a laser is detected and recorded as an electropherogram which is used to read the sequence of the gene. This flow chart was edited with biorender.com.

The second section of the Sanger sequencing is the capillary electrophoresis. The principle behind this section is that DNA fragments are negatively charged because of their phosphate group and will migrate to the anode when subjected to an electric field. The speed at which the fragments migrate through the gel is inversely proportional to its molecular weight (meaning the smaller the fragment, the faster it migrates). In the process, the molecules are injected by an electrical current into a long glass capillary filled with a gel polymer. A laser at the end of the tube excites the dye-labelled DNA fragments as they pass through a tiny window and the excited dye emits a light of a characteristic wavelength that is detected by a light sensor. The sensed signal is translated into a base call by the software, giving an electropherogram and a text file. Which are used for further identification in a BLAST search. This method is also called the gold standard method because of its high accuracy (99.99%) in sequencing nucleotides in a DNA strand (commonly less than 1,000bp in length).

1.3.6 Basic Local Alignment Tool (BLAST)

This is a bioinformatic tool to analyze sequences for their similarity and when looking at the 16S rRNA, can be used to identify an organism using the sequenced data. The sequenced data is compared with all the sequences deposited in a public domain database and presents possible matches in a 0-100% degree of identity (Ambikapathy *et al.*, 2023). 97-100% sequence similarity is acceptable for species and genus, but this can be flexible at the genus level to a range of 94-96% and above (Altschul, *et al.*, 1990). Examples of such databases are the National Center for Biotechnology Information (NCBI), the European molecular biology laboratory (EMBL), the DNA data bank of Japan, the Sequence Read Archive (SRA), *etc.*

Finally, the phylogenetic relationship between the identified bacteria is established using the Molecular Evolution Genetics Analysis software (MEGA 11) (Tamura *et al*., 2011).

1.4 Aim

This project seeks to isolate strains of Actinobacteria from marine invertebrates selectively and to optimize the existing actinobacteria isolation protocols.

1.5 Justification

Actinobacteria are slow growers and could be outcompeted by fast-dividing microbes with simple metabolic systems in a given sample. So, knowing a way to selectively isolate the organism of interest increases the success rate of the scientist and reduces the time spent in identifying colonies of the least importance. This research will contribute to the field of

marine microbial ecology, bioprospecting, and biotechnology for a better understanding of ways to selectively isolate Actinobacteria in a given marine sample. It could lead to the discovery of novel bioactive compounds with potential pharmaceutical and biotechnological applications, addressing the growing demand for innovative drugs and bioproducts, especially as more pathogenic bacteria show antibacterial resistance. Additionally, it could lead to the Identification of promising Actinobacteria strains for further bioprospecting and potential commercialization, with potential economic implications.

1.6 Objectives of this Work

- Isolation, Identification, and characterization of Actinobacteria from marine animals.
- Preserving the strains of isolated Actinobacteria for further studies.
- Investigation of different sample pre-treatments in order to increase the relative yield of actinobacteria.

2 Materials and Methods

2.1 General Methods and Materials

For the preparation of the media filtrated sea water (FSW) was prepared by the seawater supply of the Norwegian College of Fishery Science (Tromsø, Norway) by filtering the seawater from the inhouse sea water supply through a Millidisk® 40 cartridge with Durapore® 0.22µm filter membrane (Millipore, Burlington, MA, USA). All purified water (pH2O) was produced by the in-house milli-Q system at Marbio. For the sterilization of media and sterilization of agar, the liquids were autoclaved at 121°C for 30 min under increased pressure (MLS-37812, Panasonic, Kadoma, Japan).

2.2 Summary of the Workflow

This research work was done in three successive stages; the first phase was attempted isolation using the pretreatment on the five animal samples, the second phase was to test the isolation pretreatment (to test the thermal stability of the isolate) using the already identified actinobacteria isolates for confirmation and the third stage was the further investigation with freshly collected breadcrumb sponge (*Halichondria Panicea*) to continue the study since the initial original five animal samples (cryo-stocks in glycerol from 2020) were used up in the first phase.

2.3 Preparation of Media

Two cultivation media were used for this work, the selective media M1 and the subculturing media FMAP. The M1 media was prepared for Actinobacteria isolation as described by Schneider *et al*., 2022 with slight modification. The following compositions were prepared in 1L Mili-Q Water and autoclaved. To prepare M1 media for the isolation of actinobacteria, 10.0g starch, 4.0g of yeast extract, 2.0g of peptone, 40.0g Sigma Sea salt, and 20.0g agar were weighed out and dissolved in 1L Milli-Q water. The pH reading was approximately 6.70 at room temperature. The media was autoclaved and antibiotics supplements (1.0ml of Nalidixic acid 30mg/ml in 0.3M NaOH) and fungicide (5.0ml of cycloheximide 10mg/ml in EtOH) were added at a hand-warm temperature. The medium was thoroughly mixed and poured into Petri dishes under a laminar flow.

2.3.1 Preparation of Nalidixic Acid and Cycloheximide

The nalidixic acid stock was prepared by dissolving 300mg of nalidixic acid in 10 ml of 0.3M NaOH for a final concentration of 30mg/ml. The stock solution was sterile filtered into an

autoclaved glass bottle using a 25ml disposable syringe and a 25µm sterile filter under a laminar flow. Also, Cycloheximide stock solution was prepared by dissolving 200mg of cycloheximide in 20 ml of absolute ethanol for a final concentration of 10mg/ml.

2.3.2 FMAP Media Preparation

To prepare FMAP media, 20.0g Sigma Sea salt, 5.0g of peptone, 15g of difco marine broth, and 15.0g agar were weighed out and dissolved in 1L Mili-Q water. The media was autoclaved and cooled to room temperature.

2.4 Origin and Collection of Samples

Sampling was done twice in the course of this work. First were semi-liquid homogenate samples of five animals from the research cruise of Schneider *et al*., 2022. They were stored in glycerol at -80^{0}C since August 2020 and their details are given in Table 2. below. While another sample was a fresh sponge sample, *Halichondria Panicea* (breadcrumb sponge). Collected at Skavelnet shore, Uteng, Tromso with these coordinates 69.64390^{o}N, 18.73639^{o}S. they were washed twice in falcon tubes containing sterile seawater to wash off ocean-floor bacteria at the point of sampling before taking them to the lab. Under sterile conditions on a sterile bench, the sponge samples were homogenized in a falcon tube using a spatula. Two stock solutions (5ml each) were made from the homogenate; 20% glycerol stock and 20% stock with sterile filtrated sea water respectively. The glycerol stock was stored at -80^{o}C and the latter was further used for the heat and phenol treatments.

Table 2: Details of used animal samples for the first phase of the research with their identities, sampling coordinates, and the depth they were collected. These samples were semi-liquid homogenates from five animals from the research cruise of Schneider et al., 2022. The samples were stored with glycerol at -80^{0}C.

Samples	Names of Animals	Sampling Site/SST	Latitude	Longitude	Depth(m)
Animal 16	*Synoicum turgens*	SST-28	77.36985593	8.283278517	1400
Animal 18	*Dendrobeania sp.*	SST-29	77.07979728	8.863405883	2200
Animal 19	*Tegella spitzbergensis*	SST-29	76.70149475	8.773346433	2200
Animal 22	*Tricellaria ternate*	SST-29	76.15589453	8.496510833	2200
Animal 23	*Chlamys islandica*	SST-31	75.86469082	9.833769933	2300

2.5 Pre-Treatment

The sample pre-treatment is a physical or chemical treatment to increase the relative yield of the organism of interest based on the unique properties of the organism's group (in this case spore-forming bacteria like actinobacteria). It is applied to create unfavorable growth conditions for microbes and fungi that are not desired. It is used especially when the organism of interest can survive such treatment. The pre-treatments used in this work were; 10mins heat treatment at 55^0C, 65^0C, and 75^0C respectively, as well as a chemical treatment with phenol alone (3%phenol) and a combination of 3% phenol and 65^0C heating before culturing. Finally, a control group with no treatment.

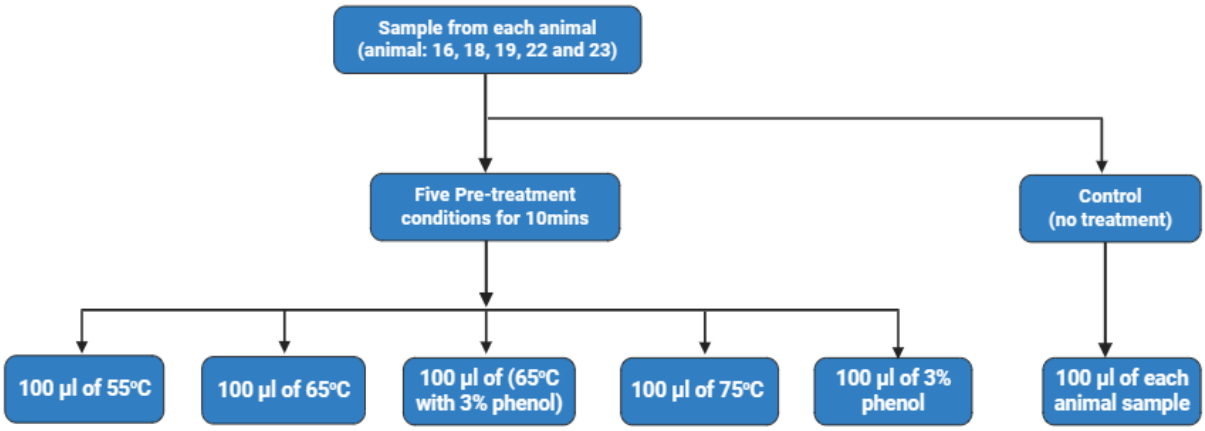


Figure 7: The pre-treatment flow chart of the five animal samples; six aliquots were prepared from each animal sample. Five were subjected to these five conditions (55^0C, 65^0C, 65^0C with 3% phenol, 75^0C, and 3% phenol) for 10mins and one aliquot had no treatment (control). 100µL from each were plated out on M1 Agar respectively.

Later in the research work some treatment conditions were included for further investigation. This was in the third phase of the research with breadcrumb sponge samples. This is because the five animal stock samples were not enough to continue the research studies. The freshly collected sponge sample was divided into three groups; unfrozen, 1_day frozen, and 15_days frozen. Heat treatment was given to them except, for the unfrozen sample which received three different phenol treatments (0.1%, 0.5%, and 1%) in addition to the heat treatment received by all. This is illustrated in the chart below (Figure 8).

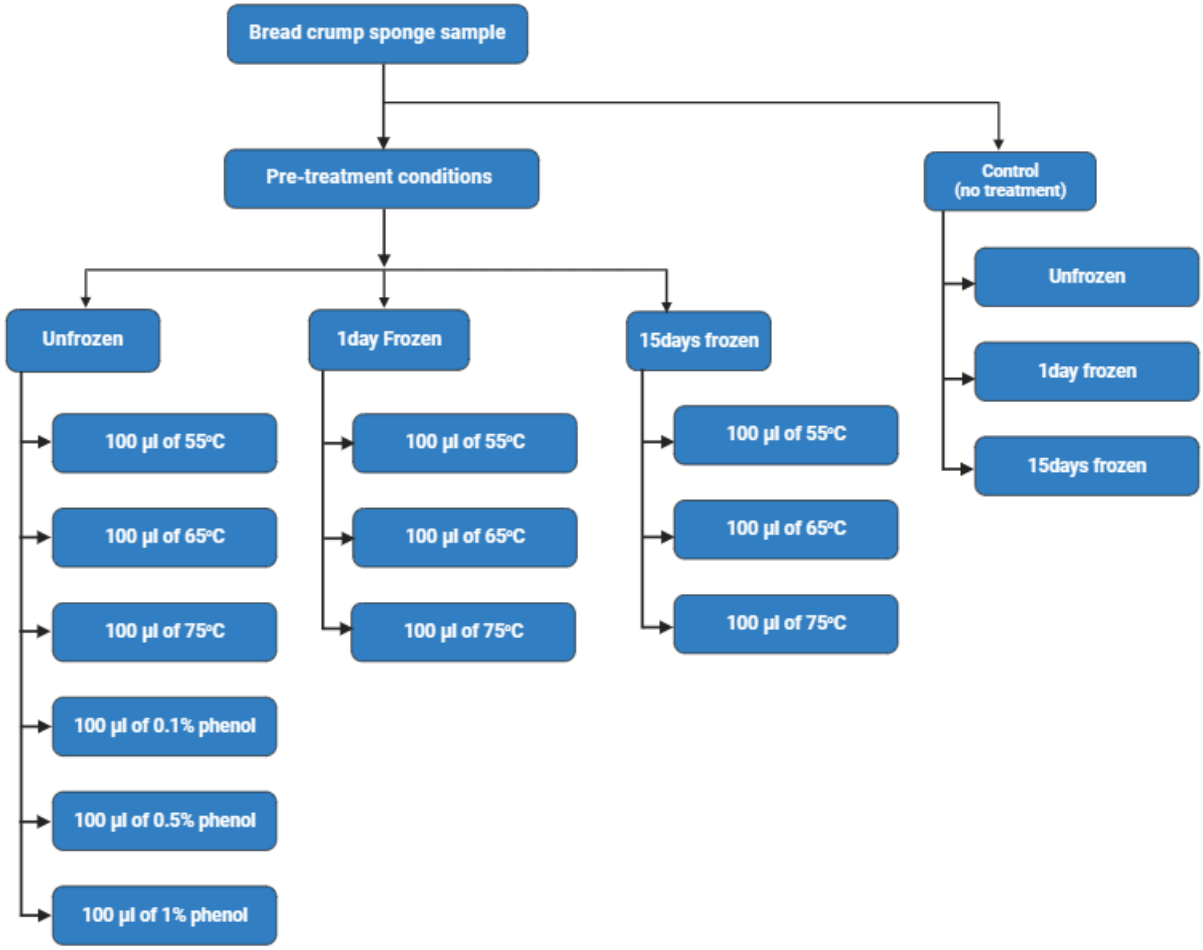


Figure 8: The pre-treatment flow chart of the bread crumb sponge sample; three conditions (unfrozen, 1 day frozen and 15 days frozen) were made with replicates of the bread crumb sponge sample to analyze the effect of freezing on the isolation. Each condition was treated with (55^{0}C, 65^{0}C, and 75^{0}C for 10mins) except for the unfrozen condition where also the phenol treatment of 0.1%, 0.5%, and 1% phenol for 10mins was applied. In addition, one sample from each without treatment was used as the control. 100uL from each were plated out on M1 Agar respectively.

100µl of each treatment condition per sample was plated out on a labeled petri dish. Each dish was sealed with a paraffin film and incubated at room temperature in the dark. After 6-weeks of incubation, morphologically distinct colonies on the plates were identified, numbered, and sub-cultured on FMAP media in order to obtain axenic isolates.

2.6 Bacteria Colony Screening

To identify bacteria colonies, morphologically distinct colonies were sub-cultured to obtain an axenic culture of the respective isolate for polymerase chain reaction (PCR) to amplify the bacteria 16S ribosomal RNA gene subunit. The amplified gene was confirmed with an agarose gel electrophoresis, and the PCR was repeated if it turned out negative. A positive PCR reaction was sequenced, and the sequenced result was presented in an electropherogram. Bioinformatic tools and gene analyzing software were used to identify the bacteria taxonomy

and analyze any mutations or variations present. Sequence alignment tools were used to compare the obtained sequences with reference sequences or databases to identify similarities or differences and possibly study their phylogenetic track to know if it is a new bacteria strain or a known bacterial species.

2.6.1 16s rRNAs Colony PCR (Polymerase Chain Reaction)

This method is used to amplify the bacteria's 16S ribosomal RNA for better identification of the bacteria. For this experiment, PCR tubes were labeled in correspondence with samples and to each test tube was added 12.5µl of 2x dream taq polymerase, 0.5µl of 10µm 27F primer (forward primer, see Tab.1), 0.5µl of 10µm 1492R primer (Reverse primer, see Tab.1), 10.5µl of graded water and 1µl of the corresponding sample to the tube. The PCR tubes were closed and spun for 5 seconds and placed in the PCR machine. The 16S-Polymerase Chain Reaction program was selected; it ran 40 cycles for 1hr:45mins.

2.6.2 Agarose gel Electrophoresis

The agarose gel electrophoresis is used to confirm and quality of the 16S PCR was successful. First, the electrophoresis glass components were rightly assembled (see Figure 4A.). To prepare the agarose gel, 1g of agar was dissolved in 100 ml of 10X TBE (Tris borate EDTA) buffer solution. The agar mixture was heated in a microwave for 3 minutes till the agar granules were completely dissolved. When it cooled down to hand-felt warmth, 5µl of DNA color dye was added and swirled gently to mix without generating bubbles. The agar mixture was poured into the glass case setup and allowed to harden. The well's comb was gently removed to create micro-wells on the agarose gel, the gel was aligned in the direction of the current flow and the buffer was added till the gel was fully covered. The DNA leather buffer was added to the first well (5µl) and 5µl of the samples were subsequently added to other wells in successions according to their numeral order. The terminals were connected and the voltage of 200-205volts was run across the gel for 25 minutes. At the end of the run, the gel was visualized and recorded using a Syngene GeneFlash gel imaging system (used to visualize and document DNA in agarose gels).

2.6.3 Sanger Sequencing

This was done once the PCR was confirmed positive. with each well plate containing a mixture of 1µl of big dye 3.0, 2µl of 5x sequencing buffer, 1µl of 1µM 27F (forward primer see Table 1.), 5.5µl of graded water and 0.5µl of the PCR sample. Electropherograms of each sample were obtained using a genetic analyzer and the gene sequence of each sample was

identified and their taxonomic tree traced using a combination of different gene analytic software and bioinformatic methods. However, samples with poor electropherogram were repeated.

2.7 Bacteria Identification

The sequence data was edited using gene editing software (BioEdit) and a basic local alignment tool (BLAST) search was done on the National Center for Biotechnology Information (NCBI) database GenBank to identify the sequenced data. Bacteria were tentatively identified at 97-100% for similarity in species/Genus, but 95% and above for Genus level. The identified bacteria were grouped according to their taxonomic class.

2.8 Preparation of Cryo-stocks

A cryo-stock of bacilli and actinobacteria isolated from this work were preserved in a liquid FMAP media with 20% glycerol and stored at -80^0C. Therefore, a sample from each pure colony was cultured in FMAP media for 6 days. To each cryo-tube, 1ml of 30% (v/v) glycerol in FMAP was added to 500µl of the isolates culture in liquid FMAP. The cryostocs for each isolate were produced in triplicates.

3 Results

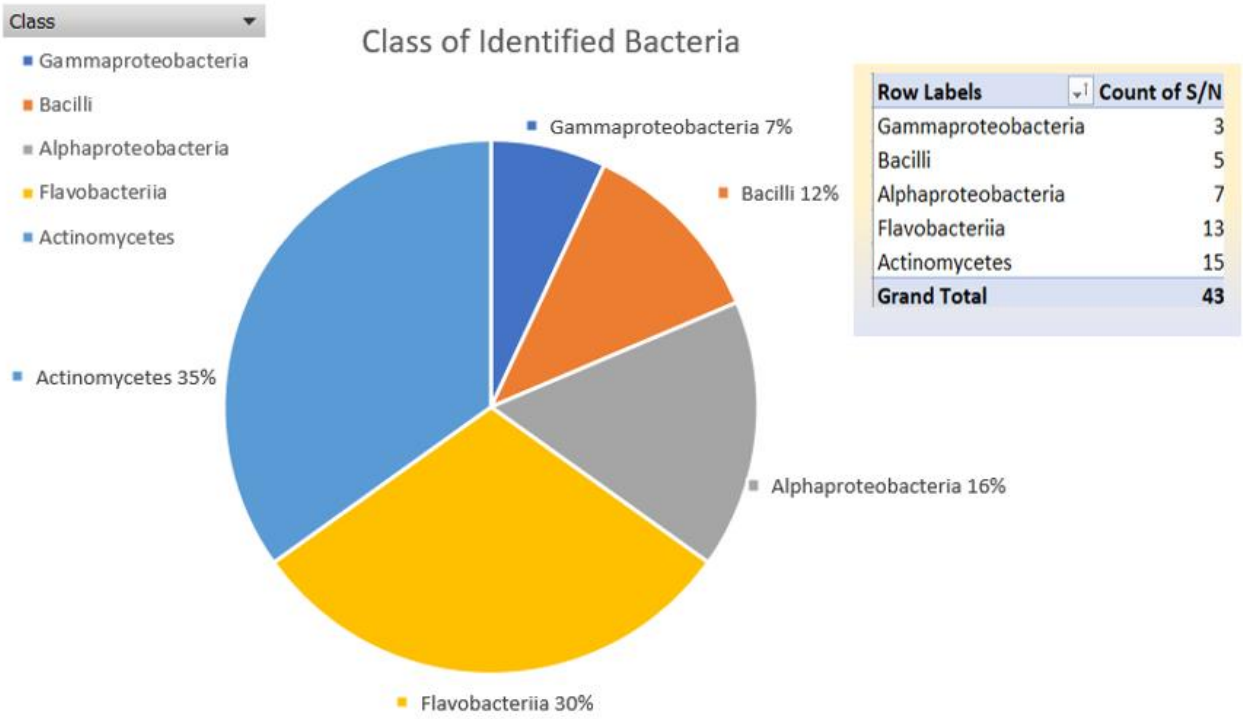
3.1 Phase1: Isolations from five Invertebrate Samples

Table 3: Showing the culture result of the five invertebrate samples, animals used and identity, pre-treatment conditions, and observations. Where G is for observed Growth and NG is for No growth observed.

Samples	Animal Names	Pre-Treatments					
		55^0C	65^0C	75^0C	65^0C + 3%phenol	3% Phenol	Control
Animal 16	*Synoicum turgens*	NG	G	NG	NG	NG	G
Animal 18	*Dendrobeania sp.*	NG	NG	NG	NG	NG	G
Animal 19	*Tegella spitzbergensis*	NG	NG	NG	NG	NG	G
Animal 22	*Tricellaria ternate*	NG	NG	NG	NG	NG	G
Animal 23	*Chlamys islandica*	NG	NG	NG	NG	NG	G

As listed in Table 3. above, there was growth in the control group of all five animal samples and the 65^0C pre-treatment of animal 16. While other pre-treatment conditions of all animal samples showed no growth.

3.2 Analysis of results of the isolated microbial strains from the five animal (invertebrate) samples



Row Labels	Count of S/N
Gammaproteobacteria	3
Bacilli	5
Alphaproteobacteria	7
Flavobacteriia	13
Actinomycetes	15
Grand Total	**43**

Figure 9: Analysis of the results from isolated microbial strains from the five animal (invertebrate) samples

In Figure 9., a table and pie-chart representation of the distribution of the bacteria classes isolated from the cultured five marine animal samples can be seen. The table shows a total of 43 isolates of which: 3 gammaproteobacteria (7%), 5 Bacilli (12%), 7 Alphaproteobacteria (16%), 13 Flavobacteriia (30%), and 15 Actinomycetes (35%).

3.3 Distribution of the classes of bacteria isolated from each animal sample.

Row Labels	Actinomycetes	Alphaproteobacteria	Bacilli	Flavobacteriia	Gammaproteobacteria	Grand Total
16	4		1	1	1	7
18	8	2	1		2	13
19	1	1		7		9
22	2	3	3	1		9
23		1		4		5
Grand Total	**15**	**7**	**5**	**13**	**3**	**43**

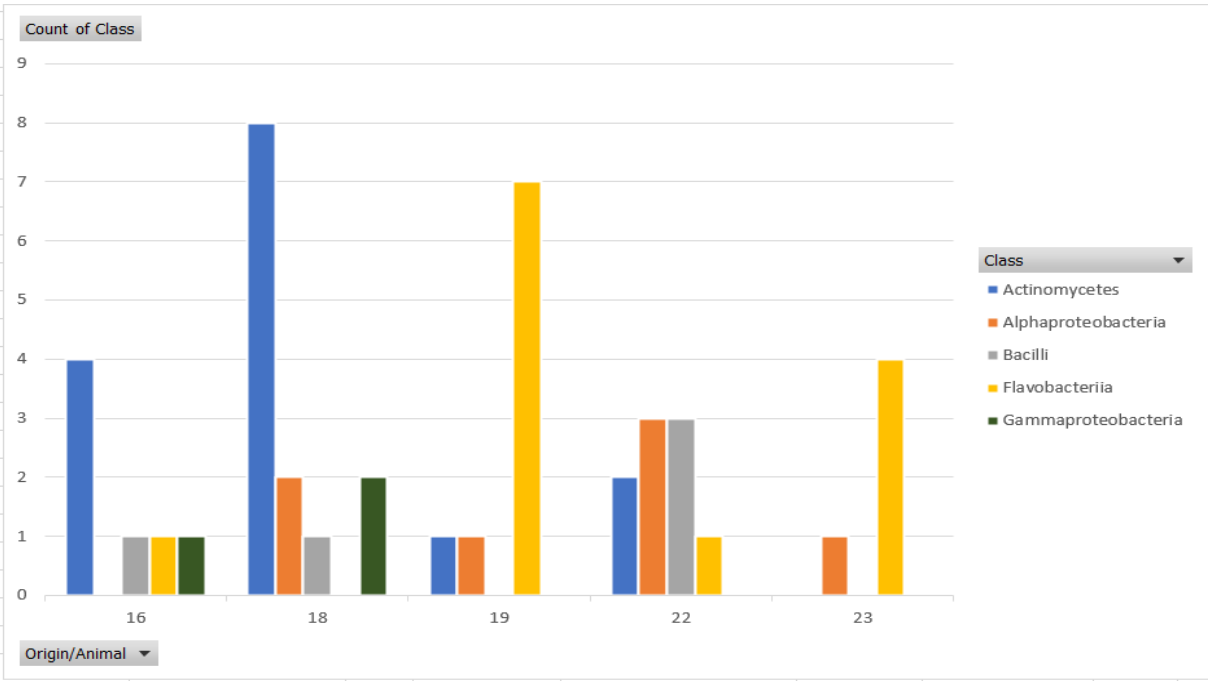


Figure 10: the respective number of bacteria Classes isolated from each animal sample.

In Figure 10., a bar chart is given to visualize the distribution of the five bacteria classes; (Actinomycetes, Alphaproteobacteria, Bacilli, Flavobacteriia, and Gammaproteobacteria) isolated from each of the animal samples; 16, 18, 19, 22 and 23. from the chart, Animals that yielded the highest number of actinobacteria isolates (16 and 18), yielded little or no flavobacteria. On the other hand, animals with a high number of flavobacteriia yield (19 and 23), were low in actinobacteria. Animal 22 however, had a little of all classes except gammaproteobacteria which were only present in animals with the highest actinobacteria production (animals 16 and 18).

3.4 Phase2: Result from Heat Treatment/Thermal Stability of Tentatively Identified Actinobacteria Isolates

Table 4: Result of the heat treatment/thermal stability of each tentatively identified class of Actinomycetes from the five animal samples. Where "G" stands for observed Growth and "NG" stands for No growth observed.

Samples ID	Treatment Conditions					
	Genus level Identification	55^{0}C	65 ^{0}C	75 ^{0}C	10X dilution Control	Control
A003	*Brevibacterium Sp.*	G	G	NG	NG	G
A010	*Microbacterium Sp.*	G	G	G	G	G
A015	*Micrococcus Sp.*	G	G	NG	G	G
A024	*Rhodococcus Sp.*	G	G	NG	G	G
A025	*Salinibacterium Sp.*	G	G	NG	G	G
A028	*Dietzia*	G	G	NG	G	G
A031	*Glutamicibacter Sp.*	G	G	NG	G	G

In Table 4., it can be seen that the tentatively identified actinobacteria grew at 55^{0}C, 65^{0}C, and Control (room temperature), but did not survive at 75^{0}C heat treatment. Except for A010 which was heat resistant and grew even after a 75^{0}C heat treatment.

3.5 Phase3: Observations from the Breadcrumb Sponge (*Halichondria panicea*)

Table 5: Observed colony growth on the isolation plates for the different treatments of the breadcrumb sponge sample, with the sample ID, the treatment for each condition, and the observed result. where "G" stands for colony Growth was observed and "NG" stands for No growth was observed.

Sample ID	Condition	Treatment	observation
B001	Unfrozen	0.1% phenol	G
B002	Unfrozen	0.5% phenol	G
B003	Unfrozen	1.0% phenol	NG
B004	Unfrozen	55	NG
B005	Unfrozen	65	G
B006	Unfrozen	75	NG
B007	Unfrozen	Control (no treatment)	G
B008	1day frozen	55	NG
B009	1day frozen	65	NG
B010	1day frozen	75	NG
B011	1day frozen	Control (no treatment)	G
B012	15day frozen	55	NG
B013	15day frozen	65	NG
B014	15day frozen	75	NG
B015	15day frozen	Control (no treatment)	G
B016	15day frozen	35	G
B017	15day frozen	45	G

The data shown in Table 5., shows that there was growth in the control group of each treatment condition and 0.1% and 0.5% of the phenol treatment for the unfrozen condition. There was also growth on the 65 treatment for the unfrozen condition, and growth was observed on the 35 and 45 heat treatments for the 15-day frozen condition while other treatments showed no growth.

3.6 Analysis of the Result from Tentatively Identified Bacteria Isolated from the Breadcrumb Sponge (*Halichondria panicea*)

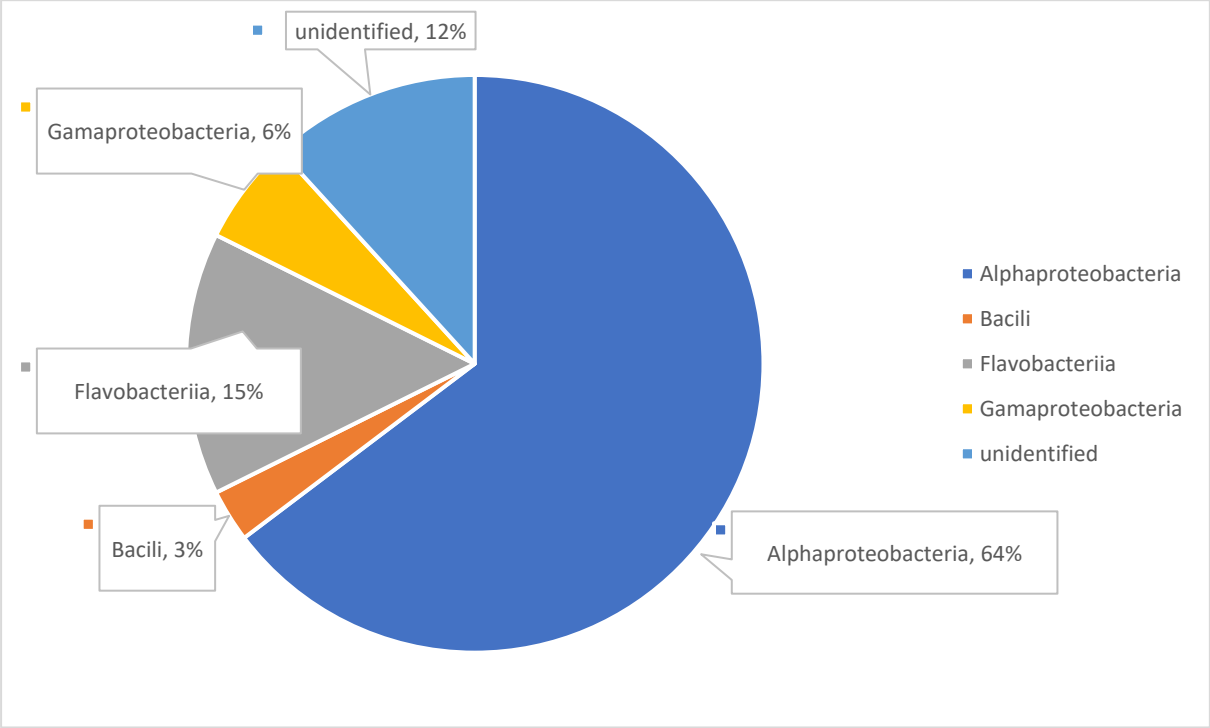


Figure 11: Analysis of the Result from tentatively identified bacteria from the breadcrumb sponge.

In Figure 11., a pie chart, showing the distribution of the organism class isolated from the breadcrumb sponge is depicted. Alphaproteobacteria made up 64%, Flavobacteriia were 15%, Gammaproteobacteria were 15%, Bacili 3%, and 12% were unknown bacteria (not yet identified).

Table 6: Results of actinobacteria yield of animals 16, 18, 19, 22, and 23 from the work of Schneider et al., 2022.

S/N	Isolate ID:	Origin:	Heat shock	*Genus level identification*
1.	T079	animal 16, *Synoicum turgens*	no	*Dietzia* sp.
2.	T080	animal 16, *Synoicum turgens*	no	*Brevibacterium* sp.
3.	T173	animal 16, *Synoicum turgens*	no	*Glutamibacter* sp.
4.	T174	animal 16, *Synoicum turgens*	no	*Arthrobacter* sp.
5.	T176-02	animal 16, *Synoicum turgens*	no	*Salinibacterium* sp.
6.	T242	animal 16, *Synoicum turgens*	yes	*Micrococcus* sp.
7.	T059	animal 18, *Dendrobeania* sp.	no	*Microbacterium* sp.
8.	T060	animal 18, *Dendrobeania* sp.	no	*Rhodococcus* sp.
9.	T076	animal 18, *Dendrobeania* sp.	no	*Citrococcus* sp.
10.	T119-01	animal 18, *Dendrobeania* sp.	no	*Dietzia* sp.
11.	T125	animal 18, *Dendrobeania* sp.	no	*Salinibacterium* sp.
12.	T134-02	animal 19, *Tegella spitzbergensis*	no	*Brevibacterium* sp.
13.	T135	animal 19, *Tegella spitzbergensis*	no	*Microbacterium* sp.
14.	T159	animal 22, *Tricellaria ternata*	no	*Microbacterium* sp.
15.	T160-02	animal 22, *Tricellaria ternata*	no	*Rhodococcus* sp.
16.	T318	animal 22, *Tricellaria ternata*	no	*Kocuria* sp.
17.	T289	animal 23, *Chlamys islandica*	no	*Rhodococcus* sp.

In Table 6., the yield of actinobacteria from animals 16, 18, 19, 22 and 23 can be seen. Animal 16 yielded six (6), animal 18 yielded four (4), animal 19 yielded two (2), animal 22 yielded three (3) and animal 23 yielded one (1) actinobacteria. However, only animal 16 yielded one (1) actinobacteria after a heat shock. Adapted from the data sheet of Schneider *et al.*, 2022.

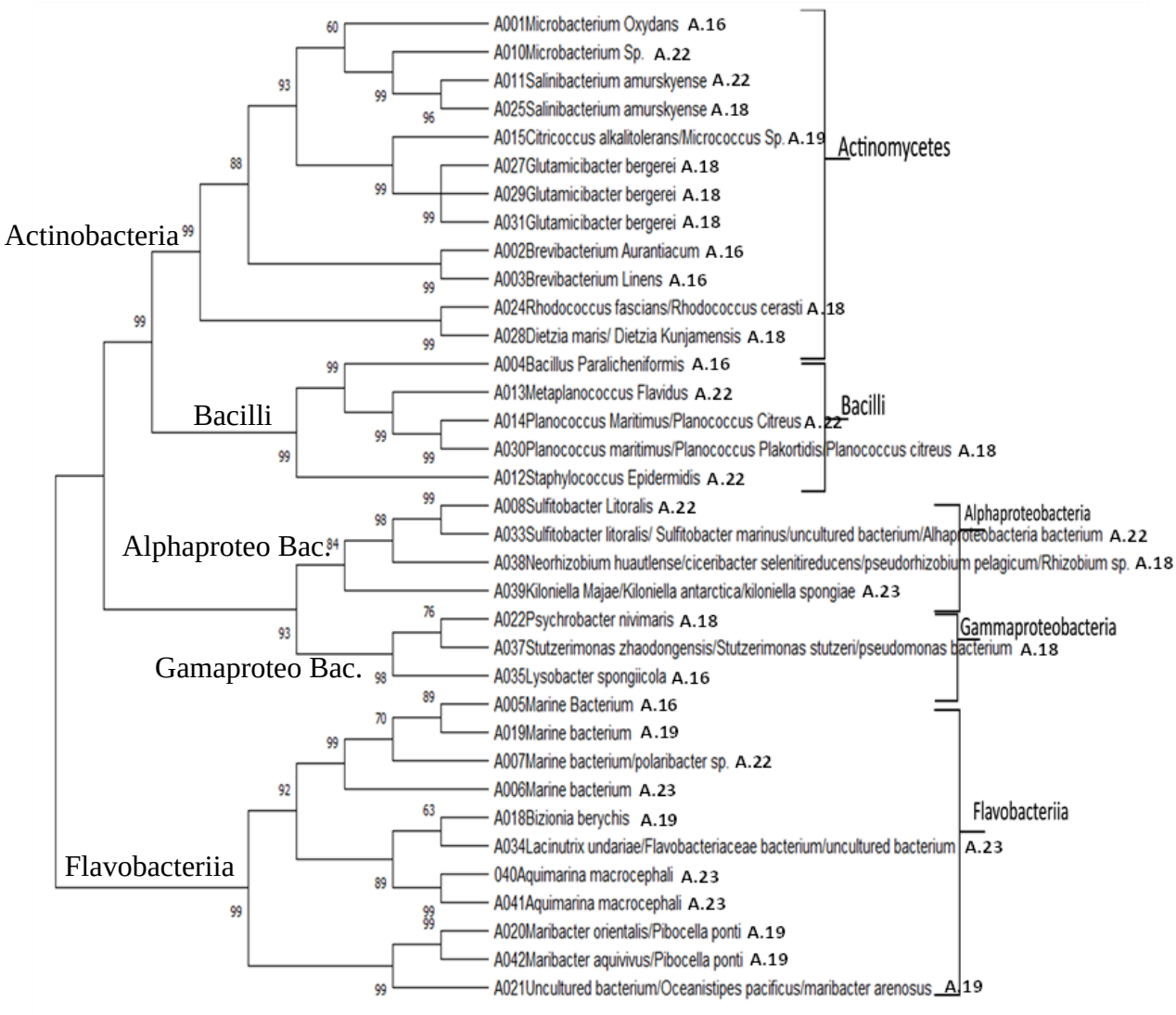


Figure 12: The phylogenetic tree of isolates from five animal samples, based on the 16S rRNA sequences, with the animal source (A.16, A.18, A.19, A.22, and A.23) tagged behind each genus.

A phylogenetic tree of the isolates based on their 16S rRNA sequences and their animal source is shown in Figure 12. to visualize the distribution of the isolates and how they are relatively related.

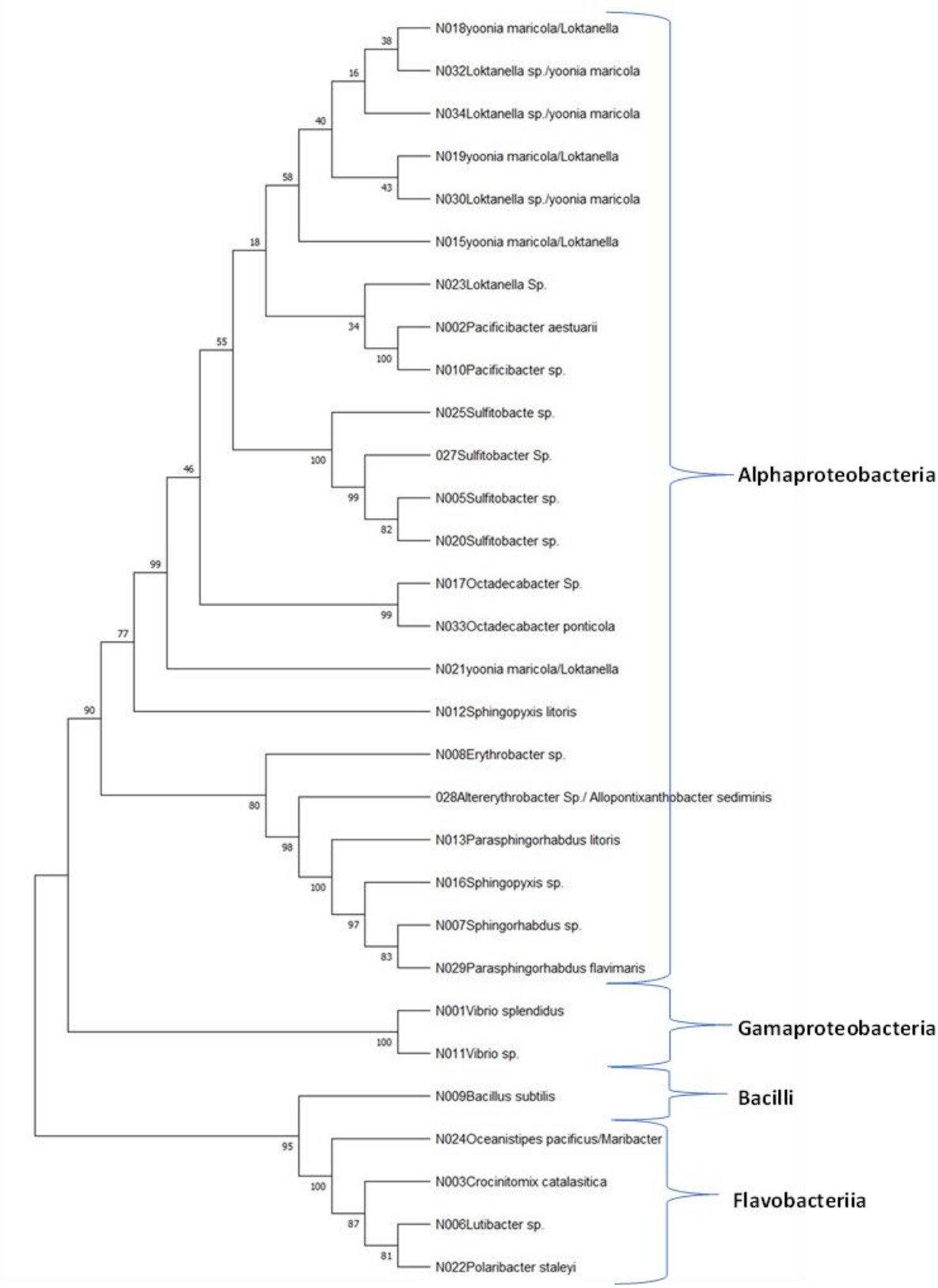


Figure 13: The phylogenetic tree of isolates from the breadcrumb sponge sample based on the 16S rRNA sequences.

4 Discussion

The results from the different heat treatments of the five animal samples did not show growth in any of the treatments except the 65^0C heat treatment where one colony of *Marine bacterium* was observed. However, there was growth in all control plates (no heat treatment) of each animal sample. Distinct colonies from these controls were sub-cultured on FMAP-agar to obtain axenic cultures of the respective isolates and identified using sequencing of a part of the 16S rRNA-gene (with primers 27F and 1492R see Table 1.). They were grouped by their taxonomic classes as previously by Schneider *et al.,* 2022. and seven distinct classes were identified; Three (3) were Gammaproteobacterial, five (5) Bacilli, seven (7) Alphaproteobacteria, thirteen (13) Flavobacteriia, and fifteen (15) Actinomycetes. Though the controls were not pre-treated with either heat or phenol, there was a high number of isolated actinomycetes. This is most likely caused by the selective media (M1) containing nalidixic acid which reduces the growth of Gram-negative bacteria, and the cycloheximide did prevent the growth of fungi successfully since no fungi were observed on the plates.

We observed that most actinomycetes came from animals 16 and 18 which also resonates with the results from Schneider *et al.,* 2022, where among all samples, animals 16 and 18 gave more actinomycetes isolates (see Table 6.) We speculate that some animals form a better symbiotic relationship with specific organisms than others. As depicted in Figure 10., animal samples with more actinomycetes (samples 16 and 18), which were Gram-positive had less of the Gram-negative bacteria while those that produced Gram-negative bacteria (samples 23 and 19) had little or no Gram-positive bacteria. Bacilli, which are Gram-positive bacteria were only isolated from animals 16 and 18. Animal 16 (*Synoicum turgens*), is an ascidian known for their prolific production of bioactive compounds. Antimicrobial peptides Turgencin A and Turgencin B with antibiotic activities on both Gram-positive and Gram-negative bacteria were isolated and characterized from *synoicum turgens* in a work by Hansen, *et al.,* (2020). Also, Ayuningrum, *et al.,* (2019) attributed the potential for producing marine natural products by ascidians partly to their associated microbiome, and Valliappan, *et al.,* (2014) reported that from analyzed 203 16S rRNA gene sequences obtained from the GenBank, it was observed that 34 genera within 16 families of the order Actinomycetales are associated with the ascidians. This was in concordance with the observed result from the ascidian, *Synoicum turgens* in this work.

Nevertheless, the growth inhibition observed in the treatment conditions sprouted more curiosity and raised more questions; Was the pre-treatment bactericidal/sporicidal? Could the prolonged storage of the five-animal samples in glycerol at -80^{0}C have had an effect? Or could it be a combination of both? These questions led to the second phase of this research. Which was to test the effect of heat shock on already isolated actinobacteria. To confirm if the heat pre-treatment was bactericidal or maybe sporicidal, isolates from each class of already isolated actinobacteria axenic cultures were subjected to the same heat treatment. As can be seen in Table 4., there was growth at 55^{0}C, 65^{0}C, control, and 10X diluted control. but the 75^{0}C showed a high level of inhibition except the isolate A010 which had high heat tolerance. This showed that actinobacteria could tolerate the heat pre-treatments of 65^{0}C, however, it is worth to note that these samples were already cultured and are not fresh samples from the field. To confirm this pre-treatment on a freshly collected sample, we obtained fresh sponge biomass from the intertidal zone because the cryo-stocks of the five animals were unfortunately used up in the previous experiments.

A breadcrumb sponge (*Halichondria panicea*) sample from Uteng in Tromsø was collected to complete this study which was its third phase. To test for the effect of freezing over a long storage period, the sample was homogenized under sterile conditions and divided into three groups of aliquots; not-frozen, one day (1) frozen, and 15 days frozen at -80^{0}C (frozen in this context means cryo-conserved at -80^{0}C in 20% glycerol). For each condition, heat pre-treatments of 55^{0}C, 65^{0}C, and 75^{0}C were applied to one aliquot, the phenol treatments were only applied to the unfrozen group at 0.1%, 0.5%, and 1.0%. in addition to this, further treatments of 35^{0}C and 45^{0}C were tested on the 15 days frozen sample.

As shown in Table 5., there was growth on the control group plates in all three conditions. Also 0.1%, and 0.5% phenol treatment, 65^{0}C of unfrozen and 35^{0}C with 45^{0}C of the 15 days frozen showed growth. Other conditions and pre-treatment showed no growth. However, a total of 37 strains were identified, of these, Alpha-proteobacteria made up 65%, Flavobacteriia was 14%, Gamma-proteobacteria was 16%, Bacilli 3%, and 12% could not be sequenced after two trials (insufficient sequencing electropherogram). As observed in the first phase of the work and also in Schneider *et al.,* 2022, phenol pre-treatment at 3% concentration was bactericidal the same was observed at 1% phenol treatment in the third phase of this research but at 0.1% and 0.5%, there was a scanty colony growth relative to the control group as intended. However, no actinobacteria was isolated from the breadcrumb sponge sample. Just as explained above in the first phase of this experiment, animal samples

with more Gram-positive bacteria had less Gram-negative and vice versa. The same pattern can be observed here since bacilli which were the only Gram-positive isolates, accounted for 3% of all isolates while 85% were Gram-negative. Even if the yet unidentified 12% were Gram-positive it would still be a ratio of 85:15. This also goes a long way to explain why it was insufficient to use just one sample to test a selective isolation treatment because of the possibility of a false negative result just as observed with the bread crumb sponge sample. That actinobacteria were isolated was not a result that depends solely on the method, but obviously also on the presence of actinobacteria or its spores within the sample. *E.g.* this result with the breadcrumb sponge could have been obtained if only animal 23 was used in the first phase of this study which yielded no actinobacteria but only 5 Gram-negative bacteria (4 flavobacteria and 1 alpha-proteobacteria) as shown in Figure 10. While the experiments on the *Halichodria* were a pilot test, a trial with three to five different sponge species like in the first phase, could have given a more convincing result on the pre-treatment method used, however, this would require a more extensive sampling campaign.

Furthermore, the period of sampling could also have affected the result obtained. Barka *et al.*, (2016) reported a study by Hiltner and Strömer, where they compared Actinobacteria obtained from a sample collected in the same area on different seasons to determine the effect of climate on the distribution of Actinobacteria showed that in winter just 13% of actinobacteria were isolated while 20% and 30% of actinobacteria were isolated in spring and Autumn respectively. They explained that in winter, frost could reduce the abundance of actinobacteria, and the sampling of the breadcrumb sponge for the third phase of the study was done in mid-November. The weather at the sampling date was well below the freezing point with ice in the intertidal zone. This was another indicator that actinobacteria seem to be cold-sensitive and fresh samples not exposed to temperatures below 0^0C are desirable for isolation.

Through a discussion with my supervisor (Hosea I. Masaki), he hypothesized that "bacteria need an optimum temperature to grow, this he called growth induction/thermal activation, but once grown, it can survive/withstand higher temperature range which he called thermal stability. And if a bacterium has a high thermal stability doesn't necessarily mean it can be cultured at that high temperature." This was well observed from the three phases of this study. In the first and third phases of this work, there was high inhibition at 55^0C, 65^0C, and 75^0C, but when the already identified isolates from the control group of the first phase were treated with the same conditions in the second phase, all the strains survived the heat shock at 55^0C

and 65^0C, with strains A010 surviving even at 75^0C. Edwards (1993), reported that the optimal growth temperature for actinobacteria is between 25^0C and 30^0C but that thermophilic Actinobacteria can grow at 50^0C to 60^0C.

The optimal growth temperature differs with the respective environment and sample source. The heat shock pretreatment used for this study was selective for actinobacteria because they are spore-forming bacteria and the treatment was intended to kill every bacteria but not spores since spores have a high-temperature resistance, this also explains the numerous yield of Bacilli when employing a 55^0C/10min heat shock in the previous study by Schneider *at al.*, 2022, since Bacilli can form endospores too. However, this pretreatment (55^0C, 65^0C, and 75^0C) was most likely optimized for soil and sediment samples and may not be perfect for animal samples. This is because, soil samples and sediments have a higher probability of containing spores which according to my supervisor Hosea Masaki, will need higher thermal activation than in animals where it is mostly the living organism/cell in active collaboration with the host animal. This agrees with the hypothesis rule proposed by Schneider *et al* (2022) where he hypothesized that over time, particles of sand, soil, weathered rocks, and animal debris, including bacterial spores, are deposited on the sea floor and slowly form layers of the sea-bed. Thus, making it a hot spot to find bacteria spores. Also using a heat-shock pre-treatment at 55^0C in his work, just one strain of actinobacteria was isolated from animal 16 (*Micrococcus sp.*) compared to sediment samples where 11strains were isolated with the same heat pre-treatment (Schneider *et al.*, 2022). This entails that selective isolation treatment should be sample-specific.

It is also worth mentioning that my supervisor, Hosea Masaki, repeated the heat treatment upon our observations but with a slight change in the method and observed some growth in the treatment samples; instead of plating out the samples immediately after 10mins of each heat treatment, he first cooled the samples down in crushed ice for two minutes before plating them out. However, due to lack of time, I couldn't repeat this method to confirm this observation.

At the end of this project, we were able to obtain a 35% yield of actinobacteria from the control group with no treatment and we observed that the proposed pre-treatment was not best suited for samples from animal sources, and we are confident that with the application of a sample-specific selection method, like membrane filter culture method, the percentage yield could be increased.

4.1 Conclusion and Recommendation

At the end of this work, we were able to establish that the isolation pretreatment could be sample-specific and that using the wrong treatment for a given sample could affect the result obtained. High heat treatment could be best for sediment and soil samples as they contain more spores than animal samples which probably contain mostly living bacteria. However, while consulting different literatures on this work, we found a selective method ("membrane filter method according to Savitha, *et al.*, 2022") that could best isolate actinobacteria from animal samples though it was not used in this work due to the time restrain. I recommend more work be done to test this method with different sample sources to ascertain its applicability limits. This method is based on the principles that actinobacteria grow mycelia and the use of a membrane with pores that are not permeable to the cells but permeable to the mycelium of actinobacteria will have other bacteria cells growing on the membrane while the mycelium of actinobacteria will remain in the media once the membrane is removed. In this method, a sterile membrane filter is placed on the media plate and the sample is added on the membrane, and after 3 to 4 weeks of incubation, the membrane is removed.

It was difficult to confirm the effect of prolonged years of cryo-storage on the viability of bacteria within an animal sample as most researchers comment that, the viability of cells is reduced over prolonged storage. However, there was no work on measuring the periodic viability of a sample over a long period and I recommend more studies to be done on this to properly guide bioprospecting scientists and microbiologists on how best to store collected animal samples over time. Further experiments on the effect of cryo-storage based upon this work and samples will be done, as several aliquots of the breadcrumb sponge sample were stored at -80^0C for further studies (repeating isolation after 1 year of storage) on this. Also, a proper account of the sample material should be taken into consideration before the commencement of any research project, to avoid suspending the study halfway or switching to an alternative source that could influence the confidence in the obtained result.

For this study, we have used cryo-conserved animal samples and freshly sampled material that could be made available at the time. However, the learnings of this work were already put into application in order to further investigate the effects of the heat shock on fresh samples. The sample pre-treatments of 55^0C and 65^0C combined with subsequent cooling of the sample (4^0C aluminum-block for 1.5mL reaction tubes) were applied on a four days research cruise in

April 2024 where, sediment, driftwood, and animal samples were processed using the same isolation agar.

Finally, a pre-treatment that could selectively isolate bacteria of interest from a sample source (in this case actinobacteria) would be undoubtedly commendable. As it saves researchers lots of time in blind bioprospecting for desired bacteria. However, applying an unsuitable method to a given sample source has been observed to affect the produced result. So, I recommend that more work should be done to identify the best methods to selectively isolate actinobacteria with consideration to the sampled source, based on observed environmental conditions or unique characteristics of the bacteria or a combination of both.

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Appendix

Table 7: Tentatively identified bacteria from sub-cultured colonies of the five animal samples, with sample ID, animal sample, treatment condition, Gen Bank ID, and tentatively identified class.

S/N	Origin/ Animal	Sample treatment	Gen Bank ID (closest match)	Per. (%) Ident	E. Value	Genus	Class
A001	16	Control	HM584226.1	100	0.0	Micobacterium	Actinomycetes
A002	16	Control	CP025333.1	99.45	0.0	Brevibacterium	Actinomycetes
A003	16	Control	MT229096.1	99.15	0.0	Brevibacterium	Actinomycetes
A004	16	Control	OR793883.1	100	0.0	Bacillus	Bacilli
A005	16	65^0C	HM100727.1	100	0.0	polariabacter	Flavobacteriia
A006	23	Control	OQ053266.1	99.63	0.0	polariabacter	Flavobacteriia
A007	22	Control	OQ053266.1	100	0.0	polariabacter	Flavobacteriia
A008	22	Control	MH478325.1	99.76	0.0	Sulfitobacter	Alphaproteobacteria
A009	22	Control	KY508328.1	99.65	0.0	Sulfitobacter/ Roseobacter	Alphaproteobacteria
A010	22	Control	MT626284.1	99.86	0.0	Microbacterium	Actinomycetes
A011	22	Control	OP316838.1	99.80	0.0	Salinibacterium	Actinomycetes
A012	22	Control	OR771407.1	100	0.0	Staphylococcus	Bacilli
A013	22	Control	MH550146.1	100	0.0	Metaplanococcus	Bacilli
A014	22	Control	MT525292.1	99.51	0.0	Planococcus	Bacilli
A015	19	Control	OQ617761.1	99.4	0.0	Citricoccus/ Micrococcus	Actinomycetes
A016	19	Control	OQ053266.1	99.52	0.0	Polaribacter Sp.	Flavobacteriia
A017	19	Control	OP623327.1	100	0.0	Oceaniglobus Sp.	Alphaproteobacteria
A018	19	Control	MK224810.1	100	0.0	Bizionia	Flavobacteriia
A019	19	Control	HM100727.1	100	0.0	Polariabacter Sp.	Flavobacteriia
A020	19	Control	OP623395.1	99.71	0.0	Mairbacter/ Pibocella	Flavobacteriia
A021	19	Control	EF108216.1	95.71	0.0	Mairbacter	Flavobacteriia
A022	18	Control	MN080178.1	99.87	0.0	Psychrobacter	Gamaproteobacteria
A023	18	Control	MK123478.1	99.86	0.0	Novosphingopyxis	Alphaproteobacteria
A024	18	Control	HE800820.1	99.66	0.0	Rhodococcus	Actinomycetes
A025	18	Control	MH929620.1	99.86	0.0	Salinibacterium	Actinomycetes
A026	18	Control	OP537143.1	99.88	0.0	Microbacterium	Actinomycetes
A027	18	Control	MH550136.1	99.88	0.0	Glutamicibacter	Actinomycetes
A028	18	Control	MT632637.1	99.85	0.0	Dietzia	Actinomycetes
A029	18	Control	MH550136.1	99.76	0.0	Glutamicibacter	Actinomycetes
A030	18	Control	MT525292.1	98.73	0.0	Planococcus	Bacilli
A031	18	Control	MH550136.1	99.75	0.0	Glutamicibacter	Actinomycetes
A032	18	Control	OQ300290.1	99.23	0.0	Microbacterium	Actinomycetes
A033	22	Control	MK224785.1	99.79	0.0	Sulfitobacter	Alphaproteobacteria
A034	23	Control	NR_136819.1	99.82	0.0	Lacinutrix	Flavobacteriia
A035	16	Control	MN931279.1	98.57	0.0	Lysobacter	Gammaproteobacte

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A036	16	Control	MT632637.1	99.07	0.0	Dietzia	Actinomycetes
A037	18	Control	LR722852.1	100	0.0	pseudomonas	Gammaproteobacteria
A038	18	Control	MF062561.1/ KY471026.1/ OQ071780.1	99.61 99.61 99.61	0.0 0.0 0.0	Neorhizobium/ Rhizobium/ pseudorhizobium	Alphaproteobacteria
A039	23	Control	LT717342.1	99.56	0.0	Kiloniella	Alphaproteobacteria
A040	23	Control	OR863783.1	99.08	0.0	Aquimarina	Flavobacteriia
A041	23	Control	OR863783.1	98.91	0.0	Aquimarina	Flavobacteriia
A042	19	Control	NR_025750.1	99.14	0.0	Maribacter	Flavobacteriia
A043	19	Control	MK224810.1	88.54	0.0	Bizionia Sp.	Flavobacteriia

Table 8: Tentatively identified bacteria from sub-cultured colonies from the breadcrumb sponge, with the sample ID, treatment, treatment condition, tentative identity, and class.

S/N Sample	S/N (Seq)	Sample treatment	Tentative Identity/ BLAST hits	Genus	Class
N001	V01	Control	*Vibro splendidus*	Vibro	Gamaproteobacteria
N002	V02	Control	*Pacificibacter aestuarii*	Pacificibacter	Alphaproteobacteria
N003	V03	Control	Crocinitomix catalasitica	Crocinitomix	Flavobacteriia
N004					unidentified
N005	V04	Control	*Sulfitobacter sp.*	*Sulfitobacter*	Alphaproteobacteria
N006	V05		*Lutibacter Sp.*	Lutibacter	Flavobacteriia
N007	V06	Control	*Sphingorhabdus sp.*	Sphingorhabdus	Alphaproteobacteria
N008	V07	Control	*Erythrobacter sp.*	Erythrobacter	Alphaproteobacteria
N009	V08	Control	*Bacillus subtilis*	Bacillus	Bacili
N010	V09	Control	Pacificibacter sp.	Pacificibacter	Alphaproteobacteria
N011	V10	Control	Vibro sp.	Vibro	Gamaproteobacteria
N012	V11	Control	Sphingopyxis litoris	Sphingopyxis	Alphaproteobacteria
N013	V12	Control	Parasphingorhabdus Litoris	Parasphingorhabdus	Alphaproteobacteria
N014					unidentified
N015	V13	Control	yoonia maricola	Yoonia	Alphaproteobacteria
N016	V14	Control	Sphingopyxis sp.	Sphigopyxis	Alphaproteobacteria
N017	V15	Control	Octadecabacter Sp.	Octadecabacter	Alphaproteobacteria
N018	V16	Control	yoonia maricola/Loktanella	Yoonia	Alphaproteobacteria
N019	V17	Control	Loktanella sp./yoonia maricola	yoonia	Alphaproteobacteria
N020	V18	Control	Sulfitobacter Sp.	Sulfitobacter	Alphaproteobacteria
N021	V19	Control	yoonia maricola	Yoonia	Alphaproteobacteria
N022	V20	Control	Polaribacter staleyi	Polaribacter	Flavobacteriia
N023	V21	Control	Loktanella tamlensis	Loktanella	Alpaproteobacteria
N024	V22	Control	Oceanistipes pacificus	Oceanistipes	Flavobacteriia
N025	V23	Control	Sulfitobacter Sp.	Sulfitobacter	Alphaproteobacteria
N026	V24	Control			unidentified
N027	V25	Control	Sulfitobacter Sp.	Sulfitobacter	Alphaproteobacteria
N028	V26	Control	Altererythrobacter Sp.	Altererythrobacter	Alphaproteobacteria
N029	V27	Control	Parasphingorhabdus flavimaris	Parasphingorhabdus	Alphaproteobacteria
N030	V28	Control	yoonia maricola/Loktanella	Yoonia	Alphaproteobacteria
N031	V29	Control			unidentified
N032	V30	Control	yoonia maricola/Loktanella	Yoonia	Alphaproteobacteria
N033	V31	Control	Octadecabacter ponticola	Octadecabacter	Flavobacteriia
N034	V32	Control	yoonia maricola	Yoonia	Alphaproteobacteria
N035	V33	Control			unidentified
N036	V34	Control			unidentified